Phosphorylation Sites of HPr

A Thesis

Submitted to the College of Graduate Studies and Research

In the Partial Fulfillment of Requirement for the

Degree of Doctor of Philosophy

In the Department of Biochemistry

University of Saskatchewan

By

Scott Napper

Fall 1999

UNIVERSITY OF SASKATCHEWAN

College of Graduate Studies and Research

SUMMARY OF DISSERTATION

Submitted in partial fulfillment

Of the requirements for the

DEGREE OF DOCTOR OF PHILOSOPHY

By

Scott Napper

Department of Biochemistry

University of Saskatchewan

Fall 1999

Examining Committee:

Dr. W. Xiao	Dean/Associate Dean, Dean's Designate, Chair College of Graduate Studies and Research
Dr. R. Khandelwal	Chair of Advisory Committee, Department of Biochemistry
Dr. L.T.J. Delbaere	Supervisor, Department of Biochemistry
Dr. E.B.W. Waygood	Department of Biochemistry
Dr. J.W. Quail	Department of Chemistry
Dr. J. Lee	Department of Biochemistry

External Examiner:

Dr. N. Strynadka

Department of Biochemistry

University of British Columbia

2146 Health Sciences Mall

Vancouver, B.C. V6T 1Z3

Abstract

The histidine-containing protein (HPr) is a central phosphotransfer component of the bacterial phosphoenolpyruvate:sugar phosphotransferase system (PTS) that transports carbohydrates across the cell membrane of bacteria. There are two HPr phosphorylation events investigated in this thesis.

Firstly HPr from Gram-positive species may undergo a regulatory phosphorylation of an absolutely conserved Ser46 residue. There are numerous metabolic consequences to this phosphorylation including inducer exclusion and expulsion, inhibition of PTS sugar uptake and catabolite repression. While HPr from Gram-negative sources cannot undergo phosphorylation of Ser46 *in vivo* or *in vitro* it is possible to mimic the phosphorylation through the Ser46Asp mutation. To determine the structural consequences of the mutation the crystallographic structure of the *E. coli* Ser46Asp HPr was determined at 1.5 Å resolution. The structure revealed that no significant structural rearrangements are induced by the mutation and the inability to accept phosphotransfer from Enzyme I is due to electrostatic disruption of the interaction of these proteins.

Phosphorylation of an absolutely conserved His15 for the purpose of phosphotransfer represents the second phosphorylation event to be investigated. The absolute requirement for histidine at the 15 position was investigated through mutagenesis. The mutation of His15Asp of *E. coli* HPr was able to accept a phosphoryl group from Enzyme I and further transfer the phosphoryl group to

Enzyme IIA^{glc}. None of the other mutations of the fifteen position were able to be phosphorylated. The His15Asp mutant had a Vmax of 0.1% and a ten-fold increase in Km with respect to wild type HPr.

As a consequence of the phosphorylation of His15Asp HPr a third protein species of higher pI than the original protein was identified. This high pI species seemed to share numerous similarities to succinimides which are known to be involved in deamidation. The inability to detect the known degradation products of succinimides suggested that the high pI species may involve isoimide formation. Isoimides have been proposed, but never experimentally demonstrated in proteins. A mechanism through which the phosphoacyl intermediate may catalyze isoimide formation is proposed. In addition the potential involvement of isoimide formation as a mechanism in physiological regulatory signaling is discussed.

Acknowledgements

Most young scientists are fortunate to have a single mentor in their academic upbringing, I've been fortunate enough to have two. I would like to express my sincere thanks to Dr. Louis Delbaere for taking a chance on me when I was a struggling undergraduate and providing me with the opportunity to focus on my studies in my fourth year. His generosity provided me the means and the confidence to pursue post-graduate training. I am also extremely grateful to Dr. Delbaere for allowing me the freedom to pursue my research interests. His willingness to allow students to follow their true passions, even when it isn't in his best interests, says a lot about him as a supervisor and a man.

I am also extremely grateful to Dr. Bruce Waygood for spotting early potential in me beyond being Kim's brother. Bruce's constant enthusiasm has been an inspiration to appreciate the magic of science.

I would like to thank the members of my advisor committee for the helpful suggestions and encouragement during my project. Your help and wisdom is greatly appreciated.

Financial support was provided by the College of Graduate Studies and Research in the form of graduate scholarship awards.

I would like to thank Murray, Al, Lindsay, Jason, Danny and Mike. I could have been done graduate studies a lot faster if it wasn't for you guys but I wouldn't trade the memories for anything.

Finally I would like to give thanks to "the best thing that ever walked up to my door", my best friend and soul-mate Krystal Baerg. Only you can really

understand what it took to complete this work. The pride and wonder you show in my achievements is more important to me than the degree I'm receiving. I can't wait to spend the rest of my life with you.

Dedication

This thesis is dedicated to my family:

To my mother for teaching me to work harder;

To my father for teaching me to work smarter;

and to my sister for reminding me they're both crazy.

And to my new family, Krystal and Levi,

you complete me.

Table of Contents

List of Tables

List of Figures

Abbreviations

Adenosine triphosphate	ATP
Catabolite control Protein A	CcpA
Catabolite gene repressor activator protein	CAP
Catabolite responsive element	Cre
Cyclic adenosine 3'-5'-monophosphate	cAMP
Dimethyl sulphoxide	DMSO
Dithiothreitol	DTT
Deoxynucleotide triphosphate	dNTP
Deoxyadenosine triphosphate	dATP
Deoxycytosine triphosphate	dCTP
Deoxyguanine triphosphate	dGTP
Deoxythymine triphosphate	dTTP
Dideoxynucleotide triphosphate	ddNTP
Dideoxyadenosine triphosphate	ddATP
Dideoxycytosine triphosphate	ddCTP
Dideoxyguanine triphosphate	ddGTP
Dideoxythymine triphosphate	ddTTP
Enzyme I	EI

Enzyme II	EII
Ethylene diamine tetraacetic acid	EDTA
Antibody fragment	Fab
Fructose-specific protein	FPr
Fructose	Fru
Glucose	Glc
N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid	Hepes
Histidine-containing Protein	HPr
Isopropylthiogalactoside	IPTG
Isoelectric focusing	IEF
Luria broth	LB.
Low melting point	LMP
Mannitol	Mtl
Matrix assisted laser diode ionization	MALDI
Melting temperature	Tm
Nicotinamide adenine dinucleotide	NADH
N-acetyl-glucosamine	Nag
Nuclear Magnetic Resonance	NMR
Polyacrylamide Gel Electrophoresis	PAGE
Phosphatase II	Pase II
Polymerase chain reaction	PCR

Phosphoenolpyruvate	PEP
Phosphoenol:pyruvate:sugar phosphotransferase system	PTS
Sodium dodecyl sulfate	SDS
Tris acetate EDTA	TAE
Tris borate EDTA	TBE
N,N,N',N'-tetramethylethylenediamine	TEMED
Tris(hydroxymethyl)aminomethane	TRIS
5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside	X-gal

Chapter 1 Introduction

1.1 The phosphoenolpyruvate:sugar phosphotransferase system

The survival and proliferation of bacteria, such as *Escherichia coli*, are dependent upon their ability to detect, migrate towards and take up sugars. The bacterial phosphoenolpyruvate:sugar phosphotransferase system (PTS) is a complex system of proteins which functions in mediating motility towards sugars and their subsequent translocation and phosphorylation (for reviews, see Meadow *et al.*, 1990; Postma *et al.*, 1993; Lengeler and Kahreis, 1996). Despite variability in the identity of the sugars which are transported by individual PTSs there is a common objective of initiation of metabolism and prevention of leakage from the cell through group translocation and phosphorylation (Kundig *et al.*, 1964,1971). The general role of the PTS can be described by the equation:

P-enolpyruvate(in) + carbohydrate(out) ——> pyruvate(in) + carbohydrate-P(in)

The PTS system is involved in a variety of other related cellular functions, such as the regulation of uptake of non-PTS sugars (for review see Saier, 1989), chemotaxis (for review see Titgemeyer, 1993; Lukat and Stock, 1993) and catabolite repression (for review see Postma *et al.*, 1993).

The PTS can be viewed as a linear arrangement of both soluble and membrane-bound phosphocarrier proteins and enzymes. The PTS functions as a series of phosphotransfer reactions with the phosphate group originating from phosphoenolpyruvate (PEP) and ultimately finishing with phosphorylation of the sugar moiety. A general representation of the pattern of phosphotransfer within the PTS is presented in Figure 1.1.

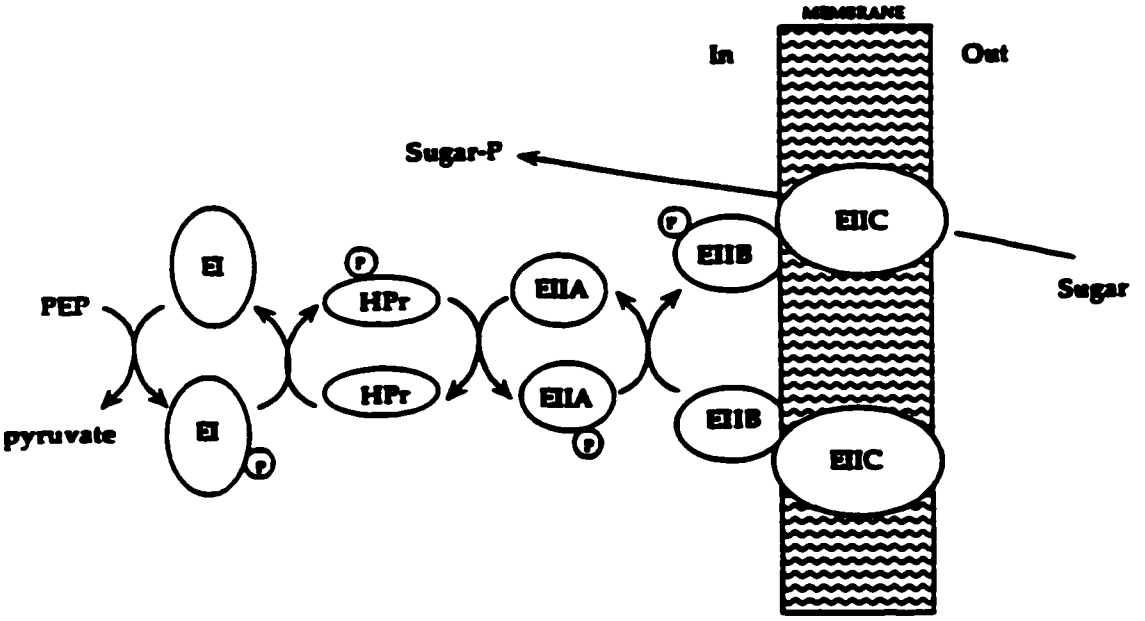


Fig 1.1: An illustration of the phosphoenolpyruvate:sugar phosphotransferase system.

The first two proteins of the PTS, Enzyme I (EI) and histidine-containing phosphocarrier protein (HPr) are soluble, non-sugar specific, energy-coupling components. Enzyme I catalyzes the transfer of a phosphoryl group from PEP to HPr. Sugar specificity of the PTS originates in the Enzyme II (EII) complexes. Each EII complex contains, a hydrophilic EIIA domain which serves to accept phosphotransfer from HPr, a hydrophilic EIIB domain which is phosphorylated by EIIA and a hydrophobic EIIC transmembrane domain which comprises the sugar binding domain. In a limited number of PTS systems such as *E. coli* mannose PTS and *Klebsiella pneumoniae* sorbose PTS, an additional membrane-bound component EIID exists (Postma *et al.*, 1993). The Enzyme II proteins can be fused or detatched depending on the sugar-transport system. A more detailed schematic illustrating the differences in the Enzyme II complexes is presented in Figure 1.2.

The PTS may be regarded as a series of protein domains, these domains retain their functional objectives whether they operate independently or as chimeras with other PTS domains. For example, the fructose PTS in *Salmonella typhimurium* and *E. coli* have a fructose inducible protein, FPr, which is a chimera of HPr and Enzyme IIA (Greese *et al.*, 1989). FPr permits strains deficient in HPr to grow on fructose (Waygood *et al.*, 1984). A chimera of Enzyme I, HPr and Enzyme II has been identified for the fructose PTS from *Rhodobacter capsulatus* (Wu *et al.*, 1990).

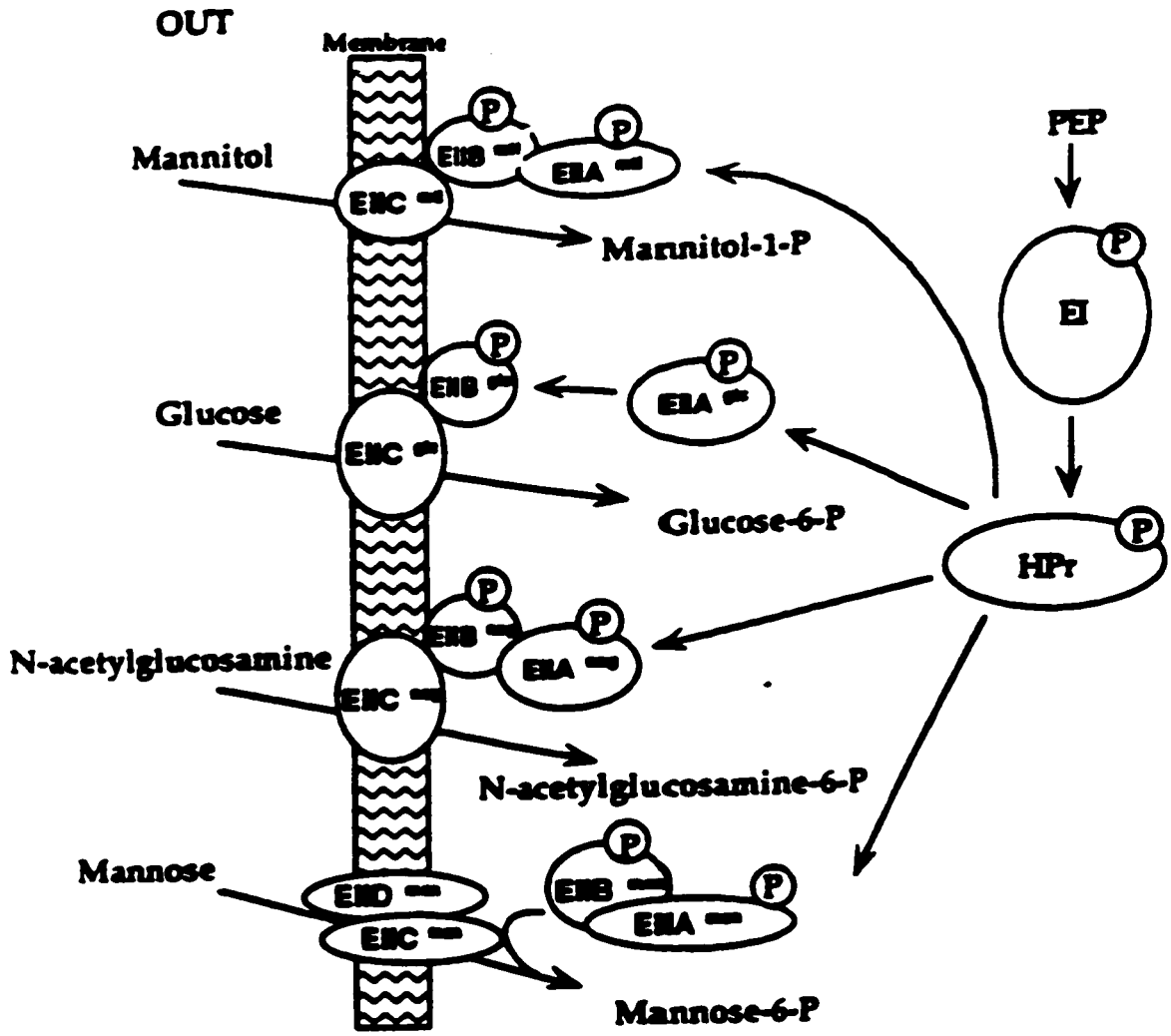


Figure 1.2 An illustration of representative Enzyme II complexes.

1.2 Components of the PTS

Due to the wealth of information available regarding the specific details and variations of each PTS protein this overview will focus only on details of immediate importance to the research of this thesis.

1.2.1 Enzyme I

The first protein component of the PTS is a common energy-coupling protein Enzyme I. Enzyme I initiates sugar-uptake by catalyzing the first PTS reaction of phosphotransfer from PEP to HPr. The importance of this reaction to PTS function has been illustrated by the diverse effects of Enzyme I mutation including disruption of PTS sugar uptake and inability to induce synthesis of catabolic enzymes. (Saier *et al.*, 1972).

1.2.1.1 Comparison of Enzyme I from different species

Enzyme I is present in all bacterial species which contain a PTS and its role and properties seem to be conserved. The sequences of Enzymes I from various species have been determined from gene sequences. Sequence alignments of Enzymes I from seven species of bacteria including *E. coli*, *Bacillus subtilis*, *Alcaligenes eutrophus*, *Staphylococcus carnosus*, *Streptococcus salivarius* and *S. typhimurium* demonstrate a high degree of homology. In particular there are six regions of Enzyme I sequence which are strongly conserved in all (Reizer *et al.*, 1993). A more specific comparison of sequences of *E. coli* and *S. typhimurium* Enzymes I illustrates the high degree of similarity; both proteins are 575 amino acids

long with only 16 substitutions (De Reuse *et al.*, 1985; LiCalsi *et al.*, 1991). Critical residues, such as the active site histidine 189, are conserved. Similarity of the active sites is further indicated by the comparable phosphohydrolysis properties of the active site phospho-histidines (Waygood *et al.*, 1987; Anderson *et al.*, 1992). In general, Enzyme I is functional as a homodimer (Waygood and Steeves, 1980; Misset *et al.*, 1980, Weigel *et al.*, 1982), the exception being Enzyme I from *Mycoplasma capricolum* which functions as a tetramer of three different subunits (Ullah *et al.*, 1977). The monomeric subunits range in weight from 58,00 to 85,000 daltons.

1.2.1.2 Enzyme I kinetics

The kinetic properties of the phosphoryl transfer from PEP to HPr by Enzyme I have been extensively characterized. In *E. coli* Enzyme I has a Km of 6.0 μM with respect to HPr and 0.18 mM with respect to PEP (Saier *et al.*, 1980; Waygood and Steeves, 1980). Similarly *S. typhimurium* Enzyme I has a K_m of 4.5 μM for HPr and 0.20 mM for PEP (Weigel *et al.*, 1982) Studies with *S. typhimurium* and *E. coli* Enzyme I indicate the kinetic mechanism of the reaction is that of a bi-bi ping-pong reaction, (Cleland, 1963, Weigel *et al.*, 1982) indicating that phosphorylation of HPr proceeds through a phosphorylated Enzyme I intermediate. This is supported by the observation that *E. coli* Enzyme I catalyzes isotope exchange between PEP and pyruvate (Saier *et al.*, 1980; Hoving *et al.*, 1981).

Collectively, studies indicate (for review see Meadow *et al.*, 1990; LiCalsi *et al.*, 1991) that the phosphorylation of Enzyme I by PEP occurs when it is dimerized. Enzyme I has two sites of phosphorylation per dimer (Weigel *et al.*, 1982; Waygood,

1986). Phosphorylation occurs on the $N^{\varepsilon 2}$ atom of a conserved His189 residue in the active site region. Despite early reports that the dimer is the active form of Enzyme I (Misset *et al.*, 1980) further investigations suggeted that the phosphorylated dimer disassociates into two phosphorylated monomers, which is the catalytic unit for phospho-transfer to HPr (Han *et al.*, 1990; LiCalsi *et al.*, 1991; Chavin *et al.*, 1992). The unphosphorylated Enzyme I monomers then reassemble into the dimer to restart the process. As the rate of dimer formation would be the rate-limiting step in initiating PTS phosphotransfer (Misset *et al.*, 1980; Han *et al.*, 1990; Chauvin *et al.*, 1992) the ratio of monomer to dimer may serve to regulate rates of sugar uptake in whole cells (Waygood *et al.*, 1977; LiCalsi *et al.*, 1991).

1.2.1.3 Structure of Enzyme I

The overall three dimensional structure of the Enzyme I has not been determined. The size of the Enzyme I is outside the current limits of structural determination by NMR and the entire protein has proven difficult to crystallize into suitable quality crystals. Recently, however, the N-terminal fragment (MW ~30 kDa) of *E. coli* Enzyme I has been determined crystallographically at 2.5 Å (Liao *et al.*, 1996). The N-terminal domain of Enzyme I contains the active site His189 and can accept a phosphoryl group from P-HPr but not from PEP (LiCalsi *et al.*, 1991). The N-terminal fragment is composed of two structural domains; a helical domain consisting of four α-helical structures arranged into two hairpins in a claw-like conformation. The second domain is comprised of a β-sandwich consisting of a three-

stranded anti-parallel β-sheet in conjuncture with a four-stranded parallel β-sheet united with three short α-helices (Liao *et al.*, 1996).

The second domain shows topological similarity to the phosphohistidine domain of the enzyme pyruvate phosphate dikinase (Herzberg *et al.*, 1996; McGuire *et al.*, 1998) as well as a moderate degree of sequence homology (Reizer *et al.*, 1991). The reaction catalyzed by the pyruvate phosphate dikinase has similarities to the Enzyme I catalyzed reaction, most importantly an autophosphorylation of a conserved histidine residue by PEP. The similarity of this domain would suggest a similar functional role in mediating the phosphorylation of the active site His189 residue by PEP. The helical domain would therefore be expected to be involved in mediating recognition and phosphotransfer to HPr (Liao *et al.*, 1996).

A structure solution of the N-terminal fragment of *E. coli* Enzyme I was determined by multidimensional NMR and reached the same conclusions as the crystallographic work (Garrett *et al.*, 1997).

1.2.1.4 Enzyme I/HPr interaction

The interaction between HPr and the N-terminal fragment Enzyme I, both of *E. coli*, has been investigated by crystallography (Liao *et al.*, 1996) and using heteronuclear magnetic resonance spectroscopy to monitor chemical shifts which emerge as a consequence of interaction of the two proteins (Garrett *et al.*, 1997, 1999). The most significant alterations which occur as a result of the interaction are in the α-helical domain of the N-terminal fragment, in particular in helices H1, H2 and H4 of Enzyme I. This is consistent with the speculation that the helical domain

represents the unique portion responsible for HPr recognition while the alpha/beta domain is involved in the interaction with PEP. The respective active site histidines, His15 of HPr and His189 of Enzyme I were speculated to interact in the shallow cleft which exists between the two functional domains of the N-terminal Enzyme I fragment. Furthermore it was demonstrated that no significant structural alterations occurred in the active site region of the N-terminal fragment as a result of the interaction with HPr (Garrett *et al.*, 1999), although it must be noted that phosphotransfer was not occurring as neither of the proteins was in the phosphorylated form.

1.2.2 Enzymes II complexes

Specificity of sugar uptake by the PTS is established by the Enzyme II complexes which link the non-sugar specific, initiating reactions to the specific uptake and phosphorylation of sugar residues. Although the Enzyme II complexes are similar in size, approximately 625 amino acids, they are the point of greatest structural diversity within the PTS (Saier *et al.*, 1988). Saier and Reizer (1992) have classified the Enzyme II complexes in six categories based upon sequence homology. Common to the Enzyme II^{sugar} complexes is the presence of at least three protein domains, operating alone or fused into chimeric structures (Saier and Reizer, 1992). The hydrophilic Enzyme IIA subunit accepts the phosphoryl group, from $N^{\delta 1}$-P-His15 of HPr, on an $N^{\varepsilon 2}$ atom of a histidine. In turn phosphorylated Enzyme IIA is able to transfer the phosphoryl group to a cysteine residue in most Enzyme IIB proteins (Pas *et al.*, 1991). The Enzyme IIB proteins of *E. coli* mannose, *B. subtilis* fructose and *K.*

pneumoniae sorbose systems are phosphorylated to form a P-$N^{\delta 1}$-histidine residue. All EIIB domains are hydrophilic. The phosphoryl group can then be donated to the sugar moiety recognized and translocated by the hydrophobic transmembrane EIIC domain (Saier and Reizer, 1992). In some Enzyme II complexes there exists a fourth protein component EIID. The EIID domain has been found in the PTSs of *E. coli* mannose (Erni, 1989), the *B. subtilis* fructose (Martin-Verstraete *et al.*, 1990) and the *K. pneumoniae* sorbose (Wehmeier *et al.*, 1995) sugar-transport systems. The EIID domain is a hydrophobic transmembrane domain which is likely involved in recognition or transport of the sugar residue. The Enzyme II permeases are often inducible enzymes to optimize utilization of currently available sugar substrates.

1.2.2.1 Enzyme II^{glc} from *Escherichia coli*

The Enzyme II^{glc} system will be examined in detail as a representative Enzyme II^{sugar} complex. Glucose is the preferred sugar of *E. coli* and the system of its uptake has been extensively studied with respect to structural, kinetic and regulatory parameters. The Enzyme II^{glc} complex of *E. coli* consists of two polypeptide chains; $EIIA^{glc}$ and $EIIBC^{glc}$.

The hydrophilic $EIIA^{glc}$ protein has a molecular weight of about 18,550 daltons. $EIIA^{glc}$ is phosphorylated by HPr on the $N^{\varepsilon 2}$ position of His90 (Meadow and Roseman, 1982; Dorschug *et al.*, 1984). Another histidine, residue 75, is not involved in accepting the phosphoryl group from HPr, but is required for donation of the phosphoryl group to $EIICB^{glc}$ (Presper *et al.*, 1989). Two forms of the EIIA protein can be purified from *E. coli*, $EIIA^{glc}$fast and $EIIA^{glc}$slow, corresponding to their

electrophoretic mobility. EIIA^{glcfast} results from the removal of seven residues from the N-terminus of the protein as a consequence of an endopeptidase reaction (Meadow and Roseman, 1982). NMR investigations indicate that no significant structural differences between the proteins (Pelton *et al.*, 1992). The phosphodonor properties of EIIA^{glc}fast are severely impaired in phosphodonation, but not acceptance, indicating the N-terminus may be involved in the interaction between EIIA^{glc} and EIIBC^{glc}.

EIIBC^{glc} is integral membrane protein with a molecular weight of 50,650 (Erni and Zanolari, 1986). The hydrophobic domain, EIIC, spans the membrane six to eight times and is predicted to contain the sugar-binding domain (Hummel *et al.*, 1992). The EIIB domain is phosphorylated by EIIA^{glc} on a cysteine at the 421 position (Nuoffer *et al.*, 1988). Given the numerous regulatory roles of EIIA^{glc} which requires the interaction of EIIA^{glc} with many other proteins the interaction between EIIA^{glc} and EIIBC^{glc} is expected to be weak and transient (Erni, 1989).

1.2.2.2 Structure of Enzyme IIA^{glc} from *E. coli*

The structure of EIIA^{glc} protein from *E. coli* has been determined by x-ray crystallography at a resolution of 2.1 Å (Worthylake *et al.*, 1991). The structure consists primarily of anti-parallel β-sheets which form a β-sandwich. The two histidines, His75 and His90, are located close together in a hydrophobic region with the $N^{\varepsilon 2}$ atom of His90 exposed to solvent. This hydrophobic region about the active centre region has been proposed to be critical in mediating interaction with a similarly electroneutral region of HPr for phosphotransfer (Herzberg, 1992b).

The structures of the phosphorylated and unphosphorylated forms of *E. coli* $EIIA^{glc}$ were determined using 3D NMR (Pelton *et al.*, 1992; Chen *et al.*, 1993). The structures indicate that no significant structural alterations arise as a consequence of phosphorylation. The structural differences are minor and limited to the immediate area about the active centre.

1.2.3 HPr

HPr, the histidine-containing protein is a small, soluble protein which occupies a central position in PTS function by acting as a phosphotransfer protein between Enzyme I and the $EIIA^{sugar}$ proteins. HPrs from different species can vary in size from 6,700 to 15,000 daltons (for review see Meadow *et al.*, 1990). HPr is present in all PTS-containing bacteria, as well as some strains which lack a functional PTS. The presence of HPr in *Lactobacillus brevis* and *Lactobacillus buchneri*, both of which lack the other PTS components, suggests that HPr is involved in other cellular, non-PTS, processes (Reizer *et al.*, 1988). This is further supported by the discovery of a gene encoding an 82-amino acid HPr-like protein in the eukaryotic mold, *Asperigillus fumigatus* (Barker *et al.*, 1991).

1.2.3.1 Comparison of HPr from different species

HPr has been extensively studied in a variety of species. The amino acid sequences of HPr from numerous organisms have been determined and comparisons made (Reizer *et al.*, 1993). While there is only approximately 30% amino acid identity between HPrs from Gram-positive and Gram-negative species, many of the

differences are conservative alterations. Conservation of sequence is particularly evident in the active centre region including the absolutely conserved His15 and Arg17 residues. A comparison of HPrs and HPr domains from the multi-domain protein FPr is presented in Figure 1.3.

A critical difference between HPrs from Gram-positive and Gram-negative species is the potential for regulation of HPr in Gram-positive bacteria (Deutscher *et al.*, 1984,1989). In Gram-positive bacteria HPr performs an important regulatory role through the phosphorylation of a conserved Ser46 residue by an ATP-dependent kinase (Deutscher *et al.*, 1983; Eisermann *et al.*, 1988). The physiological consequences of Ser46 phosphorylation of HPr in Gram-positive bacteria will be discussed later. This Ser46 phosphorylation has never been demonstrated with HPrs from Gram-negative sources, despite the conservation of the Ser46 residue and remarkably similar overall structures (Waygood *et al.*, 1984; Jia *et al.*, 1994a). It is suggested that the immediate surrounding environment about Ser46 determines the potential for the regulatory phosphorylation event (Jia *et al.*, 1994a). In Gram-positive bacteria there is considerable conservation about the Ser46 region of HPr extending from residues 40-50. This conservation is not evident in Gram-negative such as *E. coli* HPr. Structural analysis reveals that the overall positioning of Ser46 of HPr is remarkably similar in Gram-positive and Gram-negative bacteria and suggests that the inability to phosphorylate HPrs from Gram-negative bacteria with the ATP-dependent kinase may be the result of steric interference of neighboring side chains (Jia *et al.*, 1994a). In particular Gram-negative bacteria have a lysine at position 49 of HPr whereas Gram-positive bacteria have a glycine.

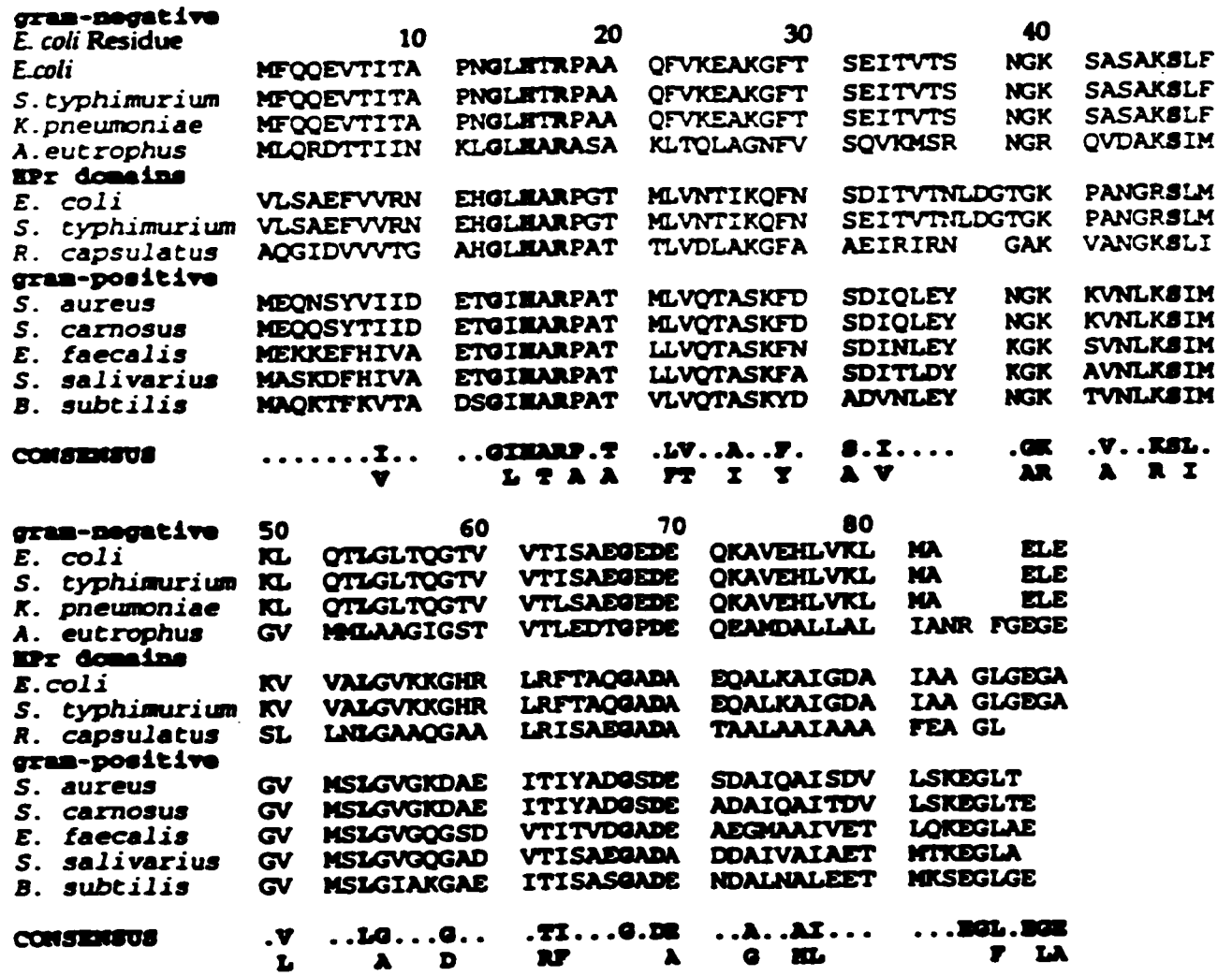


Fig 1.3 Sequences of HPrs and HPr domains from multidomain proteins from fructose-specific PTS (FPr). The alignment is adapted from Reizer *et al.*, (1993). Conserved residues are bolded. Consensus residues are defined for residues for which there are a maximum of two different residues for a given position.

A chimeric HPr protein was created in which Gram-positive *M. capriculum* HPr, was substituted in the residues 43-57 with the *E. coli* HPr sequence (Zhu *et al.*, 1998). This chimeric protein was unable to be phosphorylated by either the ATP-dependent kinase or Enzyme I. Further mutagenesis was performed to determine the critical residues for both types of phosphorylation. It was concluded that the identity of residues 51-57 was essential for His15 phosphorylation by Enzyme I and residues 48, 49 and 51-53 were distinctive in the recognition by the ATP-dependent kinase (Zhu *et al.*, 1998).

1.2.3.2 HPr structural information

HPr has been the subject of numerous structural investigations. Structures from a variety of sources having been determined by both x-ray crystallography, *E. coli* (Jia *et al.*, 1993a), *Streptococcus faecalis* (Jia *et al.*, 1993b,1994b), *B. subtilis* (Herzberg *et al.*, 1992b), *M. capricolum* (Pieper *et al.*, 1995), and NMR; *E. coli* (Klevit *et al.*, 1986), *B. subtilis* (Wittekind *et al.*, 1992) and *Staphylococcus aureus* (Kalbitzer *et al.*, 1993). In addition the phosphorylated forms of HPr have been investigated from *E. coli* (van Nuland *et al.*, 1995) and *B. subtilis* (Jones *et al.*, 1997b). The small size makes HPr ideally suited for such studies. HPr has also proven to crystallize with relative ease and has provided high quality crystals that diffract well and are remarkable stable during x-ray diffraction data collection. The abundance of mutants and the ease with which others can be produced makes HPr a model protein for investigation of issues of concern to all structural biochemists.

1.2.3.2.1 HPr NMR investigations

The first investigations of the HPr molecule through NMR were to examine the unusual pK_a of the active centre His15 residue and the phosphohistidine intermediate (Gassner *et al.*, 1977). The pKas of free amino acids can be described by a specific, constant value. In the context of proteins, the pK_a of an amino acid can be considerably different from that of the free amino acid due to effects of the microenvironment, such as electrostatic influence of nearby charged groups or helix dipole influences. Histidine, with a pK_a in the physiological range, is of interest due to the potential for a dynamic protonation state during physiological function (Sancho *et al.*, 1992). In *E. coli* HPr, the pK_a of the His15 residue was found to be 5.4, corresponding to the imidazole $N^{\delta 1}$ nitrogen atom and upon phosphorylation the pK_a shifts to 7.8, corresponding to the imidazole $N^{\varepsilon 2}$ atom (Dooijewaard *et al.*, 1979; Kalbitzer *et al.*, 1982; Anderson *et al.*, 1992). Functionally this means that at physiological pH the active centre His15 is deprotonated, providing a free pair of electrons on the imidazole $N^{\delta 1}$ nitrogen atom. These free electron pairs are essential for the nucleophilic attack on the phosphoryl group carried by Enzyme I. The protonation of the imidzole $N^{\varepsilon 2}$ atom (pK_a = 7.8), is involved in mediating the phosphoryl group into a good leaving group, facilitating transfer to the EIIA protein. The protonation state of the active centre histidine of HPr is essential for its physiological function. A schematic detailing the charge distribution which occurs during phosphotransfer is presented in Figure 1.4.

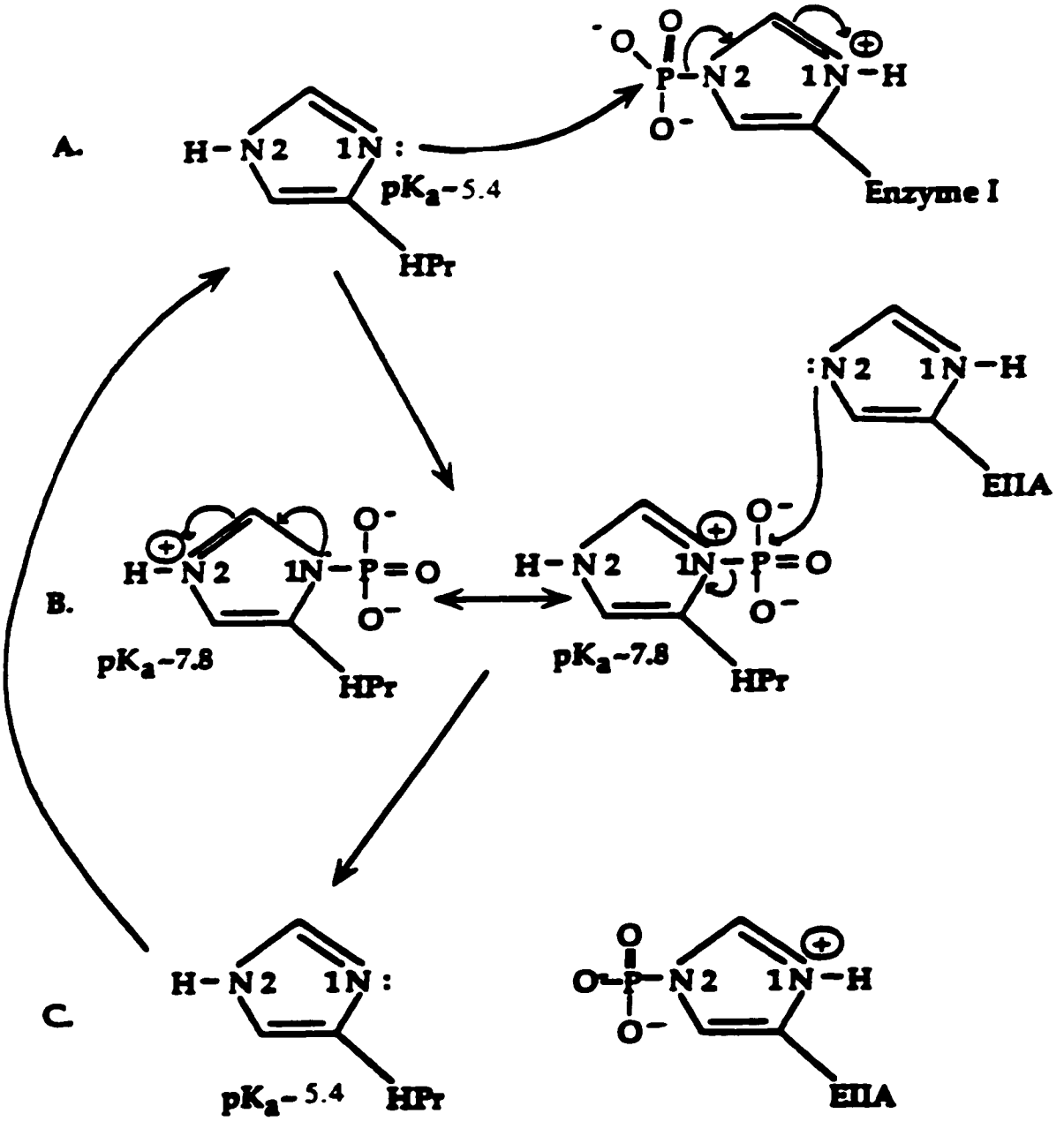


Fig. 1.4: Schematic illustration of PTS phosphohistidines during phosphotransfer: A) Deprotonated (pKa = 5.4) $N^{\delta 1}$ of His15 of HPr serves as a nucleophile to the bound phosphoryl group of Enzyme I; B) In P-HPr the imidazole is protonated (pKa = 7.8) providing a good leaving group for the attack of the free electron pair of the $N^{\varepsilon 2}$ of the imidazole of EIIA; C) Phosphoryl group transfer from HPr returns the $N^{\delta 1}$ atom of His15 to the reactive unprotonated form.

NMR has also provided information regarding the dynamic structural features of the active centre during phosphotransfer (Rajagopal *et al.*, 1994; van Nuland *et al.*, 1995,1996; Jones *et al.*, 1997b). In both *E. coli* and *B. subtilis* HPrs, phosphorylation of His15 results in a minor conformational change which allows the phosphoryl group to form hydrogen bonds with the main-chain nitrogen atoms of residues 16 and 17. The side chains of residues 12 and 17, which have long been proposed to be involved in stabilization of phosphorylated His15, do not appear to have extensive roles in this regard. Arg17, which is conserved in all HPrs, is restrained from its native mobile positioning and the guanidinium group approaches but does not interact with the phosphoryl group. Residue 12, asparagine in *E. coli* and serine in *B. subtilis*, maintain positions very similar to that of the unphosphorylated form (for review see Waygood, 1998).

NMR has also been utilized to investigate the structural alterations which occur in the regulatory Ser46 phosphorylation event. NMR investigations of *B. subtilis* HPr Ser46 phosphorylation indicate that inactivation of the protein occurs in the absence of any major structural rearrangements (Wittekind *et al.*, 1989, 1990, 1992; Pullen *et al.*, 1995).

1.2.3.2.2 HPr crystallographic investigations

There have been numerous crystallographic determinations of HPr from a variety of species; *E. coli* (Jia *et al.*, 1993a; Napper *et al.*, 1996; Prasad *et al.*, 1998), *B. subtilis* (Herzberg *et al.*, 1992b), *S. faecalis* (Jia *et al.*, 1993b,1994b) and *M. capricolum* (Pieper *et al.*, 1995). All share a similar folding topology. The overall

folding of HPr is a classic open-faced β-sandwich composed of four anti-parallel β-strands and three α-helices. The four β-strands form a single, pleated anti-parallel β-sheet on one face of the protein and the three α-helices lie on one side of the β-sheet. There are also four reverse turns and several regions of extended structure. A ribbon diagram of the *E. coli* HPr is presented in Figure 1.5.

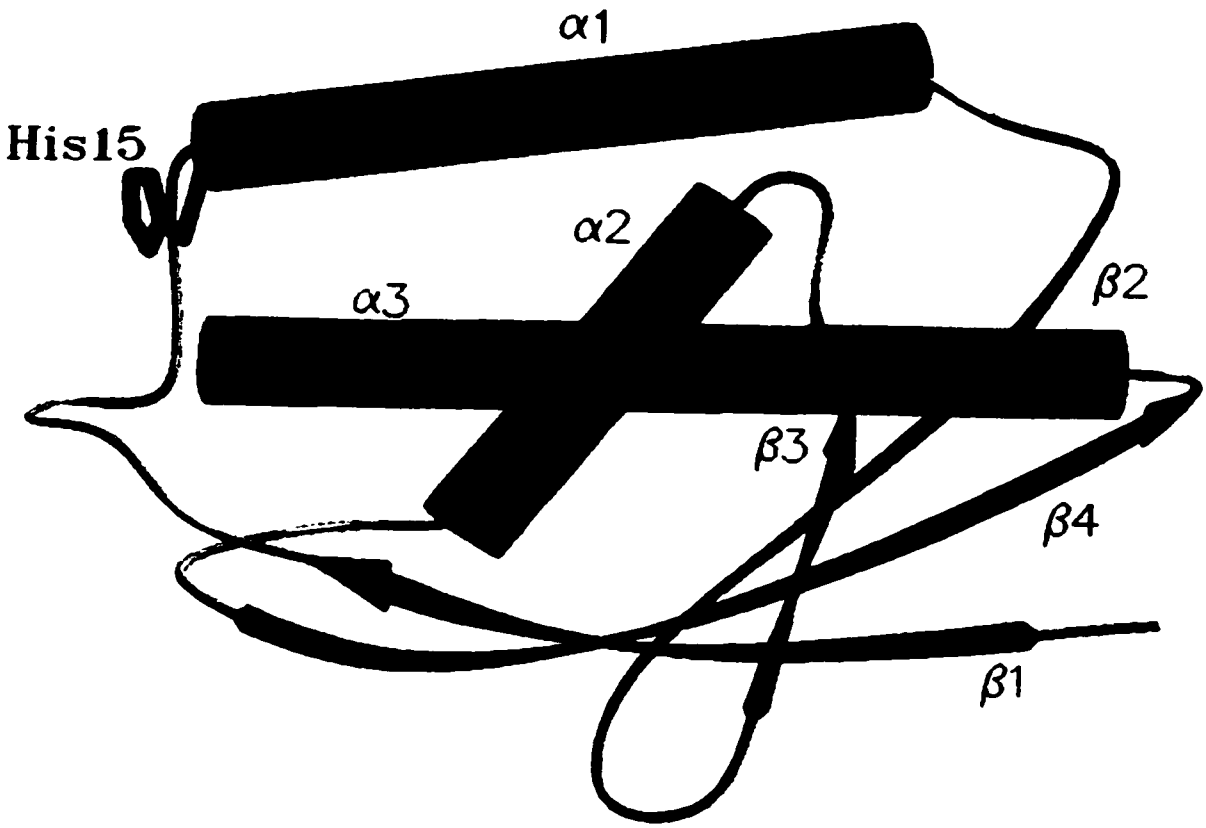


Fig 1.5: Ribbon representation of *E. coli* HPr. Diagram produced with SETOR (Evans, 1992).

The interpretation of HPr crystallographic structures must consider the pH at which the crystals were produced. Structures obtained from crystals produced at pH below 5.5 will have His15 in the improper protonation state for the unphophosphorylated species. The common occurrence of a sulfate anion in the active centre of HPr must also be interpreted with caution. While the fortuitous presence of a sulfate in the active centre region is thought to mimic the phosphorylated state, other studies have indicated that the structural interactions between proteins and sulphate and phosphate anions are not identical (Copley and Barton, 1994). Despite these considerations there is a conserved active centre secondary structure orientation. The positioning of active centre side chains is generally conserved. The active centre of *E. coli* HPr is presented in Figure 1.6 with labeling of proposed critical residues. The significance of these residues in the active centre is subsequently discussed.

1.2.3.3 Active centre of HPr

Considerable evidence has been gathered regarding the active centre residues of HPr through various structural determinations as well as site-directed mutagenesis. Phosphohydrolysis studies have also assisted in understanding which residues contribute to the properties of the active centre region (for review Anderson, 1995). The principle residues which have been implicated in the HPr active centre, His15, Arg17, Glu85 and residue 12 which has a variable identity across species, will be discussed in detail.

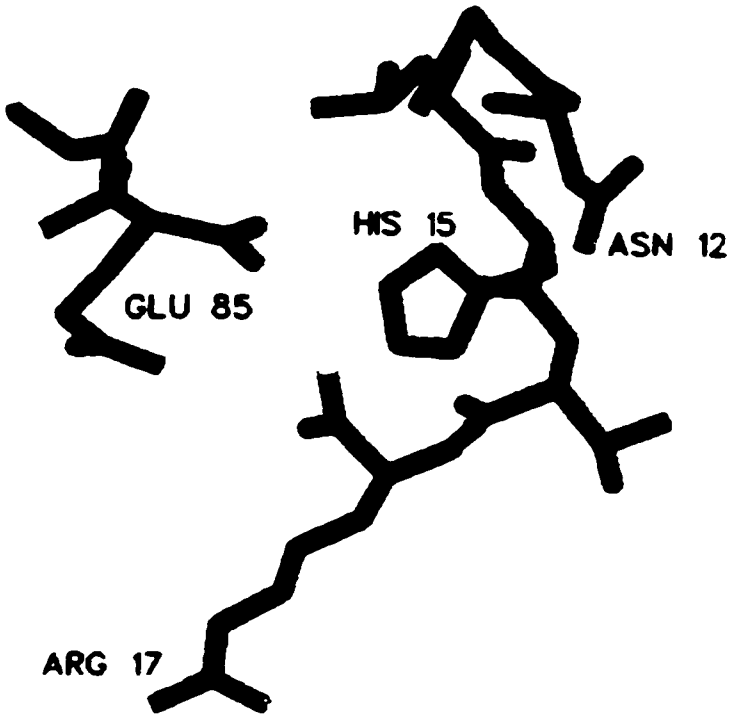


Figure 1.6: The active centre of wild type *E. coli* HPr. Diagram was produced with SETOR (Evans, 1992).

1.2.3.3.1 His15

Histidine at position 15 is absolutely conserved in all HPrs sequenced to date. His15 is phosphorylated at the $N^{\delta 1}$ position of the imidazole ring, forming an unstable, high-energy phosphoramidate (Anderson *et al.*, 1971). An essential feature of HPr phosphotransfer abilities is the unusual pKa of His 15 in the native structure. Investigations of HPrs from *E. coli* (Dooijewaard *et al.*, 1979; Kalbitzer *et al.*, 1982; Anderson *et al.*, 1991,1993), *B. subtilis*, *S. faecalis*, *Stretococcus lactis* (Kalbitzer *et al.*, 1982) and *S. aureus* (Gassner *et al.*, 1977) all report that the pKa of His15 is considerably lower than that of free histidine. The pKa of unphosphorylated *E. coli* HPr His15 is 5.4 (Anderson *et al.*, 1991, 1993). Mutagenesis studies of *E. coli* HPr illustrate that the micro-environment of the active centre region, including the helix A

dipole, are responsible for the unusual pKa (Anderson *et al.*, 1991,1993). In all structural determinations, His15 is located at the N-terminus of helix A. The tautomeric state of both HPr and P-HPr involve the protonation of the $N^{\varepsilon 2}$ atom of the imidazole ring (Rajagopal *et al.*, 1994). With the ionization of the incoming dianionic phosphoryl group results in change in charge of −1 upon phosphorylation.

The possibility of functional substitutions of the His15 residue of HPr will be investigated in this thesis. Substitutions are based on the observation of their phosphorylation in other proteins as well as structural similarity to histidine.

1.2.3.3.2 Arg17

An arginine at position 17 is conserved in all HPrs sequenced to date. The modification of Arg17 with cyclohexanedione resulting in an HPr of about 20% activity was the first indication of the importance of this residue (Kalbitzer *et al.*, 1982). The involvement of arginine side chains in phosphoryl group transfer mechanisms had been previously discussed (Knowles, 1980) and a similar role was hypothesized for the Arg17 of HPr (Waygood *et al.*, 1985). All mutations of the 17 position, including the conservative change to lysine, result in severely impaired kinetic properties with respect to both Enzyme I and EIIA proteins, with the exception of $EIIA^{Man}$, which demonstrated modest alterations (Anderson *et al.*, 1993). The kinetic properties suggest that Arg17 has dual function in mediating interaction with Enzyme I as well as in the catalytic mechanism. While it has been suggested that the role of Arg17 in phosphotransfer is to accommodate the negative charge introduced by phosphorylation through interaction of the guanidinium group, this is

not supported by NMR investigations (van Nuland *et al.*, 1995; Jones *et al.*, 1997b). While the structural evidence regarding the positioning and functional role of Arg17 in HPr phosphotransfer is far from unanimous the majority of the evidence would indicate Arg17 is mobile and adopts a more constrained position as a consequence of phosphorylation; however, this does not involve binding the phosphoryl group (for review see Waygood, 1998).

1.2.3.3.3 C-terminus

A point of disagreement between structural investigations and mutagenic analysis of the HPr active centre is with regard to the involvement of the C-terminal residue. In *E. coli*, the involvement of a glutamate residue in the active centre was suggested from the pH-dependency of phosphohydrolysis of P-HPr (Waygood *et al.*, 1985). Two dimensional NMR investigations of HPr supported the involvement of Glu85 in the active centre (Klevit and Waygood, 1986). The crystallographic determination of *E. coli* HPr at 2.0 Å resolution indicated an interaction between the C-terminal α-carboxylate, rather than the side chain γ-carboxylate, with the active centre histidine (Jia *et al.*, 1993a). A crystallographic determination of the HPr:Jel42 antibody complex at 2.5 Å resolution, in which the active centre has no intermolecular crystallographic contacts, shows no involvement of Glu85 with His15 (Prasad *et al.*, 1998). HPrs from Gram-positive bacterial species position the C-terminus, which extends beyond residue 85, away from the active centre (Herzberg *et al.*, 1992b, Jia *et al.*, 1994a). In addition mutagenic studies of *E. coli* HPr, however,

demonstrate little consequence of deleting or mutating this residue (Anderson *et al.*, 1991).

1.2.3.3.4 Residue 12

Residue 12 is not highly conserved, threonine in *S. aureus* and *S. faecalis*, serine in *B. subtilis* and asparagine in *E. coli*, yet all retain the potential and structural positioning for hydrogen bond formation with His15. In the *S. faecalis* crystallographic structure there is a hydrogen bond between the main-chain carbonyl of Thr12 and the $N^{\delta 1}$ atom of His15 (Jia *et al.*, 1994b). In the *B. subtilis* structure the hydroxyl group of Ser12 forms a hydrogen bond with the $N^{\epsilon 2}$ atom of His15 (Herzberg *et al.*, 1992b). Van Dijk *et al.* (1990) reported that the NMR spectral properties of His15 in *E. coli* HPr were consistent with hydrogen bonding to the $N^{\epsilon 2}$ atom of His15 and that Asn12 was the most likely residue to be participating in this hydrogen bond (van Nuland *et al.*, 1995). While no interaction between Asn12 and His15 were found in the *E. coli* HPr x-ray structure, the potential for hydrogen bonding was proposed through permitted rotation of Asn12 (Jia *et al.*, 1993a). Multi-dimensional NMR experiments and molecular dynamics investigations implicate Asn12 to be a likely candidate to form the $N^{\epsilon 2}$ hydrogen bond with His15 in *E. coli* HPr based on pH-dependent changes to resonances (van Nuland *et al.*, 1992) and suggest this interaction may be maintained in the P-His species (van Nuland *et al.*, 1995). The recently described 2.5 Å resolution structure of the complex of the Jel42 monoclonal antibody Fab fragment with HPr shows a hydrogen bond between the Asn12 side chain and the $N^{\epsilon 2}$ atom of His15 (Prasad *et al.*, 1998).

Also supportive of the role of Asn12 in the active centre region are the kinetic alterations, as well as changes in the phosphohydrolysis properties, which occur in HPrs which undergo deamidation at this site to form the species HPr-2; Km 15 uM and Vmax 30% (Waygood *et al.*, 1985; Sharma *et al.*, 1993). Deamidation results in the production of both L-aspartic as well as L-isoaspartic acid. The Asn12Asp mutant of *E. coli* HPr was produced to mimic HPr-2 and produced similar kinetic consequences (Sharma *et al.*, 1993).

The structural and kinetic evidence, as well as the pattern of conservation of amino acids at residue12, suggest hydrogen bonding capabilities are required at this position. The proposed hydrogen bond to the $N^{\varepsilon 2}$ atom of the His15 imidazole would serve to correctly position the His15 for phosphorylation and to specify phosphoryl transfer to the $N^{\delta 1}$ atom. It would appear from species comparison that the exact identity of the residue at the 12 position is not as important as hydrogen bonding capabilities. The importance of the hydrogen bonding capabilities of a residue at position 12 will be examined in this thesis.

1.2.3.4 Deamidation of HPr

The earliest investigations of the HPr protein reported the observation of sub-species of the protein. These species were of the same approximate molecular weight as the wild-type protein but were more negatively charged (Anderson *et al.*, 1971). These HPrs are designated HPr-1 and HPr-2, corresponding to their order of elution from a DEAE-cellulose column. Deamidation is an established mechanism of spontaneous covalent modification of proteins which occurs as a consequence of

protein aging or extreme conditions of pH or temperature. Deamidation results in the formation of protein species of more negative charge than the original protein (for review see Wright, 1991; Clarke, 1992). Based on the extent of charge difference between the wild type, HPr-1 and HPr-2 HPr proteins it was determined that HPr-1 arose as a consequence of a single deamidation event whereas HPr-2 required a second deamidation event. HPr-1 demonstrated unimpaired kinetic properties while HPr-2 showed changes to kinetic properties as well as to phosphohydrolysis rates (Waygood *et al.*, 1985).

In *E. coli* HPr there are eight residues with deamidation potential, six glutamines and two asparagines. Both asparagines are followed by glycines; Asn12-Gly13 and Asn38-Gly39. High rates of deamidation are often observed when the potential deamidating residue is in a region of high main-chain flexibility. The combination of asparagine followed by a glycine is the most commonly occurring deamidation site due to the flexibility of the main-chain imparted by glycine (for review see Wright, 1991). Site-directed mutagenesis verified that these asparagines, Asn12 and Asn38, were the sites of deamidation in *E. coli* HPr (Sharma *et al.*, 1993). The functionally inconsequential HPr-1 formation is a result of deamidation of Asn38 while deamidation of the active site Asn12 showed greater influence on kinetic and phosphohydrolysis profiles (Sharma *et al.*, 1993). It is interesting to note that the Asn38 residue is in a tight β-turn in the *E. coli* HPr structure that would not seem conductive to succinimide ring formation due to steric restraints. The steric requirements for deamidation through succinimide ring formation will be discussed later in greater detail.

1.3 Phosphoamino acids of the PTS

The post-translational modification of proteins through phosphorylation by protein kinases occurs on a limited sub-set of amino acids. These phosphorylated amino acids differ in their chemical properties and physiological roles. There are two primary classes of phosphoamino acids based upon physiological function, first those involved in regulation of enzymatic activity, these include phosphorylation of serine, threonine and tyrosine residues. These phosphoesters are stable linkages and generally serve in regulation of enzymatic catalysis without any direct involvement in the catalytic mechanism. Removal of phosphoryl groups from these phosphoesters usually requires a specific phosphatase enzyme. An example of this type of phosphorylation is seen in Gram-positive PTSs with the regulatory Ser46 phosphorylation of HPr.

The second class encompasses phosphorylation for the purpose of phosphoryl group transfer, these include aspartyl, glutamyl, histidinyl and cysteinyl phosphorylations. Phosphohistidines generally act as high-energy phospho-intermediates for the purpose of transferring the phosphoryl group from a phosphoacceptor to a phosphodonor molecule (Stock *et al.*, 1989). Examples of phosphohistidines in phosphoryl group transfer include the bacterial histidine kinase phosphotransfer components (Parkinson *et al.*, 1992) as well as the PTS.

An interesting subcategory of regulatory phosphotransfer reactions involves the transfer of a phosphoryl group to an aspartyl group. Phosphorylation of aspartate residues in this context have been reported in a number of systems such as the bacterial two-component system (for review see Stock *et al.*, 1989; Parkinson *et al.*,

1992). Bacteria contain a phosphotransfer signaling system, referred to as the two component system, to regulate adaptive responses to an extracellular stimulus. The system is comprised of two types of signal transducers, a sensor kinase and a response regulator. The sensor kinase monitors environmental signals and modulates the function of the response regulator accordingly (Nixon *et al.*, 1986). The mechanism of this regulation is through a histidinyl to aspartyl phosphotransfer (for review see Stock *et al.*, 1989; Lukat *et al.*, 1991).

The phosphotransfer events in the PTS involve phosphorylations of four different proteins, leading to three different types of phosphorylations on two different amino acids (Meadow *et al.*, 1990). The beginning to this phosphotransfer chain is the initial phosphate donating molecule phosphoenolpyruvate (PEP). The standard energy of hydrolysis of PEP (-14.7 kcal/mol) is nearly double that of ATP (-7.5 kcal/mol). The first three phosphorylations of the PTS involve the amino acid histidine. Histidine is a functionally diverse amino acid and is found in the active sites of many enzymes (Schneider, 1978). The diversity is due to the presence of the imidazole ring which bestows both hydrogen acceptor and donor properties. Moreover, the imidazole ring presents two potential phosphorylation sites, atoms $N^{\delta 1}$ and $N^{\epsilon 2}$ (Hultquist *et al.*, 1966; Hultquist, 1968). Both types of histidine phosphorylation are found in the PTS. Phosphoryl group attachment at the $N^{\delta 1}$ position of the imidazole ring of free histidine is much more labile than $N^{\epsilon 2}$ linkages in histidine due to the interaction of the $N^{\delta 1}$ phosphoryl group with the positively-charged amino group (Hultquist, 1966). In proteins the amino group is involved in a peptide bond and the phospho-stability properties of histidines differ from free

histidines. For the *E. coli* HPr P-$N^{\delta 1}$-His15 the rates of phosphohydrolysis are three times higher than that for a free P-$N^{\delta 1}$-histidine (Anderson *et al.*, 1993). The local structural and electrostatic environment of histidine in the protein may have significant influence on the chemical properties (Waygood *et al.*, 1985; Anderson *et al.*, 1993) and the properties of histidine may vary considerably in different protein contexts.

A general pattern of phosphotransfer between histidines is the alternation of points of attachment from $N^{\delta 1}$ to $N^{\epsilon 2}$ atoms and vice versa. This tendency is also true of the PTS phosphotransfers. Enzyme I is phosphorylated by PEP at the $N^{\epsilon 2}$ atom of His 189, HPr is phosphorylated at the $N^{\delta 1}$ atom of His 15 and the EIIA proteins are $N^{\epsilon 2}$ phosphorylated (for review see Postma *et al.*, 1993).

Variability exists in the type of residue which is phosphorylated in the $EIIB^{sugar}$ proteins. Cysteine is the most common site of phosphorylation in the $EIIB^{sugar}$ proteins. However, in the *E. coli* mannose, *B. subtilis* fructose and *K. pneumoniae* sorbose sugar transport systems EIIB domains form P-$N^{\delta 1}$-histidine intermediates.

1.4 Regulation by the PTS

The PTS, also controls the uptake of many non-PTS sugars and would seem an ideal point for regulation of expression of proteins involved in sugar metabolism. Through control of uptake of sugars, the PTS can efficiently indicate the appropriate enzymes which are required in the cell. Regulation of carbohydrate metabolism, by PTS components occurs indirectly in a manner which is dependent upon the state of

PTS phosphotransfer activity. Despite the common objective of optimization of carbohydrate metabolism there are considerable differences in the strategies employed by Gram-positive and Gram-negative PTSs to achieve these goals. Regulation in Gram-negative species is mediated primarily through the $EIIA^{glc}$ protein while in Gram-positive species HPr occupies a more central role.

1.4.1 PTS regulation in Gram-negative bacteria

When enteric bacteria, such as *E. coli*, are grown in glucose-containing media with other carbon sources, glucose will be preferentially metabolized (Monod, 1942). The presence of glucose therefore negates the need for the catabolic enzymes involved in metabolism of other carbohydrates and their expression is regulated accordingly. The PTS operates to optimize protein expression to best suit the carbon source currently available. In Gram-negative bacteria $EIIA^{glc}$ plays a central role in regulating the expression pattern of enzymes and permeases involved in carbohydrate metabolism (for reviews see Meadow *et al.*, 1990; Postma *et al.*, 1993).

1.4.1.1 Inducer exclusion and catabolite repression

There are two distinct methods by which Gram-negative bacteria modify the pattern of protein expression to optimize glucose utilization. Firstly, through a process termed "inducer exclusion" (Saier and Roseman, 1972,1976) the uptake of non-PTS substrates or inducers is inhibited as a consequence of glucose metabolism. Secondly, the synthesis of catabolic enzymes is regulated through modulation of levels of cyclic adenosine 3'-5'-monophosphate (cAMP) in a process termed

"catabolite repression" (Makman and Sutherland, 1965; Harwood *et al.*, 1976; Ullmann and Danchin, 1985; Saier, 1989).

Central to the process of inducer exclusion in *E. coli* is $EIIA^{glc}$. The extent of phosphorylation of $EIIA^{glc}$ is a reflection of metabolic activity and serves as the regulatory signal (Kornberg *et al.*, 1978,1980; Saier, 1989). Glucose uptake requires phosphoryl group movement through the PTS to the sugar molecule. Glucose uptake therefore, results in a higher percentage of $EIIA^{glc}$ in the unphosphorylated state. Unphosphorylated $EIIA^{glc}$ acts as a regulator of catabolic enzyme synthesis through interaction with specific enzymes or permeases which are involved in the uptake or metabolism of non-PTS sugars (for review see Saier, 1993). For example, unphosphorylated $EIIA^{glc}$ serves as a negative regulator of lactose permease through protein:protein interactions (Osumi *et al.*, 1982; Nelson *et al.*, 1983). By preventing the uptake of lactose the $EIIA^{glc}$ protein indirectly prevents expression of the *lac* operon (Nelson *et al.*, 1983).

By similar mechanisms $EIIA^{glc}$ prevents the metabolism of glycerol (Saier and Roseman, 1976). Unphosphorylated $EIIA^{glc}$ binds to and inhibits the enzyme glycerol kinase, the first enzyme in the metabolism of glycerol, preventing the production of glycerol-3-P, which is the inducer of the *glp* operon (Novotny *et al.*, 1985). In both situations the unphosphorylated form of $EIIA^{glc}$ indicates the presence of the preferred carbon source, glucose, therefore negating the need for expression of proteins involved in metabolism of other sugars (Postma *et al.*, 1984).

The second mechanism of optimization of expression of catabolic enzymes is the process of catabolite repression. In enteric bacteria many carbohydrate catabolic

enzymes are positively regulated by cAMP (for review see Botsford and Harmann, 1992). Regulation of adenylate cyclase, the enzyme which produces cAMP, by PTS components provides a general mechanism of regulation of numerous operons. When glucose is unavailable the $EIIA^{glc}$ molecule will be primarily in the phosphorylated form. Phosphorylated $EIIA^{glc}$ acts as a positive modulator of adenylate cyclase stimulating the production of cAMP (Peterkofsky *et al.*, 1989). The subsequent increase in levels of cAMP result in activation of the catabolite gene repressor activator protein (CAP) dependent genes. Transcriptional regulation by cAMP occurs through the binding of the cAMP-CAP complex to a *cis* element of the catabolic operon. The cAMP molecule serves not only as an indicator of the absence of glucose, but also as an activator of expression of carbohydrate catabolic enzymes to activate alternative systems for the uptake and metabolism of secondary energy sources (Paigen and Williams, 1970).

1.4.2 PTS regulation in Gram-positive bacteria

In contrast to Gram-negative bacteria, where $EIIA^{glc}$ occupies a central regulatory position, HPr is the primary regulatory metabolic protein in Gram-positive bacteria. A unique functional difference between the Gram-positive and Gram-negative PTSs is the potential for regulation of the HPr protein through a Ser46 phosphorylation (for review see Reizer *et al.*, 1993; Eisermann *et al.*, 1988; Saier *et al.*, 1996). P-(Ser)HPr is also found in several species which lack a functional PTS supportive of a regulatory role for the protein (Romano *et al.*, 1987; Reizer *et al.*, 1988,1993). The phosphorylation event is catalyzed by a HPr-specific ATP-

dependent kinase (Deutscher and Saier, 1983; Reizer *et al.*, 1984,1988) and dephosphorylation occurs by the action of a HPr-specific phosphatase (Deutscher *et al.*, 1984). The activity of the ATP-dependent kinase is stimulated by fructose 1,6-bisphosphate and inhibited by inorganic phosphate (Deutscher and Saier, 1983).

The Ser46 phosphorylation of HPr in Gram-positive species regulates numerous processes such as catabolite repression, inducer explusion and the uptake of PTS and non-PTS sugars. These processes are mediated by the differential interactions of HPr and HPr(Ser-P) with numerous other proteins including Enzyme I, non-PTS sugar transport proteins, sugar phosphatases and transcription factors. An overview of the proposed regulatory roles of the HPr Ser46 phosphorylation event are presented in Fig 1.7 and subsequently discussed.

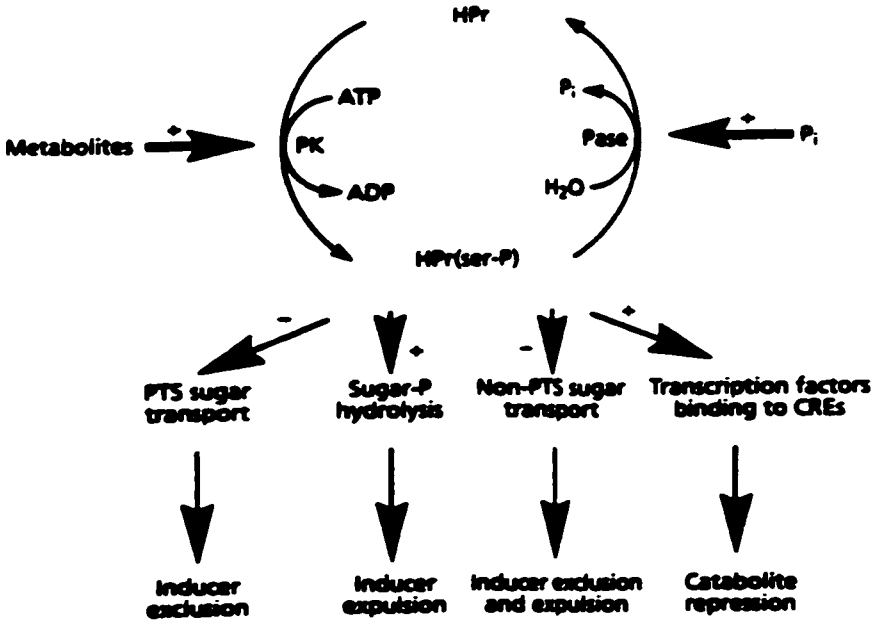


Fig. 1.7 Regulatory functions of HPr(Ser-P) in Gram-positive bacteria. Adapted from Saier *et al.*, 1996.

Regulation of both PTS and non-PTS sugar uptake in Gram-positive bacteria is regulated by HPr(Ser-P). The phosphorylation state of Ser46 HPr in Gram-positive bacteria regulates the process of inducer exclusion of both PTS and non-PTS carbohydrates. For PTS sugars the affect is mediated by rendering the HPr molecule a poor substrate for Enzyme I when it is in the Ser46 phosphorylated form. The inability of Enzyme I to phosphotransfer to HPr(Ser-P) ensures that further PTS sugar uptake will be inhibited. Uptake of non-PTS sugars are also regulated by HPr(Ser-P). For example *Lac. brevis* can take up lactose through a lactose/H+ symporter which couples the uptake of the carbohydrate to the proton-motive force. This uptake of lactose by the permease has been shown to be inhibited by HPr(Ser-P) (Ye *et al.*, 1994). The mechanism of the regulation is proposed to involve the HPr(Ser-P) mediated conversion of the sugar symporter into a sugar uniporter as illustrated in Figure 1.8. The uncoupling of sugar transport from proton transport ensures that the permeases cannot accumulate the sugar substrates against a concentration gradient.

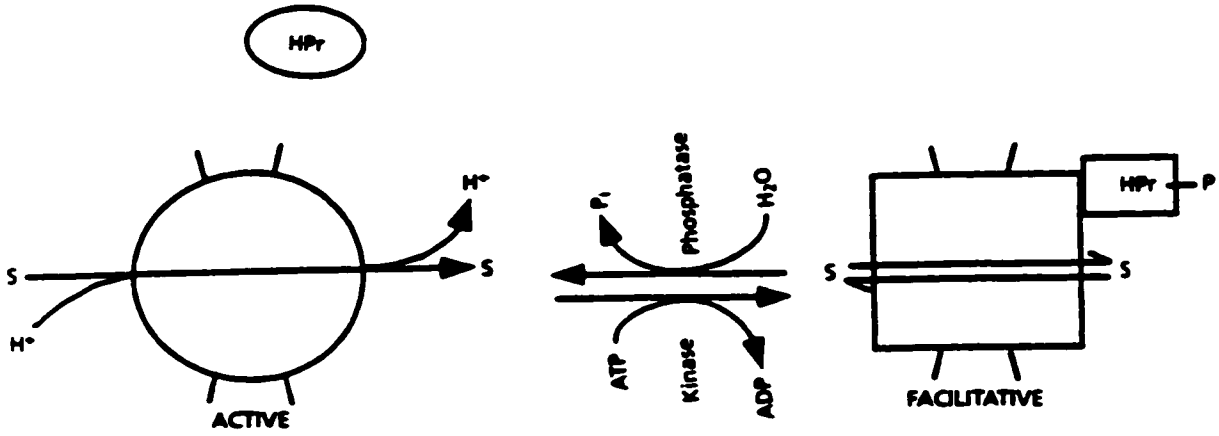


Fig. 1.8: Mechanism of regulation of HPr(Ser-P) on permease function. Adapted from Saier *et al.*, 1996.

In Gram-positive bacteria, HPr is involved in a form of catabolite repression which is dependent upon Ser46 phosphorylation (Reizer *et al.*, 1989). The Ser46 phosphorylation state of Gram-positive HPr dictates the protein's ability to interact with transcription factors involved in the regulation of expression of PTS and non-PTS carbohydrate catabolic enzymes (for review see Saier *et al.*, 1996). The enzyme gluconate kinase can be induced by inclusion of gluconate in the growth media. This induction by gluconate can be suppressed by the inclusion of PTS sugars, but not non-PTS sugars. The ability to suppress the induction was dependent upon Ser46 phosphorylation as demonstrated by the inability of the gene replacement mutant Ser46Ala HPr to suppress induction. Upon the reversal of the alanine mutation to wild type, the ability for repression was restored (Reizer *et al.*, 1989). The molecular mechanism of catabolite repression through HPr(Ser-P) has recently been elucidated.

Catabolite repression in Gram-positive bacteria is dependent on the catabolite control protein A (CcpA) and its interaction with the *cre* element. DNase I protection assays have shown that *cre* sequences are protected by CcpA in the presence of HPr phosphorylated at Ser46. HPr which was not phosphorylated at Ser46 did not offer protection to the cre sequences. CcpA binds to cre elements and prevents expression of the catabolic operons when in association with HPr (Ser)-P (Jones *et al.*, 1997a; Gösseringer *et al.*, 1997). In situations of high glycolytic activity the accumulation of fructose 1-6-bisphosphate will result in Ser46 phosphorylation of HPr which, through the interaction with CcpA, negatively regulate the expression of other carbohydrate operons as illustrated in Figure 1.9.

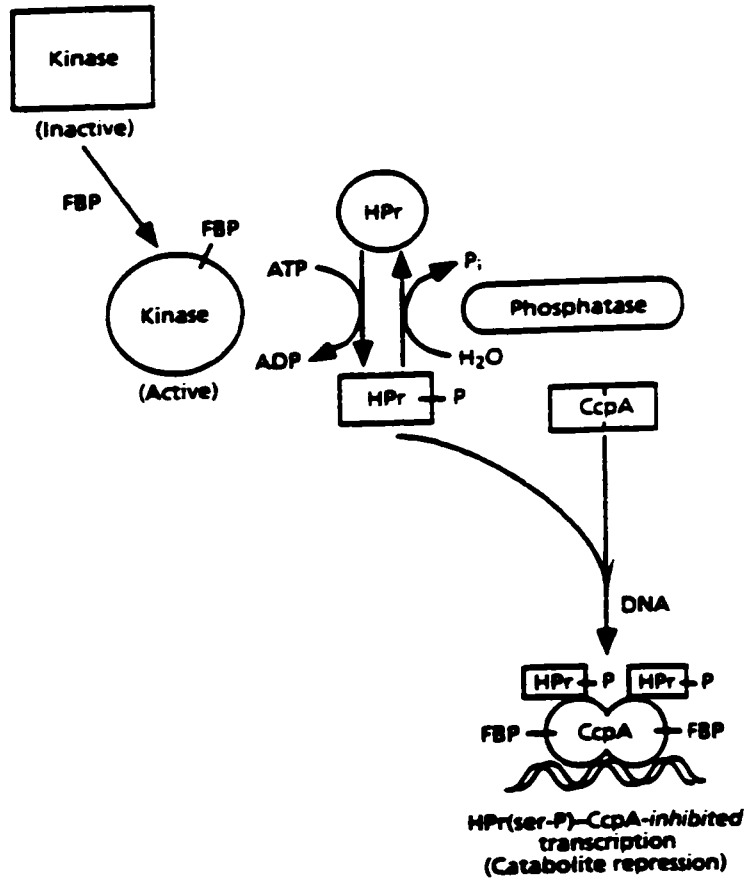


Fig. 1.9. Proposed mechanism for HPr(Ser-P) mediated catabolite repression. Adapted from Saier *et al.*, 1996.

Inducer expulsion prevents the excessive accumulation of intracellular sugar phosphates through regulated carbohydrate dephosphorlation and efflux. Once cytoplasmic sugar have been dephosphorylated they rapidly exit the cell by an energy-dependent mechanism (Sutrina *et al.*, 1988). HPr Ser46 phosphorylation serves as an activator of inducer explusion through activation of the sugar phosphate phosphatase (Ye and Saier, 1995). Dephosphorylation of intracellular sugars is mediated by a peripherally membrane-associated sugar-P phosphatase (Pase II). Pase II has been found in all bacteria which possess sugar-P hydrolysis-dependent expulsion phenomenon but not in bacteria which are lacking this mechanism (Ye *et*

al., 1996). Pase II demonstrates broad specificity for sugar-P substrates. The activity of the phosphatase is stimulated 10-fold by HPr(Ser-P) (Ye *et al.*, 1995). HPr which is not phosphorylated at the 46 position is unable to activate the phosphatase. Chemical crosslinking verified that the monomeric Pase II assosciated with the Ser46Asp phosphorylation analogue with 1:1 stoichiometry and wild type HPr did inhibit complex formation (Ye *et al.*, 1995). The involvement of HPr(Ser-P) is illustrated in Figure 1.10.

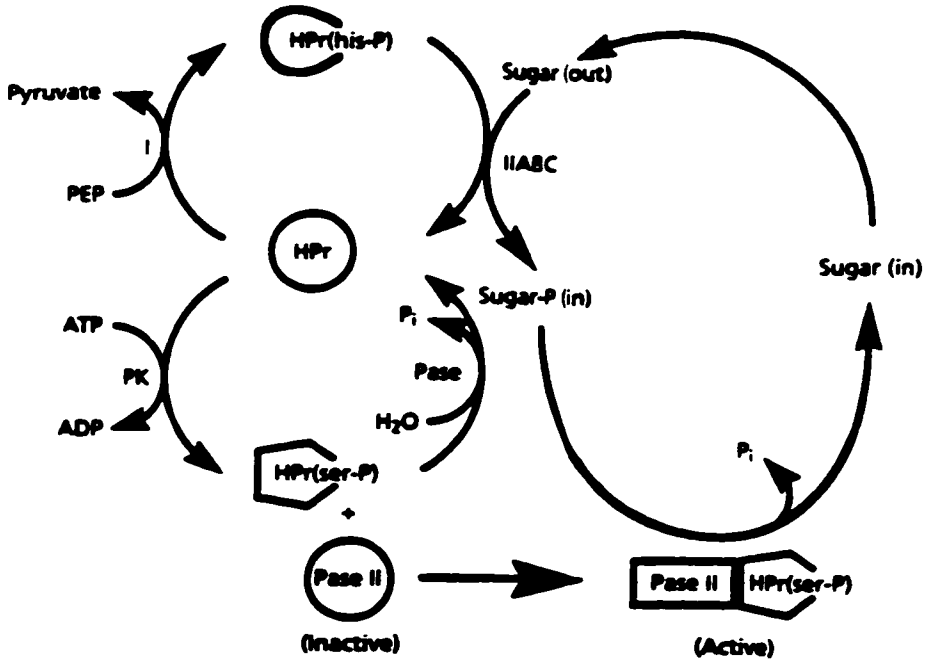


Fig. 1.10. Illustration of involvement of HPr(Ser-P) in mediating inducer expulsion through Pase II activation. Adapted from Saier *et al.*, 1996.

In addition to the regulatory roles mediated through HPr(Ser-P) there are additional roles which are independent of the serine 46 phosphorylation. In Gram-negative bacteria the activity of glycerol kinase was inhibited by $EIIA^{glc}$ in a manner

which was dependent upon the phosphorylation state of $EIIA^{glc}$, which in turn was dependent upon the activity of glucose uptake (Reizer *et al.*, 1993). In Gram-positive bacteria there is an alternate, though related mechanism for regulation of glycerol kinase involving HPr. When PTS sugars are being transported into the cell, the rate of phosphoryl transfer through the PTS will be high, resulting in a relatively high percentage of unphosphosphorylated HPr. Conversely, an absence of PTS sugar uptake will have HPr primarily in the phosphorylated state. The phosphorylated HPr is able to donate the phosphoryl group to a histidine of glycerol kinase, resulting in activation of the enzyme with subsequent production of glycerol-3-phosphate and activation of the *glp* operon (Deutscher and Sauerwald, 1986). The net result of this system is to ensure repression of the glycerol metabolizing system when PTS sugars are present and stimulation of the glycerol metabolic system in the absence of PTS sugars and presence of glycerol.

1.4.2.1 Ser46Asp HPr as a phosphorylation analog

Mimicking of serine phosphorylation through mutagenic substitution of aspartic acid has been successfully demonstrated in a number of proteins. In *B. subtilis*, where Ser46 of HPr undergoes physiological phosphorylation, it is possible to mimic this event through the Ser46Asp HPr mutation (Reizer *et al.*, 1989; Deutscher *et al.*, 1994). This mutant displays identical properties to the Ser46 phosphorylated protein. NMR investigations report similar chemical shifts for both the *B. subtilis* P-(Ser)HPr and Ser46Asp HPr, with no evidence for any large structural change in either protein (Wittekind *et al.*, 1989, 1990, 1992; Pullen *et al.*,

1995). A crystallographic determination of the Ser46Asp *B. subtilis* HPr reached a similar conclusion (Liao and Herzberg, 1994). Despite the inability to phosphorylate the Ser46 residue of Gram-negative HPr it is still possible to mimic this regulated state through the Ser46Asp mutation. The structural consequences of the *E. coli* Ser46Asp HPr mutation will be addressed by x-ray crystallography in this thesis.

1.4.2.2 Proposed mechanism of inactivation by Ser46 phosphorylation

There are only four examples of proteins which are regulated by phosphorylation where the crystallographic structure of both the regulated and non-regulated states have been determined. From these examples three alternative mechanisms through which phosphorylation can affect protein activity are presented. 1) Changes in the electrostatic surface identity of the protein to alter interactions with other substrates or proteins. In the example of isocitrate dehydrogenase, inhibition can be achieved in the absence of significant changes in the protein structure. The addition of the phosphoryl group produces an electrostatic barrier to substrate binding, effectively abolishing enzyme activity (Hurley *et al.*, 1990). 2) Localized rearrangement of side chains and regions of secondary structure. The addition of the phosphoryl group in yeast glycogen phosphorylase causes the repositioning of a short terminal segment of the protein which does not cause significant changes in the overall structure (Lin *et al.*, 1996). 3) Rearrangement of sub-units in an allosteric manner. The mammalian glycogen phosphorylase enzyme undergoes a re-orientation of two sub-units as a consequence of phosphorylation (Sprang *et al.*, 1988). While the structure of the respective sub-units are unaffected by phosphorylation, the re-

positioning of the subunits represents a significant change in overall quaternary structure of the protein. 4) Phosphorylation acting in concert with another regulatory signal. In phenylalanine hydroxylase, phosphorylation of the regulatory Ser16 residue does not produce any significant structural alterations nor does it result in enzyme activation. Activation of the enzyme requires both phosphorylation as well as the presence of the activating molecule, phenylalanine at a regulatory site. A higher concentration of phenylalanine is required for activation of the unphosphorylated enzyme suggested that the two signals work in concert (Kobe *et al.*, 1999).

The possibility of localized conformational change seemed a distinct possibility for HPr. The Ser46 residue is located at the N-cap of the minor helix B. Previous NMR investigations of the *E. coli* HPr reported this helix to be dynamic as indicated by the rate of exchange of amide protons with solvent. The backbone amide protons of helix B exchange rapidly as compared to the A and C helices in both *E. coli* and *B. subtilis* HPrs (Klevit and Waygood, 1986; Wittekind *et al.*, 1989, 1990,1992; van Nuland *et al.*, 1992). This conclusion is also supported by various crystallographic determinations of the protein (Herzberg *et al.*, 1992b; Jia, 1993a,1994b) which showed different positions of the C-terminal end of this helix for each structure.

The flexibility of the helix may arise as a consequence of an unfavorable N-capping residue (Hol, 1985). The helix hypothesis predicts that sequences with favorable N-capping and C-capping residues should be helical in nature and that favorable N-capping substitutions should afford protection of helical amides against hydrogen exchange (Sali *et al.*, 1988). Aspartate is the most common N-capping

residue as the negative charge interacts favorably with the positive charge of the helix dipole (Hol, 1985). In HPr from Gram-positive species helical stabilization could be assisted by the introduction of negative charge at Ser46 upon phosphorylation. Presumably similar helical rearrangements could occur in HPrs from Gram-positive and Gram-negative sources by the mutagenic introduction of negative charge at the N-terminal region of this helix. Stabilization of the helix may influence the ability of HPr to interact with Enzyme I for phosphotransfer. Alterations of this helix as a consequence of phosphorylation could impair the interaction with these proteins. Helix B has been proposed to be involved in the interaction with Enzyme I as well as $EIIA^{glc}$ (Herzberg, 1992a) which has been verified by the solution structure of the N-terminal fragment of Enzyme I and HPr (Garrett *et al.*, 1999).

1.5 Deamidation

The precise three-dimensional structure of a protein which is essential for function, is determined by the primary sequence of amino acids. Numerous cellular mechanisms exist to ensure the proper amino acid sequence of proteins. Proteins, however, are not chemically stable over long periods of time. Spontaneous alterations to the protein structure may occur, influencing both structure and function. The most common type of alteration is the deamidation reaction (for review see Clarke, 1987; Wright, 1991; Clarke *et al.*, 1992).

Deamidation involves the intra-molecular reactivity of the amino acids, asparagine and glutamine (Robinson and Rudd, 1974). The reaction mechanism may result in the conversion of the reactive residues to non-standard amino acids or to standard amino acids not genetically destined for that position of the protein.

Asparagine and glutamine may be converted into aspartate and glutamate respectively, with the potential to produce both the iso and D-forms of these amino acids (Clarke, 1987). These modifications may result in structural rearrangements which significantly alter both protein structure and/or function.

1.5.1 Residues involved in deamidation

Asparagine and gluatamine can undergo deamidation to produce aspartate and glutamate respectively. Deamidation also results in the production of the iso and D-iso forms of the product residues. Although aspartate and glutamate lack a side-chain amide and therefore cannot undergo deamidation they may undergo a related reaction resulting in the production of the iso as well as D and D-iso forms of the reacting side chain. Asparagine and aspartate react to produce a mixture of L-aspartate, L-isoaspartate, D-aspartate and D-isoaspartate. Similarly glutamine and glutamate may react to produce L-glutamate, L-isoglutamate, D-glutamate and D-isoglutamate. The mechanism and products of these reactions will be discussed in detail.

1.5.2 Deamidation through succinimide formation

The initial observation of the deamidations of both asparagine and glutamine residues were proposed to proceed through direct water hydrolysis for removal of the side chain amino group. However, the rates of such attacks, at neutral pH indicated that this mechanism would be far too slow to account for the rates of deamidation witnessed in proteins. The half-life of the direct hydrolysis reaction was estimated at

7 years, as compared to the rates of 70 days for asparagines and 410 days for glutamines in peptides (Geiger and Clark, 1987).

Deamidation involves the intramolecular attack of the main-chain nitrogen on the side chain of the preceding residue. The consequence of deamidation is the formation of a five membered succinimide ring with the release of ammonium ion as illustrated in Figure 1.11 (for review see Clarke *et al.*, 1992, Wright, 1991; Stephenson and Clarke, 1989). The acceptance of succinimides as the intermediate of deamidation was based largely upon the ability of the structure to explain the charges and products observed in proteins. Succinimide formation in proteins is supported by observations of protein species of higher pI and a mass verifying the loss of a water molecule from the normal protein. Succinimides have been proposed in a number of proteins based on these experimental observations; calbindin (Chazin *et al.*, 1989), calmodulin (Ota *et al.*, 1989), HPr (Brennan *et al.*, 1994), somatotropin (Violand *et al.*, 1992) and lysozyme (Tomizawa *et al.*, 1994). Recently a succinimide intermediate has been crystallographically demonstrated in lysozyme in complex with tri-N-acetylchitotriose (Noguchi *et al.*, 1998).

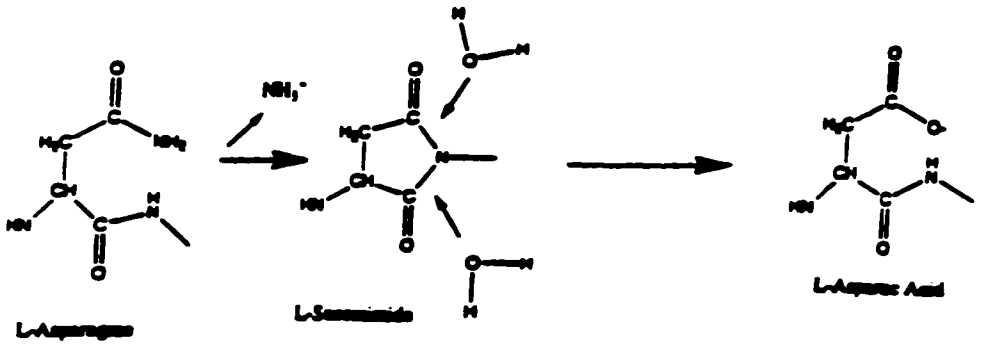


Fig 1.11: Mechanism of deamidation from asparagine.

1.5.3 Products of deamidation

Succinimides are unstable in water and will undergo subsequent water hydrolysis. The position of attack of the water molecule will determine the by-products produced. If the attack is on the carbonyl carbon of the side chain, the product of the ring cleavage will be L-aspartic acid. Conversely attack on the main-chain carbon carbonyl will result in the production of L-isoaspartic acid, in which the peptide backbone is redirected through the β-carboxyl group. The succinimide ring also has the potential to undergo limited racemization. The consequence of this is that hydrolytic cleavage of a D-succinimide ring will result in the production of D-isomer by-products. Typically succinimide ring cleavage will result in an approximate ratio of 56% L-isoaspartyl, 22% D-isoaspartyl, 15% L-aspartyl, and 7% D-aspartyl (for review see Clarke *et al.*, 1992). Considerable variation does exist in the ratios of by-products of succinimide cleavage. These differences seem to be unique to the protein and are likely the consequence of structural or electrostatic forces which influence the position of attack of the water molecule hence shifting the balance of by-products.

In situations in which aspartic acid is the attacking side chain the same mechanism of main-chain attack occurs with the same succinimide formation. The distinction being that this does not represent a deamidation reaction as there is no side chain amine group to be lost. The succinimide will behave in the same manner as those produced by aspargine attack on the main-chain. The breakdown products and ratios will be identical. Notably it is also possible for the succinimide ring to break down in a manner which returns the original aspartic acid residue to the offending position.

1.5.4 Deamidation through isoimide formation

There are examples of proteins, hemoglobin Providence (Moo-Penn *et al.*, 1976) and hypoxanthine-guanine phosphoribosyltransferase (Wilson *et al.*, 1982), in which L-aspartic acid is the only product of deamidation. There are two possible explanations for the absence of the other potential by-products of succinimide cleavage. Firstly, that electrostatic or conformational restraints of the protein prevent succinimide cleavage to produce any other products. Secondly, that these deamidation reactions may proceed through a non-succinimide intermediate. A structural intermediate which has been proposed for the second possibility is that of an isoimide (Clarke, 1987; Clarke *et al.*, 1992). The formation of this five-membered ring would require the nucleophilic attack of the main-chain carbonyl carbon with the side chain of the same residue, resulting in the removal of the amino group in situations with asparagine and the loss of a water molecule with aspartic acid as the residue of interest. The structural importance of the isoimide intermediate is that regardless of the position of attack of the incoming water molecule the breakdown product will always be L-aspartic acid as indicated in Figure 1.12.

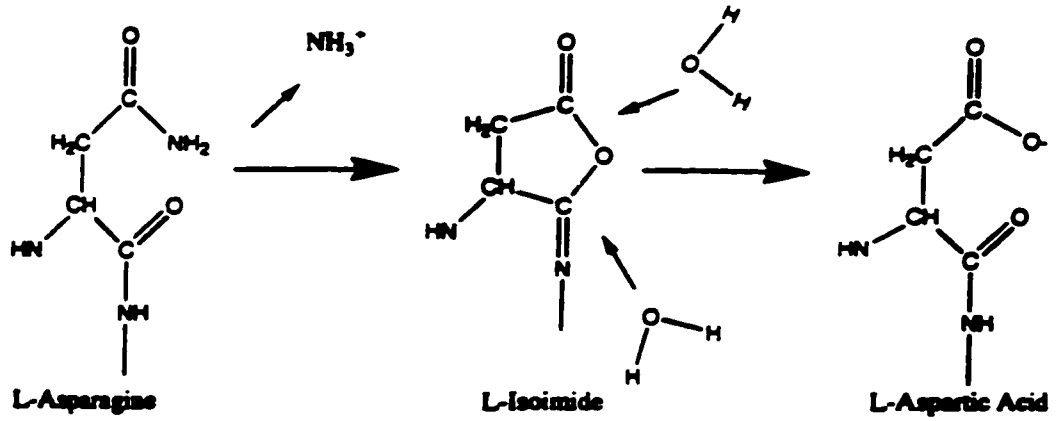


Fig 1.12: Mechanism of isoimide formation from asparagine.

1.5.5 Rates of deamidation

There is considerable variation in the rates of deamidation in proteins. This variability arises as a consequence of the numerous factors, such as pH, ionic strength and flexibility of the main-chain, which influence the rate limiting step of succinimide formation (for review see Wright, 1991; Clarke *et al.*, 1992). Despite these factors it is still possible to make some generalizations about rates of deamidation. In general, asparagine residues show the greatest rates of deamidation. Asparagines in peptides deamidate with a half-life of 1.5 days for the asparagine-glycine doublet. This compares to a deamidation half-life of approximately 41 days for a similar aspartate-glycine doublet in a protein context. The cyclization of glutamine and glutamic acid residues are approximately thirty times slower than the rates seen for their deamidation counterparts as a result of the extra carbon length of their side-chains (Clarke, 1987). There is limited information regarding rates of deamidation in proteins under moderate conditions. Representative half-lifes include cytochrome c, 81 days, aspartate aminotransferase, 22 days, serine hydroxymethyltransferase, 19 days and aldolase, 8 days (for review see Wright, 1991).

1.5.6 Factors influencing rates of deamidation

There are numerous factors, both physiological and non-physiological, which influence the rate of succinimide ring formation. The three primary non-physiological variables are pH, temperature and ionic strength.

Some of the earliest observations of deamidation arose from procedures which involved heating protein samples for the purpose of denaturation or purification. The influence of temperature arises from two distinct mechanisms. High temperatures help in overcoming the large activation energy of ring formation, approximately 21.7 kcal/mol at pH 7.5 (Ahern *et al.*, 1985). Increased temperature which results in denaturation or partial loss of tertiary structure also helps release structural restraints impeding succinimide formation. Structural restraints are a major factor in preventing deamidation (Kossiakoff, 1988). The considerably faster rates of deamidation in peptides as compared to protein are likely a direct consequence of the greater conformational flexibility in peptides.

The ability of pH to influence rates of deamidation is due to changes in the protonation state of reacting residues. In deamidation involving asparagine and glutamine there are increased rates of succinimide ring formation at basic pHs. The increased rates are a consequence of deprotonation of the attacking main-chain nitrogen, rendering it a more effective attacking group. The pH dependency of intramolecular reactivity of aspartyl and glutamyl residues is more complicated. The pH dependency of succinimide ring formation for aspartyl and glutamyl residues is a bell-shaped curve with the highest rates occurring around pH 5.0. The pH dependency is due to two opposing influences. Firstly protonation of the side-chain carboxyl group at acidic pHs makes it a better leaving group thus promoting deamidation. Conversely the main-chain nitrogen acts as a better attacking group in the unprotonated state, thus showing faster rates at higher pHs (Clarke, 1987).

The rate of succinimide formation by asparagine but not aspartate residues is also affected by the dielectric constant. The rate of deamidation at asparagine is reduced in solvents of low dielectric strength due to destabilization of the deprotonated main-chain nitrogen anion which is the attacking species in succinimide formation. The rates of aspartate succinimide formation are independent of the dielectric strength due to the destabilization of the attacking main-chain nitrogen atom being countered by increased protonation of the side chain carboxyl group (Brennan and Clarke, 1993). The dependence of asparagine deamidation on the dielectric constant may be of importance in preventing deamidation of asparagines which are situated in the interior of a protein or in a membrane spanning position.

In considering deamidation as an event of physiological importance in limiting the life-time of proteins, the most important factors to consider in influencing rates of deamidation are those which are of physiological variability. Control of these variables may be utilized as mechanisms to determine the lifespan of various proteins.

There are two distinct but related physiological variables which influence rates of deamidation, sequence context and structural variability. The sequence context in which deamidating residues appear is predictive of succinimide formation propensities (Robinson *et al.*, 1974). Glycine is the most common amino acid to follow deamidating residues and results in the fastest rates of deamidation in both peptides and proteins (for review see Clarke *et al.*, 1992). The most important property of glycine in promoting deamidation is the rotational flexibility which it imparts on the main-chain. Succinimide ring formation requires the main-chain to adopt conformations not normally seen in proteins, psi = −120°. The main-chain

flexibility offered by glycine increases the likelihood of adopting these conformations. Sequence analysis alone however, is not a reliable predictor of deamidation. In calmodulin there are two sites of deamidation, Asp-Gln and Asp-Thr, despite the presence of two Asn-Gly sequences which do not deamidate (Ota *et al.*, 1989). Rates of succinimide ring formation can also be accelerated by neighboring side chain residues, such as serine, with the ability to promote deprotonation of the main-chain nitrogen (Kossiakoff, 1988). However in glucagon, a Asp-Tyr sequence has been shown to undergo succinimide formation despite the presence of a Asp-Ser doublet (Ota and Clarke, 1987). It has also been observed that a histidine residue preceding an aspartate can promote succinimide formation by acting as a general acid in protonating the hydroxyl leaving group on the side chain carboxylic acid of the aspartate (Brennan and Clarke, 1993). There is also the possibility that side chains of distant residues may contribute to deamidation by protonating the leaving group of aspartate through tertiary structure interactions.

The second physiological determinant of rates of succinimide ring formation in proteins is the conformational flexibility of the region (Lura and Schirch, 1988). This is obviously dependent to a large extent upon the previously discussed influences of primary sequence. While there are few readily available methodologies to measure localized conformational flexibility of protein regions for the purposes of predicting deamidation, some indication of conformational flexibility may be obtained from NMR proton exchange rates with solvent as well as crystallographic B factors. It has been suggested that tendencies for deamidation can be used to indicate conformational flexibility of the surrounding region (Clarke *et al.*, 1992).

1.5.7 Physiological importance of deamidation

Rates of deamidation are known to be influenced by such factors as pH, temperature and ionic strength. As these are all factors which are commonly manipulated in protein purification it is often difficult to assess the extent of physiological deamidation as opposed to that induced by a purification protocol. There is however considerable evidence that proteins do physiologically deamidate.

A major product of deamidation, L-isoaspartyl, is recognized and methylated by the enzyme L-isoaspartyl (D-aspartate) *O*-methyltransferase (O'Connor and Germain, 1987) which catalyzes the transfer of a methyl group from S-adenosylmethionine to the α-carboxyl group of L-isoaspartyl or to the β-carboxyl group of D-aspartyl. The enzyme has been found in prokaryotes (O'Connor and Clarke, 1985) as well as eukaryotes (O'Connor *et al.*, 1984). The amino acid sequence of the protein is moderately conserved with 31% identity between the human and *E. coli* enzymes (Ingrosso *et al.*, 1989; Fu *et al.*, 1991). In vertebrates the enzyme is found in all tissues with particularly high levels in the brain (Kim *et al.*, 1997). In plants the methyltransferase activity is induced during seed production and in response to dehydration and other stresses (Mudgett and Clarke, 1994).

The L-isoaspartyl (D-aspartate) *O*-methyltransferase enzyme has approximately 700-fold more activity for L-isoasparate than for D-aspartate. The relative affinity for a particular substrate varies, likely due to the structural context in which the damaged residue occurs. For example the affinity of the enzyme for HPr-1, a species deamidated at Asn38 is poor (Km = 3.6 mM) compared to the affinity for HPr-2 (Km = 197 μM) which is deamidated at Asn12 and Asn38 (Brennan *et al.*,

1994). The enzyme can however, recognize and methylate non-standard residues in most amino acid sequence contexts (Aswad and Johnson, 1987) although to varying degrees. The methylation of the L-isoaspartyl residue initiates the conversion back into a L-aspartyl residue through the succinimide intermediate, indicating the enzyme functions to initiate the repair of damaged proteins (McFadden and Clarke, 1987). While the enzyme cannot restore asparagine to the deamidating position, the conversion of L-isoaspartate into L-aspartate removes the extra carbon in the polypeptide backbone.

The physiological significance of deamidation is best illustrated by observation of L-isoaspartyl (D-aspartate) *O*-methyltransferase deficient organisms. Mutant *Caenorhabditis elegans* which are deficient in *pcm* gene which codes for the enzyme display normal morphology and life-span as the wild type nematodes. Due to the limited twenty day life-span, deamidation would not be expected to occur to levels which would influence the organism. The worms have a dauer larval developmentally arrested stage specialized for long-term survival. During this phase in wild type nematodes there is a two-fold increase in the specific activity of L-isoaspartyl methyltransferase as well as superoxide dismutase and heat shock protein hsp90 suggesting a role in protection of cellular macromolecules. During this prolonged state the worms with the *pcm* deletion displayed considerably reduced life-spans (Kagan *et al.*, 1997). The appearance of physiological consequences to the mutation of the *pcm* gene over extended time periods is consistent with the hypothesis of deamidation as a marker of protein aging.

In *E. coli*, deletion of the *pcm* gene produced no changes in exponential-phase growth but the mutants did not survive well in extended culture into stationary phase or under conditions of elevated temperature or chemical stress (Li and Clark, 1992; Visick *et al.*, 1998). In rapidly dividing cells, the newly produced proteins can replace ones which have undergone spontaneous damage. In stationary phase protein synthesis is limited and repair of existing proteins would be expected to be of greater significance in preventing the accumulation of damaged proteins.

In mice, deletion of the enzyme resulted in dramatic accumulation of L-isoaspartyl containing proteins within the brain, heart, liver and erythrocytes. After 30 days approxiamately 6% of the brain cytosolic proteins of the knock-out mice contained an isoaspartyl residue. The *pcm-* mice showed significant growth retardation and died of fatal seizures after an average of 42 days (Kim *et al.*, 1997).

The results of these knock-out species are consistent with the hypothesis that the enzyme is responsible for the repair of damaged proteins. The accumulation of these proteins over time or under stressed conditions produces deleterious effects in all organisms tested. The accumulation of damaged proteins has been implicated to be an underlying cause of age-dependent changes in biological efficiency (Stadtman, 1992). In this regard L-isoaspartate-(D-aspartate)-methyltransferase functions to postpone aging at the cellular level by preventing the accumulation of dysfunctional proteins. These affects are likely a general consequence of accumulation of damaged proteins rather than due to compromised activity of a specific protein.

1.6 Aims and objectives

The primary objective of this research is to continue the ongoing structure/function analysis of the HPr molecule. HPr is an ideal protein for this type of analysis because: 1) the gene for HPr, *ptsH*, has been cloned and is readily available for mutagenic work, 2) the purification procedure for the HPr molecule has been established and refined, 3) there are numerous structural determinations which have been performed on the HPr molecule, providing a starting place for new structural determinations as well as a reference point to which new structures can be compared and interpreted and 4) the HPr molecule has been extensively characterized biochemically.

Previous analysis of the HPr molecule by the Waygood and Delbaere laboratories have focused on the identification and characterization of residues involved in the active centre region. These studies primarily involved site-directed mutagenesis with the resulting kinetic properties interpreted with consideration of the available tertiary structures and physiological consequences to the PTS.

In contrast, my work utilizes the HPr protein in investigations of more general interest in the field of protein structure/function. In particular the focus is on the consequences of aspartic acid substitutions as structural analogs of phosphorylation events. The ability of these substitutions to mimic the phosphorylated state is compared through kinetic comparison and the subsequent structural determinations are used to understand the dynamic physiological structural alterations which occur. There are two physiological phosphorylation events of HPr which are investigated in this manner; the His15 phosphorylation of PTS transfer and the Ser46

phosphorylation, which serves as a regulatory function in HPrs from Gram-positive species.

An unexpected finding of these investigations was that of the ability to phosphorylate the His15Asp phosphorylation analog. The consequence of this phosphorylation is the initiation of a series of intramolecular reactions, which bear a striking similarity to the deamidation aging reactions previously reported for HPr. The characteristics and molecular mechanisms of these reactions will be examined in detail. The possibility that the intramolecular rearrangement resulting from aspartyl phosphorylation may have physiological importance in mediating dephosphorylation of aspartyl phosphorylated regulatory proteins will be discussed.

2.0 Materials and Methods

2.1 Chemicals and enzymes

Lists of all specialty enzymes and chemicals used in these experiments are provided in Table 2.1 and Table 2.2.

Table 2.1: List of selected chemical supplies and enzymes

Chemical	Supplier
Acrylamide (ultra pure)	Bio-Rad
Adenosine 5'-triphosphate	Sigma
Agarose, electrophoresis grade	BRL
Agarose, low melting point (LMP)	BRL
Ammonium persulfate	Bio-Rad
Ampholine^{R} (ampholytes)	LKB/Pharmacia
N,N-Bis(2-hydroxyethyl)-glycine	ICN
5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal)	Boehringer Mannheim
Bromophenol Blue	ICN
Cesium chloride	BDH
Chloroform	BDH
Coomassie-Brilliant Blue R250	Sigma
Crocein Scarlet	ICN
2'-Deoxyadenosine 5'-triphosphate	Pharmacia
2'-Deoxycytosine 5'-triphosphate	Pharmacia
2'-Deoxyguanosine 5'-triphosphate	Pharmacia
2'-Deoxythymidine 5'-triphosphate	Pharmacia
Dithiothreitol (DTT)	Boehringer Mannheim
Dimethyl sulphoxide	Fisher
Ethidium bromide	BDH
Ethylenediamine tetraacetic acid (EDTA)	Boehringer Mannheim

N-2-Hydroxyethylpiperazine-N'-2-ethanesulphonic acid (Hepes)	Sigma
Isopropyl β-D-thiogalactopyranoside (IPTG)	Boehringer Mannheim
N,N'-Methylene-bis-acrylamide	Sigma
Oxaloacetic acid	Sigma
Phenol (glass distilled)	Toronto Research Chemicals
NADH	Boehringer Mannheim
Riboflavin 5-phosphate	Sigma
N,N,N',N'-Tetramethylethylene-diamine (TEMED)	BDH
Tris-[hydroxymethyl] aminomethane (ultra pure)	Sigma
Urea (analytical grade)	Schwartz/Mann
Radio-chemicals	**Supplier**
[γ-^{32}P]Adenosine 5'-triphosphate (3000 Ci/mmol)	NEN
[α-^{35}S] 2'-Deoxyadenosine 5'-triphosphate (500 Ci/mmol)	NEN
Chromatographic supplies	**Supplier**
DE81 Ion-exchange chromatography paper	Whatman
Sephadex G-50	LKB/Pharmacia
Q-Sepharose	Pharmacia
S-Sepharose	Pharmacia
Ultragel AcA 54	LKB/Pharmacia
Microbiological supplies	**Supplier**
Agar	BDH
Bacto-agar	Difco
Bacto-tryptone	Difco
Bacto-yeast extract	Difco
MacConkey agar	Difco

Enzymes/proteins	Supplier
Lactate Dehydrogenase (rabbit muscle)	Boehringer Mannheim
T4 DNA ligase	Pharmacia
T4 Polynucleotide kinase	Pharmacia
T7 DNA Polymerase	Pharmacia
Pfu DNA polymerase	Stratagene
*Dpn*1 exonuclease	Stratagene

Table 2.2 Name and address of suppliers

Supplier	Addresses
BDH	British Drug House, Saskatoon, Sask., Canada
Bio-Rad	Bio-Rad Laboratories, Mississauga, Ont., Canada
Boehringer Mannheim	Boehringer Mannheim Canada Ltd., Laval, Quebec, Canada
BRL	Bethesda Research Laboratories, Ontario, Canada
Difco	Difco Laboratories, Detroit, Michigan, U.S.A.
Fisher	Fisher Scientific, Winnipeg, Man., Canada
LKB	Pharmacia LKB Biotechnology group, Baie d'Urfe, Quebec, Canada
NEN (DUPONT)	DUPONT Canada Inc. Mississauga, Ont., Canada
New England Biolabs	New England Biolabs Ltd., Mississauga, Ont., Canada
Pharamcia	Pharmacia (Canada) Ltd., Dorval, Quebec, Canada
Schwarz/Mann	ICN Biomedical Canada Ltd., Mississauga, Ont., Canada
Sigma	Sigma Chemicals co., St. Louis, Missouri, U.S.A.
Whatman	Chromatographic Specialities Inc. Brockville, Ont., Canada

2.2 Bacterial strains

Bacterial strains utilized in these experiments are described in Table 2.3. Two *E. coli* strains, ESK108 and TP2811, possessing no detectable wild-type HPr activity were utilized for the overexpression of mutant HPr proteins. ESK120 was produced from *E. coli* strain CSH4 by N-methyl-N'-nitro-N-nitroguanidine mutagenesis (Waygood *et al.*, 1987). The ESK120 strain was rendered *recA* by P1 transduction using a *recA*56 mutation from strain JC10240 (Csonka and Clark, 1980). In this strain (ESK108) the *pts* gene has a TAG termination codon in the glutamine 71 position (Titgemeyer, 1993). The *E. coli* strain TP2811 carries a deletion of the entire *pts* operon (Levy et al., 1990).

Enzyme IIA^{glc} was expressed in *E. coli* strain ESK262, which is Kan^R::*ptsH*. This strain was created by ligating the Kan^R gene from pUC4 into the *Pst*I restriction endonuclease site in *ptsH* in pAB65 (Lee *et al.*, 1982). The linearized plasmid was used to transform *E. coli* strain DPB271 (Russel *et al.*, 1989) which is *rec*D and a *ptsH* gene replacement derivative was selected by kanamycin resistance. This *E. coli* strain ESK150 had no HPr detectable by assay, phosphorylation or immunoreactivity as determined by standard methods (Waygood *et al.*, 1987). *E. coli* strain ESK262 was a derivative of strain BL21 plysS (Studier *et al.*, 1990) which was transduced with P1-phage grown on strain ESK150 to produce a Kan^R::*ptsH* strain ESK262. The construction of the ESK262 strain was performed by James Talbot.

Table 2.3 List of bacterial strains, genotypes and references

Strain	Genotype	Reference
CSH4	*trp lacZ rpsL thi*	Miller, 1972
ESK108	*F' trp thi rpsL ptsH*465 *recA*56	Waygood *et al.*, 1987
TP2811	*F-xyl arg* HI *ΔlacX74 aroB ilvA ΔptsHptsIcrr Kan*^{R}	Levy *et al.*, 1990
ESK262	F- *ompT gal* [*dcm*] [*lon*] *hsdSb* (r_B-m_B-; an *E. coli* B strain)*Kan*^{R}::*pts*H	Napper *et al.*, 1999
XL1-Blue	*RecA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac*[*F' proAB lacI*^{q}*ZΔM15Tn10* (*Tet*)]	Stratagene

2.3 Plasmid vectors

Plasmid vectors are listed in Table 2.4.

Table 2.4 List of Plasmid Vectors

Name	Source	Reference
PT7-7	Pharmacia	Tabor and Richardson, 1985
PUC19	Pharmacia	Yanisch-Perron *et al.*, 1985

2.4 Bacterial growth media

Media preparation as described by Millar (1972). Liquid Luria broth (LB) media is composed of 10 g of Bacto tryptone, 10 g NaCl and 5 g yeast extract in one liter of distilled water. Solutions were autoclaved at 125 °C and 18 psi. Filtered (0.22 μm filter) ampicillin (50 μg/mL) added to the media after the

autoclaved material had cooled to room temperature. For solid agar media, 16 g/L of Bacto agar was added to the liquid media prior to autoclaving.

MacConkey agar media (50 g/L) was dissolved in deionized water at autoclaved at 125 °C and 18 psi. Antibiotics (50 μg/mL) and sugars (0.5%w/v) were added to the cooled media prior to pouring into petri dishes.

2.5 Hazardous materials

2.5.1 Ethidium bromide

Ethidium bromide is a potent mutagen if inhaled, swallowed or in contact with skin. Ethidium bromide was mixed with deionized water to an approximate concentration of 10 mg/mL. Latex gloves were worn to ensure no contact with skin occurred. Agarose gels stained with ethidium bromide were disposed of in a designated ethidium bromide waste container (Quillardet and Hofnung, 1988).

2.5.2 Phenol

Phenol is toxic and harmful if inhaled, swallowed or in contact with skin. Phenol was prepared through equilibration with three volumes distilled water overnight. The water layer was then removed and the phenol re-equilibrated with two volumes of 0.5 M Tris-HCl buffer, pH 8.0. The aqueous layer was again removed and the phenol equilibrated with two volumes of 0.1 M Tris-HCl buffer, pH 8.0. Use of phenol was always conducted in well-ventilated areas and contact minimized with latex gloves. Samples containing phenol were collected with chloroform waste in a glass bottle and discarded as chemical waste.

2.5.3 Chloroform:isoamyl alcohol

Inhalation of chloroform is toxic and may result in kidney and liver damage. Chloroform solutions were prepared by mixing twenty-four parts chloroform with one part isoamyl alcohol. Work involving chloroform was conducted in a fumehood wearing a laboratory coat and latex gloves. Chloroform-containing samples were collected in a glass bottle with phenol waste and treated as chemical waste..

2.5.4 Acrylamide

Acrylamide is a potent neurotoxin in the unpolymerized form if inhaled, swallowed or in contact with skin. Acrylamide solutions (40%) were prepared by dissolving 38.0 g of acrylamide and 2.0 g of bis-acrylamide in 100 mL of deionized water with vigorous mixing and heat. Acrylamide solution preparation was conducted in well ventilated area with a facial filtering mask, latex gloves and a laboratory coat to minimize exposure. Acrylamide solution were polymerized prior to disposal as regular waste.

2.6 Non-commercial chemicals and proteins

Purified *E. coli* phosphoenolpyruvate carboxykinase was provided by Dr. H. Goldie, University of Saskatchewan. The S46D and H15E *E. coli* HPr mutant proteins were initially provided by Dr. J W. Anderson, University of Saskatchewan, N12D HPr was provided by Dr. S. Sharma. Phosphoenolpyruvate

(monocyclohexylammonium salt) was prepared by the method of Clark and Kirby (1963).

2.7 Mutagenic primers

Mutagenic primers were synthesized by Dr. F. Georges of the Plant Biochemical Institute, Saskatoon, Saskatchewan.

2.7.1 Mutagenic primer design

Mutagenic primers were designed to introduce specific alteration to a specific region of a DNA template. While each primer must be specifically produced for the template and mutation desired, some characteristics are common in the design of all mutagenic primers. The primers are typically between twenty and twenty-five nucleotides in length, the point of the mismatch between the primer and the template is situated such that there are 10 nucleotides of correct sequence on either side of the mismatch. If possible the primers should have a minimum GC content of 40% and primers should always terminate with at least one or more C or G base. The nucleotide composition is also important in calculating the annealing temperature (Tm) of the primer using the formula:

$$Tm = 81.5 + 0.41\ (\%GC) - 675/N - (\%mismatch)$$

Where N is the number of base pairs in the primer. All primers utilized in this thesis are listed in Table 2.4.

Table 2.4: Mutagenic primer sequences

His15 Mutagenic Primers

His15Ser-a	5' GGCGGGTCGACAGACCGT 3'
His15Ser-b	5' ACGGTCTGTCGACCCGCC 3'
His15Gln-a	5' GGCGGGTCTGCAGACCGT 3'
His15Gln-b	5' ACGGTCTGCAGACCCGCC 3'
His15Thr-a	5' GGCGGGTGGTCAGACCGT 3'
His15Thr-b	5' ACGGTCTGACCACCCGCC 3'
His15Tyr-a	5' GGCGGGTGTACAGACCGT 3'
His15Tyr-b	5' ACGGTCTGTACACCCGCC 3'
His15Asn-a	5' GGCGGGTCTGCAGACCGT 3'
His15Asn-b	5' ACGGTCTGCAGACCCGCC 3'
His15Cys-a	5' GGCGGGTCAGCAGACCGT 3'
His15Cys-b	5' ACGGTCTGTGCACCCGCC 3'

Asn12 Mutagenic Primers

Asn12Ala-a	5' CCGCTCCGGCCGGTCTGCACACC 3'
Asn12Ala-b	5' GGTGTGCAGACCGGCCGGAGCGG 3'
Asn12Ser-a	5' CCGCTCCGAGCGGTCTGCACACC 3'
Asn12Ser-b	5' GGTGTGCAGACCGCTCGGAGCGG 3'
Asn12Thr-a	5' CCGCTCCGACCGGTCTGCACACC 3'
Asn12Thr-b	5' GGTGTGCAGACCGGTCGGAGCGG 3'

Asn38 Mutagenic Primers

Asn38Ala-a	5' CACTTCCGCCGGCAAAAGCGCC 3'
Asn38Ala-b	5' GGCGCTTTTGCCGGCGGAAGTG 3'

Double Mutant Primers

His15Asp Asn12Ala-a	5' CCGCTCCGGCCGGTCTGGACACC 3'
His15Asp Asn12Ala-b	5'GGTGTCCAGACCGGCCGGAGCGG 3'
His15Asn Asn12Ala-a	5' CCGCTCCGGCCGGTCTGAACACC 3'
His15Asn Asn12Ala-b	5'GGTGTTCAGACCGGCCGGAGCGG 3'

2.8 Site-directed mutagenesis

Site-directed mutagensis was preformed using both the Polymerase Chain Reaction (PCR) method as described by Landt *et al.*, (1990) and by the QuikChange methodology.

2.8.1 Quikchange site-directed mutagenesis

Site-directed mutagenesis through the Quikchange technique involves the utilization of a complementary set of mutagenic primers to each strand of the template to be modified. Primer extension is achieved through thermal cycling with a vast excess of the primer pairs with respect to the template. Typically fifteen cycles involving the denaturation at 95 °C, followed by annealing of the primers at 55 °C and primer extension at 68 °C. Both the annealing and denaturation steps were thirty seconds long and primer extension was allowed to proceed for two minutes for each kilobase of plasmid. Primer extension was catalyzed by the enzyme *Pfu* DNA polymerase. The critical features of this enzyme are its thermal stability as well as the inability to displace the mutagenic primer after complete extension of the primer around the plasmid. The inability

for primer displacement is essential for maintaining the mismatch introduced by the primer.

Following completion of mutagenic primer extension, digestion of the parental DNA strands is achieved through the addition of the enzyme *Dpn*1 to the thermal cycling reaction mixture. The *Dpn*1 restriction enzyme is specific for digestion of methylated DNA. Incubation of the digestion mixture at 37 °C for one hour is sufficient for complete digestion of the methylated, non-mutated parental DNA. The only DNA which is resistant to the action of the *Dpn*1 is the mutated, non-methylated extended primers.

For each mutagenic reaction 50 μL of Epicuran Coli XL1-Blue super-competent cells was dispensed to a pre-chilled Falcon 2059 polypropylene tube and 1 μL of the *Dpn*1 digested DNA from each thermal cycling tube was added. The mixture was left on ice for thirty minutes. A specific heat-shock at 42 °C for exactly 45 seconds was then performed followed by chilling on ice for 2 minutes. Half a millilitre of 42 °C LB media was then added to the cell mixture and incubated at 37 °C for 1 hour in a shaking incubator. Following incubation 250 μL of this mixture was plated onto a LB-agar plate containing ampicillin (50 μg/mL). Incubation at 37 °C typically produced colonies after 16-24 hours.

2.8.2 Site-directed mutagenesis using PCR

Initially PCR mutagenesis was conducted using the methodology described by Landt *et al.* (1990). This method which involves two separate PCR reactions and two DNA extractions from agarose gels was considerable more

cumbersome and time consuming than the Quikchange method. The efficiency of mutation was also considerably lower and a greater number of false positives were produced.

2.8.2.1 First PCR reaction

The first PCR reaction serves to elongate the mutagenic primer to the end of the gene. On the opposing strand at the end of the gene a common non-mutagenic primer elongates to the point of annealing of the mutagenic primer. A consequence of these reactions is the production of extended mutagenic primer fragments for use in the second PCR reaction.

Specifically the first PCR reaction required the following components in the following order of addition: sterile water to 50 μL, 5 μL of 10X commericially supplied Vent Buffer (100 mM KCl, 100 mM $(NH_4)_2SO_4$, 200 mM Tris-HCl buffer, pH 8.8, 20 mM $MgSO_4$ and 0.1% Triton X-100), dNTP mix (10 mM of each dATP, dGTP, dCTP and dTTP), mutagenic primer (100-150 pmol), reverse sequencing primer (100-150 pmol), pUC19 HPr template (2 nM) and Vent polymerase (2 units). This solution was mixed and covered with 35 μL of sterile mineral oil to eliminate evaporation. The thermal cycling protocol typically involved 30 cycles of 1 min at 95°C for separation of template strands, 1 min at 55°C for primer-template annealing, and 1 min at 72°C for primer extension. There is variability in the annealing temperature as a consequence of the different primer characteristics. The formula for calculation of individual primer annealing temperature is described in section 2.7.1. The PCR reaction was analyzed by a

low melting point agarose gel electrophoresis. A species corresponding to the correct predicted size of the extended primer was then excised from the gel using a razor blade and placed in an Eppendorf tube. Tris-EDTA (TE) buffer (10 mM Tris-HCl , pH 7.3 with 1 mM ethylene diamine tetra acetic acid (EDTA)) (3 volumes) was added and the tube was incubated in a 65 °C water bath for 10 min to melt the agarose. After the solution had cooled to room temperature the DNA was recovered by ethanol precipitation described in the 'mini-prep' procedure.

2.8.2.2 Second PCR reaction

The conditions of the second PCR reaction were identical to that of the first reaction with the exception of the primers. In the second PCR reaction the extended mutagenic primers of the first PCR reaction were used in combination with the universal primer to produce an amplified product corresponding to the entire mutated gene. The desired PCR product was then isolated and sub-cloned into a pUC19 plasmid vector.

2.9 Transformation of mutant plasmids

HPr and mutant HPr were expressed in *E. coli* strain ESK108 using the pUC(*ptsH*) plasmids with HPr under the control of its own promoter. The ESK108 strain when plated on MacConkey plates containing 1.0% (w/v) mannitol produces colonies of distinctive color dependent on the presence of a functional active HPr. The presence of a plasmid encoding an active HPr restores PTS activity resulting in the uptake and metabolism of mannitol with the subsequent

pH change turning the color indicator, Neutral Red, red. In contrast cells without active HPr will remain white. This color selection mechanism provided a valuable initial screening process of active site mutations which were presumed to be inactive in PTS complementation.

2.10 Dideoxynucleotide sequencing

Sanger sequencing (Sanger *et al.*, 1977) was performed using the T7 Sequenase kit. Double-stranded template (1-2 pmol/reaction mixture) was mixed with an appropriate primer (5 pmol/reaction mixture). Primers were selected on the basis of the location of the potential mutation. Dimethyl sulphoxide (DMSO) (1 μL) was then added to this 8 μL mixture and then incubated at 95 °C for 3 min to facilitate separation of the DNA strands. This was then immediately quenched in a –80 °C ethanol solution until completely frozen. The mixture was then hand-thawed, 2 μL of annealing buffer added and pulse centrifuged. The mixture was then allowed to stand at room temperature for 5 minutes following which 7.5 μL of labeling mix (1 μL of 0.1 M DTT, 3 μL labeling mix "A", 1 μL [^{35}S] dATP (500 Ci/mmol), 1 μL T7 DNA polymerase, 1.0 μL DMSO and water to 7.5 μL) was added. The mixture was allowed to sit at room temperature for 5 min. Following the five min incubation, 4.5 μL this reaction mix was dispensed into each of the pre-warmed (37 °C) termination tubes containing the sequencing mixes (T, C, G and A) and allowed to incubate at 37 °C for five minutes. 'T' mix (840 μM each of dATP, dCTP and dGTP; 93.5 μM dTTP; 14 μM ddTTP; 40 mM Tris-HCl, pH 7.6, and 50 mM NaCl), 'C' mix (840 μM each of dATP, dTTP and

dGTP; 93.5 μM dCTP; 17 μM ddCTP; 40 mM Tris-HCl, pH 7.6), 'G' mix (840 μM each of dATP, dCTP and dTTP; 93.5 μM dGTP; 14 μM ddGTP; 40 mM Tris-HCl, pH 7.6, and 50 mM NaCl), 'A' mix (840 μM each of dTTP, dCTP and dGTP; 93.5 μM dATP; 14 μM ddATP; 40 mM Tris-HCl, pH 7.6, and 50 mM NaCl). Reactions were terminated by the addition of 5 μL of "stop mix" (0.3% each Bromophenol Blue and Xylene Cyanol; 10 mM EDTA, pH 7.5 and 97.5% deionized formamide) to each tube. The reactions were either loaded immediately unto a polyacrylamide sequencing gel or frozen for later use.

2.10.1 Urea-polyacrylamide gradient gels

DNA-sequencing reactions were analyzed by running them on urea-polyacrylamide gradient gels as described by Biggin *et al.*, (1982). Glass sequencing plates were first cleaned using 95% ethanol and lint-free kimwipes. Plates were squared with respect to each other and separated by 0.4 mm plastic spacers before being clamped. Two different gel mixes are required to form the gradient gel; 0.5x gel mix is composed of 2 mL TBE buffer (89 mM Tris-HCl, 89 mM boric acid and 2 mM EDTA, pH 8.0) added to 6 mL of 40% [w/v] deionized acrylamide (38 g acrylamide and 2.0 g bis-acrylamide mixed in 100 mL water) and 18.4 g urea. The second mix, 5.0x gel mix is composed of 20 mL of 10x TBE, 6 mL of 40% acrylamide and 18.4 g urea. For a single gel 50 mL of 0.5x gel mix and 15 mL of 5.0x gel mix were required, polymerization was initiated by the addition of 100 μL and 30 μL of ammonium persulfate (25% w/v) respectively, followed by 50 μL and 15 μL of N,N,N',N'-tetramethylethylenediamine

(TEMED) respectively. After initiation of the polymerization reaction approximately 35 mL of 0.5x gel mix was drawn into a 60 mL syringe, the remainder was drawn into a 25 mL pipette using a rubber suction bulb, with the same pipette the 5.0x gel mixture was also drawn up to form a gradient buffer. The solution in the pipette was then slowly and evenly dispensed in between the glass plates. The solution contained in the syringe was then also added in between the plates. The plates were then laid flat and the flat side of a shark's tooth comb was inserted between the plates into the solution. The gel was then left for 1.5 h to allow for polymerization. After the gel had polymerized and immediately prior to running the gel the shark's tooth comb and lower spacer were carefully removed and excess dried acrylamide was washed away.

2.10.2 Electrophoresis and autoradiography

The polyacrylamide gel was then clamped to a BRL sequencing gel electrophoresis apparatus and the upper and lower chambers filled ¾ full with 1x TBE buffer. The gel was warmed with a pre-run of approximately 30 min with constant watts (50 W). During this time the sequencing samples were incubated in a 95 °C water bath for 3 min then placed on ice. The gel apparatus was then turned off and excess urea was rinsed from the upper and lower spaces between the gel plates. The shark's tooth comb was then reinserted into the top space with the points imbedded into the gel. Samples were then loaded into the arcs of the comb. Samples were electrophoresed at a constant watts (50 W) until the Bromophenol blue marker dye reached the bottom of the gel, usually about 2.5 h.

The power supply was then disconnected and the upper and lower chambers were carefully suctioned into a sink. The glass plates were then pried apart and the gel transferred to a 3M Whatman filterpaper. Gels were dried by covering them with Saran wrap and placing them in a Bio-Rad slab gel drier at 80 °C with suction for 2 h. The dried gels were then exposed to X-ray film (Kodak-X-omat Ar) for 24 h in a x-ray cassette. Films were developed using an AFP Imaging Mini Med/90 X-ray Film Processor.

2.10.3 Automated sequencing

Automated DNA sequencing was performed by the Plant Biotechnology Institute, NRC Canada, Saskatoon, using an Applied Biosystems 373 (Stretch) DNA sequencer.

2.11 Isolation of plasmid DNA through 'mini-prep'

DNA plasmid isolation was performed through the 'mini-prep' procedure (Birnboim and Doyle, 1979; Maniatis *et al.*, 1982). *E. coli* cells containing the proper plasmid were grown overnight at 37 °C with constant shaking in LB media with the antibiotic ampicillin (50 μg/mL). Cells were isolated from the cultured media by centrifugation (12,000 x g for 5 min) of a 1.5 mL aliquot in a microfuge tube. The supernatant was carefully removed by suction. The pellet was then resuspended in 100 μL of Solution I (50 mM glucose, 25 mM Tris-HCl buffer pH 8.0 and 10 mM EDTA) and placed on ice for 1 min until 200 μL of freshly prepared solution II (0.2N NaOH and 1% Sodium dodecyl sulfate (SDS)) was

added. Following this addition the tube was gently swirled to ensure that the liquid came into contact with all parts of the tube. The tube was then stored on ice for 5 min following which 150 μL of solution III (5M sodium acetate) was added and vigorously vortexed. Following a 5 min incubation of this solution on ice the tube was centrifuged for 5 min at 12,000 x g. The supernatant was then transferred to a fresh Eppendorf tube and equal parts (250 μL) of phenol and isoamyl alcohol:chloroform (1:24) were added. This solution was then mixed by inversion and centrifuged for 2 min at 12,000 x g. The upper aqueous layer was then transferred to a new Eppendorf tube and the dsDNA was precipitated with the addition of 2 volumes (1 mL) of cold 95% ethanol. The precipitation was placed on ice for at least 10 min before being centrifuged at 12,000 x g for 15 min at 4 °C. The supernatant was carefully poured off taking care not to disrupt the pellet. The pellet was then washed with cold 70% ethanol followed by another centrifugation at 12,000 x g for 15 min at 4 °C. The supernatant was again poured off and the pellet dried in a 37 °C incubator. The dried pellet was then resuspended in 30 μL of TE buffer with 1 μL of RNAse (10 mg/mL solution, boiled 10 min). Mini-preps are stored at –20 °C.

2.12 Protein purification

The experiments of this thesis required the purification of three proteins; HPr, Enzyme I and $EIIA^{glc}$.

2.12.1 Protein overexpression and cell harvesting

Different cell strains were used for the overexpression of the different proteins; HPrs were produced in strain ESK108 utilizing a pUC(*ptsH*) plasmid, $EIIA^{glc}$ in strain ESK262 with a pT7-7(*crr*) plasmid and Enzyme I from TP2811 with a pT7-7(*ptsI*) plasmid. In each case freshly transformed cells were grown in a 5 mL LB overnight culture at 37 °C with ampicillin at 50 μg/mL. A sterile 500 mL flask with 100 mL LB and 50 μg/mL ampicillin was then inoculated with the overnight culture and grown to mid-log phase with shaking at 37 °C. This was further used to inoculate 6 L of LB with 100 μg/mL ampicillin (1.5 L in each flask). Cells were allowed to grow for a period of 16-20 h. With IPTG inducible plasmids, IPTG was added to a final concentration of 0.5 mM 3 h prior to cell harvesting. Cells were harvested by centrifugation, 7,500 x g for 10 min in a GS-3 rotor in a Sorvall RC2-B refrigerated centrifuge. Cell pellets were washed in 0.85% saline solution and frozen and –20 °C.

For the HPr and $EIIA^{glc}$ cell disruption thawed cells were resuspended in 10 mM Tris-HCl buffer, pH 7.3 with 1 mM EDTA. In both cases 3.0 mL of buffer were used for each gram of wet cell weight. Cells were broken by passing them through a cooled (~4 °C) French pressure cell (~25,000 psi) two times. Cell debris was removed from the crude extract by a low-speed centrifugation (8,000 x g for 10 min) followed by a high speed centrifugation at 200,000 x g for 1.5 h in a Beckman Ti60 rotor. The resulting supernatant was used immediately in the appropriate purification procedure.

2.12.2 Purification of HPr proteins

HPrs could be purified to homogeneity in a single gel filtration purification step. The supernatant from the high speed spin, approximately 100 mL, was loaded onto an Ultragel AcA54 molecular sieve column (10 cm x 100 cm) which was equilibrated with 10 mM Tris-HCl buffer, pH 7.3. Buffer was pumped through the column at a rate of ~ 300 mL/h and fractions corresponding to 1.5 h were collected. Isoelectric focussing gels were used to determine which fractions contained HPr, typically fraction 5 or 6. This HPr was greater than 90 % pure and could be used without further purification. The HPr containing fractions were pooled and dialyzed (pore size <3,500 MW dialysis tubing) against 30 L water overnight with two changes of the water. Samples were then frozen and lyophilized.

2.12.2.1 Ion-exchange chromotography purification of HPr species

The three different species of His15Asp *E. coli* HPr which emerge during the course of phosphorylation, normal, phosphorylated and high pI, could be separated and isolated using a 1 mL Mini-Q anion exchange column in conjuncture with the Gradifrac system. All solutions were filtered (0.22 μM filter) prior to use. The buffer system was comprised of 10 mM citrate-phosphate buffer, pH 4.6. This was the sole component of the "A" buffer. The elution "B" buffer also contained 0.2 M NaCl in addition to the buffer. A typical run involved a flow rate of 2 mL/min with a increase in salt gradient of 2.5%/min. Fractions of 0.5

mL were collected and the absorbency of the elutant was monitored with a UV detector set at wavelength 214 nm.

A 500 µl sample of the His15Asp *E. coli* HPr phosphorylation mixture (3 mg His15Asp HPr, 0.2 mg Enzyme I, 5 mM PEP and 5 mM $MgCl_2$) was injected into the system. Three distinct peaks were detectable and confirmed by isoelectric focusing to correspond to the three HPr species produced as a consequence of His15Asp phosphorylation.

2.12.3 Enzyme I purification

The Enzyme I purification has been previously described (Waygood and Steeves, 1980). Following pelleting the cells by the first low speed centrifugation described earlier cells were resuspended, 3 mL buffer for each gram wet cell weight, in 1x M9 salts (42.5 mM Na_2PO_4, 22.0 mM KH_2PO_4, 8.5 mM NaCl and 18.5 µM $FeCl_3 \bullet 6H_2O$) with the inclusion of (p-aminobenzamidine, 2-deoxyglucose and KF, each to a final concentration of 10 mM) this mixture was then incubated at 37 °C for 15 min. $ZnCl_2$ was then added to a final concentration of 1 mM, the cells frozen, thawed and passed through a cooled (~4 °C) French pressure cell (~ 25,000 psi). Streptomycin sulfate was then added to a final concentration of 1.3 % (w/v). This extract was then subjected to the high speed centrifugation protocol described earlier.

The Enzyme I crude supernatant was applied to a 300 mL DEAE-cellulose column (3.0 cm x 25 cm) which had been equilibrated with 10 mM potassium phosphate buffer pH 6.5, 1 mM EDTA and 0.5 mM DTT. The column was then

eluted using a salt gradient of 0-0.5 M KCl in the equilibration buffer, fractions of 20 mL were collected. Typically Enzyme I would elute from the column at approximately 0.4 M KCl after essentially all other proteins had been removed. Enzyme I containing fractions were then pooled and dialyzed (pore size <35,000 MW) overnight against equilibration buffer. Following equilibration, PEP and $MgCl_2$ were added to the protein solution to the concentration of 5 mM each. This was then incubated at 37 °C for 15 min to ensure all Enzyme I was in the phosphorylated form. This solution was then divided into aliquots and frozen at –20 °C.

2.12.4 Enzyme IIA^{glc} purification

Approximately 75 mL of the $EIIA^{glc}$ crude supernatant from the high-speed centrifugation described earlier was applied to a 300 mL DEAE-cellulose column (3.0 cm x 25 cm) which had been equilibrated with 10 mM potassium phosphate buffer pH 6.5, 1 mM EDTA and 10 mM p-aminobenzamidine. The column was then eluted using a salt gradient of 0-0.5 M KCl within the equilibration buffer, fractions of 20 mL were collected. Typically $EIIA^{glc}$ would elute from the column at approximately 0.2 M KCl. Fractions containing $EIIA^{glc}$ were determined by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (SDS-PAGE). Appropriate fractions were then pooled and dialyzed (pore size <3,500 MW) overnight against the equilibration buffer. Samples were aliquoted and frozen at –20 °C.

2.13 HPr protein determination

HPr protein concentrations were determined by the spectrophotometric method of Waddell (1956) (1OD[OD_{215}-OD_{225}] = 0.144 mg/mL), and by the lactate dehydrogenase depletion assay (Waygood *et al.*, 1979). Lactate dehydrogenase depletion assays were performed using a Gilford 2400 recording spectrophotometer at 340 nm. A volume of the HPr in deionized water was added to a cuvette containing 0.1 M N-2-hydroxyethyl-piperazine-N'-2-ethane sulfonic acid (Hepes) buffer, pH 7.0, 0.10 mM NADH, 15 mM PEP, 5 mM $MgCl_2$, and 35 units of lactate dehydrogenase. The volume of the reaction mixture was made to 0.995 mL with deionized water, a basal activity was recorded and then the reaction was initiated by the addition of 5.0 µL of Enzyme I (1.0 mg/mL). The reaction was allowed to proceed for approximately 10 min.

The reliability of kinetic data is dependent upon accurate protein concentration dterminations. The spectrophotometric assay of Waddell (1956) is sensitive to contaminants which affect measurements at 215 and 225 nm. The lactate dehydrogenase depletion assay (Waygood *et al.*, 1979), is specific for HPr was carried out in conjuncture with the spectrometric assay of Waddell (1956). Duplicate assays with Enzyme I gave Km values within 10% variation with consistent results over a two-year period. The Enzyme I Vmax for each mutant HPr was monitored with each investigation by carrying out, simultaneously, assays with wild type HPr as a control experiment.

2.14 N-terminal sequencing

HPr protein sequencing was performed by Dr. S. Mackenzie of the Plant Biotechnology Institute, National Research Council of Canada, Saskatoon. Protein sequencing was performed using an Applied Biosystems, Inc. model 471A sequencer equipped with a model MG5 microgradient pump and Blott cartridge for PVDF-type membranes. Data were acquired and analyzed using an Apllied Biosystems Inc. model 601A data system.

2.15 Phosphohydrolysis of HPr

Phosphohydrolysis experiments were performed as described by Waygood *et al.*, (1985). Phosphohydrolysis was performed to determine the relative phosphohydrolysis of a HPr as a function of pH at a single time point. The buffers ranged in pH from 1.5 to 10.0. The buffers used were as follows: pH 1.5-2.5, HCl-KCl; pH 3-7.5, citrate-phosphate; pH 8.0-9.5 potassium phosphate; pH 10.0, borate. Phosphorylated HPr samples 1 mL (1.0 mg HPr, 0.1 mg Enzyme I, 5.0 mM $MgCl_2$, 3 mM PEP, 1 μL [^{32}P]PEP (specific activity ~1000 Ci/mmol) and 10 mM Hepes buffer, pH 7.5) were added to the appropriate buffer system, final buffer concentration 50 mM, incubated at 37 °C for 20 min. Samples of 100 μL were taken and added to ice cold 0.2 M Na_2CO_3, vortexed and put on ice. The mixture (200 μL) was carefully spotted to one end of a DEAE-cellulose paper (Whatman DE81) strip (3.0 cm x 15 cm) approximately 4 cm from the end of the strip. Ascending chromatography was then performed by placing the end of the strip to which the sample had been applied in ascending buffer (35% ethanol, 50 mM KCl, 10 mM Tris-HCl, pH 7.5 and 1 mM EDTA) for approximately 90 min.

The strips were air dried and cut (3.0 cm below the solvent front) into two pieces. The upper portion contained [^{32}P]P*i* which travels with the solvent front, the lower portion contains the [^{32}P]P-HPr which remains stationary. The strips were placed in scintillation vials, 7.0 mL of scintillation cocktail added, capped and counted in a scintillation counter. Percent hydrolysis could be calculated as a ration of the [^{32}P]P*i* to the [^{32}P]P-HPr.

2.16 Enzyme assays and reactions

2.16.1 Enzyme I kinetic assays

Enzyme I kinetics were measured by a continuous spectrophotometric assay. Assays were conducted at 340 nm using a Gilford 2400 recording spectrophotometer and a 1 mL quartz cuvette. The constant features of the reaction were 50 mM Hepes buffer, pH 7.0, 0.15 mm NADH, 10 mM PEP, 5 mM $MgCl_2$, and 35 units of lactate dehydrogenase. HPr, or mutant HPr concentration was varied. All components of the reaction mixture were filtered through 0.22 μm filters. The reaction was started with the addition of 5 μl of Enzyme I (1 mg/mL) diluted 1:200 in 50 mM Hepes buffer, pH 7.3, with 5 mM both $MgCl_2$ and PEP, left at room temperature for 30 min. prior to assay. Initial velocities would be determined for a variety of HPr concentrations. For HPr mutants of severely compromised activity, such as His15Asp, higher concentrations (200X) of Enzyme I were required.

2.16.2 HPr phosphorylation reaction

Phosphorylation of HPr and HPr mutants by Enzyme I without kinetic considerations were conducted as follows. In an Eppendorf tube 2.5 mg HPr was mixed with 50 μg of Enzyme I in a 20 μL solution of 5 mM PEP, 5 mM $MgCl_2$ and 10 mM Hepes buffer, pH 7.0. This mixture was incubated at 37 °C for 15 min then placed on ice for immediate use.

2.16.3 Enzyme IIA^{glc} phosphorylation

The determination of $EIIA^{glc}$ phosphorylation by [^{32}P]HPr was determined by audioradiography of SDS-PAGE gels. Phosphorylation was achieved through a similar Enzyme I phosphorylation mixture in which 10 μg of the $EIIA^{glc}$ protein was included in a 20 μL reaction mixture. In addition 5 μL of the [^{32}P]PEP mixture described above was included. The phosphorylation reaction was halted with the addition 2x SDS loading buffer and electrophoresed through a 12% SDS-PAGE gel. Following the run the gels were covered with commercial food wrap, and exposed to X-ray film (Kodak-X-omat Ar) for 16 h at –80°C.

2.17 Isoelectric focusing (IEF) gels

Isoelectric focusing gels were constructed using a Bio-Rad capillary tray as described by Mattoo *et al.* (1984). Glass plates (10 cm x 10 cm) were cleaned with 95% ethanol and placed on the side of the tray corresponding to the 0.8 mm (thin gel) side. Sucrose (1.1 g) was added to a 100 mL flask containing 4.8 mL deioinized water and 5.5 mL of acrylamide solution (24.25 g acrylamide and 0.75 g bis-acrylamide in 250 mL of deionized water). The solution was swirled until

the sucrose dissolved. Ampholytes (600 μL of 20% pH 3.0-10 and 40% of each pH 3.5-5.0 and pH 5.0-7.0), 50 μL of 5% ammonium persulfate, 25 μL of riboflavin 5-phosphate (2.5 mg/mL) and 5.0 μL of TEMED were then added in that order. The mixture was mixed by swirling and poured between the glass plate and the casting tray and placed under a light source for 30 min for photo polymerization to occur.

The polymerized gels were removed from the tray by prying them free with a spatula. Electrode wicks soaked with 1 M NaOH (cathode) and 1 M phosphoric acid (anode) were positioned at either end of the gel with half the wick touching the gel, the other the glass plate. A plastic sample applicator was positioned at the cathode end of the gel. The gel was placed, glass side down, on a pre-cooled (4 °C) flat bed Pharmacia IEF apparatus and samples (~20 μL) were loaded onto the gel. The gel is run for 2 h at 8 watts with a 1000 V upper limit.

Staining of the gels was performed as described by Crowle and Cline (1977). The gel was stained for 15 min with fixing stain (5.0 g $CuSO_4$, 0.4 g Coomassie-brilliant Blue R250; 0.5 g of Crocein Scarlet, 270 mL isopropanol, 100 mL acetic acid in 640 mL of distilled water). Destaining was performed by soaking the gel in destaining solution (140 mL acetic acid and 240 mL isopropanol in 1620 mL of distilled water). Destaining solution was changed every several hours until non-specific staining of the gel was removed.

2.18 SDS-PAGE

SDS-PAGE gels were prepared by the method of Laemmli (1970), and run in a discontinuous buffer system, as described by Sambrook *et al.*, (1989). Gels were (80 mm X 100 mm X 0.5 mm) with a final acrylamide concentration of 15%. Gels for monitoring protein purification were run at a constant voltage of 150 V for approximately 90 min at room temperature. Gels analyzing protein phosphorylation reactions were run in a 4 °C cold-room.

For non-radioactive samples gels were stained for 15 min in Coomassie Brilliant Blue (45% methanol, 10% glacial acetic acid, 0.25% w/v Coomassie Brilliant Blue R250) and destained in destaining solution (7.5% methanol, 10% glacial acetic acid) overnight. When samples had been labeled with [^{32}P]PEP, one plate was removed from the gel sandwich and the exposed gel covered with Saranwrap. X-ray film was placed directly against the Saranwrap and stored in a film cassette at –80 °C overnight or longer. X-ray films were developed using a AFP Imaging Mini-med/90 X-ray Film Processor.

2.19 Production of [^{32}P]PEP

[^{32}P]PEP was made by the incubation of 0.2 mCi, of [γ-^{32}P]ATP (specific activity 3000 Ci/mmol), 0.1 mg of phosphoenolpyruvate carboxykinase, 1 mM ATP, 12.5 mM KF, 5.0 mM $MgCl_2$ and 1 mM oxaloacetate in 50 mM Hepes buffer (pH 7.5) with a final volume of 250 µL at 37 °C for 10 min. The reaction was initiated with the addition of oxaloacetate. This preparation was used without further purification.

2.20 Crystallography

2.20.1 Ser46Asp HPr crystallization

The Ser46Asp *E. coli* HPr protein was provided by Dr. J. W. Anderson (University of Saskatchewan) and used without further purification. Crystals were grown by the hanging drop vapor diffusion method at 14 °C. A hanging drop was typically composed of a final concentration of 5 mg/mL HPr, 20-25% saturated ammonium sulfate and 50 mM citrate phosphate buffer pH 4.6-4.8. The initial crystals were obtained after a period of 7-14 days although they were of insufficient quality for x-ray data collection. More suitable crystals were obtained through the use of a seeding solution composed of crushed Ser46Asp HPr crystals. This solution was utilized in promoting crystal growth by touching a pipette tip into the seeding solution then touching it into the hanging drop prior to sealing of the plate. Using this procedure to transfer micro-crystal nucleation centers large, uniform crystals could be obtained in less than a week

2.20.1.1 Ser46Asp HPr data collection

Diffraction data were collected by Dr. L. T. J. Delbaere and Dr. W. Wilson Quail to 1.5 Å resolution with a MAR image plate scanner using a wavelength of 0.927 Å from a synchrotron light source (X11, DORIS ring) at the EMBL Outstation, DESY, Hamburg, Germany. Data were collected at room temperature. Rotations of 2.5° were used to obtain high-resolution data and 4.0° to collect high-intensity, low-angle diffractions which overloaded the image plate on the high- resolution scans. A total of 9806 unique reflections were collected between 8.0 and 1.5 Å resolution to produce a 96.7% complete data set. Data

were processed using DENZO and SCALEPACK (Otwinowski, 1993) and the CCP4 suite (Collaborative Computational Project #4, 1994).

2.20.1.2 Ser46Asp HPr structure solution and refinement

Molecular replacement using the wild type HPr molecule as the probe molecule was performed using the Amore package of molecular replacement (CCP4, 1994) and provided consistent rotation solutions across a variety of resolution shells. The consistent appearance of a rotation peak in a series of resolution ranges is a reliable indicator of the correctness of a rotation solution. The rotation solution was applied to the starting probe molecule to correctly position the molecule within the new unit cell. No translation search was necessary since the space group is P1. Least squares refinement using the X-PLOR 3.1 package (Brunger, 1992) was performed. The refinement protocol of (1) rigid body refinement; (2) preparation stage (backbone-restrained least-squares refinement); (3) slow cooling refinement (simulated annealing and unrestrained least-squares refinement) and (4) temperature factor refinement.

2.20.2 His15Asp HPr crystallization

Crystals were grown by the hanging drop vapor diffusion method at 14 °C. A hanging drop was typically composed of a final concentration of 5 mg/mL HPr, 20-25% saturated ammonium sulfate and 50 mM citrate phosphate buffer pH 4.6. The initial crystals were obtained after a period of a few weeks although they were of insufficient quality for x-ray data collection. More suitable crystals were

obtained through the use of a seeding solution composed of crushed Ser46Asp HPr crystals. This solution was utilized in promoting crystal growth by touching a pipette tip into the seeding solution then touching it into the hanging drop prior to sealing of the plate.

2.20.2.1 His15Asp HPr data collection

Synchrotron diffraction data were collected by Dr. L. Tari, Dr. W. Quail and Dr. L. Prasad with a Brandeis CCD detector at the Brookhaven National Laboratory, New York. Data were collected at room temperature to 1.6 Å resolution with 7751 unique reflections comprising a 96.0% complete data set. Data were processed using DENZO and SCALEPACK (Otwinowski, 1993) and the CCP4 suite (Collaborative Computational Project #4, 1994).

2.20.2.2 His15Asp HPr structure solution and refinement

The His15Asp HPr mutant was solved using the Amore molecular replacement package. *E. coli* wild type HPr, in which His15 was substituted for an alanine was used as a probe structure. A consistent rotation solution was obtained for all shells of resolutions investigated. In addition it was necessary to perform a translational search to determine the correct translation for the rotated molecule in the unit cell. The translational search was performed at 3.0 Å resolution and a single sharp peak was obtained. The initial model was refined through rigid body refinement and subsequent electron density maps demonstrated clear and continuous electron density for the protein and an aspartate residue was evident at

the fifteen position. Further refinement was performed by the same methods as described for the Ser46Asp HPr mutant.

2.20.3 High pI His15Asp HPr crystallization

Crystals were grown by the hanging drop vapor diffusion method at 14 °C. A hanging drop was typically composed of a final concentration of 5 mg/mL HPr, 20-25% saturated ammonium sulfate and 50mM citrate phosphate buffer pH 4.6. The initial crystals were obtained after a period of a week to two weeks although they were of insufficient quality for x-ray data collection. More suitable crystals were obtained through the use of a seeding solution composed of crushed Ser46Asp HPr crystals. This solution was utilized in promoting crystal growth by touching a pipette tip into the seeding solution then touching it into the hanging drop prior to sealing of the plate.

2.20.3.1 High pI His15Asp HPr data collection

The high pI form of *E. coli* His15Asp HPr was collected by Dr. L T. J. Delbaere, Dr. W. Quail and Dr. L. Tari at the Photon Factory in Japan on beamline BL18B using a screenless Weissenberg camera and a wavelength of 1.0 Å. Data were collected at room temperature. A total of 5670 unique reflections were collected to a resolution of 1.8 Å comprising a 97.5% complete data set. Data were processed using DENZO and SCALEPACK (Otwinowski, 1993) and the CCP4 suite (Collaborative Computational Project #4, 1994).

2.20.3.2 High pI His15Asp HPr structure solution and refinement

The solution structure was obtained through the same process as described for the His15Asp HPr mutant, utilizing the same probe molecule of *E. coli* wild type HPr with His15 truncated to an alanine. The initial electron density map demonstrated clear and continuous density through-out the molecule and an aspartate was evident at the fifteen position. No additional density was present in the region of residue fifteen to indicate the presence of unusual structures such as succinimides or iso-aspartyl residues.

Chapter 3
E. coli Ser46Asp HPr

3.1 *E. coli* Ser46Asp HPr

While HPrs from Gram-negative organisms are unable to undergo phosphorylation of the Ser46 residue it is still possible to mimic the effects of phosphorylation through the Ser46Asp mutation. The Ser46Asp mutant of *E. coli* HPr demonstrated kinetic properties remarkably similar to the Ser46 phosphorylated forms of HPrs from Gram-positive species. Due to the high degree of structural similarity between HPrs from Gram-positive and Gram-negative sources, it is reasonable to assume that the inactivation is occurring through similar mechanisms. In this portion of the thesis the structural mechanism of inactivation by Ser46Asp *E. coli* HPr will be described.

3.2 Kinetic properties of the Ser46 mutants of *E. coli* HPr

A series of mutations of residue 46 of *E. coli* HPr were characterized by Dr. J. W. Anderson with respect to their ability to act as substrates for Enzyme I. The results of this kinetic analysis are presented in Table 3.1. The mutants created involving the introduction of negative charge, *e.g.* aspartic and glutamic acid, had the most dramatic consequences, particularly on Km. The alteration of Km as opposed to Vmax would suggest that the catalytic mechanism of the protein, hence active site conformation is unaffected by the mutation. The Ser46Asp HPr

most closely resembled the kinetic properties of Gram-positive Ser46 phosphorylated HPr.

Table 3.1 Enzyme I kinetics of Ser46 alterations

HPr	Km (μM)	Vmax (%wild type)	K_{cat}/Km (relative)
Wild type	6	100	16.7
Ser46Ala	15	100	6.67
Ser46Arg	40	100	2.50
Ser46Asn	65	100	1.54
Ser46Asp	~4000	~30	~0.0075
Ser46Glu	~1500	100	~0.067
Ser46Gly	6	100	16.7
B. subtilis HPr ¶	23	100	4.4
B. subtilis P-(Ser) HPr¶	345	16	0.046

* data from Napper *et al.*, 1996
¶ data from Reizer *et al.*, 1992

3.3 Crystallographic parameters of the Ser46Asp structure

The Ser46Asp HPr mutant of *E. coli* was determined by x-ray crystallography at a resolution of 1.5 Å. The space group was P1 with unit cell dimensions of a=25.85 Å, b=27.33 Å, c=27.60 Å, α=66.90°, β=75.71° and γ=70.00°. The structure was refined to a final R-factor of 0.182 over a resolution range of 10.0 – 1.5 Å with no sigma cut off. $R = \Sigma |Fo| - |Fc| / \Sigma |Fo|$. The structure has a bond length rms deviation of 0.0012 Å and a bond angle rms deviation of 2.4°. The average restrained isotropic B-factors are 12.0 Å, 15.3 Å and 14.6 Å for the main chain, side chain and solvent atoms respectively. The final model contains a single sulfate ion at the active centre and 75 water molecules.

3.4 Structural analysis of the Ser46Asp mutation

The Ser46Asp *E. coli* HPr structure is a classic open-faced β-sandwich consisting of four antiparallel β-strands and three alpha-helices. A representative ribbon diagram of the Ser46Asp *E. coli* HPr is presented in Figure 3.1. The β-sheet is comprised of four β-strands as defined by the hydrogen-bonding pattern; β1 (residues 2-8), β2 (residues 32-36), β3 (residues 40-44) and β4 (residues 58-65). The hydrogen-bonding pattern defines three alpha helicies; α1 (residues 15-27), α2 (residues 46-54) and α3 (residues 69-84). It is important to note that in helix B a 3_{10} helical conformation is adopted after residue 48.

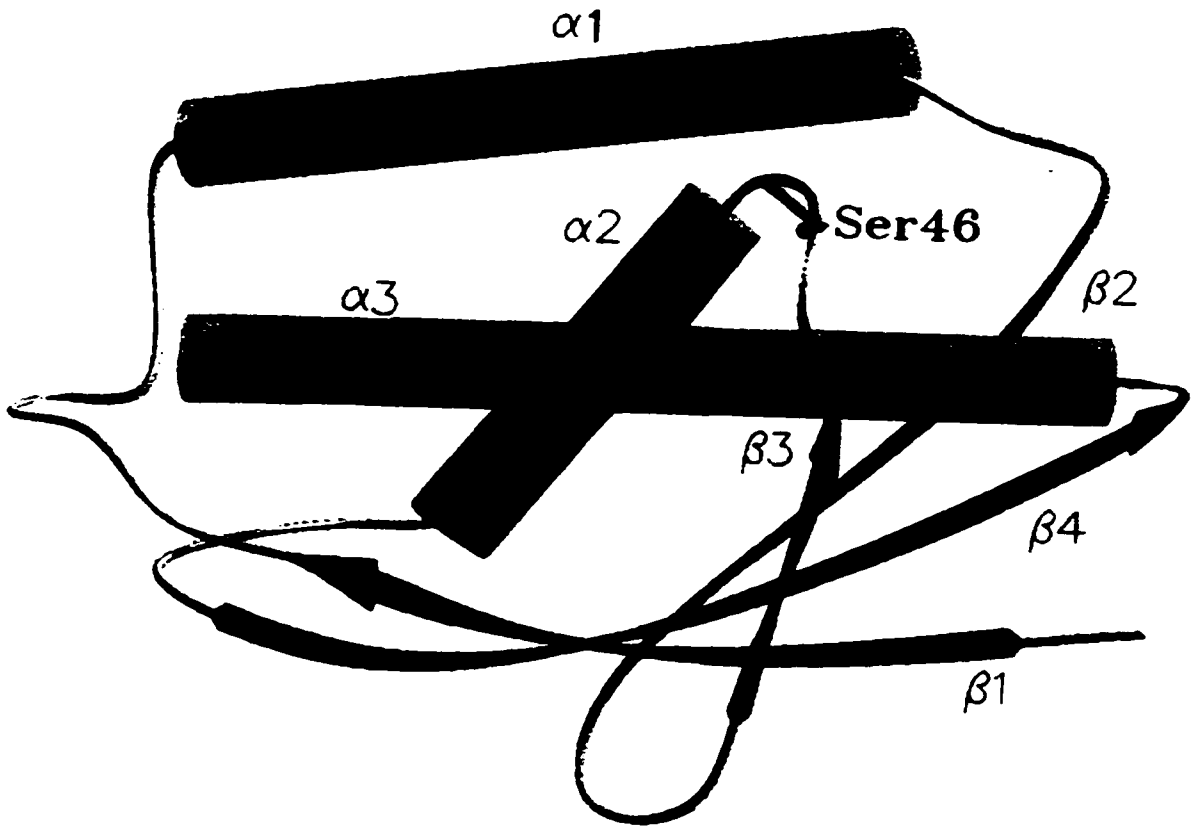


Fig. 3.1: A ribbon representation of *E. coli* Ser46Asp HPr. Diagram produced using SETOR (Evans, 1992).

3.5 Comparison of the wild type and Ser46Asp *E. coli* HPr structures

The Ser46Asp HPr has remarkable overall structural similarity to the *E. coli* wild type structure (Jia, 1992), indicating that no major conformational changes are required for inactivation. An overlay comparison of the backbone atoms of the wild type and Ser46Asp HPrs is presented in Figure 3.2. Both the wild type and Ser46Asp HPr structures have a similarly positioned sulfate anion present in the active centre region meaning that both structures are His15 phosphorylated state analogs. The wild type HPr structure has a second unique, sulfate anion which interacts with the regions of Gly54 and Asn38 but this was not found in Ser46Asp HPr.

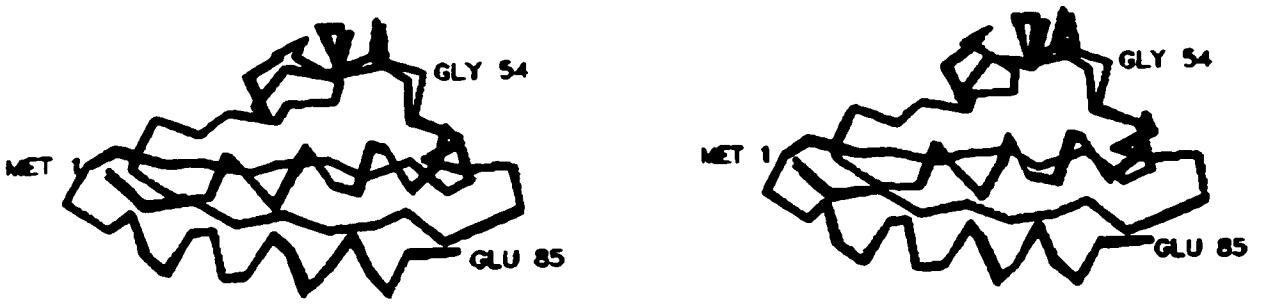


Fig. 3.2: Stereo diagram of backbone atoms of wild type (green) and Ser46Asp (blue) HPrs. Diagram produced using SETOR (Evans, 1992).

3.5.1 Active centre comparison

The main chain conformation of the active centre region in the wild type and mutant protein are indistinguishable and the specific orientation of proposed critical active centre side chains are not significantly altered in the Ser46Asp HPr. Active center water molecules are comparably positioned and the differences are believed to arise as a consequence of the different space groups rather than a consequence of the mutation. An overlay active centre comparison between Ser46Asp and wild type HPr is presented in Figure 3.3. In particular, the His15, Arg17 and Asn12 side chains occupy similar orientations. This would indicate that the catalytic capabilities of the active centre are not influenced by the mutation.

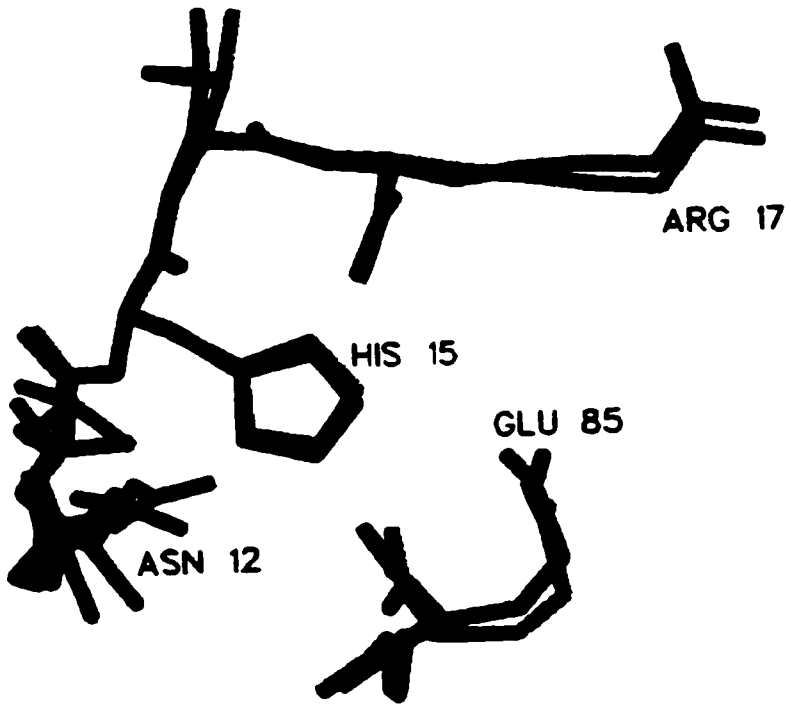


Fig. 3.3: Overlay comparison of active centre residues of wild type (green) and Ser46Asp (blue) HPrs. Diagram produced using SETOR (Evans, 1992).

Glu85, which was proposed to be involved in the active centre on the basis of the *E. coli* wild type HPr structure (Jia *et al.*, 1993a), occupies an identical position in Ser46Asp HPr. The residue forms hydrogen bonds between the terminal α-carboxylate group and the $N^{\varepsilon 2}$ atom of the imidazole ring of His15.

In both the wild type and Ser46Asp HPr structures there is a sulfate group located in the active centre region. The presence of sulfate in the active centre is a consequence of the structural and electrostatic features of region and the physiological role of this area in accommodating a phosphoryl group (Copley *et al.*, 1994). The presence of a sulfate group, which is structurally similar to phosphate, in the active centre region is considered structurally comparable to His15 phosphorylation. The positioning of the sulfate is similar in both the wild type and Ser46Asp HPr structures with interactions involving the $N^{\delta 1}$ atom of the imidazole ring of His15 as well as the main-chain amide atoms of Thr16 and Arg17. This is also the observed positioning of the phosphoryl group in P-(His)HPr (van Nuland *et al.*, 199; Jones *et al.*, 1997b). The interaction of the sulfate anion with Ser46Asp HPr in a manner analogous to that determined for the wild type protein is indicative that the catalytic capabilities of the active centre region are not influenced by the mutation.

3.6 Structural differences

A plot of the rms deviations, which provides a residue to residue distance comparison, between the Ser46Asp and wild type *E. coli* HPrs confirms the overall similarity of the structures and yet illustrates a point of structural deviation

corresponding to the residues 53-54. In addition the region about Pro11 shows differences from the wild type structure. The significance of both of these differences will be discussed in detail.

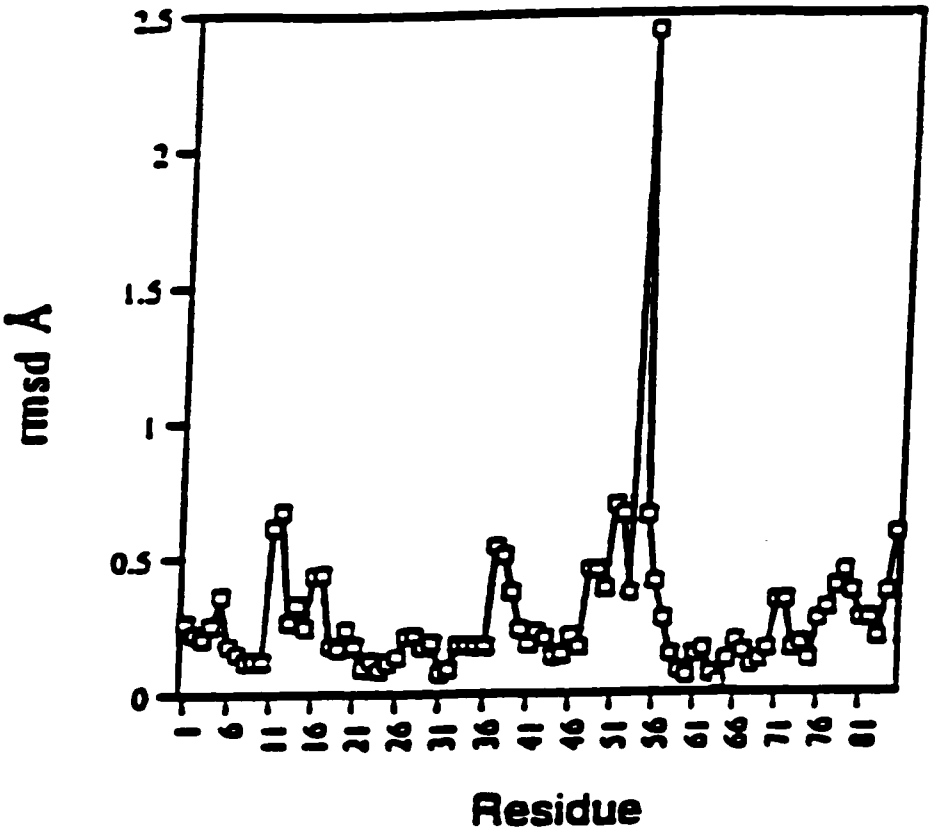


Fig. 3.4: RMSD comparison of α-carbon atoms of wild type and Ser46Asp HPr.

3.6.1 Proline 11 and torsion angle strain

Due to the inability to rotate about the peptide bond the folding of proteins is limited to movements about the phi (Φ) and psi (Ψ) bonds. While all

conformations are theoretically possible, many are disallowed due to steric strain. The best indicator of permitted rotations is the Ramachandran plot which describes the allowed torsion angle regions as a function of the phi and psi angles. In the wild type *E. coli* HPr structure, which was determined at 2.0 Å resolution, there was the observation of partial torsion angle strain at residue 16; $\Phi = -43°$, $\Psi = -65°$. This torsion angle strain was interpreted to be of physiological importance in conjuncture with the proposed model of phosphotransfer in which the stored energy of the torsion angle strain was required for acceptance of the phosphoryl group at His15 of HPr (Jia *et al.*, 1993b). This torsion angle strain was very pronounced in the *S. faecalis* HPr crystallographic structure (Jia *et al.*, 1994b). Molecular dynamics simulations suggest that torsion angle strain may be present in *E. coli* HPr upon phosphorylation (van Nuland *et al.*, 1996) but there is no evidence for this is *B. subtilis* (Jones *et al.*, 1997).

The Ser46Asp mutant HPr structure was solved at a higher resolution, 1.5 Å, with clear and continuous electron density throughout the molecule. In the 2.0 Å resolution structure of wild-type *E. coli* HPr structure, there was some ambiguity in the electron density in the region of Pro 11. In contrast the Ser46Asp HPr is clearly defined in this region and orients the Pro11 carbonyl group in a different conformation. The electron density of the Pro11 region of Ser46Asp HPr is presented in Figure 3.5 along with the wild type and Ser46Asp positions of the Pro11 carbonyl.

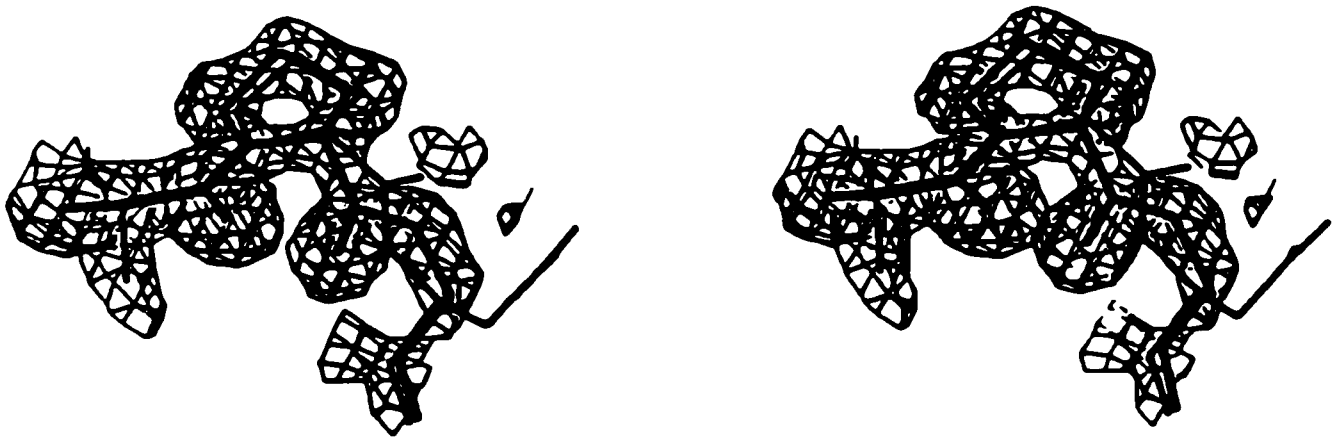


Fig. 3.5: Stereo diagram of proline 11 electron density (2Fo-Fc). Map contoured at 1.5 σ.

This revision of the Pro11 carbonyl group leads to Φ= -56° and Ψ= -50° angles for Thr16, for which there is no torsion angle strain.

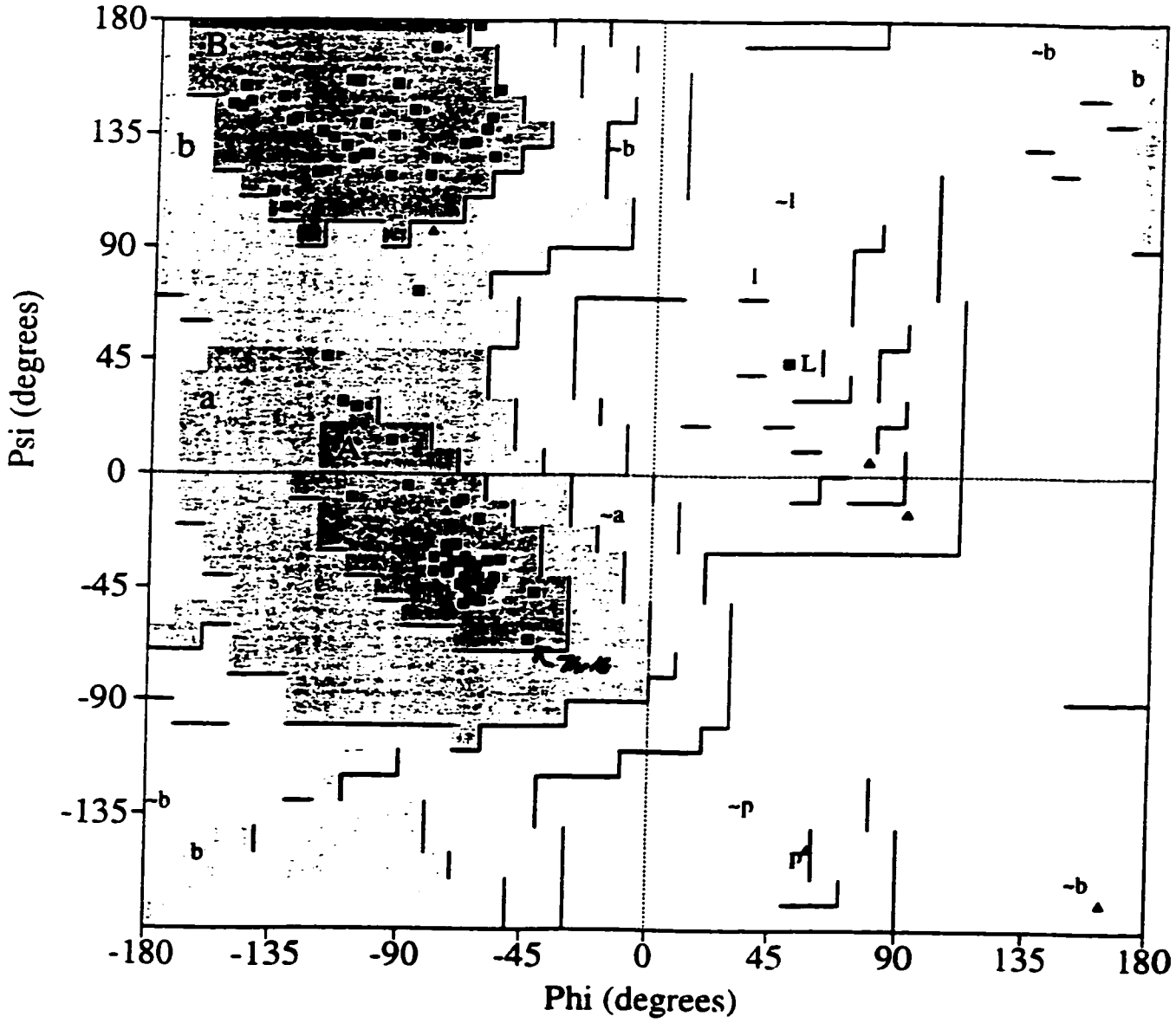


Fig. 3.6: Ramachandran plot (Ramakrishnan and Ramachandran, 1965) highlighting residue 16 in *E. coli* wild type HPr.

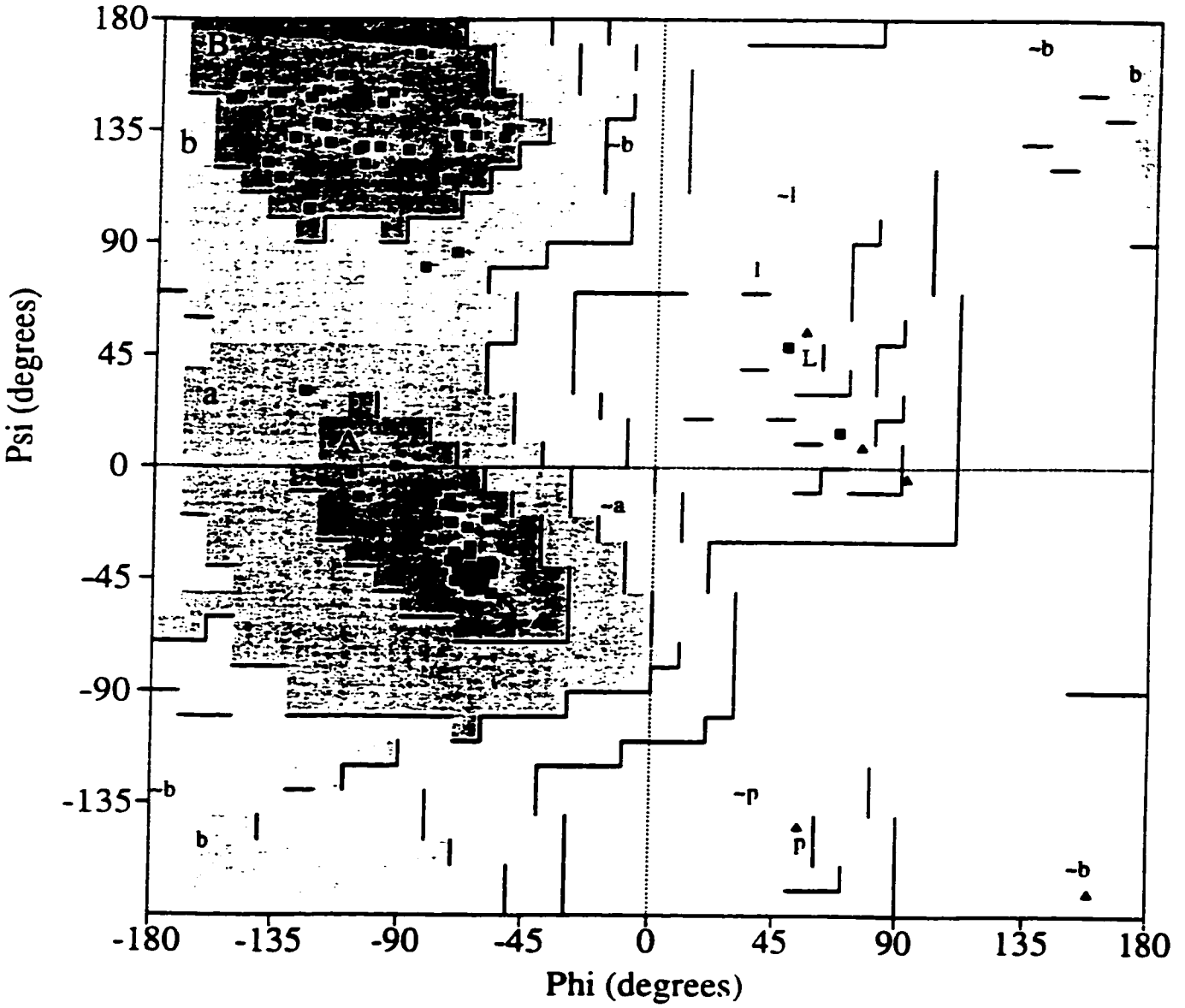


Fig. 3.7: Ramachandran plot (Ramakrishnan and Ramachandran, 1965) highlighting residue 16 in Ser46Asp HPr.

The different conformation of the Pro11 carbonyl does not arise as a consequence of the Ser46Asp mutation in HPr, but rather from the higher resolution of the structure. The lack of torsion angle strain in *E. coli* HPr would therefore suggest that the torsion angle strain model proposed by Jia *et al.* (1993b) for *S. faecalis* HPr does not apply to *E. coli* HPr.

3.6.2 Alterations to helix B

A second structural difference between the wild type and Ser46Asp HPr structures exists in helix B. Ser46 N-caps this small, irregular helix; residues 46 and 47 conform to alpha-helical structure, residue 48 represents a transition point and residues 49-54 are in 3_{10} helical conformation in Ser46Asp HPr. A comparison of the various HPr structures shows variability exists in the positioning of the final residues of this minor helix despite the fact that Leu53-Gly54 are conserved in most HPrs (Jia *et al.*, 1994a). These residues, which C-cap helix B, seem to adopt one of two conformations on the basis of whether the HPr is derived from a Gram-positive or Gram-negative species. The underlying structural explanation of this species-specific difference relates to sequence differences at residue 37, which is oriented perpendicular to the helix. In HPrs from Gram-positive species, residue 37 is often a tyrosine while in HPrs from Gram-negative species serine is more frequently found. In the Gram-positive species, the Gly54 region of HPr is in a helical conformation while in the *E. coli* HPr, Gly54 adopts a more random, nonhelical conformation and is hydrogen bonded with the Ser37 residue (Jia *et al.*, 1994a). In the *E. coli* Ser46Asp HPr,

Gly54 is in 3_{10} helical conformation similar to that seen in HPrs from Gram-positive species as illustrated in Figure 3.8. Since HPrs from Gram-positive species can be phosphorylated by *E. coli* Enzyme I reasonably efficiently, it would appear unlikely that this small change has any physiological consequences. The alterations of this helix are not limited to the recruitment of Gly54; in addition there is a tightening of the hydrogen bonding pattern through-out the helix-B as indicated by the shortening of the hydrogen bond lengths. The hydrogen-bonding pattern and lengths of the wild type and Ser46Asp HPr B-helices is presented in Table 3.2.

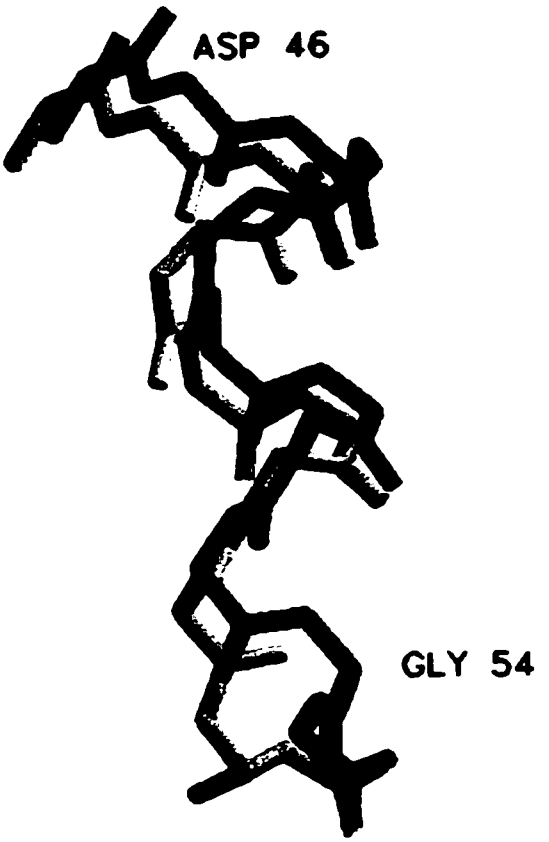


Fig. 3.8: Comparison of helix B from wild type (grey) and Ser46Asp (green) HPr. The helix in Ser46Asp HPr has a general tightening and Gly54 adopts a 3_{10} helical conformation. Diagram produced using SETOR (Evans, 1992).

Table 3.2: Hydrogen bond lengths in Helix B.

Donor Atom	Acceptor Atom	Distance in wild Type (Å)	Distance in Ser46Asp	Change (Å)
S(D)46 O	50 N	3.11	2.93	0.18
L47 O	Q51 N	3.19	3.03	0.16
F48 O[a]	Q51 N	3.35	3.44	-0.09
	T52 N	3.95	3.52	0.43
K49 O	T52 N	3.19	3.06	0.13
L50 O	L53 N	3.18	3.08	0.10
Q51 O[b]	G54 N		3.37	

[a]At residue 48 the helix changes from α-helix to 3_{10} helix.
[b]In wild type HPr Gly54 is incompatible with helix conformation.

It would appear that the introduction of negative charge at the N-capping position of helix-B results in a stabilization of this dynamic helix. This conclusion is supported by both NMR and conformational stability studies of the Ser46Asp HPr protein which were carried out in concert with this work (Thapar *et al.*, 1996) and by a similar body of work on *B. subtilis* HPr (Herzberg *et al.*, 1992b).

3.6.3 Local side chain movements

The region about the point of mutation does not undergo any significant rearrangements. The substituted aspartate side chain adopts a position very similar to that of the Ser46 in the wild type HPr structure. Both form hydrogen bonds with the main chain nitrogen of Lys49. Only Phe48 shows a modest alteration in position as compared to the wild type structure as shown in Figure 3.9. In the wild type protein, the Phe48 side chain is situated with the plane of the phenyl ring running parallel to the axis of helix B. In the Ser46Asp HPr mutant, the phenyl ring is perpendicular to the B-helix axis. This minor adjustment is unlikely to be

the cause of the disruption of HPr interaction with Enzyme I. It would be expected that the Arg46 and Asn46 mutants would result in similar crowding of the phenyl ring from its wild type position yet they do not display the dramatic kinetic consequences seen with Ser46Asp.

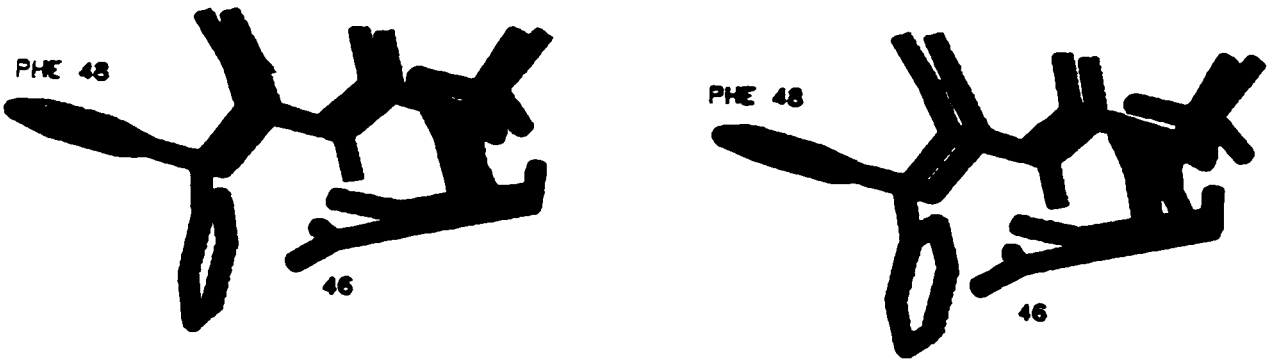


Figure 3.9: Stereo diagram of the 46 region for wild type (blue) HPr and Ser46Asp (green) HPr. Diagram produced using SETOR (Evans, 1992).

3.6.4 Electrostatic alterations

The surface region of the Ser46 region can be described as a hydrophobic patch with some regions of basic potential. An electrostatic surface potential diagram for the wild type and Ser46Asp HPrs is presented in Figure 3.10. The electroneutral region about Ser46 is believed to be involved in the interaction with Enzyme I. The electrostatic identity is likely to be a critical determinant in mediating the interaction. Disruption of this hydrophobic docking site through the introduction of negative charge, through either phosphorylation or mutagenesis, impairs the interaction with Enzyme I.

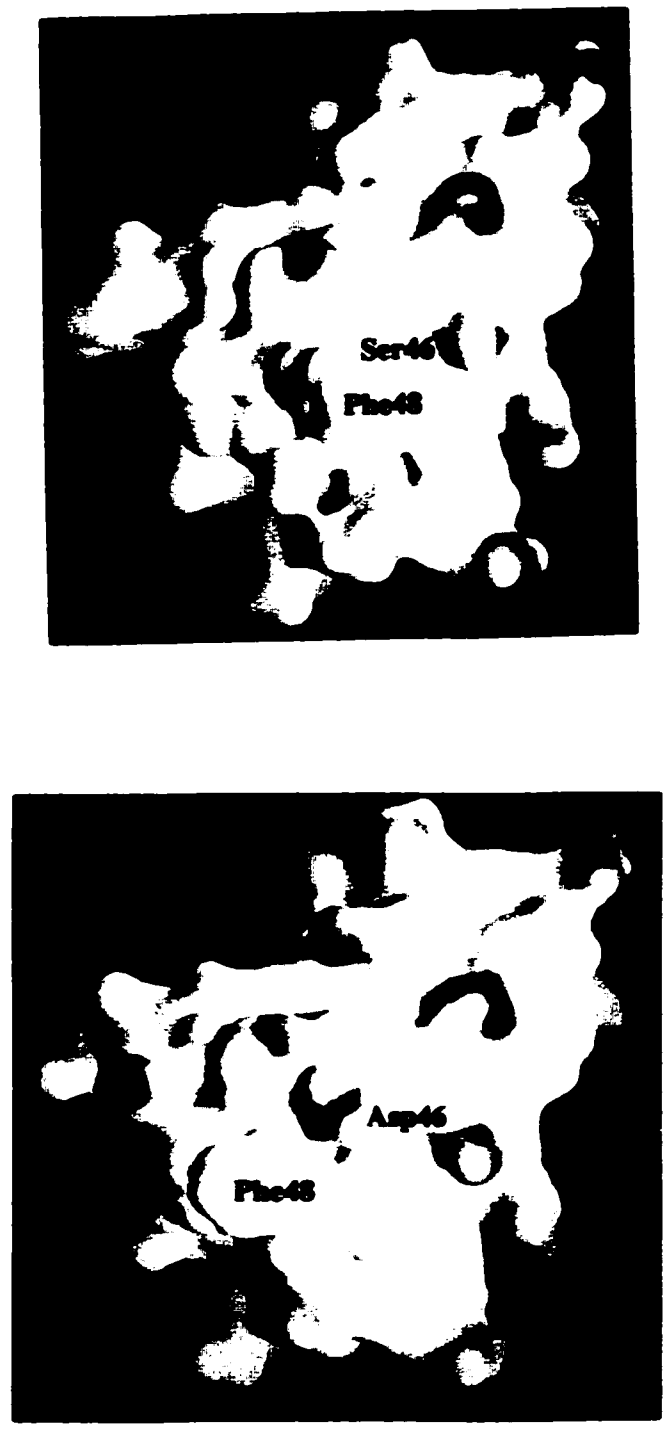


Fig. 3.10: Electrostatic surface environments of wild type HPr (top) and Ser46Asp HPr (bottom). Calculated by the GRASP program (Nicholls *et al.*, 1992) with an assumed pH of 7.0. Electrostatic potentials are as follows: blue, positive; red, negative; white, neutral.

Chapter 4
Involvement of Asn12 in the Active Center of HPr

4.1 Involvement of position 12 in the active center of *E. coli* HPr

While residue 12 of HPr is not conserved; serine in *B. subtilis*, threonine in *S. faecalis* and *S. aureus* and asparagine in *E. coli*, there is conservation in the ability for formation of hydrogen bonds. Structural determinations of the various HPrs report the presence of, or potential for, hydrogen bond formation between the side chain of residue 12 and His15 (Jia *et al.*, 1994a).

There is also kinetic evidence for the involvement of asparagine 12 in the active center of *E. coli* HPr. Deamidation of this asparagine results in impaired kinetic properties with respect to the interactions with both Enzyme I and EIIA proteins (Sharma *et al.*, 1993). This species of HPr protein which is referred to as HPr-2, also displays markedly different phosphohydrolysis properties, again indicative of a role in the active site (Sharma *et al.*, 1993).

The roie of Asn12 in the active center of *E. coli* HPr was addressed by Dr. S. Sharma through the mutant Asn12Asp HPr. The purpose of this mutant was to mimic the deamidation event rather than to address the importance for hydrogen bond formation from residue 12. The mutant displayed similar properties with respect to Enzyme I kinetics as HPr-2 with an approximate doubling of Km and halving of Vmax (Sharma, 1992).

In order to examine further the importance of hydrogen bonding potential at the 12 position, the mutants Asn12Ser, Asn12Thr and Asn12Ala were created

in *E. coli* HPr. The mutants were purified to homogeneity as assayed in the lactate dehydrogenase assay.

Table 4.1 Kinetic parameters of Asn12 substitutions of *E. coli* HPr.

HPr	Km(μM)	Vmax(%)	Relative k_{cat}/Km
Wild type	6	100	100
Asn12Ser	6	65	65
Asn12Thr	6	65	65
Asn12Ala	12	50	25
Asn12Asp*	15	60	25

*Result from Sharma *et al.*, (1993)

The kinetic results of Table 4.1 indicate that residue 12 has limited involvement in interaction with Enzyme I and an equally modest role in the catalytic mechanism of phosphotransfer. In particular, the mutant Asn12Ala, which lacks hydrogen-bonding capabilities shows similar kinetic parameters as does Asn12Asp HPr.

Chapter 5
His15Asp HPr Phosphorylation and Subsequent Ring Formation

5.1 Rationale for the His15Asp *E.coli* HPr mutation

The mutant His15Asp and His15Glu HPrs were created by Dr. J. W. Anderson for the purpose of producing a His15 phosphorylation analog for the investigation of the interaction between HPr and $EIIA^{sugar}$ during phosphotransfer. The expectation was that the "permanently phosphorylated" HPr and a EIIA protein would form a stable complex suitable for crystallization. The mutant, however, proved to be unsuccessful in undergoing stable complex formation with $EIIA^{glc}$ (J. Remmington, University of Oregon, unpublished results).

5.1.1 Phosphorylation of His15Asp HPr

Isoelectric focusing band shifts can be used to demonstrate phosphorylation of proteins. The His15Asp, His15Glu and wild type HPrs were incubated under phosphorylation conditions described in *Materials and Methods* and analyzed by isoelectric focusing shown in Figure 5.1. Incubation of the His15Asp HPr under phosphorylation conditions produced a novel protein species of increased negative charge, indicative of phosphorylation. In addition, there is the appearance of a third species of more positive charge than the original His15Asp protein. The wild type HPr protein, acting as a positive control, shows a phosphorylation band shift. The

wild type protein, however, does not display the appearance of the third higher pI species.

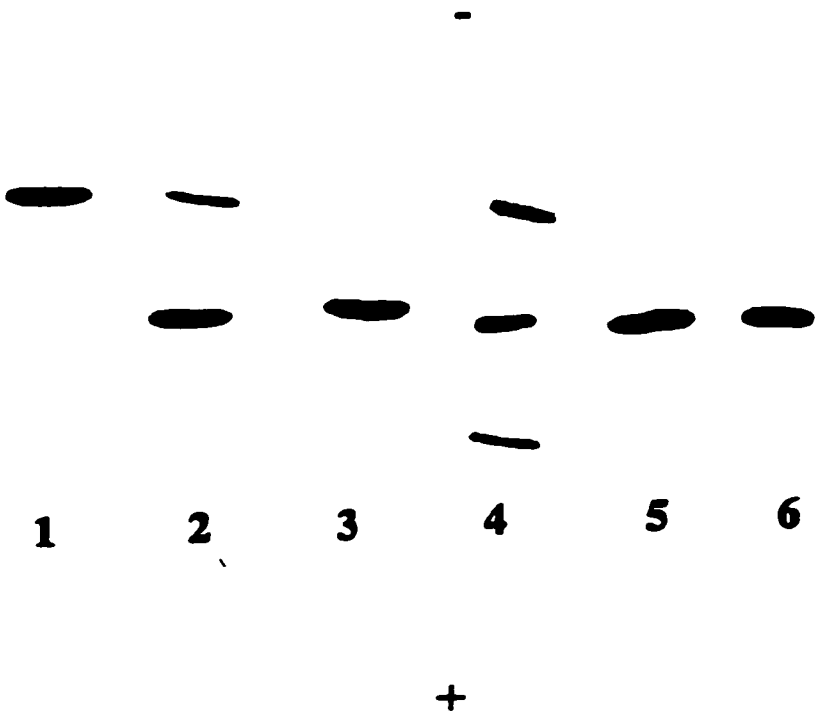


Fig. 5.1 Isoelectric focusing gel of His15Asp HPr phosphorylation. HPrs were incubated under phosphorylation conditions described in Materials and Methods and separated on isoelectric focusing gels. Lane 1 wild type HPr; Lane 2 wild type HPr under phosphorylation conditions; Lane 3 His15Asp HPr; Lane 4 His15Asp HPr under phosphorylation conditions; Lane 5 His15Glu HPr; Lane 6 His15Glu HPr under phosphorylation conditions.

5.1.2 Confirmation of His15Asp phosphorylation

When phosphorylation reactions with [^{32}P]PEP were analyzed by isoelectric focusing the lower pI band was shown by autoradiography to be phosphorylated; the higher pI band did not have a phosphoryl group. A comparison with the stained gel is presented in Figure 5.2A.

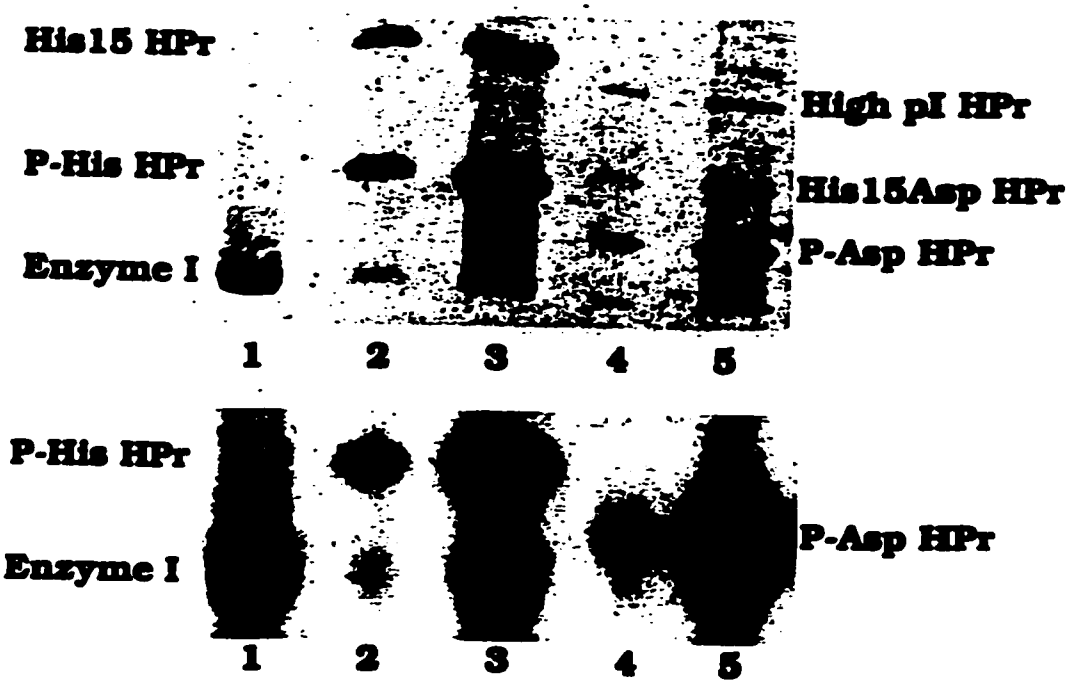


Fig. 5.2. Confirmation of HisAsp phosphorylation with [^{32}P]PEP. Phosphorylations performed as described in Materials and Methods using [^{32}P]PEP. The phosphorylation mixture was then analyzed by IEF. The gel was analyzed by autoradiograph (bottom figure) then IEF stained (top figure).(All lanes were incubated under phosphorylation conditions using 0.1 mM [^{32}P]PEP. Lane 1 enzyme I; Lane 2 wild type HPr (5 μg); Lane 3 wild type HPr (20 μg); Lane 4 His15Asp HPr (5 μg); Lane 5 His15Asp HPr (20 μg).

5.1.3 Requirements for phosphorylation of His15Asp HPr

To verify that phosphorylation of the His15Asp HPr was occurring through Enzyme I mediated phosphotransfer, phosphorylation trials were conducted with systematic elimination of each reaction component to confirm a similar dependence as the phosphorylation of wild type HPr.

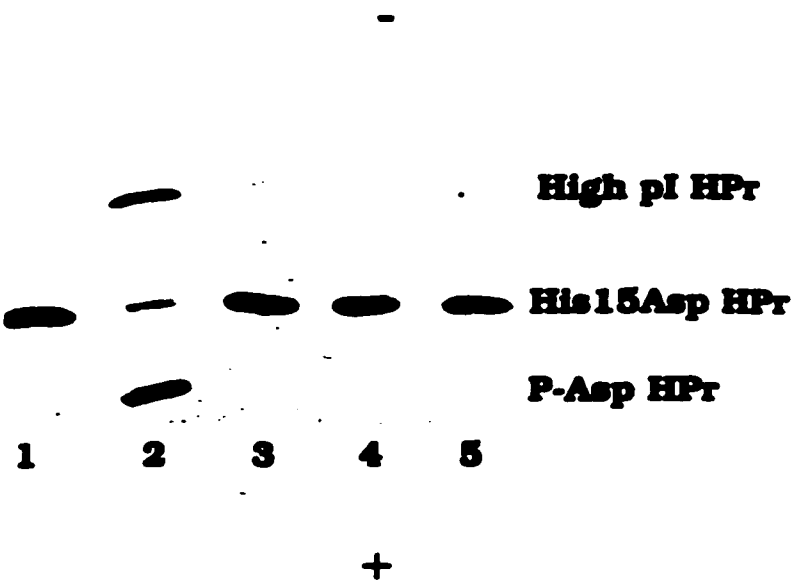


Fig. 5.3 His15Asp HPr phosphorylation requirements. Phosphorylation incubations performed as described in Materials and Methods and separated by isoelectric focusing. Lane 1 His15Asp HPr no phosphorylation incubation; Lane 2 His15Asp HPr full phosphorylation incubation; Lane 3 His15Asp HPr phosphorylation without Mg^{2+}; Lane 4 His15Asp HPr phosphorylation without PEP; Lane 5 His15Asp HPr phosphorylation without Enzyme I.

As indicated by Figure 5.3 the full complement of phosphorylation reaction components is required for phosphorylation of the His15Asp HPr mutant, confirming that the introduction of the phosphoryl group is a specific enzymatic addition by Enzyme I. Specific phosphorylation of His15Asp HPr indicates that the location of

phosphorylation is conserved. In addition it is noted that the appearance of the high pI species is dependent upon phosphorylation.

5.1.4 Kinetics of His15Asp phosphorylation

The kinetic measurement of phosphorylation of the His15Asp HPr protein by Enzyme I was carried out by the continuous spectrophometric assay described in Materials and Methods. The Vmax was determined to be 0.1% that of wild type HPr while the Km for the mutant was 66 μM, approximately an order of magnitude higher than that of wild type HPr.

5.1.5 His15Asp HPr phosphotransfer to Enzyme IIA^{glc}

As His15Asp HPr is phosphorylated by Enzyme I, the ability for further phosphotransfer from P-(Asp) His15Asp HPr to EIIA^{glc} was investigated. Due to the impairment of the Enzyme I reaction, proper kinetic measurements are impossible due to the impractical amounts of Enzyme I which would be required in the reaction mixture. Qualitative detection of EIIA^{mgsr} phosphorylation by HPr mutants is possible using [^{32}P]-labeling reactions with subsequent SDS-PAGE and autoradiography.

Demonstration of phosphorylation of EIIA^{glc}, which is dependent upon the addition of HPr, required that all proteins be purified from *E. coli* strains which did not produce HPr as described in Materials and Methods. Purification of EIIA^{glc} from strains containing HPr activity resulted in apparent contamination of EIIA^{glc} with HPr. As a consequence of this contaminating HPr, at high Enzyme I concentrations,

phosphorylation of EIIA^{glc} was independent of the addition of HPr. The [^{32}P]-phosphorylation of the EIIA^{glc} protein shown in Figure 5.4 indicates that with purification of EIIA^{glc} from a HPr deficient strain, phosphorylation of EIIA^{glc} by Enzyme I does not occur without addition of HPr to the reaction mixture. The ability of phosphorylation of EIIA^{glc} by His15Asp HPr is also demonstrated indicating that the mutant is functional in the abilities of PTS phosphotransfer from Enzyme I to EIIA^{glc}.

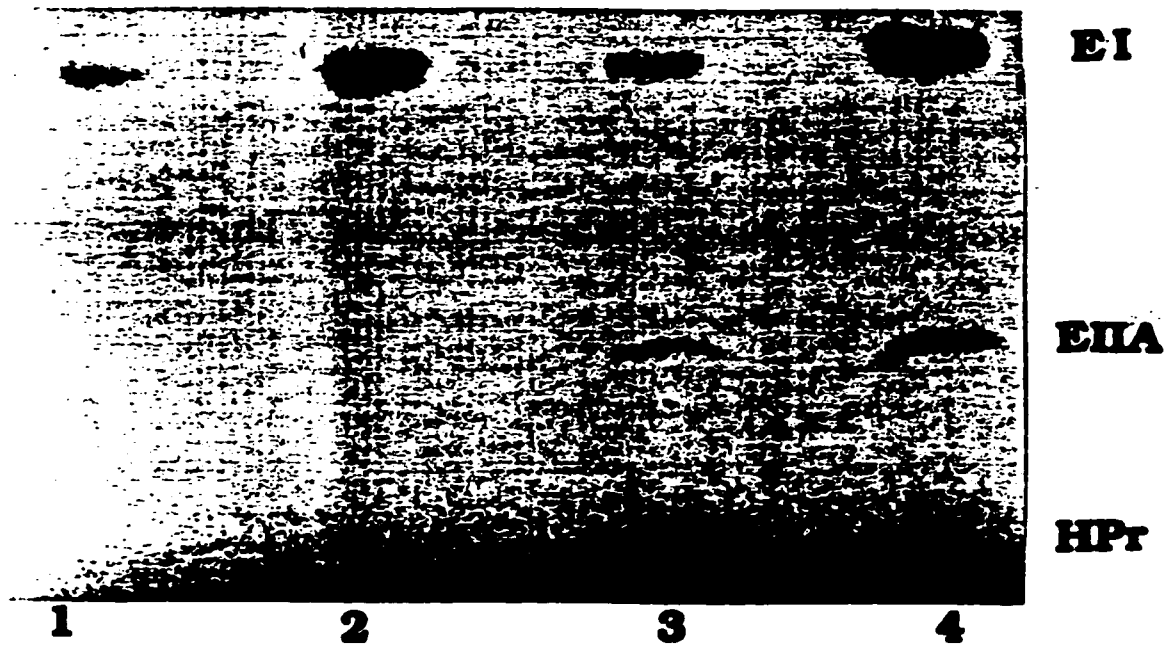


Fig. 5.4 His15Asp HPr phosphorylation of EIIA^{glc}. Phosphorylation reactions were performed as described in Materials and Methods with the addition of 0.1 mM [^{32}P]-PEP. Lane 1 enzyme I; Lane 2 Enzyme I and EIIA^{glc}; Lane 3 Enzyme I, wild type HPr and EIIA^{glc}; Lane 4 Enzyme I, His15Asp HPr and EIIA^{glc}.

5.1.6 Physiological PTS complementation by His15Asp HPr

$EIIA^{glc}$ does not represent all the enzymes IIA^{sugar}. The potential for phosphotransfer to other $EIIA^{sugar}$ proteins was investigated by *in vivo* complementation studies. The *ptsH E. coli* strain ESK108, overexpressing the His15Asp HPr protein, were grown on MacConkey plates with a series of PTS sugars; mannose, mannitol, glucose, fructose and N-acetylglucosamine. Uptake of these sugars is dependent upon phosphotransfer between His15Asp HPr and the respective $EIIA^{sugar}$ protein. His15Asp HPr failed to complement the uptake of any of the sugars tested indicating that at best His15Asp HPr has limited ability to phosphotransfer to the respective $EIIA^{sugar}$ proteins. In contrast Ser46Asp HPr, another severely impaired HPr with a ~1000-fold level of impairment, results in a delayed fermentation response when over-produced under similar conditions.

5.1.6 Crystallographic determination of His15Asp HPr

The structural consequences of the His15Asp HPr leading to the retention of phosphotransfer abilities were investigated. Retention of phosphotransfer capabilities suggests a conservation of the active centre. Furthermore the positioning of the aspartyl side chain would be expected to be similar to His15 in order to maintain the spatial orientation of the site of phosphorylation.

5.1.6.1 His15Asp HPr x-ray structure at 1.6 Å resolution

The structure of the His15Asp mutant of *E. coli* HPr was determined by x-ray crystallography at a resolution of 1.6 Å. The space group was $P2_1$ with unit cell dimensions of a= 25.37 Å, b=45.34 Å, c= 27.62 Å, α=90.0°, β=104.0° and γ=90.0°. The structure was refined to a final R-factor of 19.1% over a resolution range of 10.0 to 1.6 Å with no sigma cut off. The structure has a bond length rms deviation of 0.007 Å and a bond angle rms deviation of 1.4°. The quality of the electron density is represented in Figure 5.5 with a $2F_o$-F_c electron density map of the His15Asp site of mutation. The His15Asp mutation is clearly defined by the electron density. The final model contains 73 water molecules.

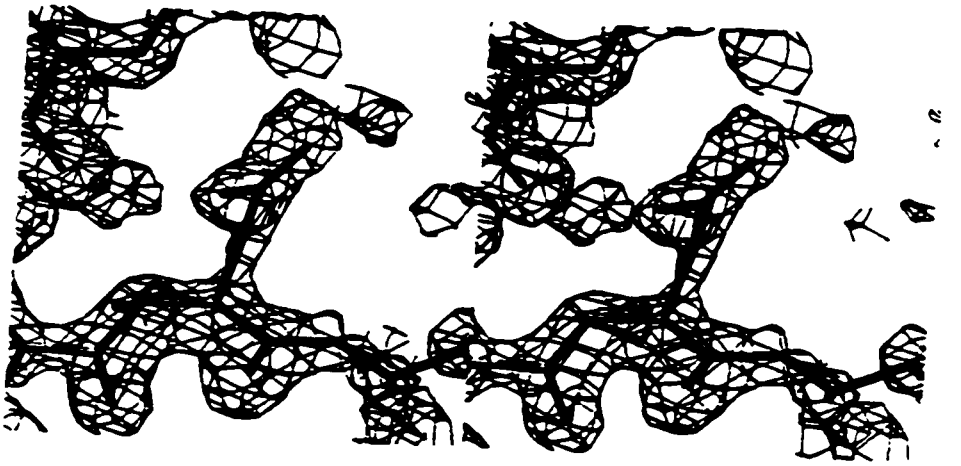


Fig. 5.5 Stereo electron density ($2F_o$-F_c) map of the Asp15 residue. The map is contoured at 1.5 σ.

The *E. coli* His15Asp HPr structure is remarkably similar to the wild type with an identical overall conformation of a open-faced β-sandwich consisting of four antiparallel β-strands and three alpha-helices. The overall structure of the His15Asp HPr is presented in Figure 5.6 with a backbone diagram. The average rmsd for mainchain atoms of the wild type and His15Asp HPrs is 0.18 Å. The β-sheet is comprised of four β-strands as defined by the hydrogen-bonding pattern; β-A (residues 2-8), β-B (residues 32-36), β-C (residues 40-44) and β-D (resides 58-65). The hydrogen-bonding pattern defines three alpha helices; α-A (residues 15-27), α-B (residues 46-51) and α-C (residues 69-84). The minor B-helix is in a conformation similar to that seen in the wild type HPr structure.

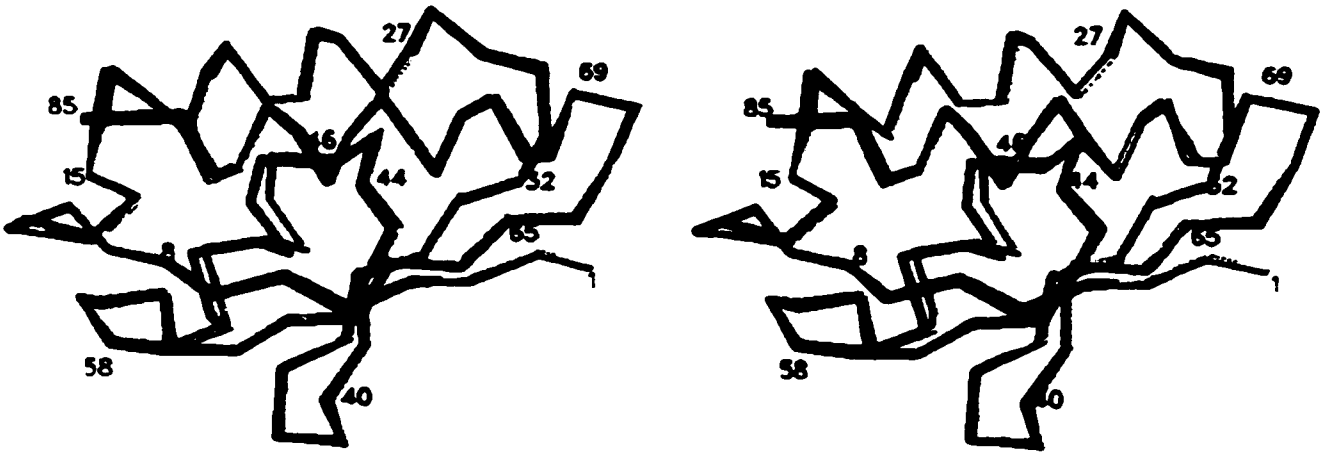


Fig. 5.6 Stereo overlay comparison of the backbone atoms of the wild type (yellow) and His15Asp (blue) HPrs. Diagram produced using SETOR (Evans, 1992).

The geometry of the active centre is preserved and the orientation of the Asp15 side chain is nearly identical to that of His15 in the wild type structure as indicated by Figure 5.7. One of the oxygen atoms of the Asp15 carboxyl group is in essentially the same position as the $N^{\delta 1}$-atom in the His15 imidazole ring of the wild type structure; the distances between the His15 $N^{\delta 1}$ atom in the wild type and the two carboxyl atoms are 1.0 Å and 2.0 Å. The Arg17 side chain adopts an identical position to that found in the wild type structure. Glu85 is positioned towards the active centre region in a similar manner to that seen in the wild type structure, but outside the range to form a hydrogen bond with Asp15. The maintenance of the conformation of the active centre region is apparently central to the success of the mutant in retention of phosphotransfer abilities.

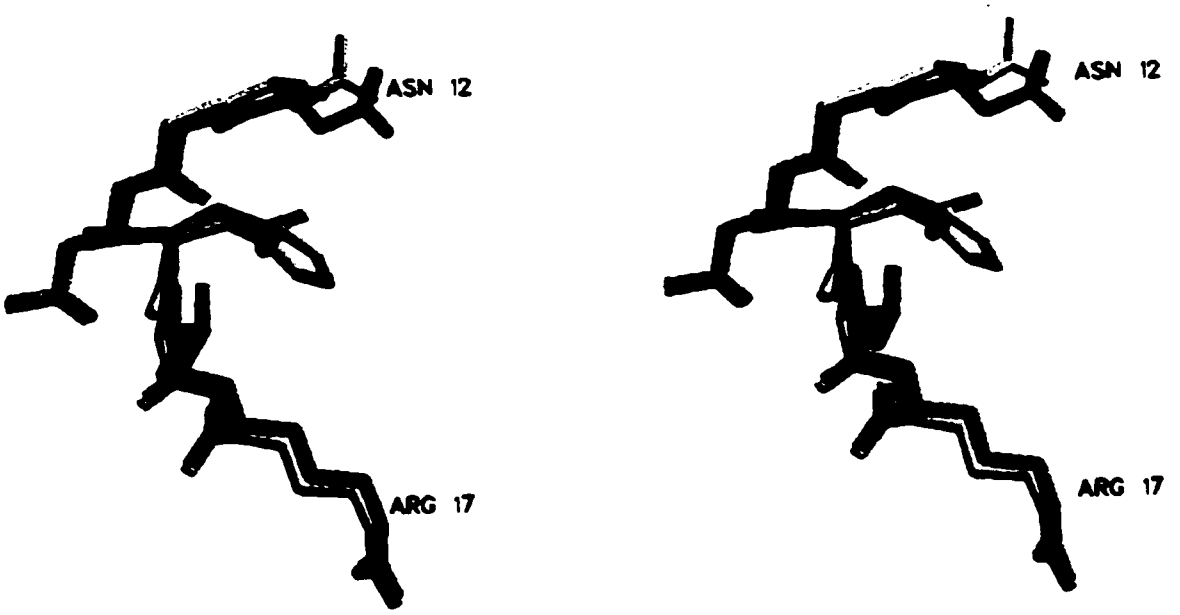


Fig. 5.7 Stereo overlay comparison of the active centre residues of wild type (yellow) and His15Asp (blue) HPrs. Diagram produced using SETOR (Evans, 1992).

The active centre region was investigated to determine the structural potential for the formation of succinimide or isoimide structures. Formation of succinimide or isoimide structures have predictive main chain Ψ angles and side chain χ dihedral angles: Ψ = -120°, χ_1 = +60° for succinimides, Ψ = +60°, χ_1 = +120° for isoimides. The Asp15 residue of His15Asp HPr has values of Ψ = −170°, χ_1 = +61°, which would favour succinimide formation.

As residue 15 is the N-capping residue of Helix A it was of interest to see if the introduction of negative charge influenced helical properties. The helix does not appear to be affected as a consequence of the mutation, as indicated by a conservation of hydrogen bonding pattern and lengths.

The absence of alterations of the active centre or helix A suggests that no significant structural rearrangements need occur in the protein to accommodate the introduction of negative charge to the region, in agreement with the conclusions reached by NMR investigations of the P-(His) HPr from *E. coli* (van Nuland *et al.*, 1995).

5.2 Investigation of other His15 HPr substitutions

The potential for phosphorylation of other His15 substitutions was investigated. Serine, cysteine, threonine and tyrosine are commonly phosphorylated in other proteins. Asparagine, glutamine and glutamic acid, were chosen as a result of similar structural or chemical properties to either histidine or aspartic acid. All of these substitutions (Ser, Cys, Thr, Tyr, Asn, Glu and Gln) were made of His15 in *E.*

coli HPr and mutants were purified to homogeneity before being tested for phosphorylation ability. These mutant HPrs demonstrated no IEF band shifting, no labeling by [^{32}P]PEP, nor activity in an lactate dehydrogenase coupled assay. Of the mutant proteins only His15Asp HPr was capable of being phosphorylated.

5.3 High pI form of His15Asp HPr

As a consequence of phosphorylation of the His15Asp HPr there is the appearance of a higher pI species in approximately equal proportions to the normal and phosphorylated forms of the mutant protein as illustrated in Fig 5.1. Omission of any of the phosphorylation reaction components such that phosphorylation does not occur also fails to produce the high pI form as presented in Fig 5.3, indicating that the two events were closely related. The properties and chemical identity of this high pI form of His15Asp *E. coli* HPr are discussed in this portion of the thesis.

5.3.1 Proposed mechanism of high pI formation

The earliest assumption about the chemical identity of the high pI form of His15Asp HPr was based on previous experience of deamidation in HPr. Deamidation reactions are known to proceed through a succinimide ring intermediate. Succinimide ring formation from an aspartic acid residue results in a species of greater positive charge than the original protein as presented in Figure 5.8. The occurrence of a succinimide in His15Asp HPr as a consequence of phosphorylation would account for the three species demonstrated in the phosphorylation reaction mixture.

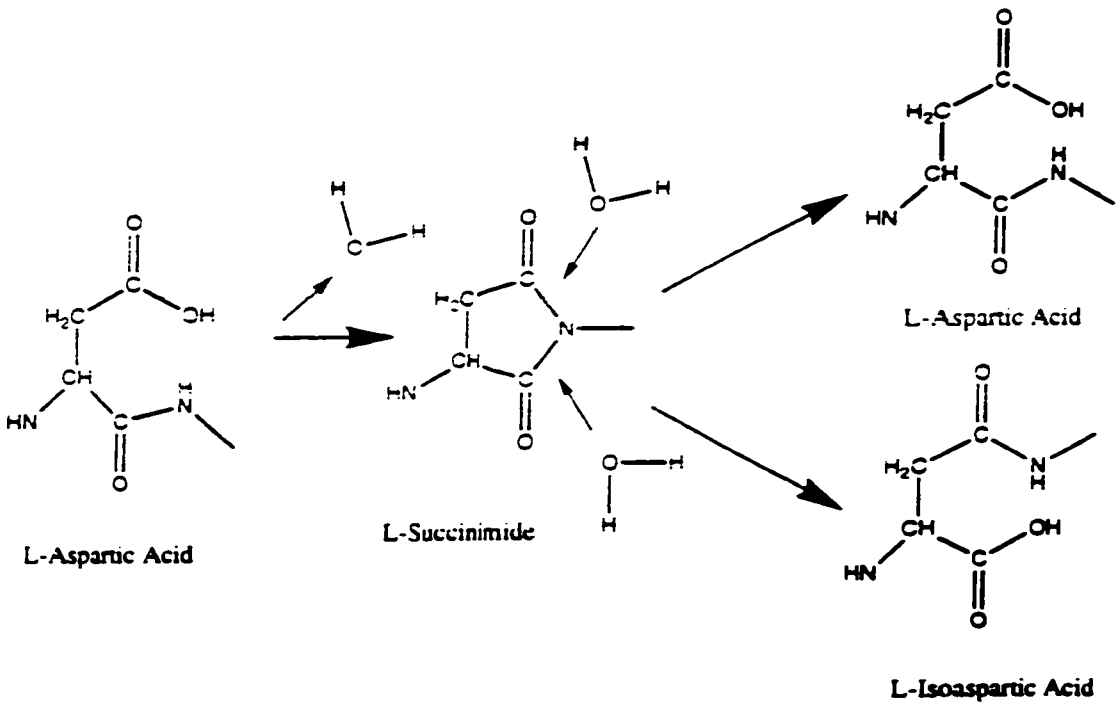


Fig. 5.8 Mechanism of succinimide formation from an L-aspartic acid residue with degradation to produce L-aspartic and L-isoaspartic acid. Not shown is the formation of small amounts of D-aspartic acid and D-isoaspartic acid which also occurs.

The rates of succinimide formation from aspartate residues are limited by the ability of the carboxyl oxygen to act as a leaving group. This ability can be enhanced through such modifications as protonation as well as methylation (for review see Clarke *et al.*, 1992). It was postulated that the phosphorylation of the aspartic acid residue would create a highly reactive group for ring formation. Phosphorylation of aspartic acid catalyzes succinimide ring formation. The chemical nature of the high pI form was investigated with the intent of verifying the succinimide hypothesis.

5.3.2 Temperature dependence of high pI formation

Increased temperature is known to promote succinimide formation by providing additional thermal energy as well as greater conformational flexibility. The contribution of elevated temperature to high pI formation during phosphorylation was investigated. Phosphorylation reactions were performed at both 37 °C and 22 °C and examined by isoelectric focusing.

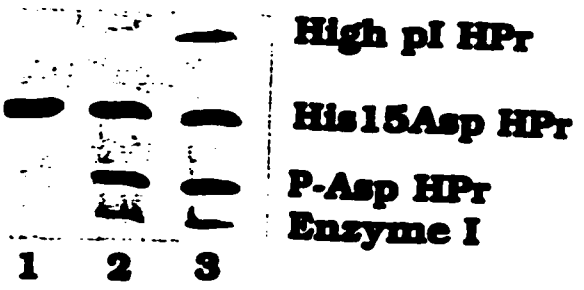


Fig. 5.9 Temperature dependence of high pI formation. Phosphorylation of His15Asp HPr: Lane 1 no phosphorylation; Lane 2 (22 °C) phosphorylation reaction; Lane 3 (37 °C) phosphorylation reaction.

Phosphorylation of the His15Asp HPr showed some temperature dependency with comparable levels of phosphorylation, as indicated by the band intensities in Figure 5.9, occurring at both temperatures investigated. Formation of the high pI

species however, was greatly reduced at lowered temperatures and did not occur at all at 37 °C in the absence of phosphorylation. This would indicate that both temperature and the addition of the phosphoryl group are contributing factors to the formation of the high pI species. Phosphorylation is a required initiating event from which elevated temperatures contribute to high pI formation.

5.3.3 pH sensitivity

The pH sensitivity of the high pI species was investigated because succinimides are known to be susceptible to base cleavage. Treatment of the His15Asp *E. coli* HPr phosphorylation reaction mixture under a range of pHs indicated that the high pI form was unstable under basic conditions (Fig 5.10). The high pI species is unstable and the reaction is reversible. Under conditions in which the high pI species was absent, only the phosphorylated and normal species could be visualized indicating that the break-down of the high pI species returns the protein to one of these forms, most likely the normal. The ability of the high pI species to return to the normal state indicates that the phosphorylation, high pI formation and return to normal could continue indefinitely under phosphorylation conditions. Succinimide theory would predict that break-down of the high pI species should produce a mixture of products, including L-isoAsp15 in the resulting product protein. It has been demonstrated in HPr (Sharma *et al.*, 1993) that the appearance of the L-isoaspartate containing proteins forms are distinguishable from L-aspartate on isoelectric focusing

with the appearance of a doublet. No such doublets are apparent for the His15Asp HPr.

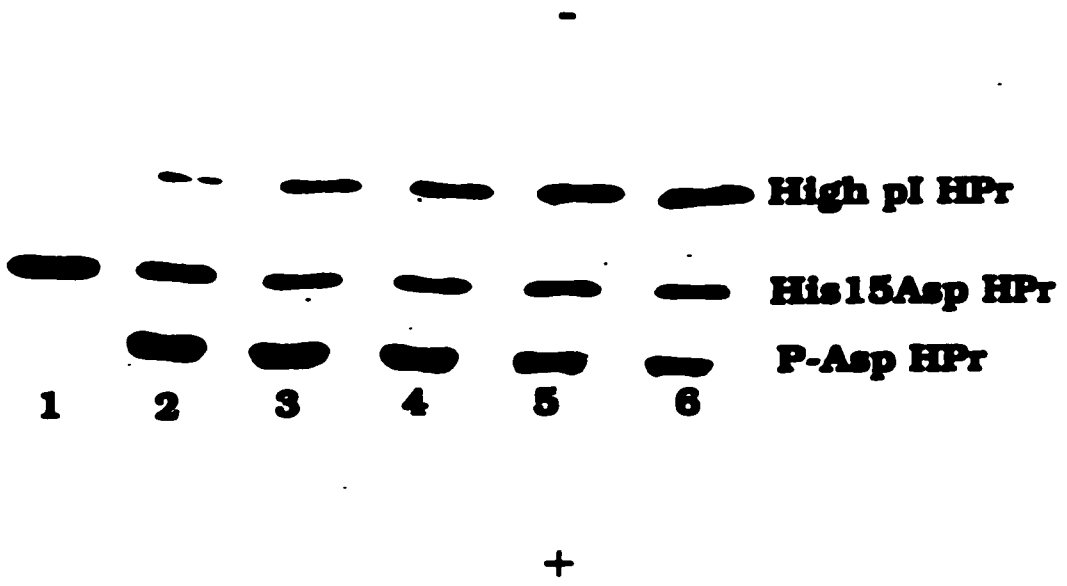


Fig 5.10 pH stability of the His15Asp HPr species. Phosphorylation reactions were performed as described in Materials and Methods. Following a 10 min incubation appropriate buffers were added to a final concentration of 50 mM. Samples were then analyzed through IEF. Lane 1 His15Asp, Lanes 2-10 His15Asp phosphorylation mixture at pHs 2.5, 3.0, 4.2, 7.6, 8.0 and 10.0.

In addition, acid hydrolysis of the phosphorylated form was observed at acidic pHs. The pH stability of the P-His15Asp was determined by phosphohydrolysis at a range of pHs as is compared to the P-His15 HPr in Figure 5.11. In the physiological range of pHs the P-Asp HPr showed an approximate 10-fold increase in stability.

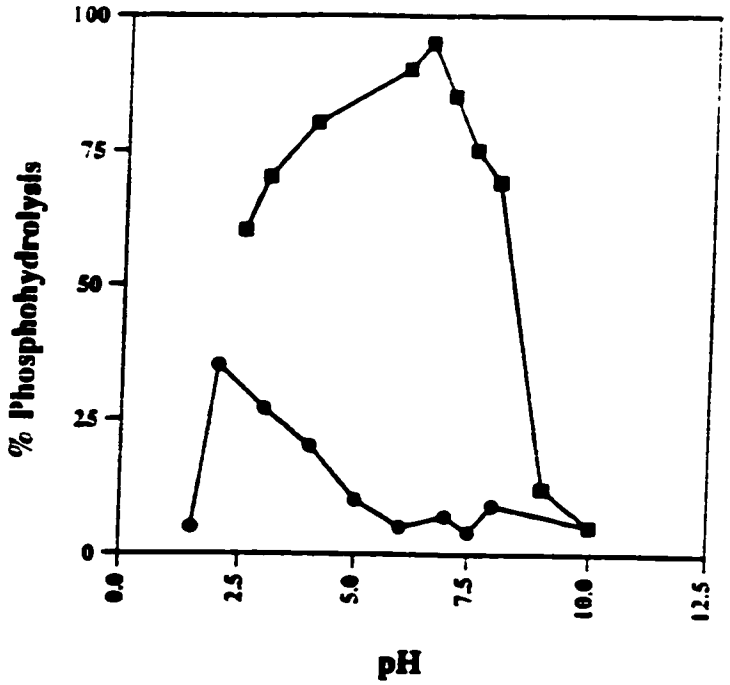


Fig. 5.11 Phosphohydrolysis (20 min at 37 °C) of P-Asp HPr (circles) was compared to P-His HPr (squares) as described in Materials and Methods.

5.3.3 Purification

Separation of the three species within the His15Asp HPr phosphorylation mixture is described in the Materials and Methods section. Isolation of the three His15Asp species of the phosphorylation mixture is demonstrated by the isoelectric focusing gel analysis of the three resolved peaks of the purification procedure (Figure 5.12). Comparatively little of the phosphorylated form could be isolated through this procedure as demonstrated by the peak height and isoelectric focusing gel band density. This is due to the instability of the phosphorylated species under the acidic condition of the purification. The phosphorylated form would be converted to the high pI species through intramolecular reaction or to the unphosphorylated species through simple phosphohydrolysis.

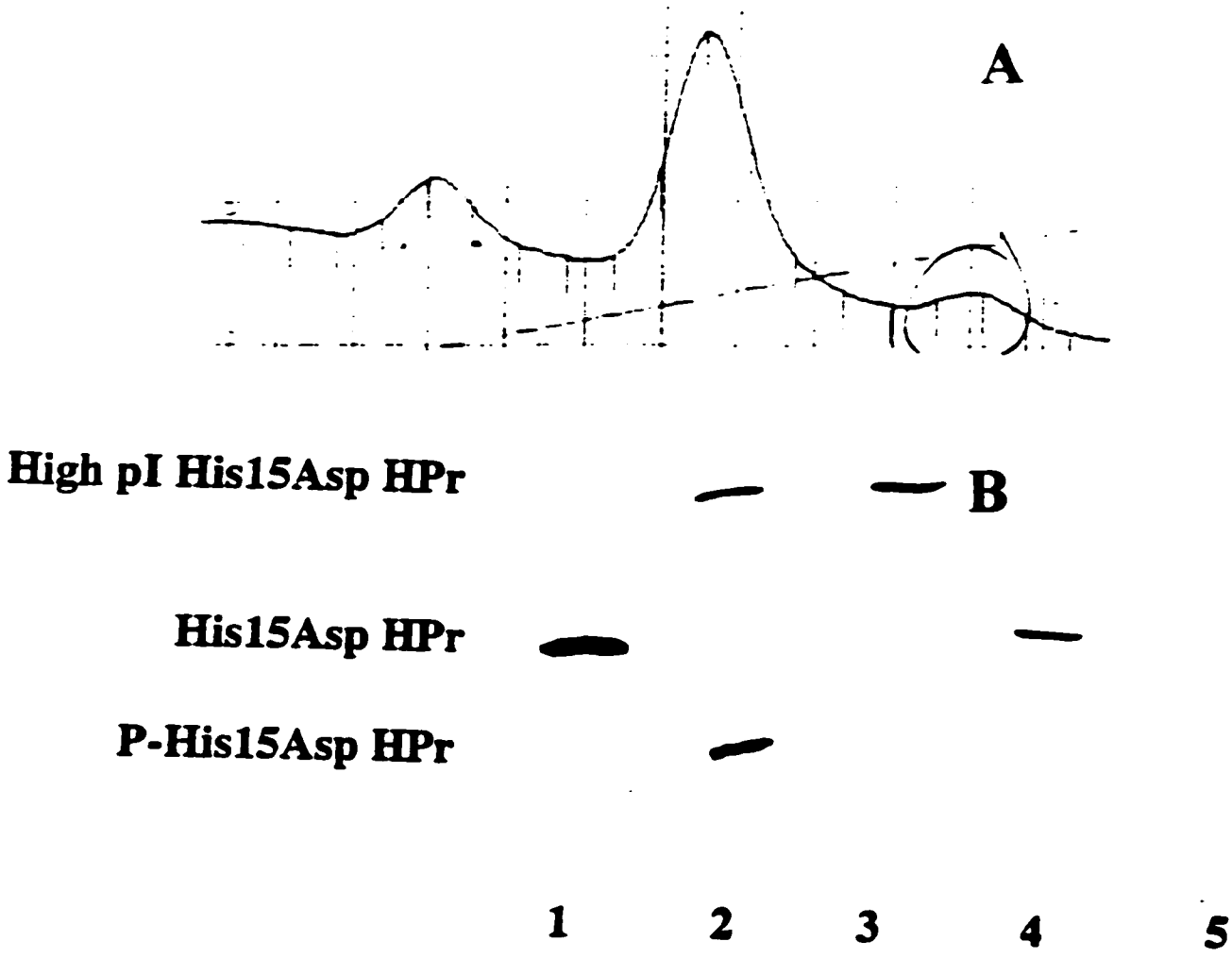


Fig. 5.12 Purification of high pI His15Asp HPr. A. Chart recording of separation of His15Asp HPr phosphorylation reaction species as described in Materials and Methods B. Isoelectric focusing analysis of separated peaks; Lane 1 His15Asp HPr; Lane 2 His15Asp phosphorylation mix; Lane 3 first peak; Lane 4 second peak ; Lane 5 third peak.

5.3.4 Mass spectrometry

Succinimide theory predicts that the high pI species should weigh 18 daltons less than the normal protein, corresponding to the loss of a water molecule. Matrix assisted laser diode ionization (MALDI) mass spectrometric analysis was performed by Lawerence Hogge of the Plant Biochemical Institute of Saskatoon, Saskatchewan. From the sequence, the His15Asp protein was calculated to have a weight of 9099 daltons, which is in agreement with the experimentally-determined value of 9098 daltons. The phosphorylation reaction mixture was initially examined to determine the ability of the technique to distinguish the three species of the mutant HPr molecule. Mass spectrometric analysis of the mixture, shown in Figure 5.13, verified the presence of three species, one of 9103 daltons in agreement with the normal protein, another of 9184 daltons an increase in weight of 80 daltons in agreement with that predicted by phosphorylation and a third species of 18 daltons less than that predicted for the normal protein, presumably corresponding to the loss of a water molecule. The molecular mass of the His15Asp HPr is ~ 5 daltons heavier than the predicted mass of the protein based on the amino acid sequence. While the changes in mass to both the phosphorylated and high pI species correspond exactly to the gain of a phosphoryl group and loss of a water molecule respectively they are equally affected by the initial putative miscalibration of ~5 daltons. This discrepancy in mass is due to a lack of suitable standard in the mass spectrometric analysis. Another mass spectrometric analysis utilizing the same His15Asp HPr protein sample gave a mass which corresponded exactly with that which was predicted by the amino acid content.

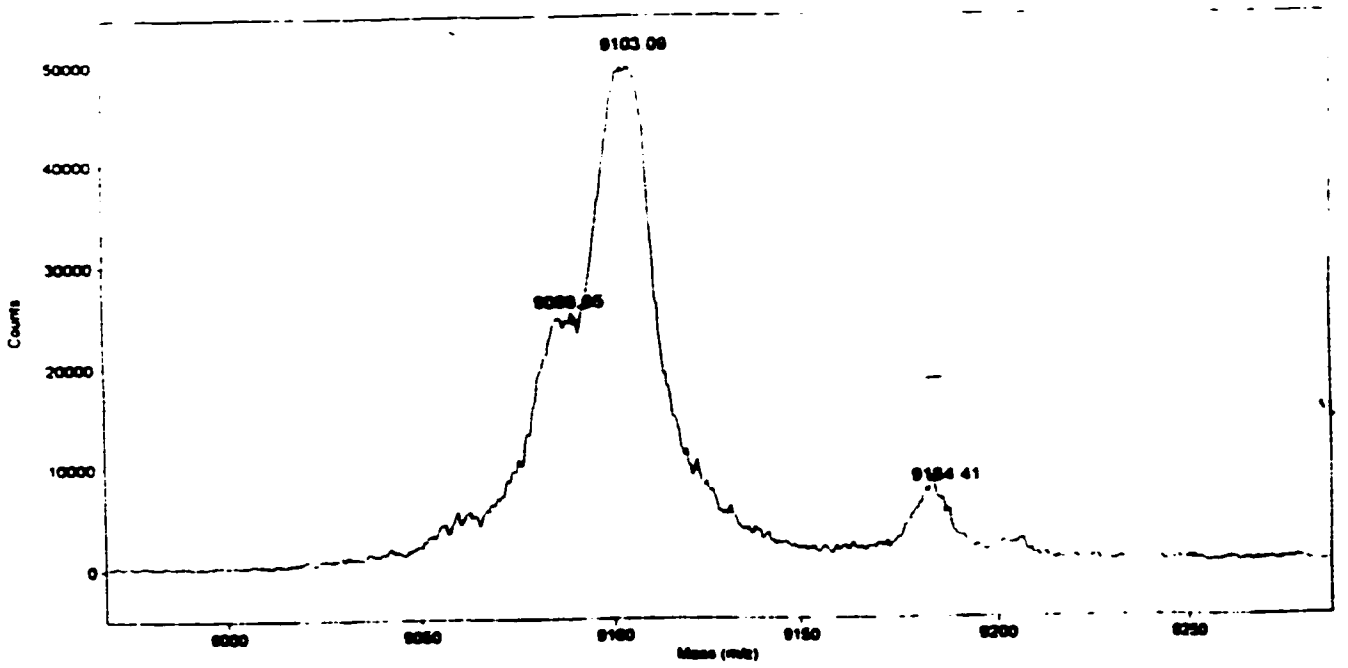


Fig. 5.13 Mass Spectrometric analysis of the His15Asp HPr phosphorylation reaction mixture.

MALDI mass spectrometric analysis was also conducted on the isolated high pI form and the results presented in Figure 5.14. The analysis again verified that this

form was 18 daltons lighter than the calculated weight of the mutant, suggestive of the loss of a water molecule in agreement with ring formation theory.

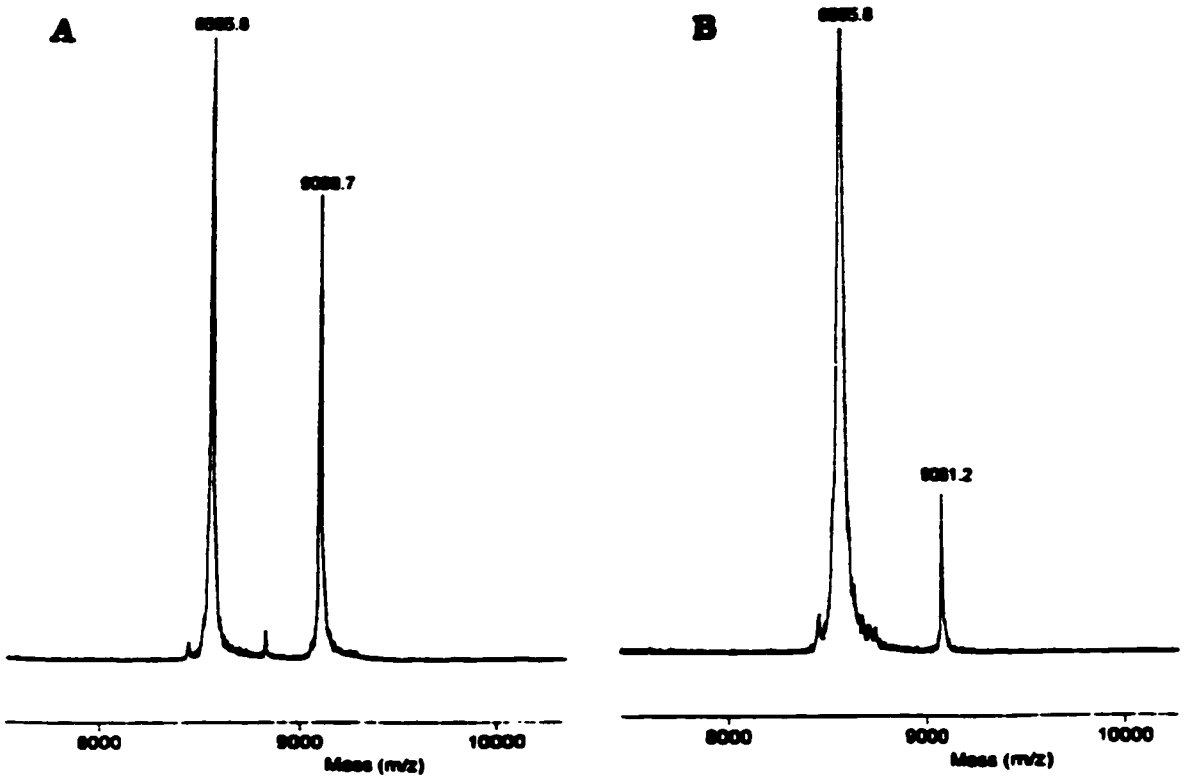


Fig. 5.14 Mass spectrometry of His15Asp and high pI HPrs. A. His15Asp HPr. B. Pure high pI form of His15Asp HPr. The standard (Mass 8566) was ubiquitin.

5.3.5 Amino acid analysis

Amino acid analysis through Edman degradation is dependent upon the presence of standard peptide bonds for the sequential removal of residues from the N-terminal portion of the protein. The presence of a non-standard linkage in the

polypeptide backbone, such as succinimide or an iso-linkage, will halt sequencing at the unique residue.

Amino acid analysis was performed on the isolated high pI sample in order to test for the presence of non-standard linkages. As predicted the analysis failed completely after the fourteenth round (data not shown), indicating the specific site of abnormality in the protein. This experiment was supportive of the hypothesis that the chemical identity of the high pI form consisted of an internal modification at the 15 position which involved the main-chain atoms.

Amino acid analysis was also performed on a sample of the isolated high pI form of the His15Asp mutant protein which had been resolved by base treatment to a species which migrated with the same pI as the original protein. Succinimide theory would predict that a considerable portion, typically two thirds, of this resolved sample should exist in the iso-form at the 15 residue, resulting in a reduction in the amino acid sequencing signal corresponding to the percentage of the protein which contained the isoaspartyl form. Amino acid analysis proceeded normally through the 15 region without any reduction in signal and indicated the sole presence of a normal L-aspartic acid residue (data not shown).

5.3.6 Methyl-transferase activity

The enzyme L-isoaspartyl (D-aspartic acid)-methyltransferase serves the physiological function of repair of deamidation by-products. The enzyme can be utilized in an assay to detect the presence of isoaspartyl and D-aspartyl residues in

proteins. Analysis of the resolved high pI form of the His15Asp HPr failed to demonstrate the presence of the normal by-products of succinimide break-down (Performed by Dr. S. Clarke, UCLA). The failure of the methyltransferase assay, as well as amino acid sequencing, to detect the formation of any non-standard residues can be explained by two alternative hypotheses. Either the mechanism of high pI formation was not through the succinimide ring intermediate, or that steric or electrostatic influences of the protein strongly favored the return to the L-aspartyl residue. There have been previous examples of proteins which fail to produce either the D or iso-forms of aspartic acid upon hydrolysis of the high pI ring; *e.g.* hemoglobin Providence (Moo-Penn *et al.*, 1976) and hypoxanthine-guanine phosphoribosyltransferase (Wilson *et al.*, 1982). The unusual behaviors of these proteins was rationalized in terms of physical properties of the protein which limited the products of ring cleavage.

5.3.7 Potential involvement of Asn12

Previous structural investigations of *E. coli* HPr indicated the possible involvement of Asn12 in the active centre region. This raised the interesting possibility that ring formation as a consequence of phosphorylation was not occurring through the main-chain nitrogen of the peptide bond but rather through covalent interaction with the amide side chain of Asn12. Further analysis of the *E. coli* His15Asp HPr structure verified the orientations of Asn12 and Asp15 would permit

such a linkage. The chemistry of formation of the covalent linkage between the side chains and the products of cleavage would be:

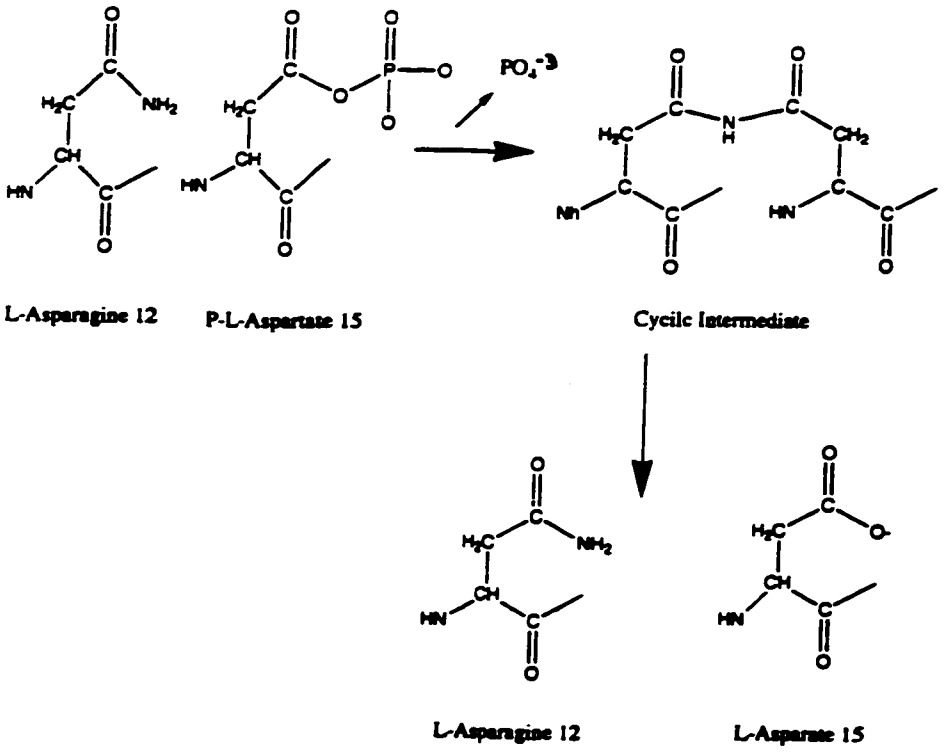


Fig. 5.15 Proposed mechanism of the involvement of Asn12 in high pI formation.

Potential ring formation between the Asp15 and Asn12 side chains was addressed through site-directed mutagenesis in which the Asn12 was substituted with alanine. The double mutant Asn12Ala, His15Asp was then subjected to phosphorylation by Enzyme I with subsequent IEF analysis. The double mutant retained the ability for both phosphorylation as well as high pI formation indicating that Asn12 was not responsible, nor involved in phosphorylation or ring formation.

5.3.8 X-ray analysis

In an effort to determine the structural basis of the high pI form of His15Asp HPr a crystallographic structural determination was attempted. Due to the known instability of the high pI species, time and pH considerations were of primary concern. The high pI species was isolated by the previously described methods and used for crystal growth in identical set-ups as used for the His15Asp HPr. Crystals were obtained within a week and data were immediately collected to a resolution of 1.8 Å. The space group was $P2_1$ with unit cell dimensions of a=25.92 Å, b= 45.34 Å, c= 27.62 Å, α= 90.0°, β= 104.2 ° and γ= 90.0 °. The structure was refined to a final R-factor of 20.0% over the resolution range of 10.0 to 1.8 Å range with no sigma cut off. The final model contains 43 water molecules.

Despite efforts to reduce the rate of break-down of the ring structure the crystallographic determinations failed to reveal any unusual structures at the 15 position. The electron density of the region is clearly that of an aspartic acid indicating the ring had converted back to the instigating residue. The absence of

5.4 Isoimide formation

An alternative structural explanation of the high pI form of His15Asp is the possibility of isoimide formation. The mechanism of isoimide formation is presented in Figure 5.17. Chemically this ring differs from the succinimide in that the point of cyclization with the main chain is through the neighboring carbonyl group rather than nitrogen atom.

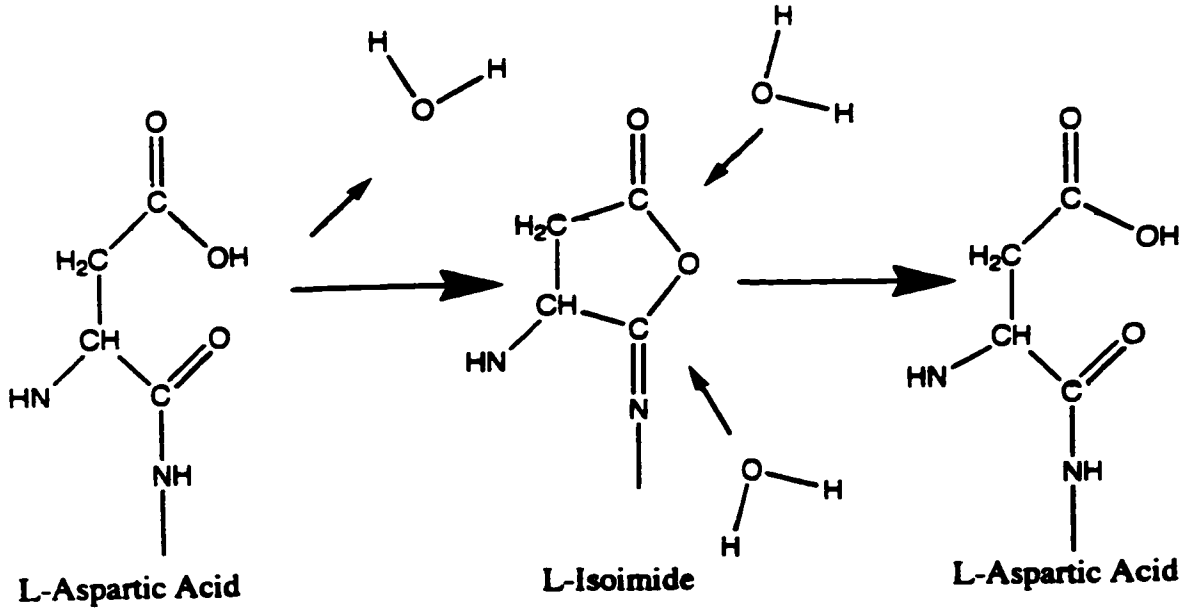


Fig. 5.17 Proposed mechanism of isoimide formation from a L-aspartyl residue.

The isoimide would be expected to share similar properties as the succinimide in terms of pH stability, weight change and disruption of Edman degradation and would therefore be supported by all the previously presented results.

The critical difference is that hydrolysis of the isoimide ring produces only L-aspartic acid.

5.5 Ring formation in the absence of phosphorylation

In order to try to discriminate between succinimide and isoimide formation the potential for high pI formation over a range of temperatures and pHs was investigated. The rates of succinimide formation from asparagine and aspartic acid residues are known to be pH dependent. Asparagines favor deamidation at basic pHs due to de-protonation of the main-chain attacking amide group whereas aspartic acid residue show the highest levels of cyclization at physiological pH's due to the combined influences of protonation of the aspartic acid side chain coupled with de-protonation of the main-chain amide group (Wright, 1991). Both asparagines as well as aspartic acid show a temperature dependency. Treatment of proteins at extremes of temperature and pH are established mechanisms for promoting succinimide formation in proteins. In order to remove background levels of deamidation at Asn12 and Asn38, these residues were mutated to alanines. Two triple mutant HPrs were created Asn12Ala His15Asp Asn38Ala and Asn12Ala His15Asn Asn38Ala. Both mutant proteins were investigated for formation of a high pI species as well as for potential by-products through incubation at 60 °C at variety of pH's followed by isoelectric focussing analysis (Figure 5.18).

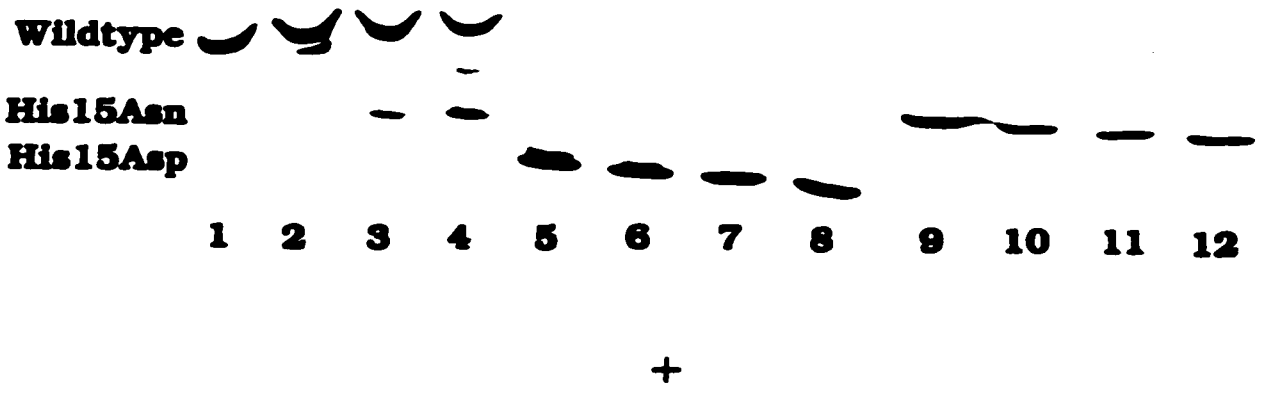


Fig. 5.18 The stability of Asn15 and Asp15 in HPr. Wild type HPr (lanes 1-4) and HPr with triple mutations, Asn12Ala, Asn38Ala with either His15Asp (lanes 5-8) or His15Asn (lanes 9-12). In each group, the first sample is unheated, the next three were heated at 60 °C at pHs from 5.0, 7.0 and 10.0 for 90 min. Samples were separated on isoelectric focusing gels which show deamidation (lower pI as in lanes 3 and 4) or cyclization (higher pI).

While the wild type control demonstrated the formation of the previously investigated HPr-1 and HPr-2 species corresponding to deamidation of residues 12 and 38, neither of the triple mutant HPrs demonstrated the formation of any unique species at pHs ranging from 5.0-10.0. Deamidation of Asn15 would result in a band shift which was not observed despite the fact that the main-chain and side chain position of Asp15 (and presumably Asn15) were near optimal for succinimide formation. The inability of these Asn15 and Asp15 side chains to be rendered chemically reactive under conditions known to favor succinimide formation is suggestive, in conjuncture with other experimental evidence, that isoimide ring formation is occurring.

6.0 Discussion

6.1 Regulation through Ser46 HPr phosphorylation

The PTS system is responsible for mediating the uptake and phosphorylation of numerous sugars in many species of bacteria (for reviews see Meadow *et al.*, 1990; Postma *et al.*, 1993). HPrs from different species share a ubiquitous function within this system of mediating phosphoryl group transfer from Enzyme I to EIIA^{sugar}. As would be suggested by the common function, HPrs from across species show a high degree of structural similarity (Jia *et al.*, 1994a). In spite of similar overall structures, HPrs from Gram-positive and Gram-negative bacteria do not interchange well in *in vitro* assays (Simoni *et al.*, 1973, Reizer *et al.*, 1989). This inability to interchange Gram-negative and Gram-positive HPrs is representative of the importance of subtle structural differences in mediating unique HPr functions.

In Gram-positive bacteria HPr may also function as a regulatory protein of carbohydrate metabolism. This role is dependent upon the phosphorylation of an absolutely conserved Ser46 residue by an ATP-dependent kinase (Deutscher and Saier, 1983). This kinase is not present in Gram-negative bacteria, nor can it phosphorylate Gram-negative HPrs *in vitro* (Waygood *et al.*, 1984) despite the presence of an absolutely conserved Ser46 residue and considerable overall structural homology (Jia *et al.*, 1994a). The addition of *E. coli* HPr to crude extracts of *S. salivarius* inhibits the formation of *S. salivarius* P-(Ser)HPr

indicating that the *E. coli* HPr has enough structural similarity to act as a competitive inhibitor of the ATP-dependent kinase (Waygood *et al.*, 1986b).

There are several physiological consequences to this post-translational modification. First, the P-(Ser) HPr becomes a poor substrate for Enzyme I in accepting phosphoryl transfer. In *B. subtilis* HPr Ser46 phosphorylation results in an increase in the Km value with respect to Enzyme I phosphorylation from 23 μM to 345 μM and the Vmax of the reaction is decreased six-fold (Reizer *et al.*, 1989). In addition to inhibition of PTS phosphotransfer, P-(Ser) HPr is able to mediate a variety of physiological affects through protein:protein interactions (Ye *et al.*, 1995). Immediate metabolic effects are seen with the stimulation of inducer exclusion and expulsion through activation of a cytoplasmic sugar phosphate phosphatase. P-(Ser) HPr also serves to regulate transcriptional machinery through interaction with the catabolite control protein, CcpA, repressor homologue. The complex is able to bind a catabolite responsive element, *cre*, for negative modulation of transcription (Ye and Saier, 1995).

6.2 Ser46Asp HPr phosphorylation analog

The mimicking of serine phosphorylations through aspartate substitutions has been demonstrated in a number of proteins. A series of mutations of the Ser46 site of *B. subtilis* HPr revealed that the regulatory phosphorylation event could be mimicked with the mutant Ser46Asp (Reizer *et al.*, 1989; Deutscher *et al.*, 1994).

The structural mechanisms of inactivation through Ser46 phosphorylation or Ser46Asp mutagenesis were investigated by NMR. Wittekind *et al.*, found that

the ^{1}H-NMR spectra of the P-(Ser46) and of the Ser46Asp *B. subtilis* HPrs were similarly perturbed relative to the spectrum of the wild type unphosphorylated protein. They reported that the backbone chemical shifts extend to localized regions of the protein distant to the 46 site and that the changes are not purely a result of through-bonds effects caused by the addition of negative charge (Wittekind *et al.*, 1990, 1992; Pullen *et al.*, 1995).

In contrast a more recent characterization of the *B. subtilis* P-(Ser46) HPr and Ser46Asp mutant have been performed by NMR as well as solvent denaturation studies. The results indicate that phosphorylation of Ser46 or its conversion to aspartate does not result in a far-reaching conformational change but rather a local stabilization of the B-helix of which 46 is the N-capping residue. Amide exchange rates are increased and the protein is stabilized against both thermal and solvent denaturation by 0.7-0.8 kcal/mol as a consequence of phosphorylation or mutagenesis (Thapar *et al.*, 1996).

The minor B-helix has been a point of diversion in HPr determinations, in particular the residues 53-54, despite the fact they are highly conserved. In Gram-positive HPr residues 53 and 54, which C-cap the minor B-helix tend to adopt a helical positioning, possibly as a consequence of steric crowding by Tyr37 which is conserved in Gram-positive bacteria. In Gram-negative HPr the 53-54 region is more random, possibly as a result of hydrogen bonding with Ser37 which is conserved in Gram-negative bacteria (Jia *et al.*, 1994a). All NMR determinations of this region has been shown to be quite dynamic compared to the A and C α-helicies as judged by the rate of exchange of amide protons with solvent (Thapar

et al., 1996). It has also been proposed that helix B represents a critical region of interaction with both Enzyme I as well as IIA^{sugar} proteins as indicated by shift perturbations during protein:protein interaction (Herzberg, 1992).

Although *E. coli* HPr cannot be phosphorylated by the ATP-dependent kinase it was postulated that the Ser46Asp mutation had the potential to mimic this regulated state. The Ser46Asp mutation of *E. coli* HPr has similar *in vitro* properties as the P-(Ser) or Ser46Asp *B. subtilis* HPrs. The Km with respect to Enzyme I phosphorylation is 4000 μM as compared to 6 μM for the wild type. However due to the concentrations of HPr required to perform the assays this number is an extrapolation and should therefore be interpreted with caution. The Vmax shows a moderate decrease to about 30% of the wild type levels, indicating that the active site is unaffected by the mutation. The kinetic results would therefore suggest that the effects were mediated primarily through a disruption in the ability of the protein to interact with Enzyme I.

Three monoclonal antibodies, Jel 323, Jel 42 and Jel 44, exist for *E. coli* HPr and their epitopes have been characterized (Sharma *et al.*, 1991). Of these only Jel 323 includes the 46 region in its epitope and is the only one to show compromised affinity to the Ser46Asp HPr. The comparable binding affinities of Jel 42 and Jel 44 for the Ser46Asp *E. coli* HPr suggest that no major structural rearrangements underlie the compromised kinetics.

6.3 *E. coli* Ser46Asp HPr at 1.5 Å resolution

The *E. coli* Ser46Asp HPr was examined by x-ray crystallography to determine the specific details of any structural alterations which may be occurring. The structure was solved at 1.5 Å resolution and showed little deviation from the wild type. As predicted from the kinetic results the main-chain conformation of the active center region was unaffected by the mutation. The specific orientation of proposed critical active center side chains are not altered. In particular the His15, Arg17 and Asn12 side chains occupy similar orientations, indicating that the catalytic capabilities of the protein are not influenced by the mutation.

In both the wild type and Ser46Asp HPr structures there is a sulfate group located in the active center region. The presence of a sulfate group, which is chemically similar to phosphate, is taken to be structurally comparable to His15 phosphorylation. The positioning of the sulfate is similar in both the wild type and Ser46Asp structures with interactions with the protein involving the imidazole ring of His15 as well as the main-chain atoms of Thr16 and Arg17. The ability of the Ser46Asp HPr to interact with the sulfate anion in a manner analogous to the proposed mechanism of His15 phosphorylation is indicative that the catalytic capabilities of the active center region are not influenced by the mutation.

Glu85, which was proposed to be involved in the active center on the basis of the *E. coli* wild type structure (Jia *et al.*, 1993), occupies an identical position in Ser46Asp. The α-carboxylate group forms hydrogen bonds between the terminal carboxyl group and the $N^{\varepsilon 2}$ atom of the imidaloze ring of His15. This

interaction has been investigated through mutation and deletion with the conclusion that the positioning is not of catalytic importance.

The requirement of torsion angle strain in HPr for phosphotransfer was proposed by Jia *et al.*, (1993b) based on the observation of disallowed phi and psi angles at residue 16 in the *S. faecalis* and partially disallowed torsion angle strain in the wild type *E. coli* HPr structures. In the Ser46Asp structure an alternate positioning of the carbonyl group of Pro11, which was misplaced in the original structure, relieves the torsion angle strain from residue 16 indicating that the proposed torsion angle strain model does not apply to *E. coli* HPr.

In agreement with NMR and protein stability investigations of *B. subtilis* and *E. coli* HPr, structural stabilization occurs in the α-helix B as a consequence of the mutation. In wild type *E. coli* HPr, Gly54 adopts a random, non-helical conformation and is hydrogen bonded with the Ser37 residue. In the Ser46Asp structure there is a readjustment of Gly54 to a 3_{10} helical conformation similar to HPrs from Gram-positive species. Asp46 N-caps this small, irregular helix; residues 46 and 47 conform to alpha-helical structure, residue 48 represents a transition point and residues 49-54 are in 3_{10} helical conformation. The alterations are not limited to the recruitment of Gly54 to helical conformation, in addition there is a tightening of the hydrogen bonding pattern throughout the helix as indicated by the shortening of the hydrogen bond lengths. It would appear that the introduction of negative charge at the N-capping position results in a solidification of this previously dynamic helix. While the alterations which occur in the helix are interesting from the perspective of protein structure/function they

are not believed to be the underlying cause of kinetic impairment. While this helical region has been proposed to be involved in the interaction with both the Enzyme I and $EIIA^{sugar}$ proteins it is doubtful that the minor rearrangements would be sufficient to mediate the considerable kinetic effects. Furthermore it is noted that the Ser46Asn mutant, which also results in helix stabilization as indicated by denaturation experiments (Thapar *et al.*, 1996), has only moderate effects, 10-fold, on Km.

The residue 46 region does not undergo any significant rearrangements with respect to neighboring side chains. The substituted aspartate side chain adopts a positioning very similar to that of the serine in the wild type structure, forming hydrogen bonds with the main chain nitrogen of lysine 49. Phe48 shows a modest alteration from the wild type structure where the phenyl ring is situated parallel to the axis of the B helix. In the Ser46Asp structure this phenyl ring is perpendicular to the B-helix axis. This minor adjustment is not thought to be the causative agent in the disruption of HPr interaction with Enzyme I. It would be expected that the Arg46 and Asn46 mutants would result in similar crowding of the phenyl ring from its wild type position yet they do not display the dramatic kinetic consequences seen with Ser46Asp.

The surface region of the Ser46 region can be described as a hydrophobic patch which is involved in the interaction with a complimentary hydrophobic patch of Enzyme I (Herzberg 1992; Garrett *et al.*, 1999). The electrostatic identity of both molecules is likely to be a critical determinant in mediating the interaction. Disruption of this hydrophobic docking site through the introduction of negative

charge, through either phosphorylation or mutagenesis, impairs the interaction with Enzyme I. This is supported by the recent solution structure determination of the N-terminal fragment of Enzyme I with HPr. The complex demonstrates a glutamate residue of Enzyme I in proximity of the Ser46 region of HPr. Phosphorylation of Ser46 would result in an electrostatic repulsion between these residues. The electrostatic disruption of the interaction between Enzyme I and HPr through Ser46 phosphorylation is also supported by kinetic results which imply an disruption of binding rather than impairment of catalytic mechanism.

The collective agreement on the mechanism through which phosphorylation affects HPr activity can be added to the limited base of knowledge regarding the structural mechanism of regulation through phosphorylation. Reversible protein phosphorylation is a common mechanism for the regulation of enzyme activity in both prokaryotes and eukaryotes and regulates both metabolic pathways as well as transcriptional activation signals. While numerous kinase/phosphatase systems have been investigated with regards to the enzymes regulated, the amino acid recognition motifs and kinetic consequences of phosphorylation, little is known about the structural mechanisms through which phosphorylation influences activity. There have been only four examples of proteins which are regulated through phosphorylation for which the crystallographic structure of both the phosphorylated and unphosphorylated forms have been elucidated; *E. coli* isocitrate dehydrogenase (Hurley *et al.*, 1990), mammalian (Sprang *et al.*, 1988) and yeast glycogen phosphorylase (Lin *et al.*, 1996) and phenylalanine hydroxylase (Kobe *et al.*, 1999).

Based on these three examples there is no definitive structural explanation for the mechanism through which phosphorylation modifies enzyme activity. In isocitrate dehydrogenase (Hurley *et al.*, 1990), inhibition through phosphorylation can be achieved in the absence of significant changes in the protein structure. The addition of the phosphoryl group establishes an electrostatic block to substrate binding, effectively abolishing enzyme activity. In contrast, both the mammalian (Sprang *et al.*, 1988) and *Saccharamyces cerevisiae* glycogen phosphorylase (Lin *et al.*, 1996) enzymes show an alteration in structure as a result of phosphorylation. The mammalian glycogen phosphorylase enzyme, when phosphorylated, undergoes a re-orientation of the protein's two sub-units. While the individual structures of the sub-units are unaffected, the re-positioning represents a significant change in overall structure. Yeast glycogen phosphorylase undergoes a localized re-folding upon the addition of the phosphoryl group by re-positioning a short, terminal fragment which doesn't cause significant changes in the protein away from this localized region. In phenylalanine hydroxylase, phosphorylation of the regulatory Ser16 residue does not produce any significant structural alterations nor does it result in enzyme activation. Activation of the enzyme requires phosphorylation as well as the presence of the activating molecule, phenylalanine. A higher concentration of phenylalanine is required for activation of the unphosphorylated enzyme suggested that the two signals work in concert (Kobe *et al.*, 1999).

Thus the four previously determined examples of understanding regulation through phosphorylation present three unique mechanisms; alteration in

electrostatic identity, localized structural changes and re-orientation of sub-units. Regulation of HPr through phosphorylation most closely resembles that of isocitrate dehyrdogenase with inactivation resulting from an alteration of electrostatic identity with no significant alterations of structure.

6.4 Residue requirements in the active centre of *E. coli* HPr

The active center of *E. coli* HPr has been extensively characterized by the Waygood, Delbaere and Klevit laboratories in efforts to determine the critical contributing residues which contribute to the phosphotransfer abilities and properties. These efforts have been continued in this thesis through investigations of the proposed involvement of Asn12 as an important hydrogen bonding partner for the active center histidine. In addition the absolute requirement for histidine as the phosphotransfer residue of HPr is investigated.

6.4.1 Hydrogen bonding requirements at position 12

In order to specify phosphoryl transfer to the $N^{\delta 1}$ atom of the His15 imidazole ring a hydrogen bond would be predicted to the $N^{\epsilon 2}$ atom. Numerous structural determinations have cited the existence or potential for such an interaction between the side chain of residue 12 and the His15 imidazole. In addition all HPrs have an amino acid at position 12 with the potential for formation of a hydrogen bond (Herzberg *et al.*, 1992; Kalbitzer and Hengstenberg, 1993; Jia *et al.*, 1993a; van Nuland *et al.*, 1995). The Asn12 residue of *E. coli* HPr has recently been reported to form a hydrogen bond with

the $N^{\varepsilon 2}$ atom of His15 in a structural determination in which the active center was free from crystallographic contacts which would influence active center structural properties (Prasad *et al.*, 1998).

The substitution of *E. coli* HPrs Asn12 with either serine or threonine has little influence of the kinetic properties of phosphorylation by Enzyme I. Serine is found at the 12 position of *B. subtilis* HPr and threonine at the 12 position in *S. aureus* and *S. faecalis* HPrs. The removal of hydrogen bonding capabilities through the mutant Asn12Ala *E. coli* HPr has only moderate influence of phosphotransfer capabilities with an approximate four-fold change in k_{cat}/Km. These results suggest that the hydrogen bonding capabilities of the 12 position are not a requirement in the phosphorylation mechanism of *E. coli* HPr. This conclusion is in agreement with the lack of direct evidence in NMR spectra for a hydrogen bond between His15 and residue 12 (van Nuland *et al.*, 1994a, 1995; Jones *et al.*, 1997).

6.4.2 Phosphorylation of *E. coli* His15Asp HPr

In contrast to the permitted flexibility of mutations at position 12 it was anticipated that histidine would be an absolute requirement at position 15. Nevertheless the His15Asp *E. coli* HPr showed retained potential for phosphorylation in the *E. coli* HPr. In retrospect there are numerous observations to predict the success of this mutant.

Undoubtedly a critical factor in retaining phosphorylation ability is the structural similarity of the substituted residue to the original residue. Structural

flexibility of the active center region is likely a further contributing factor. The geometry of aspartic acid and histidine are quite similar with respect to the positioning of points of phosphorylation. As illustrated in Figure 6.1 the carboxyl group of aspartic acid occupies a position similar to the $N^{\delta 1}$ atom of histidine.

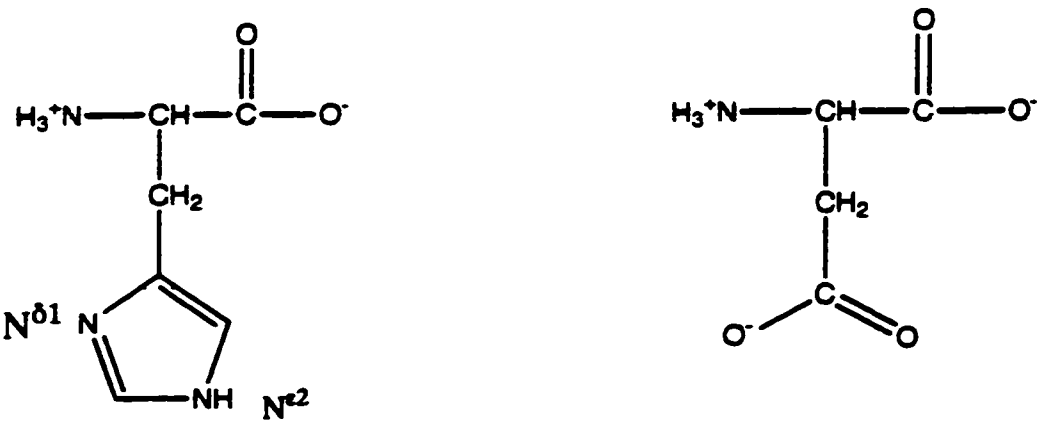


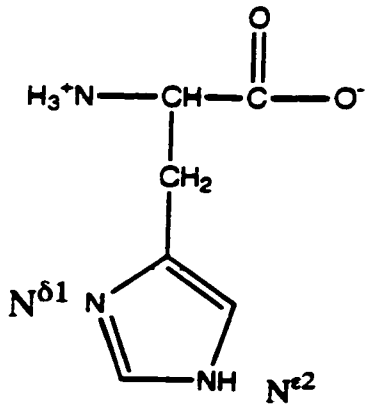
Figure 6.1:Comparison of histidine and aspartate.

Structurally histidine and aspartic acid are quite similar with respect to the position of the site of phosphorylation, $N^{\delta 1}$ of histidine and the carboxyl group of the aspartic acid. The crystallographic determination of the His15Asp *E. coli* HPr at 1.6 Å resolution verifies the similar positioning of the points of phosphorylation of the aspartic acid and histidine side chains at position 15. One of the oxygen atoms of the Asp15 carboxyl group is in essentially the same position as the $N^{\delta 1}$-atom on the His15 imidazole of the wild type structure. In addition the overall conformation of the active center is not significantly influenced by the mutation.

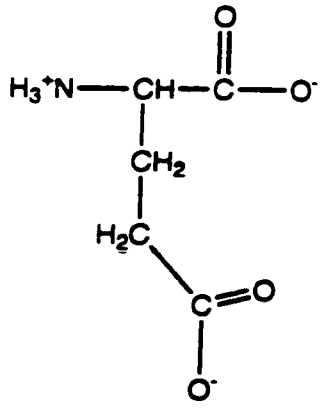
The retained ability for phosphorylation of the His15Asp *E. coli* HPr is also predicted by numerous examples in which phosphotransfer is mediated between histidines and aspartic acid residues. In the bacterial two-component system phosphotransfer signaling systems utilize His-to-Asp phosphotransfer to mediate signal transduction between the sensor and the corresponding regulator (Stock *et al.*, 1989). For example reactions in chemotaxis involve transfer of phosphoryl groups between P-$N^{\varepsilon 2}$-histidne in CheA (Hess *et al.*, 1988) and aspartic acid in CheY (Sanders *et al.*, 1989). Similar His-to-Asp signaling pathways have also been found in eukaryotic species such as Arabidopsis (Imamura *et al.*, 1998).

The ability for Enzyme I to mediate phosphotransfer to and from acyl-phosphates has been demonstrated with the example of acetate kinase. Acetate kinase, in which a γ-glutaminyl phosphate is formed (Todhunter and Purich, 1974), phosphotransfers to enzyme I to form a $N^{\varepsilon 2}$-P-histidine (Fox and Roseman, 1986; Fox *et al.*, 1986).

The success of the His15Asp *E. coli* HPr mutant raises the possibility that perhaps histidine and aspartic acid may be interchangeable as sites of phosphorylation when the histidine phosphorylation is on the $N^{\delta 1}$-atom. Furthermore it is noted that glutamic acid has structural phosphorylation geometry similar to histidines which are phosphorylated at the $N^{\varepsilon 2}$-atom as indicated in Figure 6.2. Perhaps in situations of phosphorylation of the $N^{\varepsilon 2}$–atom of histidine a His-to-Glu mutation might be successful. These possibilities are being investigated in other PTS proteins.



Histidine



Glutamate

Figure 6.2: Comparison of histidine and glutamate.

6.4.3 Abilities and limitations of His15Asp HPr

The typical measure of a mutant's properties comes through analysis of the key kinetic parameters of Km and Vmax. The His15Asp mutant can also be judged on such criteria, although perhaps the more appropriate measure of success of such a dramatic alteration is simply in the retained activity of the protein, a qualitative rather than quantitative standard. With respect to the ability of the His15Asp *E. coli* HPr mutant to act as a substrate for Enzyme I the Km was measured at 66 μM as compared to 6 μM for the wild type protein. The Vmax for

the phosphoryltransfer reaction was measured at a level of 0.1% that of the wild type HPr.

The His15Asp also retains the ability for further phosphotransfer to at least one of the Enzyme IIA proteins. In an isolated *in vitro* assay the mutant showed the ability to phosphorylate the $EIIA^{glucose}$ molecule. Due to the already low rates of phosphorylation of the mutant HPr by Enzyme I in conjuncture with the low rates of transfer to the $EIIA^{glc}$ no quantitative measures of this phosphotransfer could be determined.

The ability of the His15Asp to phosphotransfer to other $EIIA^{sugar}$ proteins was investigated through *in vivo* complementation analysis. Within the sensitivity of the experiment no phosphotransfer activity to any of the other $EIIA^{sugar}$ proteins was detected. The physiological competence will be more closely examined in the future with investigation of phosphotransfer abilities in isolated *in vitro* systems as well as through chromosomal substitution of the His15Asp HPr.

6.5 Other His15 substitutions of *E. coli* HPr

The observation that His15Asp *E. coli* HPr is functional in its ability to accept phosphoryl transfer from Enzyme I, additional substitutions of 15 position with the potential for phosphorylation were created. Amino acid substitutions to the fifteen position were based firstly on their known ability to be phosphorylated: serine, threonine, cysteine and tyrosine and secondly due to structural similarity to histidine and aspartic acid, these choices included asparagine, glutamic acid and glutamine. None of these mutations demonstrated

the ability for phosphorylation by Enzyme I by any of the available methods indicating the uniqueness of the His15Asp mutation.

In many enzyme IIB^{sugar} domains, P-cysteine is found, while those enzymes with an extra IID domain have a $N^{\delta 1}$-P-histidine in the IIB^{sugar} domain suggesting that phosphohistidine is not the only functional possibility. Because His15Cys is not phosphorylated, the HPr active center is not likely to be a model for these IIB domains.

6.6 Structural explanation of the high pI form of His15Asp HPr

The His15Asp HPr mutant provides an interesting lesson in the potential to dramatically alter a key residue while still retaining function. It also provides an important lesson into considerations which need to be taken into account in designing mutants. The initial phosphorylation efforts of the His15Asp HPr mutant demonstrated the appearance of a high pI species of the protein which seemed to emerge as a consequence of phosphorylation. Similar behaviors had been observed in HPr as a consequence of protein aging and were known to involve the formation of succinimide intermediates, which represent the high pI species (Wright, 1991). Cyclization reactions to form succinimides, as illustrated in Figure 6.3, are established for both asparagine and aspartate, and the production of isoaspartyl from the hydrolysis of these cyclic compounds is well documented (Clarke, 1987; Wright *et al.*, 1991; Clarke *et al.*, 1992).

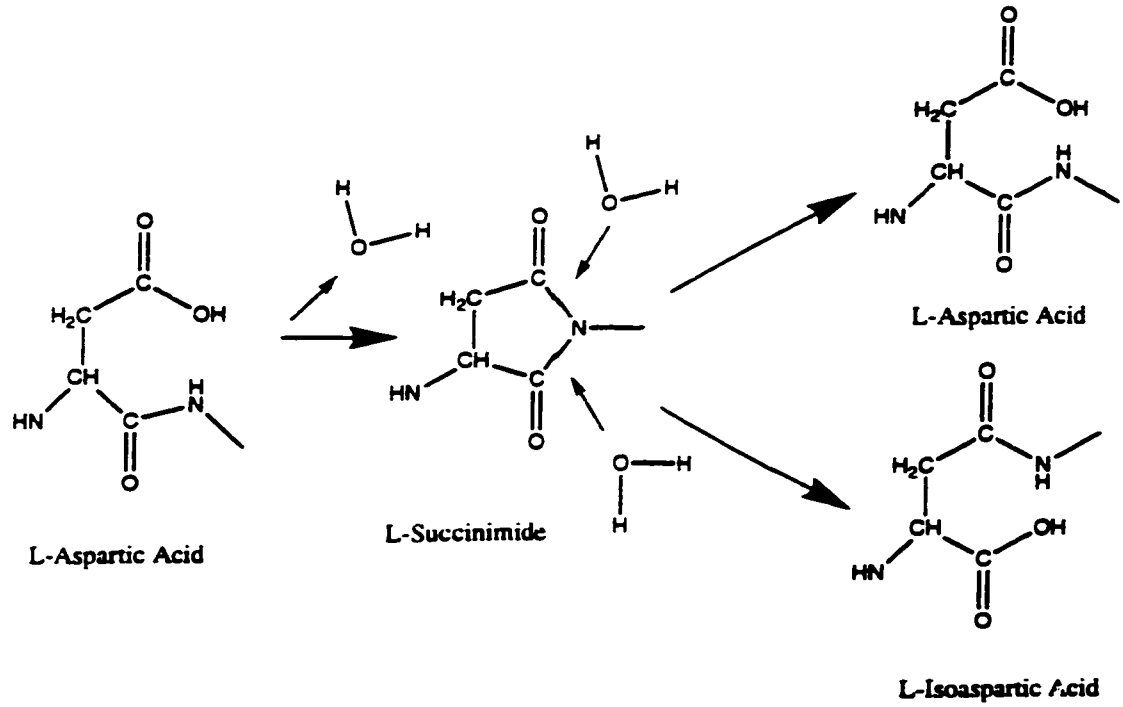


Fig. 6.3 Mechanism of succinimide ring formation.

Isolation of the high pI form of the His15Asp HPr protein demonstrated the similar behavior of this species to that of succinimide containing proteins (Tomizawa *et al.*, 1994; Chazin *et al.*, 1989; Violand *et al.*, 1992), namely the reduction in weight corresponding to the loss of a water molecule and the inability of amino acid sequencing to proceed past the site of internal reactivity. Uncharacteristic, though not unprecedented (Moo-Penn *et al.*, 1976; Wilson *et al.*, 1982), of succinimide investigations was that the break-down of the ringed form failed to result in D or iso forms of the reacting residue. The reverted form of the protein demonstrated equal abilities for further phosphorylation and amino acid

analysis did not indicate the presence of any non-standard residues at position 15. The observation in other proteins, which also failed to produce these non-standard amino acids as a consequence of deamidation, were rationalized by the explanation that steric or electrostatic restraints of the protein prevented the formation of products other than L-aspartic acid (Clarke, 1987).

A second explanation for the inability to produce the predicted products of succinimide cleavage is that the identity of the ring formed is an isoimide rather than succinimide (Clarke, 1987). The mechanism and products of formation of an isoimide from an aspartate residue are presented in Figure 6.4.

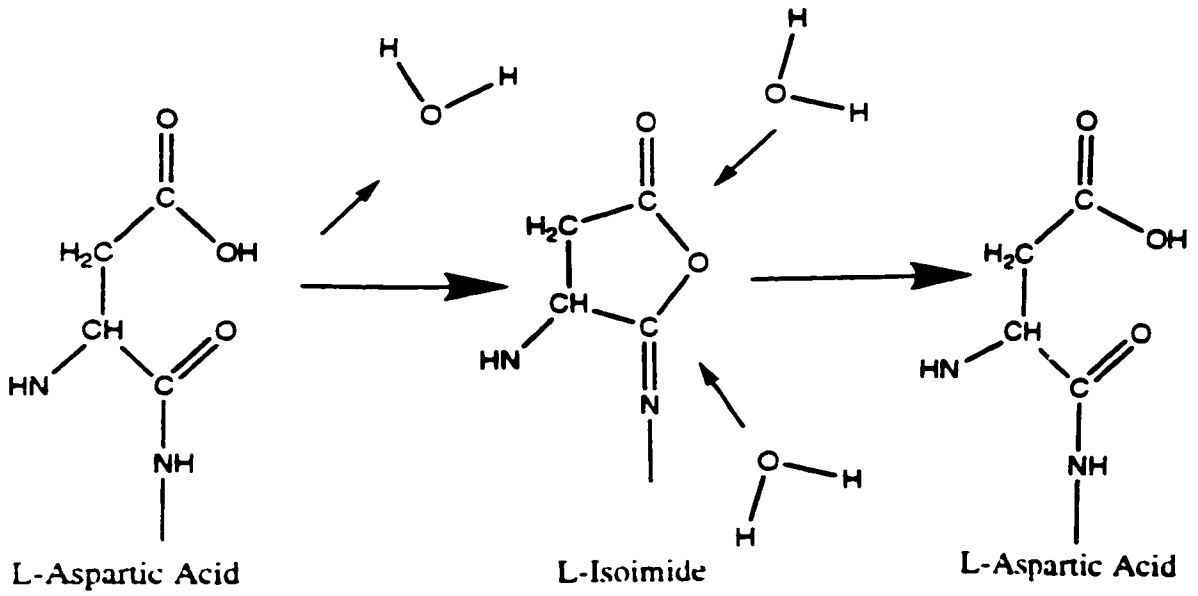


Fig. 6.4 Mechanism of isoimide formation.

Isoimides are possible protein structures which could emerge through a chemical process similar to succinimide formation. While isoimides have been demonstrated in peptides (Clark *et al.*, 1992) they have never been demonstrated in proteins. The primary functional distinction between succinimide and isoimide rings is that the break-down of isoimide rings will produce a single product of L-aspartic acid as would appear to be the case for the His15Asp HPr.

The predicted structural arrangement of a local polypeptide region for isoimide formation is atypical of that normally adopted in protein structures ψ = +60°, χ_1 = +120°. The Asp15 region of His15Asp HPr is not in a conformation predictive of isoimide formation ψ = -170°, χ_1 = +61°. These structural arguments against isoimide formation must be interpreted with some caution. Firstly succinimide formation has been shown to occur in locations which would not be predicted based upon conformational analysis. For example the ideal geometry for succinimide formation has been described as ψ = -120° χ_1 = +60°, Asn12 of HPr which shows considerable deviation from this positioning has been shown to deamidate through succinimide ring formation (Sharma *et al.*, 1993). Secondly the active center region of HPr has been shown to be quite flexible (Jia *et al.*, 1994a). This flexibility would be expected to be particularly evident during phosphorylation which might allow positioning more suitable for isoimide formation.

The occurrence of an isoimide is difficult to experimentally differentiate from a succinimide. The presence of an isoimide in the His15Asp HPr is supported by three circumstantial pieces of evidence.

Firstly, the absence of degradation products outside that of the L-aspartyl residue. The explanation of structural constraints limiting breakdown of succinimides into a single product does not fit with the known flexibility of the active center region of HPr demonstrated by NMR investigations as well as the variances in positioning of side chains in crystallographic determinations (Jia *et al.* 1994a). The active site region surrounding position 15 is exposed to solvent and would allow attack of an incoming water molecule against the succinimide to produce the isoaspartyl product.

Secondly the mutants Asn12Ala His15Asn Asn38Ala and Asn12Ala His15Asp Asn38Ala are unable to undergo ring formation of any kind even under the extremes of pH and temperature which are known to promote succinimide formation. Typically the energetic barriers limiting succinimide formation from aspartates can be overcome with conditions of elevated temperature and pH 5.0. Asparagines favor succinimide formation at elevated temperature and basic pHs. The inability of the Asn12Ala His15Asn Asn38Ala mutant to deamidate under heated, basic conditions indicating that the active center region is not structurally conducive to succinimide formation. This also suggests that the phosphorylation of aspartate 15 is a way by which the cyclization to an isoimide is catalyzed.

6.7 Catalyzed ring formation

Rates of succinimide and presumably isoimide formation from aspartyl residues under physiological conditions typically measure in the time course of weeks to months; the rate of high pI formation in the His15Asp HPr during

phosphorylation occurs in a matter of seconds. The catalyzed ring formation is thought to arise as a direct consequence of phosphorylation. Previous investigations indicate that modification of the carboxyl group of aspartic acid influences the rate of ring formation by providing a better leaving group. The formation of B-methyl ester derivatives has been shown to form succinimides 10,000 to 24,000 times faster than the corresponding unesterified aspartyl residues (Clark *et al.*, 1992). Similarly the phosphoryl group provides a strong leaving group to facilitate the intra-molecular attack.

In wild type HPr, the phosphoryl group bound to the $N^{\delta 1}$-atom of His15 has hydrogen bonding interactions with the amide nitrogens of residues 16 and 17. This has been shown in both *E. coli* and *B. subtilis* HPrs by NMR (van Nuland *et al.*, 1995; Pullen *et al.*, 1995; Jones *et al.*, 1997). It seems reasonable to assume that the same interactions between the phosphoryl group and the amide nitrogens of residues 16 and 17 are occurring in the P-(Asp) His15Asp HPr. This would mean that the main chain amide atom of residue 16, which is normally involved in nucleophilic attack for succinimide formation is rendered unavailable due to involvement in hydrogen bonding to the phosphoryl group. Furthermore the interaction of the phosphoryl group with the main chain amide of position 16 instigates a concerted reaction resulting in the formation of an isoimide structure. The details of the reaction include: a) the phosphoryl group extracts the proton from the amide nitrogen of residue 16; b) this results in the phosphoryl group becomes a better leaving group; and c) that the carbonyl of residue 16 is more reactive in attacking the carbonyl of residue 15 d) which in turn is activated by the

phosphorylation. The proposed concerted reaction for the formation of an isoimide from a phosphorylated aspartate residue is presented in Figure 6.5.

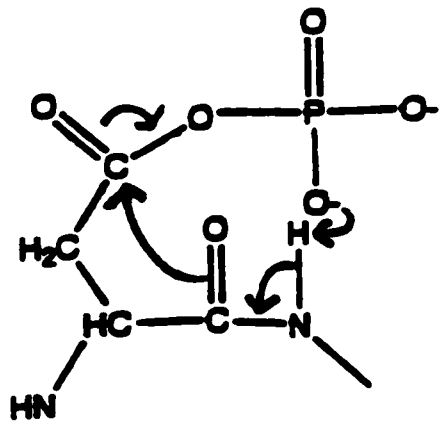


Fig. 6.5: Proposed mechanism for isoimide formation from phosphorylation of aspartate.

Collectively the cumulative evidence and chemical logic of the proposed mechanism provide strong arguments to indicate the presence of an isoimide structure in the HPr protein. This would be the first reported case of a isoimide in the context of a protein.

6.8 Phosphoacyls in Proteins

The proposed mechanism of isoimide formation from a phosphorylated aspartate residue could potentially apply to other proteins which undergo aspartic acid phosphorylation. Protein regulation through phosphorylation of aspartates is present in both prokaryotic and eukaryotic systems. In particular, the two-component system of prokaryotes are modulated in a manner which is dependent

upon the phosphorylation of an aspartic acid residue of the response regulator protein (for review see Stock *et al.*, 1989). Two-component systems consist of a sensor kinase protein which typically autophosphorylates from ATP on a histidine residue. In some systems the sensor kinase is directly responsible for the detection and subsequent phosphorylation response to a given stimulus. In other systems additional sensor proteins may be required for detection of the signal and initiation of the phosphorylation response. The sensor kinase domain, once phosphorylated, then transfers the phosphoryl group onto an aspartic acid residue of the corresponding response regulator. It is interesting to note that the dephosphorylation of these response regulator proteins is primarily achieved through autophosphatase activity (for review see Stock *et al.*, 1989). The rates of dephosphorylation of these proteins is faster than would be expected for an acyl-phosphate indicating that features of the protein are responsible for mediating a catalyzed dephosphorylation. The lifetimes of the phosphorylated forms vary considerably from seconds (Hess *et al.*, 1988) to minutes (Weiss and Magasanik, 1988) to hours (Igo *et al.*, 1989) and the stability of phosphorylation is compatible with physiological function of the individual signal. For example chemotaxis, which requires a rapid response time, is correlated with a phosphorylation signal lifetime which is measured in seconds.

The chemotaxis two-component system of bacteria is one of the most well studied two-component systems. Bacteria govern their swimming behavior in response to environmental stimulus by regulating the frequency of which the flagellar rotation is reversed. By suppressing reversal of the flagellar rotation a

smooth swimming behavior ensues to direct the bacteria towards a stimulus. In contrast by increasing the frequency of reversal of the flagellar rotation "tumbly" swimming redirects bacterial momentum away from negative stimuli (Macnab *et al.*, 1972).

In the chemotaxis system the sensor kinase, CheA, is a multimeric complex on the interior of the cell wall in association with a membrane-spanning receptor. An additional protein, CheW, functions in mediate interaction between CheA and the CheY response regulator. There are a variety of receptors and ligand pairs, such as amino acids, peptides and some sugars, which are involved in regulating bacterial motility. The receptors possess an extracellular domain for ligand binding and an intracellular domain which interacts with the CheA sensor kinase. The intracellular portion of the receptors contain two sites which undergo reversibly methylation at glutamate residues. P-CheB is the active form of an enzyme that demethylates the glutaminyl residues. Binding of an attractant to the extracellular receptor and subsequent methylation of the intracellular domain results in inhibition of CheA autokinase activity. The reduction in autokinase activity of CheA results in reduced levels of P-CheY. P-CheY associates with the flagellar switching apparatus to cause clockwise rotation with subsequent tumbling swimming (for review see Stock *et al.*, 1989). The presence of a stimulant results in a suppression of random swimming and allows the bacteria to swim towards the attractant. A schematic of the chemotaxis system is presented in Figure 6.6.

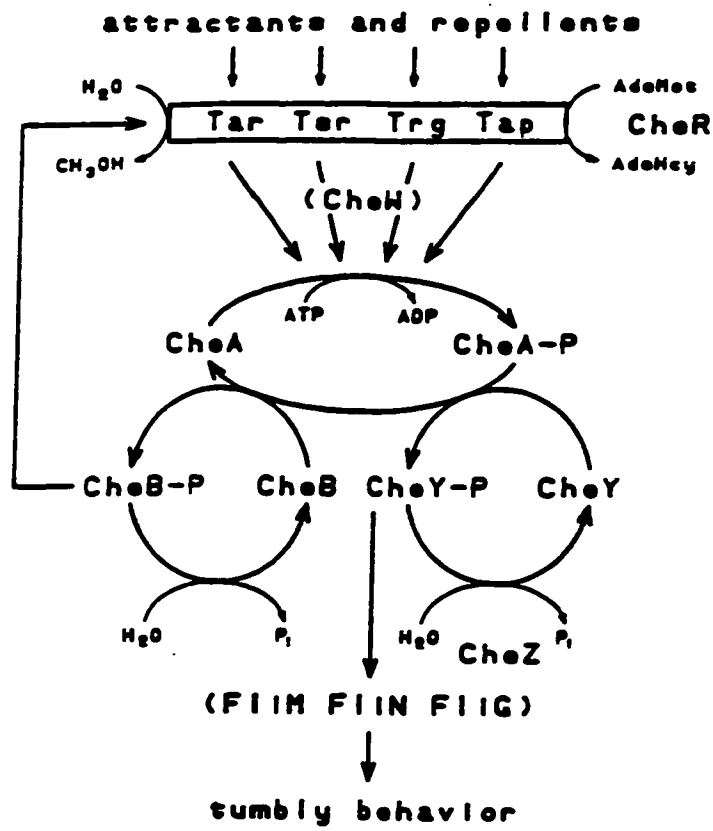


Fig. 6.6: Chemotaxis system of *Escherichia coli*. Diagram from Stock *et al.*, 1989.

P-CheY possesses an inherent autophosphatase activity that is accelerated by the auxiliary protein CheZ. Autophosphatase activities have been reported for other response regulators (for review see Stock *et al.*, 1989). Under physiological conditions CheY-P has a half-life of about 10s (Bourret *et al.*, 1989). An interesting possibility which arises from the studies of the P-Asp in His15Asp HPr is that the autophosphatase activity of the CheY, and perhaps other two-component response regulators, is achieved through the formation of an isoimide ring structure. Formation of an isoimide ring could function as an autocatalytic mechanism for the dephosphorylation of aspartyl residues as a consequence of their intra-molecular reactivity. The subsequent breakdown of the isoimide would

return the original L-aspartyl residue. The structural ability for ring formation from the active site Asp57 of CheY is supported by mutagenic investigations of *E. coli* CheY. The mutation Asp57Asn was created to investigate the possibility of secondary sites of phosphorylation on the CheY protein. The authors report that upon prolonged incubation of this mutant with CheA phosphorylation would occur. This 'secondary' phosphorylation was assumed to occur at an alternate point of phosphorylation on the protein and assumed not to be of physiological importance (Bourret *et al.*, 1990). The Asp57Asn mutation was also created for the *S. typhimurium* CheY protein and a similar secondary phosphorylation event observed (Lukat *et al.*, 1991). An alternative explanation is that the Asp57Asn mutant is undergoing deamidation upon prolonged incubation to reverse the introduced mutation. This would indicate the propensity of ring formation in the active center.

6.9 Future Studies

The possibility that the autophosphatase activity of CheY, and perhaps other response regulators, is achieved through isoimide formation has been considered and preliminary experiments conducted. Mass spectrometric analysis of the CheY protein phosphorylated by acetyl phosphate, an alternative source of phosphoryl groups for CheY, shows the appearance of a species of 18 daltons less than the untreated protein. This apparent loss of a water molecule is consistent with the theory of succinimide or isoimide formation. It is important to note that the dephosphorylation of CheY through intramolecular ring formation would

require that L-aspartic acid be exclusively returned to the active site position to ensure the resultant CheY remains functional. This could only be guaranteed through the formation of an isoimide rather than succinimide structure. Other response regulator proteins will be examined to investigate the possibility of ring formation as a mechanism of autophosphatase activity of the two-component system.

The lifetime of the two-component signal is regulated by the lifetime of phosphorylation of the response regulator proteins. The varying rates of dephosphorylation of the different response regulators will be examined with considerations to sequence context of the phosphorylated aspartate as well as tertiary structural features which may influence rates of ring formation.

The example of His15Asp HPr suggests the ability of aspartate to functionally substitute for histidines which undergoes phosphorylation of the $N^{\delta 1}$-atom. Furthermore, glutamate would seem a reasonable substitute for histidines which undergo phosphorylation of the $N^{\epsilon 2}$-atom. The viability of these substitutions will be addressed in other PTS proteins which undergo phosphorylation at a histidine residue.

References

Ahern, T. J. and Klibanov, A. M. (1985). The Mechanism of Irreversible Enzyme Inactivation at 100°C. Science, 228, 1280-1284.

Anderson, B., Weigel, N., Kundig, W. and Roseman, S. (1971). Sugar Transport. III. Purification and Properties of a Phosphocarrier Protein (HPr) of the Phosphoenolpyruvate-Dependent Phosphotransferase System of *Escherichia coli*. J. Biol. Chem., 246, 7023-7033

Anderson, J. W. (1995). HPr: a Model Protein. Biochem. Cell Biol., 73, 219-222

Anderson, J. W., Bhanot, P., Georges, F., Klevit, R. E. and Waygood, E. B. (1991). Involvement of the Carboxy-Terminal Residue in the Active Site of Histidine-Containing Protein, HPr, of the Phosphenolpyruvate:Sugar Phosphotransferase System of *Escherichia coli*. Biochemistry, 30, 9601-9607.

Anderson, J. W., Pullen, K., Georges, F., Klevit, R. E. and Waygood, E. B. (1993). The Involvement of the Arginine-17 Residue in the Active Site of the Histidine-Containing Protein, HPr, of the Phosphoenolpyruvate:Sugar Phosphotransferase System of *Escherichia coli*. J. Biol. Chem., 268, 12325-12333.

Anderson, J. W., Saier, M. H., Jr., Reizer, J. and Waygood, E. B. (1992). Properties of Phosphorylated Protein Intermediates of the Bacterial Phosphoenolpyruvate:Sugar Phosphotransferase System. Biochem. Cell Biol., 70, 242-246.

Aswad, D. W. and Johnson, B. A. (1987). The Unusual Substrate Specificity of Eukaryotic Protein Carboxyl Methyltransferase. Trends in Biochem. Sci., 12, 155-158.

Barker, S., Matthews, R., Lee, W., Bostock, A. and Burnie, J. (1991). Identification of a Gene Encoding an HPr-Like Protein in *Aspergillus fumigatus*. J. Med. Vet. Mycology, 29, 381-386.

Biggen, M. D., Gibson, J. and Hong, G. F. (1982). Buffer-gradient Gels and ^{35}S-Label as an Aid to Rapid DNA Sequence Determination. Proc. Natl. Acad. Sci. U.S.A., 80, 3963-3965.

Birnboim, H. C. and Doyle, J. (1979). A Rapid Alkaline Extraction Procedure for Screening Recombinant Plasmid DNA. Nucleic Acids Res., 7, 1513

Botsford, J. L. and Harman, J. G. (1992). Cyclic AMP in Prokaryotes. Microbiol. Rev., 56, 100-122.

Bourret, R. B., Hess, J. F. and Simon, M. I. (1990). Conserved Aspartate Residues and Phosphorylation in Signal Transduction by the Chemotaxis Protein CheY. Proc. Natl. Acad. Sci. U.S.A., 87, 41-45.

Brennan, T. V., Anderson, J. W., Jia, Z., Waygood, E. B. and Clarke, S. (1994). Repair of Spontaneously Deamidated HPr Phosphocarrier Protein Catalyzed by the L-Isoaspartate-(D-Aspartate) *O*-Methyltransferase. J. Biol. Chem., 269, 24586-24595.

Brennan, T. V. and Clarke, S. (1993). Spontaneous Degradation of Polypeptides at Aspartyl and Asparaginyl Residues: Effects of the Solvent Dielectric. Protein Science, 2, 331-338.

Brennan, T. V. and Clarke, S. (1995). Effect of Adjacent Histidine and Cysteine Residues on the Spontaneous Degradation of Asparaginyl- and Aspartyl-containing Peptides. Int. J. Peptide Protein Res., 45, 547-553.

Brünger, A.T. (1992). X-PLOR (version 3.1) Manual, Yale University, New Haven, CT 06511, U. S. A.

CCP4 (1979) The SERC (U.K.) Collaborative Computing Project No. 4: A Suite of Programs for Protein Crystallography, Daresbury Laboratory, Warrington, U.K.

Chavin, F., Toptysin, D., Roseman, S. and Brand. L. (1992). Time-resolved Intrinsic Flourescence of Enzyme I. The Monomer/Dimer Transition. Biophys-Chem., 44, 163-173.

Chazin, W. J., Kordel, J., Thulin, E., Hofmann, T., Drakenberg, T. and Forsen, S. (1989). Identification of an Isoaspartyl Linkage Formed Upon Deamidation of Bovine Calbindin D_{9K} and Structural Characterization by 2D ^1H NMR. Biochemistry, 28, 8646-8653.

Chen, Y., Fairbrother, W. J. and Wright, P. E. (1993). Three-Dimensional Structures of the Central Regulatory Proteins of the Bacterial Phosphotransferase System, HPr and Enzyme IIA^{glc}. J. Cell Biochem., 51, 75-82.

Clark, V. M. and Kirby, A. J. (1963). An Improved Synthesis of Phosphoenol Pyruvate. Biochim. Biophys. Acta., 78, 732.

Clarke, S. (1987). Propensity for Spontaneous Succinimide Formation from Aspartyl and Asparaginyl Residues in Cellular Proteins. Int. J. Peptide Protein Res., 30, 808-821.

Clarke, S., Stephenson, R. C. and Lowenson, J. D. (1992). Lability of Asparagines and Aspartic Acid Residues in Proteins and Peptides. Spontaneous Deamidation and Isomerization reactions. In: Stability of Protein Pharmaceuticals. Part A.

Chemical and Physical Pathways of Protein Degradation. Ch. 1. Pg. 1-29. Ahern, T. J. and Manning, M. C., (eds). Plenum Press, New York.

Cleland, W. W. (1963). The Kinetics of Enzyme-Catalyzed Reactions With Two or More Substrates or Products: Nomenclature and Rate Equations. Biochim. Biophys. Acta., 67, 104-137.

Copley, R. R. and Barton, G.J. (1994). A Structural Analysis of Phosphate and Sulfate Binding Sites in Proteins. J. Mol. Biol., 242, 321-329.

Crowle, A. J. and Cline, L. G. (1977). An Improved Stain for Immunodiffusion Tests. J. Immunol. Methods, 17, 379-381.

Csonka, L. N., and Clark, A. V. (1980). Construction of an HPr Strain Useful for Transferring *recA* Mutation Between *Escherichia coli* Strains. J. Bacteriol., 143, 529-530.

De Reuse, H., Roy, A. and Danchin, A., (1985). Analysis of the *ptsH*, *ptsI*, *crr* Region in *Escherichia coli* K-12: Nucleotide Sequence of the *ptsH* Gene. Gene, 35, 199-207.

Deutscher, J., Kessler, V., Alpert, C. A. and Hengstenberg, W. (1984). Bacterial Phosphoenolpyruvate-Dependent Phosphotransferase System: P-Ser-HPr and its Possible Regulatory Function. Biochemistry, 23, 4455-4460.

Deutscher, J. and Saier, M. H., Jr., (1983). ATP-Dependent Protein Kinase-Catalyzed Phosphorylation of a Seryl Residue in HPr, a Phosphate Carrier Protein of the Phosphotransferase System in *Streptococcus pyogenes*. Proc. Natl. Acad. Sci. U.S.A., 80, 6790-6794.

Deutscher, J., Sossna, G. and Treboul, G. G. (1989). Regulatory Functions of the Phosphocarrier Protein HPr of the Phosphoenolpyruvate-Dependent Phosphotransferase System in Gram-Positive Bacteria. FEMS Microbiol. Rev., 63, 167-174.

Deutscher, J. and Sauerwald, H. (1986). Stimulation of Dihydroxyacetone and Glycerol Kinase Activity in *Streptococcus faecalis* by Phosphoenolpyruvate-Dependent Phosphorylation by Enzyme I and HPr of the Phosphotransferase System. J. Bacteriol., 166, 829-836.

Deutscher, J., Reizer, J., Fischer, C., Galinier, A., Saier, M. H., Jr. and Steinmetz, M. (1994). Loss of Protein Kinase Catalyzed Phosphorylation of HPr, a Phosphocarrier Protein of the Phosphotransferase System by Mutation of the *ptsH* Gene Confers Catabolite Repression Resistance to Several Catabolic Genes of *Bacillus subtilis*. J. Bacteriol., 176, 3336-3344

Dorschug, M., Frank, R., Kalbitizer, R. H., Hengstenberg, W. and Deutscher, J. (1984). Phosphoenolpyruvate-Dependent Phosphorylation Site in Enzyme III^{glc} of the *Escherichia coli* Phosphotransferase System. Eur. J. Biochem., 114, 113-119.

Dooijewaard, G., Roosien, F. F. and Robillard, G. T. (1979). *Escherichia coli* Phosphoenolpyruvate-Dependent Phosphotransferase System. Copurification of HPr and 1-6 Glucan. Biochemistry, 18, 2990-2996.

Eisermann, R., Deutscher, J., Treboul, G. G. and Hengstenberg, G. W. (1988). Site-Directed Mutagenesis with the *ptsH* gene of *Bacillus subtillis*. Isolation and Characterization of Heat Stable Proteins Altered at the ATP-Dependent Regulatory Phosphorylation Site. J. Biol. Chem., 263, 17050-17054.

Erni, B. (1989). Glucose Transport in *Escherichia coli*. FEMS Microbiol. Rev., 63, 13-24.

Erni, B. and Zanolari, B. (1986). Glucose Permease of the Bacterial Phosphotransferase System. Gene Cloning, Overproduction and Amino Acid Sequence of Enzyme II^{glc}. J. Biol. Chem., 261, 16398-16403.

Evans, S. V. (1992). SETOR: Hardware-lighted Three-dimensional Solid Model Representations of Macromolecules. J. Mol. Graphics. 11, 134-138.

Fox, D., Meadow, N. D. and Roseman, S. (1986) Phosphate Transfer Between Acetate Kinase and Enzyme I of the Bacterial Phosphotransferase System. J. Biol. Chem., 261, 13498-13503.

Fox, D. K. and Roseman, S. (1986). Isolation and Characterization of Homogenous Acetate Kinase from *Salmonella typhimurium* and *Escherichia coli*. J. Biol. Chem., 261, 13487-13497.

Fu, J. C., Ding, L. and Clarke, S. (1991). Purification, gene cloning, and Sequence Analysis of an L-isoaspartyl Protein Carboxyl Methyltransferase from *Escherichia coli*. J. Biol. Chem., 266, 14562-14572.

Gassner, M., Stehlik, D., Schrecker, O., Hengstenberg, W., Maurer, W. and Ruterjans, H. (1977). The Phosphoenolpyruvate-dependent Phosphotransferase System of *Staphylococcus aureus*. ^{1}H and ^{31}P Nuclear-magnetic Resonance Studies on the Phosphocarrier Protein HPr, Phosphohistidines and Phosphorylated HPr. Eur. J. Biochem., 75, 287-296.

Garrett, D. S., Seok, Y., Peterkofsky, A., Gronenborn, A.M. and Clore, G.M. (1999). Solution structure of the 40,000 Mr Phosphoryl Transfer Between the N-terminal Domain of Enzyme I and HPr. Nature Struct. Biol., 6, 166-173.

Garrett, D.S., Seok, Y., Liao, D I., Peterkofsky, A., Gronenborn, A. M. and Clore, G. M. (1997). Solution structure of the 30 kDa N-terminal Domain of Enzyme I of the *Escherichia coli* phosphoenolpyruvate:sugar phosphotransferase System by Multidimensional NMR. Biochemistry, 36, 2517-2530.

Geiger, T. and Clarke, S. (1987). Deamidation, Isomerization, and Racemization at Asparaginyl and Aspartyl Residues in Peptides. J. Biol. Chem., 262, 785-794.

Gösseringer, R., Küster, E., Galinier, A., Deutscher, J. and Wolfgang, H. (1997). Cooperative and Non-cooperative DNA Binding Modes of Catabolite Control Protein CcpA from *Bacillus megaterium* Result from Sensing Two Different Signals. J. Mol. Biol., 266, 665-676.

Greese, R. H., Izzo, F. and Postma, P. W. (1989). The PEP:Fructose Phosphotransferase System in *Salmonella typhimurium*: FPr Combines Enzyme III^{fru} and Pseudo-HPr Activities. Mol. Gen. Genet., 216, 517-525.

Han, M. K., Knutson, J. R., Roseman, S. and Brand, L. (1990). Sugar Transport by the Bacterial Phosphotransferase System. Flouresence Studies of Sub-units Interactions of Enzyme I. J. Biol. Chem., 265, 1996-2003.

Harwood, J. P., Gazdar, C., Prasad, C., Peterkofsky, S. J., Curtis, S. J. and Epstein, W. (1976). Involvement of the Glucose Enzymes II of the Sugar Phosphotransferase System in the Regulation of Adenylate Cyclase by Glucose in *Escherichia coli*. J. Biol. Chem., 251, 2462-2468.

Herzberg, O. (1992a). An Atomic Model for Protein-Protein Phosphoryl Group Transfer. J. Biol. Chem., 34, 24819-24823.

Herzberg, O., Chen, C. C., Kapadia, G., McGuire, M., Carroll, L. J., Noh, S. J. and Dunaway-Mariano, D. (1996). Swiveling-domain Mechanism for Enzymatic

Phosphotransfer Between Remote Reaction Sites. Proc. Natl. Acad. Sci. U. S. A., 93, 2652-2657.

Herzberg, O., Reddy, P., Reizer, J. and Kapadia, G. (1992b). Structure of the Histidine-Containing Phosphocarrier Protein HPr from *Bacillus subtilis* at 2.0 Å Resolution. Proc. Natl. Acad. Sci. U.S.A. 89, 2499-2503.

Hess, J. F., Bourret, R. B. and Simon, M. I. (1988a) Histidine Phosphorylation and Phosphoryl Group Transfers in Bacterial Chemotaxis. Nature, 336, 138-143.

Hess, J. F., Oosawa, K., Kaplan, N. and Simon, M. I. (1988b). Phosphorylation of Three Proteins in the Signaling Pathway of Bacterial Chemotaxis. Cell, 53, 79-87

Hol, W. (1985). The Role of the α–Helix Dipole in Protein Function and Structure. Prog. Biophy. Molec. Biol., 45, 149-195.

Hoving, H., Koning, J. H. and Robillard, G. T. (1981). *Escherichia coli* Phosphoenolpyruvate-Dependent Phosphotransferase System: Equilibrium Kinetics and Mechanism of Enzyme I Phosphorylation. Biochemistry, 20, 87-93.

Hultquist, D. E. (1968). The Preparation and Characterization of the Phosphorylated Derivatives of Histidine. Biochim. Biophys. Acta., 153, 329-340.

Hultquist, D., Moyer, R. W. and Boyer, P. D., (1966). The Preparation and Characterization of 1-Phosphohistidine and 3-Phosphohistidine. Biochemistry, 5, 322-331.

Hummel, U., Nuoffer, C., Zanolari, B. and Erni, B. (1992). A Functional Protein Hybrid Between the Glucose Transporter and the N-acetylglucosamine Transporter of *Escherichia coli*. Protein Science, 1356-1362.

Hurley, J. H., Dean, A. M., Thorsness, P. E., Koshland, D. E., Jr. and Stround, R. M. (1990). Regulation of Isocitrate Dehydrogenase by Phosphorylation Involves No Long-range Conformational Change in the Free Enzyme. J. Biol. Chem., 265, 3599-3602.

Igo, M. M., Ninfa, A. J., Stock, J. B. and Silhavy, T. J. (1989). Phosphorylation and Dephosphorylation of a Bacterial Transcription Activator by a Transmembrane Receptor. Genes Dev., 3, 1725-1734.

Imamura, A., Hanaki, N., Umeda, H., Nakamura, A., Suzuki, T., Ueguchi, C. and Mizuno, T. (1998). Response Regulators Implicated in His-to-Asp Phosphotransfer Signaling in *Arabidopsis*. Proc. Natl. Acad. Sci. U.S.A., 95, 2691-2696.

Ingrosso, D., Fowler, A. V., Bleibaum, T. and Clark, S. (1989). Sequence of the D-aspartyl/L-isoaspartyl Protein Methyltransferase from Human Erythrocytes. J. Biol. Chem., 264, 20131-20139.

Jia, Z. (1992). Crystallographic Studies of HPr from *Streptoccus faecalis* and *Escherichia coli*. Ph. D. thesis, University of Saskatchewan.

Jia, Z., Vandonselaar, M., Hengstenberg, W., Quail, J. W. and Delbaere, L. T. J. (1994a). The 1.6 Å Structure of Histidine-containing phosphotransfer protein HPr from *Streptococcus faecalis*. J. Mol. Biol., 236, 1241-1355.

Jia, Z., Vandonselaar, M., Quail, J. W. and Delbaere, L. T. J. (1993a). Active-Centre Torsion-Angle Strain Revealed in 1.6 Å Resolution Structure of Histidine-Containing Phosphocarrier Protein. Nature, 361, 94-97.

Jia, Z., Quail, J. W., Waygood, E. B. and Delbaere, L. T. J. (1993b). The 2.0 Å Resolution Structure of *Escherichia coli* Histidine-Containing Phosphocarrier Protein HPr. A Redetermination. J. Biol. Chem., 268, 22490-22501.

Jia, Z., Quail, J. W., Waygood, E. B., Delbaere, L. T. J. and Waygood, E. B. (1994b). Structural comparison of the Histidine-Containing Phosphocarrier Protein HPr. Biochem. Cell Biol., 72, 202-217.

Jones, B. E., Dossonnet, V., Küster, E., Hillen, W., Deutscher, J. and Klevit, R. E. (1997a). Binding of the Catabolite Repressor Protein CcpA to its DNA Target is Regulated by Phosphorylation of its Corepressor HPr. J. Biol. Chem., 272, 26530-26535.

Jones, B. E., Rajagopal, P. and Klevit, R. E. (1997b). Phosphorylation on Histidine is Accompanied by Localized Structural Changes in the Phosphocarrier Protein, HPr from *Bacillus subtilis*. Protein Science, 6, 2107-2119.

Kagan, R. M., Niewmierzycka, A. and Clarke, S. (1997). Targetted Gene Disruption of the *Caenorhabditis elegans* L-isoaspartyl Protein Methyltransferase Impairs Survival of Dauer Stage Nematodes. Arch. Biochem. and Biophys., 348, 320-328.

Kalbitzer, H. R. and Hengstenberg, W. (1993). The Solution Structure of the Histidine-Containing Protein (HPr) from *Staphyloccus aureus* as Determined by Two-Dimensional ^{1H}NMR Spectroscopy. Eur. J. Biochem., 216, 205-214.

Kalbitzer, H. R., Hengstenberg, W., Rosch, P., Muss, P., Bernsmann, P., Engelmann, R., Dorschug, M. and Deutscher, J. (1982). HPr Proteins of Different Organisms Studied by Hydrogen-1-High Resolution of Nuclear Magnetic Resonance: Similarities of Structures and Mechanisms. Biochemistry, 21, 2879-2885.

Kim, E., Lowenson, J. D., MacLaren, D. C., Clarke, S. and Young, S. G. (1997). Deficiency of a Protein-repair Enzyme Results in the Accumulation of Altered Proteins, Retardation of Growth, and Fatal Seizures in Mice. Proc. Natl. Acad. Sci. U.S.A., 94, 6132-6137.

Klevit, R. E. and Waygood, E. B. (1986). Two-Dimensional 1H NMR Studies of Histidine-containing Protein from *Escherichia coli*. Biochemistry, 25, 7774-7781.

Knowles, J. R. (1980). Enzyme Catalyzed Phosphoryl Transfer Reactions. Annu. Rev. Biochem., 49, 877-919.

Kobe, K., Jennings, I. G., House, C. M., Michell, B. J., Goodwill, K. E., Santarsiero, Stevens, R. C., Cotton, R. G. H. and Kemp., B. E. (1999). Structural Basis of Autoregulation of Phenylalanine Hydroxylase. Nat. Struct. Biol., 6, 442-448.

Kornberg, H. L. and Watts, P. D. (1978). Roles of *crr*-gene Products in Regulating Carbohydrate Uptake by *Escherichia coli*. FEBS Lett., 104, 313-316.

Kornberg, H. L., Watts, P. D. and Brown, K. (1980). Mechanisms of Inducer Exclusion by Glucose. FEBS Lett., 1717, K28-K36.

Kossiakoff, A. A. (1988). Tertiary Structure is a Principal Determinant to Protein Deamidation. Science, 248, 191-194.

Kundig, W. S., Ghosh, S. and Roseman, S. (1964). Phosphate Bound to a Histidine in a Protein as an Intermediate in a Novel Phosphotransferase System. Proc. Natl. Acad. Sci. U.S.A., 52, 1067-1074.

Kundig, W. S. and Roseman, S. (1971). Sugar Transport: Isolation of a Phosphotransferase System from *Escherichia coli*. J. Biol. Chem., 246, 1393-1406.

Laemmli. L. (1970). Cleavage of Structural Proteins During the Assembly of the Head of Bacteriophage T4. Nature, 227, 680-685.

Landt, O., Grunert, H. P. and Hahn, U. (1990). A General Method for Rapid Site-Directed Mutagenesis using the Polymerase Chain Reaction. Gene, 96, 125-128.

Lee, L. G., Britton, P., Parra, F., Boronet, A. and Kornberg, H. L. (1982). Expression of the *ptsH*$^{-}$ Gene of *Escherichia coli* Cloned on Plasmid pBR322. FEBS Lett., 149, 288-292.

Levy, S., Zeng, G. Q. and Danchin, A. (1990). Cyclic AMP Synthesis in *Escherichia coli* Strains Bearing Known Deletions in the *pts* Phosphotransferase Operon. Gene, 31, 27-33.

Lengeler, J. and Kahreis, K. (1996). Phosphotransferase Systems or PTSs as Carbohydrate Transport and as Signal Transduction Systems. *In*: Handbook of Biological Physics. Vol. 2, Ch. 25, pp 573-598. Konings, W.N., Kaback, H.R. and Lolkema, J.S. (eds). Elsevier Science B.V.

Li, C. and Clarke, S. (1992). A Protein Methyltransferase Specific for Altered Aspartyl Residues is Important in *Escherichia coli* Stationary-Phase Survival and Heat-shock Resistance. Proc. Natl. Acad. Sci. U.S.A., 89, 9885-9889

Liao, D. I., Silverton, E., Seok, Y. J., Lee, B. R., Peterkofsky, A. and Davies, D. R. (1996). The First Step in Sugar Transport: Crystal Structure of the Amino Terminal Domain of Enzyme I of the *E. coli* PEP:sugar Phosphotransferase

System and a Model of the Phosphotransfer Complex with HPr. Structure, 4, 861-872.

Liao, D. and Herzberg, O. (1994). Refined Structures of the Active Ser83Cys and impaired Ser46Asp Histidine-containing phosphocarrier proteins. Structure, 2, 1203-1216.

LiCalsi, C., Crocenzi, T. S., Frire, E. and Roseman, S. (1991). Sugar Transport by the Bacterial Phosphotransferase System: Structural and Thermodynamic Domains of Enzyme I of *Salmonella typhimurium*. J. Biol. Chem., 266, 19519-19527.

Lin, K., Rath, V. L., Dai, S.C., Fletterick, R. J. and Hwang, P.K. (1996). A Protein Phosphorylation Switch at the Conserved Allosteric Site in GP. Science, 273,1539-1541.

Lukat, G. S., Lee, B. H., Mottonen, J. M., Stock, A. M. and Stock, J. B. (1991). Roles of the Highly Conserved Aspartate and Lysine Residues in the Response Regulator of Bacterial Chemotaxis. J. Biol. Chem., 266, 8348-8354.

Lukat, G. S. and Stock, J. (1993). Response Regulation in Bacterial Chemotaxis. J. Cell Biochem., 51, 41-46.

Lura, R. and Schirch, V. (1988). Role of Peptide Conformation in the Rate and Mechanism of Deamidation of Asparaginyl Residues. Biochemistry, 27, 7671-7677.

Macnab, R. M. and Koshland, D. E. Jr. (1972). The Gradient Sensing Mechanisms in Bacterial Chemotaxis. Proc. Natl. Acad. Sci. U.S.A., 69, 2509-2512.

Makman, R. S. and Sutherland, E. W. (1965). Adenosine 3-5'-Phosphate in *Escherichia coli*. J. Biol. Chem., 240, 1309-1314.

Maniatis, T., Fritsch, E. F., and Sambrook, J. (1982). Molecular Cloning: A Laboratory Manual. Cold Springs Harbor Lab., Cold Springs Harbor, NY.

Martin-Verstraete, I., Debarbouille, M., Klier, A. and Rapoport, G. (1990). Levanase Operon of *Bacillus subtilis* Includes a Fructose-specific Phosphotransferase System Regulating Expression of the Operon. J. Mol. Biol., 214, 657-671.

Mattoo, R. L., Khandelwahl, R. L. and Waygood, E. B. (1984). Isoelectrophoretic Separation and the Detection of Soluble Proteins Containing Acid Labile Phosphate: Use of the Phosphoenolpyruvate: Sugar Phosphotransferase System as a Model System for N^1-P-Histidine and N^3-P-Histidine Containing Proteins. Anal. Biochem., 139, 1-16.

McFadden, P. N. and Clarke, S. (1987). Conversion of Isoaspartyl Peptides to Normal Peptides: Implications for the Cellular Repair of Damaged Proteins. Proc. Natl. Acad. Sci. U.S.A., 84, 2595-2599.

McGuire, M., Huang, K., Kapadia, G., Herzberg, O. and Dunaway-Mariano., P. (1998). Location of the Phosphate Binding Site with *Clostridium symbiosum* Pyruvate Phosphate Dikinase. Biochemistry, 37, 13463-13474.

Meadow, N. D., Fox, D. K. and Roseman, S. (1990). The Bacterial Phosphoenolpyruvate:Glucose Phosphotransferase System. Annu. Rev. Biochem., 59, 497-542.

Meadow, N. D. and Roseman, S. (1982). Sugar Transport by the Bacterial Phosphotransferase System. J. Biol. Chem., 257, 14526-14537.

Miller, J. H. (1972). Experiments in Molecular Genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York

Misset, O., Brouwer, M. and Robillard, G. T. (1980). *Escherichia coli* Phosphoenolpyruvate-Dependent Phosphotransferase System. Evidence that the Dimer is the Active form of the Enzyme I. Biochemistry, 19, 833-890.

Monod, J. (1942). Recherches sur la croissance des cultures bacteriennes. PhD thesis. Paris: Hermann

Moo-Penn, W. F., Jue, D. L., Bechtel, K. C., Johnson, M. H. and Schmidt, R. M. (1976). Hemoglobin Providence. A Human Hemoglobin Variant Occurring in Two Forms *In Vivo*. J. Biol. Chem., 251, 7557-7562.

Mudgett, M. B. and Clarke, S. (1994). Hormonal and Enviromental Responsiveness of a Developmentally Regulated Protein Repair L-isoaspartyl Methyltransferase in Wheat. J. Biol. Chem., 269, 25605-25612.

Napper, S., Anderson, J. W., Georges, F., Quail, J. W., Delbaere, L. T. J. and Waygood, E. B. (1996). Mutation of Serine-46 to Aspartate in the Histidine-Containing Protein of *Escherichia coli* Mimics the Inactivation by Phosphorylation of Serine-46 in HPrs from Gram-Positive Bacteria. Biochemistry, 35, 11260-11267.

Napper, S., Delbaere, L. T. J. and Waygood, E. B. (1999). The Aspartyl Replacement of the Active Site Histidine-containing Protein, HPr, of the *Escherichia coli* Phosphoenolpyruvate:Sugar Phosphotransferase System can Accept and Donate a Phosphoryl Group. J. Biol. Chem., Accepted for Publication (May, 1999)

Nicholls, A., Sharp, K. and Honing, B. (1992). GRASP Manual, Columbia University, New York, New York

Nixon, B. T., Ronson, C. W. and Ausubel, F. M. (1986). Two-component Regulatory Systems Responsive to Environmental Stimuli Share Strongly Conserved Domains with the Nitrogen Assimilation Regulatory Genes *ntrB* and *ntrC*. Proc. Natl. Acad. Sci. U.S.A., 83, 7850-7854.

Noguchi, S., Miyawaki, K. and Satow, Y. (1998). Succinimide and Isoaspartate Residues in the Crystal Structures of Hen Egg-white Lysozyme Complexed with Tri-N-acetylchitotriose. J. Mol. Biol., 278, 231-238.

Nelson, S. O., Wright, J. K. and Postma, P. W. (1983). The Mechanism of Inducer Exclusion. Direct Interaction Between Purified III^{glc} of the Phosphoenolpyruvate:Sugar Phosphotransferase System and the Lactose Carrier of *Escherichia coli*. EMBO J., 2, 715-720.

Novotny, M. J., Frederickson, W. L., Waygood, E. B. and Saier, M. H., Jr. (1985). Allosteric Regulation of Glycerol Kinase by Enzyme III^{glc} of the Phosphotransferase System in *Escherichia coli* and *Salmonella typhimurium*. J. Bacteriol., 162, 810-816.

Nuoffer, C., Zanolari, B. and Erni, B. (1988). Glucose Permease of *Escherichia coli*. The Effect of Cysteine to Serine Mutations on the Function, Stability and Regulation of Transport and Phosphorylation. J. Biol. Chem., 263, 6647-6655.

O'Connor, C. M. (1987). Regulation and Subcellular Distribution of a Protein Methyltransferase and Its Damaged Aspartyl Substrate Sites in Developing *Xenopus* Oocytes. J. Biol. Chem., 21, 10398-10403.

O'Connor, C. M., Aswad, D. W. and Clarke, S. (1984). Mammalian Brain and Erythrocyte Carboxyl Methyltransferase are Similar Enzymes that Recognize both D-Aspartate and L-iso-Aspartate Residues in Structurally Altered Protein Substrates. Proc. Natl. Acad. Sci. U.S.A., 81, 7757-7761.

O'Connor, C. M. and Clarke, S. (1985). Specific Recognition of Altered Polypeptides by Widely Distributed Methyltransferase. Biochem. Biophys. Res. Acta., 132, 1144-1150.

Osumi, T. and Saier, M. H., Jr. (1982). Regulation of Lactose Permease Activity by the Phosphoenolpyruvate:Sugar Phosphotransferase System: Evidence for Direct Binding of the Glucose-Specific Enzyme III to the Lactose Permease. Proc. Natl. Acad. Sci. U.S.A., 79, 1457-1462.

Ota, I. M. and Clarke, S. (1989). Enzymatic Methylation of L-Isoaspartyl Residues Derived from Aspartyl Residues in Affinity-purified Calmodulin. J. Biol. Chem., 264, 54-60.

Ota, I. M., Ding, L. and Clarke, S. (1987). Methylation of Specific Altered Asparatate and Asparagine Residues in Glucagon by the Erythrocyte Protein Carboxylase Methyl Transferase. J. Biol. Chem., 262, 8522-8531.

Otwinowski, Z. (1993). Oscillation Data Reduction Program. In Data Collection and Processing: Proceedings of the CCP4 Study Weekend, 29-30 January, 1993. (Sawyer, L., Issacs, N. and Bailey, S. eds.), pp. 56-62, Science and Engineering Research Council, Daresbury Laboratory, Warrington, UK.

Paigen, K. and Williams, B. (1970). Catabolite Repression and Other Control Mechanisms in Carbohydrate Utilization. Adv. Microbiol. Physiol., 4, 251-324.

Parkinson, J. S. and Kofoid, E. C. (1992). Communication Modules in Bacterial Signaling Proteins. Annu. Rev. Genet., 26, 71-112.

Pas, H. H., Meyer, G. H., Kruizinga, W. H., Tamminga, K. S., van Weeghel. R. P. and Robillard, G. T. (1991). ^{31}Phospho-NMR Demonstration of Phosphocysteine as a Catalytic Intermediate on the *Escherichia coli* Phosphotransferase System Enzyme II^{mtl}. J. Biol. Chem., 266, 6690-6692.

Pelton, J., Torchia, D., Meadow, N.D. and Roseman, S. (1992). Structural Comparisons of Phosphorylated and Unphosphorylated Forms of III^{glc}, a Signal Transducing Protein from *Escherichia coli*, Using Three-Dimensional NMR Techniques. Biochemistry, 31, 5215-5224.

Peterkofsky, A., Svenson, I. and Amin, N. (1989). Regulation of *Escherichia coli* Adenylate Cyclase Activity by the Phosphoenolpyruvate:Sugar Phosphotransferase System. FEMS Micobiol. Rev., 63, 103-108.

Pieper, U., Kapadia, G., Zhu, P. P., Peterkofsky, A. and Herzberg, O. (1995). Structural Evidence for Evolutionary Divergence of *Mycoplasma* from Gram-positive Bacteria: the Histidine-containing Phosphocarrier Protein Structure. Structure, 3, 781-790.

Postma, P. W., Epstein, W., Schuitema, A. R. J. and Nelson, S. O. (1984). Interaction Between III^{glc} of the Phosphoenolpyruvate: Sugar Phosphotransferase System and Glycerol Kinase of *Salmonella typhimurium*. J. Bacteriol., 158, 351-353.

Postma, P. W., Lengler, J. W. and Jacobson, G. R. (1993). Phosphoenolpyruvate:Carbohydrate Phosphotransferase System in Bacteria. Microbiol. Rev., 57, 543-594.

Prasad, L., Waygood, E. B., Lee, J. S. and Delbaere, L. T .J. (1998) The 2.5 Å Resolution Structure of the Jel 42 Fab Fragment/HPr Complex. J. Mol. Biol., 280, 829-845.

Presper, K .A., Wong, C. Y. and Liu, L. (1989). Site-Directed Mutagenesis of the Phosphocarrier Protein, III^{glc}, a Major Signal-Transducing Protein in *Escherichia coli*. Proc. Natl. Acad. Sci. U.S.A., 86, 4052-4055.

Pullen, K., Rajagopal, P., Branchini, B. R., Huffine, M. E., Reizer, J., Saier, M. H., Jr., Schotlz, J. M. and Klevit, R. E. (1995). Phosphorylation of Serine-46 in HPr, a Key Regulatory Protein in Bacteria, Results in Stabilization of its Solution Structure. Protein Science, 4, 2478-2486.

Quillardet, P. and Hofnung, M. (1988). Ethidium Bromide and Safety-Readers suggest Alternative Solutions. Letter to Editor. Trends Genet., 4, 89-90

Rajagopal, P., Waygood, E. B. and Klevit, R. E. (1994). Structural Consequences of Histidine Phosphorylation. NMR Characterization of the Phosphohistidine form of Histidine-containing Protein from *Bacillus subtilis* and *Escherichia coli*. Biochemistry, 33, 15271-15282.

Reizer, J., Hoischen, C., Reizer, A., Pham, T. N. and Saier, M. H. Jr. (1993). Sequence Analyses and Evolutionary Relationships Among the Energy-Coupling Proteins Enzyme I and HPr of the Bacterial Phosphoenolpyruvate:sugar Phosphotransferase System. Protein Sci., 2, 506-521.

Reizer, J. W., Novotny, M. J., Hengstenberg, W. and Saier, M. H., Jr. (1984). Properties of ATP-Dependent Protein Kinase from *Streptococcus pyrogenes* that Phosphorylates a Seryl Residue in HPr, a Phosphocarrier Protein of the Phosphotransferase System. J. Bacteriol., 160, 333-340.

Reizer, J., Peterkofsky, A. and Romano, A. H. (1988). Evidence for the Presence of Heat Stable Protein (HPr) and ATP-Dependent HPr Kinase in Heterofermentative *Lactobacilli* Lacking Phosphoenolpyruvate:Glycose Phosphotransferase Activity. Proc. Natl. Acad. Sci. U.S.A., 85, 2041-2045.

Reizer, J., Romano, A. H. and Deutscher, J. (1993). The Role of Phosphorylation of HPr, a Phosphocarrier Protein of the Phosphotransferase System in the Regulation of Carbon Metabolism in Gram-Positive Bacteria. J. Cell Biochem., 51, 19-24.

Reizer, J., Sutrina, S. L., Saier, M. H., Jr., Stewart, G. C., Peterkofsky, A. and Reddy, P. (1989). Mechanistic and Physiological Consequences of HPr (Ser) Phosphorylation on the Activities of the Phosphoenolpyruvate:Sugar Phosphotransferase System in Gram-Positve Bacteria: Studies with Site-Specifc Mutants of HPr. EMBO J., 8, 2111-2120.

Reizer, J., Sutrina, S. L., Wu, L., Deutscher, J., Reddy, P. and Saier, M. H., Jr. (1992). Functional Interactions between Proteins of the Phosphoenolpyruvate:sugar Phosphotransferase Systems of *Bacillus subtilis* and *Escherichia coli*. J. Biol. Chem., 267, 9158-99169.

Robinson, A. B. and Rudd, C. J. (1974). Deamidation of Glutaminyl and Asparanginyl Residues in Peptides and Proteins. Curr. Top. Cell. Regul., 8,247-294.

Robinson, A. B., McKerrow, J. H. and Legaz, M. (1974). Sequence Dependent Deanidation Rates for Model Peptides of Cytochrome c. Int. J. Peptide Protein Res., 6, 31.

Roseman, S. and Meadow, N. D. (1990). Signal Transduction by the Bacterial Phosphotransferase System. J. Biol. Chem., 265, 2993-2996.

Russel, C. B., Thaler, D. S. and Dahlquist, F. W. (1989) Chromosomal Transformation of *Escherichia coli recA* Strains with Linearized Plasmids. J. Bacteriol., 171, 2609-2613.

Saier, M. H., Jr., (1989). Protein Phosphorylation and Allosteric Control of Inducer Exclusion and Catabolite Repression by the Bacterial Phosphoenolpyruvate:Sugar Phosphotransferase System. Microbiol. Rev., 53, 109-120.

Saier, M. H., Jr., (1993). Regulatory Interactions Involving the Proteins of the Phosphotransferase System in Enteric Bacteria. J. Cell. Biochem., 51, 62-68.

Saier, M. H., Jr., Chauvaux, S., Cook, M. G., Deutscher, J., Paulsen, T. I., Reizer, J. and Ye, J. (1996). Catabolite Repression and Inducer Control in Gram-positive Bacteria. Microbiology, 142, 217-230.

Saier, M. H., Jr. and Roseman, S. (1972). Inducer Exclusion and Repression of Enzyme Synthesis in Mutants of *Salmonella typhimurium* Defective in Enzyme I of the Phosphoenol:Sugar Phosphotransferase System. J. Biol. Chem., 247, 972-975.

Saier, M. H., Jr. and Roseman, S. (1976). Sugar Transport. Inducer Exclusion and Regulation of the Melibiose, Glycerol, and Lactose Transport Systems by the Phosphoenolpyruvate: Sugar Phosphotransferase System. J. Biol. Chem., 251, 6606-6615.

Saier, M. H., Jr., Schimdt, M. H. and Lin, P. (1980). Phosphoryl Exchange Reaction Catalyzed by the Enzyme I of the Bacterial Phosphoenolpyruvate:sugar Phosphotransferase System. J. Biol. Chem., 245, 5870-5873.

Saier, M. H., Jr., and Reizer, J. (1992). Proposed Uniform Nomenclature for the Protein Domains of the Bacterial Phosphoenolpyruvate:sugar Phosphotransferase System. J. Bacteriol., 174, 1433-1438.

Saier, M. H., Jr., Yamada, M., Erni, B., Serda, K., Lengeler, J., L Ebner, R., Argos, P., Rak, B., Schetz, K., Lee, C. A., Stewart, G. C., Breidt, F., Jr., Waygood, E. B., Peri, K. G. and Doolittle, R. F. (1988). Sugar Permeases of the Bacterial Phosphoenolpyruvate-Dependent Transferase System: Sequence Comparison. FASEB J., 2, 199-208.

Sali, D., Bycroft, M. and Fresht, A.R. (1988). Stabilization of Protein Structure by Interaction of α-helix Dipole with a Charged Side Chain. Nature, 335, 740-745.

Sambrook, J., Firtsch, E. F. and Maniatis, T. (1989). Molecular Cloning: A Laboratory Manual. (second edition) Cold Spring Harbor Lab., Cold Spring Harbor, NY.

Sancho, J., Serrano, L. and Fersht, A. R. (1992). Histidine Residues at the N- and C-Termini of α–Helices: Perturbed pK_as and Protein Stability. Biochemistry, 31, 2253-2258.

Sanders, D. A., Gillece-Castro, B. L., Stock, A. M., Burlingame, A. L. and Koshland, D. E., Jr. (1989) Identification of the Site of Phosphorylation of the Chemotaxis Response Regulator Protein CheY. J. Biol. Chem., 264, 2177-2178.

Sanger, F., Nicklen, S. and Coulson, A.R. (1977). DNA Sequencing With Chain Terminating Inhibitors. Proc. Natl. Acad. Sci. U.S.A., 74, 5463-5467.

Schneider, F. (1978). Histidine in Enzyme Active Centers. Angew. Chem. Int. Ed. Engl., 17, 583-592.

Sharma, S. (1992). Epitope Mapping of Monoclonal Antibodies Specific for the Histidine-Containing Protein, HPr, by Site-Directed Mutagenesis. Ph.D. thesis, University of Saskatchewan.

Sharma, S., Georges, F., Delbare, L. T. J., Lee, J., Klevit, R. E. and Waygood, E. B. (1991). Epitope Mapping by Mutagenesis Distinquishes Between the Two Tertiary Structures of the Histidine-Containing Protein HPr. Proc. Natl. Acad. Sci. U.S.A., 88, 4877-4881.

Sharma, S., Hammen, P. K., Anderson, J. W., Leung, A., Georges, F., Hengstenberg, W., Klevit, R. E. and Waygood, E. B. (1993). Deamidation of HPr, a Phosphocarrier Protein of the Phosphoenolpyruvate:Sugar Phosphotransferase System, Involves Asparagine 38 (HPr-1) and Asparagine 12 (HPr-2) in Isoaspartyl acid Formation. J. Biol. Chem., 268, 17695-17704.

Simoni, R. D., Nakazawa, T., Hays, J. B. and Roseman, S. (1973). Sugar Transport. IV. Isolation and Characterization of the Lactose Phosphotransferase System in *Staphylococcus aureus*. J. Biol. Chem., 248, 932-940.

Sprang, S. R., Archarya, K. R., Goldsmith, E. J., Struart, D. I., Varvill, K., Frellerick, R. J., Madson, N. B. and Johnson, L. N. (1988). Structural Changes in Glycogen Phosphorylase Induced by Phosphorylation. Nature, 336, 215-223.

Stadtman, E. R. (1992).Protein Oxidation and Aging. Science, 257, 1220-1224.

Stephenson, R. C. and Clarke, S. (1989). Succinimide Formation from Aspartyl and Asparaginyl Peptides as a Model for the Spontaneous Degradation of Proteins. J. Biol. Chem., 264, 6164-6170.

Stock, J., Ninfa, A. and Stock, A. M. (1989). Protein Phosphorylation and Regulation of Adaptive Responses in Bacteria. Microbiol. Rev., 53, 450-490.

Studier, F. W., Rosenberg, A. H., Dunn, J. H. and Dubendorff, J. W. (1990) Use of T7 RNA Polymerase to Direct Expression of Cloned genes. Methods Enzymol., 185, 60-89.

Sutrina, S., Reizer, J. and Saier, M. H. Jr. (1988). Inducer Expulsion in *Strepococcus pyogenes*; Properties and Mechanism of the Efflux Reaction. J. Bacteriol., 170, 1874-1877.

Tabor, S. and Richardson, C. C. (1985). A Bacteriophage T7 RNA Polymerase/Promoter System for Controlled Expression of Specific Genes. Proc. Natl. Acad. Sci. U.S.A., 82, 1074-1078.

Thapar, R., Nicholson, E. M., Rajagopal, P., Waygood, E. B., Scholtz, J. M. and Klevit, R. E. (1996). Influence of N-Cap Mutations on the Structure and Stability of *Escherichia coli* HPr. Biochemistry, 35, 11268-11277.

Titgemeyer, F. (1993). Signal Transduction in Chemotaxis Mediated by the Bacterial Phosphotransferase System. J. Cell. Biochem., 51, 69-74.

Todhunter, J. A. and Purich, D. L. (1974). Evidence for the Formation of a Gamma-phosphorylated Glutamyl Residue in the *Escherichia coli* Acetate Kinase Reaction. Biochem. Biophys. Res. Commun., 60, 273-280.

Tomizawa, H., Yamada, H., Udea, T. and Imoto, T. (1994). Isolation and Characterization of 101-Succinimide Lysozyme That Possesses the Cyclic Imide at Asp1-1-Gly102. Biochemistry. 33, 8770-8774.

Thompson, J., Turner, K. W. and Thomas, T. D. (1978). Catabolite Inhibition and Sequential Metabolism of Sugars by *Streptococcus lactis*. J. Bacteriol., 133, 1163-1174.

Ullah, A. H. J. and Cirillo, V. P. (1977). Mycoplasma Phosphoenolpyruvate-Dependent Sugar Phosphotransferase System: Purification and Characterization of Enzyme I. J. Bacteriol., 131, 988-996.

Ullman, A. and Danchin, A. (1985). Role of Cyclic AMP in Bacteria. Adv. Cyclic Nucleotide Res., 15, 2-53.

van Dijk, A. A., De Lange, L. C. M., Bachovin, W. W. and Robillard, G. T. (1990). Effect of Phosphorylation on Hydrogen-Bonding Interactions of the Active Site Histidine of the Phosphocarrier Protein HPr of the Phosphoenolpyruvate-Dependent Phosphotransferase System Determined by ^{15}N NMR Spectroscopy. Biochemistry, 29, 8164-8171,

van Nuland, N. A., Boelens, R., Scheek, R. M. and Robillard, G. T. (1995). High-Resolution Structure of the Phosphorylated Form of the Histidine-containing Phosphocarrier Protein HPr from *Escherichia coli* Determined by Restrained Molecular Dynamics from NMR-NOE Data. J. Mol. Biol. 135, 180-189.

van Nuland, N. A., van Dijk, A. A., Dijkstra, K., van Hoesel, R., Scheek, R. M. and Robillard, G.T. (1992). Three-Dimensional ^{15}N-^{1}H-^{1}H and ^{15}N-^{13}C-^{1}H Nuclear-Magnetic Resonance Studies of HPr a Central Component of the Phosphoenolpyruvate-Dependent Phosphotransferase System from *Escherichia coli*. Assignments of Backbone Resonances. Eur. J. Biochem., 203, 483-491.

van Nuland, N. A., Wiersma, J. A., Vanderspeil, P., DeGroot, B. C., Scheek, R. M. and Robillard, G. T. (1996). Phosphorylation Induced Torsion-angle Strain in the Active Center of HPr, Detected by NMR and Restrained Molecular Dynamics Refinement. Protein Sci., 5, 442-446.

Vieira, J. and Messing, J. (1982). The pUC Plasmid an M13mp7 Derived System for Insertion Mutagenesis and Sequencing with Synthetic Universal Primers. Gene, 19, 259-268.

Violand, B. N., Schlittler, M. R., Kolodziej., E. W., Toren, P. C., Cabonce, M. A., Siegel, N. R., Duffin, K. L., Zobel, J. F., Smith, C. E. and Tou, J. S. (1992). Isolation and Characterization of Porcine Somatotrpoin Containing a Succinimide in Place of Aspartate. Protein Sci., 1, 1634-1641.

Visick, J. E., Cai, H. and Clarke, S. (1998). The L-isoaspartyl Protein Repair Methyltransferase Enhances Survival of Aging *Escherichia coli* Subjected to Secondary Environmental Stresses. J. Bacteriol., 180, 2623-2629.

Waddell, W. J. (1956). A Simple Ultraviolet Spectrophotometric Method for Determination of Protein. J. Lab. Clin. Med., 48, 311-314.

Waygood, E. B. (1986a). Enzyme I of the Phosphoenolpyruvate:Sugar Phosphotransferase System has Two Sites of Phosphorylation Per Dimer. Biochemistry, 25, 4085-4090.

Waygood, E. B. (1998). The Structure and Function of HPr. Biochem. Cell Biol., 76, 359-367.

Waygood, E. B., Erickson, E., El-Kabbani, O. A. L. and Delbaere, L. T. J. (1985). Characterization of Phosphorylated Histidine-Containing Protein (HPr) of the Bacterial Phosphoenolpyruvate:sugar Phosphotransferase System. Biochemistry, 24, 6938-6945.

Waygood, E. B., Matto, R. L. and Peri, K. G. (1984). Phosphoproteins and the Phosphoenolpyruvate:Sugar Phosphotransferase System in *Salmonella tryphimurium* and *Escherichia coli*: Evidence for III^{man}, III^{fru}, $III^{glucitol}$, and the Phosphorylation of Enzyme $II^{mannitol}$, and Enzyme$II^{N\text{-}acetylglucosamine}$. J. Cell. Biochem., 25, 139-159.

Waygood, E. B., Matto, R. L., Erickson. E. and Vadeboncoeur, C. (1986b). Phosphoproteins and the Phosphoenolpyruvate:sugar Phosphotransferase System of *Streptococcus salivarius*. Detection of Two Different ATP-dependent Phosphorylations of the Phosphocarrier Protein HPr. Can. J. Microbiol., 32, 310-318.

Waygood, E. B., Meadow, N. D. and Roseman, S. (1979). Modified Assay Procedures for the Phosphotransferase System in Enteric Bacteria. Anal. Biochem., 95, 293-304.

Waygood, E. B., Reiche, B., Hengenstenberg, W. and Lee, J. S. (1987). Characterization of Mutant Histidine-Containing Proteins of Phosphoenolpyruvate:sugar Phosphotransferase Systems of *Escherichia coli* and *Salmonella tryphimurium*. J. Bacteriol., 169, 2810-2818.

Waygood, E. B. and Steeves, T. (1980). Enzyme I of the Phosphoenolpyruvate:sugar Phosphotransferase System of *Escherichia coli*. Purification to Homogeneity and Some Properties. Can. J. Biochem., 58, 40-48.

Wehmeier , U. F., Wohol, B. M. and Lengeler, J. W. (1995). Molecular Analysis of the Phosphoenolpyruvate-dependent L-Sorbose Phosphotransferase System from *Klebsiella pneumoniae* and it's Multidomain Structure. Mol. Gen. Genetics, 246, 610-618.

Weigel, N., Powers, D. A. and Roseman, S. (1982). Sugar Transport by the Bacterial Phosphotransferase System. Primary Structure and Active Site of a

General Phosphocarrier Protein (HPr) from *Salmonella typhimurium*. J. Biol. Chem., 257, 14499-14509.

Weiss, V. and Magasanik, B. Phosphorylation of Nitrogen Regulator1 (NR1) of *Escherichia coli*. (1988). Proc. Natl. Acad. Sci. U.S.A., 85, 8919-8923

Wilson, J. M., Landa, L. E., Koayashi, R. and Kelley, W. (1982). Human Hypoxanthine-Guanine Phosphoribosyltransferase. J. Biol. Chem., 257, 14830-14834.

Wittekind, M., Reizer, J., Deutscher, J., Saier, M. H. Jr. and Klevit, R. E. (1989). Common Structural Changes Accompany the Functional Inactivation of HPr by Seryl Phosphorylation or by Serine to Aspartate Substitution. Biochemistry, 28, 9908-9912

Wittekind, M., Reizer, J. and Klevit, R. E. (1990). Sequence Specific ^{1}H-NMR Resonance Assignments of *Bacillius subtillis* HPr: Use of Spectra Obtained from Mutants to Resolve Spectral Overlap. Biochemistry, 29, 7191-7200

Wittekind, M., Rajagopal, P., Branchini, B., Reizer, J., Saier, M. H. Jr. and Klevit, R. E. (1992). Solution Structure of the Phosphocarrier Protein HPr from *Bacillus subtilis* by Two-Dimensional NMR Spectroscopy. Protein Sci., 1, 1363-1376.

Worthylake, D., Meadow, N. D., Roseman, S., Liao, D., Herzberg, O. and Remington, S. J. (1991). Three-Dimensional Structure of the *Escherichia coli* Phosphocarrier Protein III^{glc}. Proc. Natl. Acad. Sci. U.S.A., 88, 10382-10386.

Wright, H. T. (1991). Nonenzymatic Deamidation of Asparaginyl and Glutaminyl Residues in Proteins. Crit. Rev. Biochem. Mol. Biol., 26, 1-52.

Wu, L., Tomich, J. M. and Saier, M. H., Jr. (1990). On the Evolutionary Origins of the Bacterial Phosphoenolpyruvate:Sugar Phosphotransferase System. J. Mol. Biol., 213, 687-703.

Yanisch-Perron, C., Vierra, J. and Messings, J. (1985). Improved M13 Phage Cloning Vectors and Host Strains: Nucleotide Sequence of the M13mp18 and pUC19 vectors. Gene, 33, 103-119.

Ye, J. J., Minarick, J. R. and Saier, M. H. Jr. (1996). Inducer Expulsion and the Occurance of an HPr(Ser-P)-activated Sugar-P Phosphatase in *Enterococcus faecalis* and *Streptococcus pyogenes*. Microbiology, 142, 585-592.

Ye, J. J. and Saier, M. H., Jr. (1995). Purification and Characterization of a Small Membrane-assosciated Sugar Phosphate Phosphatase that is *Lactococcus lactis* Allosterically Activated by HPr(Ser(P)) of the Phosphotransferase System in. J. Biol. Chem., 28, 16740-16744.

Zhu, P., Herzberg, O. and Peterkofsky, A. (1998). Topography of the Interaction of HPr(Ser) Kinase with HPr. Biochemistry, 37, 11762-1170.