STRUCTURE AND FUNCTION OF ENZYME I OF THE PTS

A Thesis Submitted to the College of Graduate Studies and Research
in Partial Fulfillment of the Requirements for the Degree of
Doctor of Philosophy in the Department of Biochemistry
University of Saskatchewan, Saskatoon

By
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Fall 2000

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The phosphoenolpyruvate: sugar phosphotransferase system (PTS) is responsible for the uptake and concomitant phosphorylation of many sugars in several species of bacteria. This key system also plays indirect roles in the regulation of other aspects of bacterial metabolism and physiology. The first step of the PTS involves the phosphorylation of histidine containing phosphocarrier protein (HPr) by enzyme I (E.C. 2.7.3.9), with phosphoenolpyruvate (PEP) serving as the phosphoryl donor. Enzyme I has logically been viewed as a potential target for regulation of the PTS.

This thesis presents important information regarding the structure and function of enzyme I of *Escherichia coli* and *Salmonella typhimurium*. Fluorescence polarization analysis indicated that the interactions of HPr with enzyme I and HPr with enzyme IIA^B^ are of low affinity, with a $K_d$ of roughly 10 - 100 µM. An enzyme I binding site on HPr was determined by a kinetic assay, using site-directed mutants of HPr as substrates for enzyme I. Key HPr residues involved in this interaction are His15, Thr16, Arg17, Lys24, Lys27, Lys40, Ser46, Leu47, Lys49, Gln51, and Thr56. This site of interaction agreed very well with that found in the NMR solution structure of the complex of HPr with the N-terminal domain of enzyme I (Garrett *et al.*, 1999), with a few important differences.

Genes encoding enzyme I mutants were cloned from *S. typhimurium* strains and the purified proteins were analyzed. The Arg126Cys mutant was defective in phosphotransfer, while the Gly356Ser and Arg375Cys mutants were defective in dimerization and PEP-binding. Intragenic complementation was observed between purified Arg126Cys and Gly356Ser or Arg375Cys enzymes I, through formation of a
heterodimer. This heterodimer was unstable, and stability depended upon concentrations of the enzyme and of PEP. Other site-directed mutants of *E. coli* enzyme I were constructed, which showed the importance of the residues Asn352 and Leu355 in dimerization, and Arg296 in PEP-binding. The active site mutants His189Glu and His189Asp were also shown to have a small phosphotransfer activity. These data led to the conclusions that dimerization and PEP-binding of enzyme I are closely linked cooperative events, and that the monomer:dimer equilibrium of enzyme I, with the dimer being the most active form, may have significant physiological importance. Regulation of the monomer:dimer equilibrium may provide a key target for modulation of the activity of enzyme I, and thus regulation of the PTS.
ACKNOWLEDGEMENTS

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shudder to think at the amount of coffee and beer imbibed in your collective company, but it has always been a great time.

Lastly I would like to thank my family for their love and support. Mom, Dad, Richard and Walter, you have always been there for me. I hope I can always be there for you too.
For my Mom and Dad
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<th>Description</th>
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<tbody>
<tr>
<td>bp</td>
<td>Base pair</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>cAMP</td>
<td>Adenosine-2',3'-cyclic monophosphate (cyclic AMP)</td>
</tr>
<tr>
<td>CAP</td>
<td>Cyclic AMP activator protein</td>
</tr>
<tr>
<td>CRE</td>
<td>Catabolite responsive element</td>
</tr>
<tr>
<td>CRP</td>
<td>Cyclic AMP receptor protein</td>
</tr>
<tr>
<td>C-terminus</td>
<td>Carboxy terminus</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethylsulfoxide</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylene diamine tetraacetic acid</td>
</tr>
<tr>
<td>EI</td>
<td>Enzyme I</td>
</tr>
<tr>
<td>EIC</td>
<td>Cloned C-terminal domain of enzyme I</td>
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<td>EIN</td>
<td>Cloned N-terminal domain of enzyme I</td>
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<tr>
<td>Hepes</td>
<td>N-2-hydroxyethyl-piperazine-N'-2-ethanesulfonic acid</td>
</tr>
<tr>
<td>HPr</td>
<td>Histidine-containing phosphocarrier protein</td>
</tr>
<tr>
<td>IEF</td>
<td>Isoelectric focusing</td>
</tr>
<tr>
<td>IPTG</td>
<td>Isopropyl-β-D-thiogalactopyranoside</td>
</tr>
<tr>
<td>Kₐ</td>
<td>Association constant</td>
</tr>
<tr>
<td>Kₖ</td>
<td>Dissociation constant</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilodalton</td>
</tr>
<tr>
<td>Kₘ</td>
<td>Michaelis-Menten constant</td>
</tr>
<tr>
<td>LDH</td>
<td>Lactate dehydrogenase</td>
</tr>
<tr>
<td>MCP</td>
<td>Methyl-accepting chemotaxis protein</td>
</tr>
<tr>
<td>mP</td>
<td>Millipolarization units</td>
</tr>
<tr>
<td>MTP</td>
<td>Multiprophosphoryl transfer protein</td>
</tr>
<tr>
<td>NADH</td>
<td>Nicotinamide adenine dinucleotide, reduced form</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance</td>
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<tr>
<td>NOE</td>
<td>Nuclear Overhauser effect</td>
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<tr>
<td>N-terminus</td>
<td>Amino terminus</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>P-X</td>
<td>Phosphorylated compound</td>
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<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
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<td>Protein data bank</td>
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<td>PEG</td>
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<tr>
<td>PEP</td>
<td>Phosphoenolpyruvate</td>
</tr>
<tr>
<td>PEPCK</td>
<td>Phosphoenolpyruvate carboxykinase</td>
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<td>PPDK</td>
<td>Pyruvate phosphate dikinase</td>
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<td>PTS</td>
<td>Phosphoenolpyruvate:sugar phosphotransferase system</td>
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<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
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<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
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<tr>
<td>TEMED</td>
<td>N,N,N',N'-tetramethylenediamine</td>
</tr>
<tr>
<td>T&lt;sub&gt;m&lt;/sub&gt;</td>
<td>Melting temperature</td>
</tr>
<tr>
<td>Tris</td>
<td>Tris-(hydroxymethyl) aminoethane</td>
</tr>
<tr>
<td>V&lt;sub&gt;max&lt;/sub&gt;</td>
<td>Maximum velocity</td>
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1.0 INTRODUCTION

1.1 The bacterial phosphoenolpyruvate:sugar phosphotransferase system (PTS)

The phosphoenolpyruvate:sugar phosphotransferase system (PTS), discovered in 1964 (Kundig et al., 1964) is the key system responsible for the uptake of carbohydrates into many bacterial cells. It catalyzes the transport of many monosaccharides and disaccharides across the cytoplasmic membrane, as well as the concomitant phosphorylation of the transported sugar. The PTS also influences other aspects of bacterial physiology in processes such as catabolite repression, inducer exclusion, and chemotaxis. For recent general reviews of the PTS, see Postma et al. (1993) and Postma et al. (1996). The PTS is found in many prokaryotic species, which emphasizes its importance in carbohydrate metabolism.

1.1.1 Overview and PTS components

The PTS, regardless of the sugar involved, catalyzes the overall reaction:

\[ \text{PEP} + \text{sugar}_{\text{out}} \xrightarrow{\text{PTS}} \text{pyruvate} + \text{sugar-P}_{\text{in}} \]  \hspace{1cm} (1.1)

The PTS is composed of several protein components (Figure 1.1), which are involved in phosphotransfer reactions. Enzyme I (EI), one of two general cytosolic proteins of the PTS, is autophosphorylated, with phosphoenolpyruvate (PEP) serving as the phosphoryl donor. Phospho-enzyme I then in turn donates its phosphoryl group to the second general cytosolic protein, histidine-containing protein (HPr). A sugar-specific enzyme II protein is then phosphorylated, with phospho-HPr serving as the phosphodonor. Enzymes II are
Figure 1.1 A schematic illustration of the phosphoenolpyruvate:sugar phosphotransferase system, adapted from: Anderson, 1994.

(°) Represents the phosphorylated form.
multidomain, and in many cases multisubunit proteins which vary in composition (Saier and Reizer, 1992). In general, enzymes II are composed of a IIA domain, or often a separate soluble polypeptide, which is phosphorylated by HPr, along with a IIB phosphoryl transfer domain and an integral membrane sugar transporter made up of one type of subunit (IIC) or two different subunits (IIC and IID). The full role of an intact enzyme II is to translocate, and simultaneously phosphorylate, a specific sugar.

1.1.2 Thermodynamic considerations of the PTS

The phosphate transfer potentials of the phosphoprotein components of the PTS are among the highest of all known biological phosphorylated derivatives (Stock et al., 1990). The free energy (\(\Delta G^\circ\)) of hydrolysis of PEP is \(-61.5\) kJ/mol (Atkinson and Morton, 1960), and this energy potential is well conserved in phospho-enzyme I and phospho-HPr of Escherichia coli and Salmonella typhimurium, with free energies of phosphate hydrolysis of \(-60.7\) kJ/mol and \(-54.3\) kJ/mol, respectively (Weigel et al., 1982a). Considerable energy is lost in the latter part of the PTS, as the free energy of hydrolysis of a typical phosphorylated sugar is much lower at about \(-12.5\) kJ/mol (Atkinson and Morton, 1960). However, this energy usage is not wasted as the sugar is both translocated and phosphorylated by the PTS. For example, the net product of PTS-mediated glucose transport is glucose-6-phosphate. These phosphorylated sugars are readily available for utilization by glycolysis or other processes. In addition, the phosphorylation of the sugar sequesters it in the cytoplasm, as the negative charges of the phosphoryl group renders it incapable of diffusing out across the plasma membrane. This transport and concomitant phosphorylation of sugars by the PTS is accomplished at the expense of the conversion of a single molecule of PEP into pyruvate.
1.1.3 Phosphohistidines involved in the PTS

Histidine residues are commonly found in active sites of enzymes. In the physiological range of pH, histidine is able to either accept or donate a proton, which makes it suitable to participate in various catalytic mechanisms. However, histidine is also a site for covalent modification such as, in the case of the PTS, phosphorylation. A histidine residue is available for phosphorylation at either of the two nitrogen atoms on the imidazole ring, \( N^1 \) and \( N^2 \), also called N-1 and N-3, respectively, forming a high energy phosphoramidate bond.

Both \( E. \) coli and \( S. \) typhimurium enzymes I are autophosphorylated on His189 at the \( N^2 \) position (Figure 1.2). Phospho-enzyme I then catalyzes the phosphorylation of HPr at the \( N^1 \) position of His15. This phosphoryl group is then transferred to an \( N^2 \) atom of a histidine in a IIA molecule, and the phosphoryl group is then subsequently passed onto an amino acid residue of the IIB molecule, commonly a cysteine, but in some cases \( N^1 \)-histidine. The phospho group is then transferred to the sugar molecule as it is transported into the cytoplasm by the sugar transporter portion of the EII complex, commonly the IIC domain (Postma et al., 1993).

1.1.4 The PTS operon

In \( E. \) coli and \( S. \) typhimurium, the genes encoding HPr, enzyme I, and enzyme \( \text{IIA}^\text{glucose} \) (IIA\text{\_lec}), \( ptsH, ptsI \), and \( \text{crr} \), respectively, are found in a single operon. This operon is located at min. 52 on the \( E. \) coli chromosome (Cordaro and Roseman, 1972; De Reuse et al., 1985; De Reuse and Danchin, 1988). Two promoters, designated P0 and P1, separated by 100 base pairs, are located upstream of the transcription initiation site of the \( E. \) coli PTS operon (Figure 1.3), and control from these promoters can occur independently of each other (De Reuse and Danchin, 1991). De Reuse et al. (1992) identified two sequences, one within each promoter region, which were similar to the consensus of the cyclic-AMP (cAMP) activator protein (CAP) binding site. These two
Figure 1.2 A schematic representation of the phosphohistidines of enzyme I and HPr of the PTS, and the phosphoryl transfer reactions involving the two proteins.
Figure 1.3 A diagram of the PTS operon of *E. coli*, including the three transcribed genes and the 5' promoter region. The boxed area represents the operon organization on the bacterial chromosome. The shaded regions represent intergenic, nontranslated regions of the operon. The horizontal arrows indicate the approximate location of the three transcripts, as described in the text.
CAP binding sites, CAPa and CAPb, are important for the regulation of PTS operon expression. However, the transcription of the PTS operon is also complex. Three major RNA species have been identified (De Reuse and Danchin, 1988); one tricistronic mRNA which encompasses all three genes, *ptsH*, *ptsl*, and *crr*, a second mRNA which includes *ptsH* but is terminated within *ptsl*, and a third transcript which contains only *crr*. The first two mRNA species originate from the region upstream of *ptsH*, involving P0 or P1. The mRNA encoding only *crr* originates from a separate initiation site, P2, within *ptsl*. More recently, another factor mediating PTS operon expression in *E. coli* has been identified. Mlc (for Making large colonies), a transcriptional regulator with multiple targets, binds to P0 and inhibits transcription (Kim *et al.*, 1999; Plumbridge, 1999). The exact function of Mlc remains to be elucidated.

1.1.5 Secondary Roles of the PTS

In *E. coli*, the PTS plays an active role not only in sugar transport, but in a multitude of other physiological processes, including the regulation of many aspects of metabolism (Saier *et al.*, 1990; Postma *et al.*, 1993, 1996; Saier and Reizer, 1994; Saier, 1996).

1.1.5.1 The PTS and regulation of carbon metabolism in *E. coli*

When *E. coli* is grown in a liquid medium containing two different carbohydrates, the bacteria will preferentially utilize one sugar instead of another as an energy source. This phenomenon, known as diauxie, is controlled by regulation processes called catabolite repression and inducer exclusion, which directly involve the PTS, and in particular the IIA^bc^ protein (Saier, 1989; Roseman and Meadow, 1990). When *E. coli* cells are grown on a PTS sugar such as glucose, the level of phosphorylation of IIA^bc^ on its active site histidine residue is relatively low. Phospho-IIA^bc^, indicative of a PTS that is not actively translocating and phosphorylating sugars, is able to bind adenylate cyclase,
the enzyme which catalyzes the conversion of ATP to cyclic AMP (cAMP), and adenylate cyclase is subsequently activated (Liberman et al., 1986; Peterkofsky et al., 1989). The cAMP subsequently produced is an important signaling molecule, and may then be bound by the cyclic AMP activator protein (CAP), also known as the cyclic AMP receptor protein (CRP). The transcription of many inducible carbohydrate catabolic enzymes, such as those found in the lactose operon, is positively regulated by the cAMP-CAP receptor complex. In the presence of an active PTS, cellular levels of cAMP are relatively low, and operons such as the lac operon are repressed.

This catabolite repression process (Figure 1.4) can only partially explain the multitude of effects that growth on PTS sugars exerts on the uptake and catabolism of non-PTS substrates. There also exists a second group of phenomena, organized into a category termed “inducer exclusion” (Saier and Roseman, 1972; Saier and Crasnier, 1996). The major protein responsible for inducer exclusion is, once again, IIA^glc. An unphosphorylated IIA^glc molecule, indicative of an active PTS, is able to bind various cellular targets including many non-PTS sugar permeases, such as lactose permease (Osumi and Saier, 1982) and maltose permease (Dean et al., 1990), resulting in the allosteric inhibition of the transporter. Thus the transport of non-PTS substrates can be directly inhibited in favour of transport of PTS substrates. Unphosphorylated IIA^glc also binds enzymes responsible for catabolism of non-PTS substrates, such as glycerol kinase (Postma et al., 1984), resulting in the allosteric inactivation of the enzyme. Phospho-IIA^glc is unable to bind to these protein targets to initiate this process of inducer exclusion.

Even though IIA^glc is the most important regulatory PTS protein, binding interactions between other E. coli PTS proteins and metabolic protein targets have also been observed. Seok et al. (1997), identified and described a novel regulatory interaction between HPr and glycogen phosphorylase. Two non-PTS proteins capable of interacting with E. coli enzyme I have also been identified. Fox et al. (1986) demonstrated a phosphoryl exchange between catalytic residues of acetate kinase and enzyme I. More
Figure 1.4 A schematic illustration of the role of II\textsubscript{A}\textsuperscript{glc} in catabolite repression and inducer exclusion in \textit{E. coli} and other enteric bacteria.
recently in the same laboratory, Dannelly and Roseman (1996) discovered a novel ATP-dependent kinase, with an apparent cofactor requirement for NAD\(^+\) or NADP\(^+\), that reversibly phosphorylates enzyme I at its active site residue. In each case, possible links between PTS-driven sugar transport and other metabolic pathways were proposed.

The PTS of \textit{E. coli} and other enteric bacteria is needed not only for its direct role in sugar transport, but also for the overall global regulation of bacterial metabolism. The availability of glucose and other preferentially utilized PTS sugars, an important indicator of overall energy potential of the cell, is directly sensed by bacteria in the phosphorylation state of cytosolic PTS proteins. Other proteins important in metabolism can then be elegantly controlled in part by their interactions with those PTS proteins.

### 1.1.5.2 Regulation of carbon metabolism in Gram-positive bacteria

The preceding section discussed the regulation of carbon metabolism in Gram-negative enteric bacteria such as \textit{E. coli} and \textit{S. typhimurium}. Gram-positive bacteria such as \textit{Bacillus subtilis} have a much different method of regulation of carbon metabolism (Saier et al., 1995). Unlike \textit{E. coli}, catabolite repression and inducer exclusion in \textit{B. subtilis} does not involve protein:protein interactions of IIA\(^{\text{II}}\). Instead, the key regulatory PTS protein is HPr. \textit{B. subtilis} possesses an ATP-dependent regulatory HPr kinase, which is able to phosphorylate HPr, not at the active site His15 residue, but rather at the Ser46 regulatory residue (Reizer et al., 1993a). High cellular concentrations of key metabolites such as glucose-6-phosphate and fructose-1,6-bisphosphate, indicative of an active PTS, allosterically activate the regulatory HPr(Ser) kinase (Deutscher and Saier, 1983). The consequences of phosphorylation of \textit{B. subtilis} HPr on Ser46 are many.

Phosphorylation on Ser46 of \textit{B. subtilis} HPr strongly inhibits overall PTS activity and sugar transport. It was revealed, using studies with site-directed mutants of \textit{B. subtilis} HPr (Reizer et al., 1989), that the negative charge imparted by phosphorylation results in an increased \(K_m\) and a much lower \(V_{\text{max}}\) for interactions with both enzyme I and
Thus, Ser46 phosphorylation of HPr directly inhibits the PTS as the primary level of regulation of carbon metabolism.

Like IIA^{ac} in *E. coli*, *B. subtilis* HPr(Ser-P) is also responsible for other secondary regulation of carbohydrate metabolism, mediated through protein:protein interactions with non-PTS proteins. However, *B. subtilis* and other Gram-positive bacteria do not possess an adenylate cyclase enzyme or significant levels of cAMP. Instead, catabolite repression is mediated by a transcription factor called the CcpA protein (Henkin, 1996). CcpA binds to a cis-acting element, called CRE (catabolite repression element) of many catabolic operons, thereby inhibiting transcription (Kim and Chambliss, 1997). When CcpA is complexed by HPr(Ser-P), the binding of CcpA to a CRE is dramatically enhanced. Through this interaction, Gram-positive bacteria such as *B. subtilis* are capable of catabolite repression.

Gram-positive bacteria are also capable of regulation of carbohydrate metabolism through inducer exclusion and a similar process called inducer expulsion (Saier *et al.*, 1996), both of which are again mediated by phosphorylation of HPr on Ser46. In several Gram-positive bacteria such as *Lactococcus lactis*, HPr(Ser-P) binds to and allosterically activates a membrane associated hexose-6-P phosphatase (Ye and Saier, 1995). Cytoplasmic sugar-P may then be converted to free sugar which is able to rapidly diffuse through the cytoplasmic membrane and out of the cell — hence the name “inducer expulsion”. In other Gram-positive species, such as *Lactobacillus brevis*, the PTS is not involved in transport of sugars, but these organisms possess an HPr. In these organisms, HPr(Ser-P) is capable of binding to sugar-proton symport permeases, such as lactose permease, resulting in their allosteric inactivation (Ye *et al.*, 1994). This inducer exclusion process is directly comparable to that mediated by the binding of unphosphorylated IIA^{ac} to lactose permease in *E. coli*.

In both Gram-positive and Gram-negative bacterial species, the regulation of carbon metabolism is very complex, and this remains an area of intense study. However,
it is evident that in all cases the PTS plays a direct role in this regulation. The key regulatory proteins may be different, IIA^K in Gram-negative species, and HPr in Gram-positive species, but the targets of their regulation and the overall global picture of regulation of carbohydrate metabolism remains very similar.

1.1.5.3 Involvement of the PTS in chemotaxis in E. coli

The chemotactic process in bacteria such as E. coli refers to the ability of the microorganism to adjust its swimming behaviour in response to chemical attractants or repellents (Armitage, 1999). The general chemotaxis pathway in E. coli (Figure 1.5) involves, in part, the activation and autophosphorylation of an ATP-dependent protein “sensor kinase” called CheA. CheA, phosphorylated at a histidyl residue, subsequently donates its phosphoryl group to the aspartyl residue of the “response regulator” CheY (Ninfa et al., 1991), and P-CheY interacts with the flagellar motor to cause the bacterium to adjust its swimming pattern. This histidine to aspartate phosphotransfer is a key feature of a large homologous group of signal transduction proteins, collectively called two component systems (Appelby et al., 1999). P-CheA can also interact indirectly with the integral membrane chemotactic receptors, better known as methyl accepting chemotaxis proteins (MCPs). The methylation state of these receptors is then altered, which modifies the activity of the receptors (Armitage, 1999).

Chemotaxis to PTS sugars can occur independently of MCPs and other chemotaxis components. However, phosphorylation of CheA and CheY is required. This chemotactic pathway requires the interaction of protein components of the PTS with those of the general chemotactic pathway (Titgemeyer, 1993). Lux et al. (1995) demonstrated a direct interaction between enzyme I and CheA, in that enzyme I in its unphosphorylated state (indicative of an active PTS) inhibits CheA autophosphorylation. Thus the PTS can provide a sensing mechanism for PTS sugars which can be directly transmitted to the chemotaxis pathway. However, there is no direct phosphoryl transfer between enzyme I
Figure 1.5 A schematic diagram of the principal regulatory interactions of chemotaxis.
and CheA (Johnson et al., 1995), and the exact mechanism of CheA inhibition by enzyme I is still under investigation (Lux et al., 1999). Still, the link between the PTS and chemotaxis remains certain, and once again emphasizes the utmost importance given by *E. coli* and other bacteria to the acquisition and catabolism of PTS sugars as a primary energy source.

1.2 The sugar-specific enzymes II

Many enzymes II of the PTS are known, each of which is responsible for acquiring a phosphoryl group from HPr and, through a series of phosphoryl transfer reactions, transfer it to a specific sugar which the enzyme II has transported into the cytoplasm. As previously discussed, the enzymes II are a heterologous group of proteins, possessing significant differences in structure and function, but they can be grouped into several general categories. For reviews of the PTS enzymes II, see Lengeler et al. (1994) and Robillard and Broos (1999).

1.2.1 Structural and functional classification of enzymes II

The sugar-specific enzymes II (Figure 1.6) have a minimum of three domains, named IIA, IIB, and IIC (Saier and Reizer, 1992). The IIA domain accepts a phosphoryl group from HPr on an N\(^{6}\) atom of a histidine residue. The phosphoryl group is then passed to the IIB domain, commonly on a cysteine residue. The IIC domain is responsible for the translocation and eventual phosphorylation of the specific sugar. These domains may be found on a single polypeptide, as in the case of enzyme II\(^{\text{mannitol}}\) of *E. coli*, thus named IIBC\(^{\text{mannitol}}\). The II\(^{\text{man}}\) is written in that order to indicate the order of domains on the polypeptide from the amino terminus to the carboxy terminus. The three domains may also be found on two separate polypeptides, as in the case of enzyme II\(^{\text{glucose}}\) of *E. coli* – IIA\(^{\text{glc}}\) and IIBC\(^{\text{glc}}\), or three separate polypeptides, such as enzyme II\(^{\text{cellulose}}\) of *E. coli* – IIA\(^{\text{cel}}\), IIB\(^{\text{cel}}\), and IIC\(^{\text{cel}}\). In older nomenclature, a soluble, separate IIA
Figure 1.6 An illustration of the various types of enzymes II involved in the E. coli PTS. Figure adapted from Lengeler et al., 1994. Note: The domains of enzyme II\textsuperscript{mannitol} are shown in the order of phosphotransfer for purposes of clarity, not the order in which they are found on the polypeptide chain (see text).
polypeptide was called an enzyme III or factor III. Another variant is the addition of a fourth domain, IID, as in the case of enzyme II$^\text{manose}$ of *E. coli*, which is found in the format of IIAB$^\text{man}$, IIC$^\text{man}$, and IID$^\text{man}$. On the basis of similarities in domain structure and sequence homology, most enzymes II can be grouped together into four families (Table 1.1). Whether with one or more polypeptides, an entire IIABC protein is made up of approximately 630 amino acids (Postma *et al*., 1993). In *E. coli*, *crr*, the gene encoding IIA$\text{ec}$, is found within the PTS operon (De Reuse and Danchin, 1988), whereas *ptsG*, the gene encoding IICB$\text{ec}$, is found elsewhere on the chromosome. This gene and all other genes encoding enzymes II are found scattered throughout the *E. coli* chromosome (Postma *et al*., 1993).

Two exceptions to the general classification of enzymes II are important to note. The fructose PTS in *E. coli* and *S. typhimurium* involves a separate protein called FPr (Waygood, 1980; Geerse *et al*., 1989). FPr contains an N-terminal IIA$\text{fru}$ domain, which is grouped within the same family as IIA$\text{mut}$. FPr also contains a central linker domain and a C-terminal domain with homology to HPr. In fact, PTS mediated transport of fructose does not involve HPr; instead enzyme I phosphorylates FPr on its "pseudo-HPr" domain, followed by interdomain phosphoryl transfer to the IIA portion of FPr, followed by intersubunit phosphoryl transfer to the IIB domain of the IIBC$\text{fru}$ sugar transporter. In *Rhodobacter capsulatus*, PTS mediated transport of fructose involves a multiphosphoryl transfer protein (MTP). This large 827 residue soluble protein contains an amino-terminal IIA$\text{fru}$ domain, a central HPr-like domain, and a C-terminal domain resembling enzyme I (Wu *et al*., 1990). Indeed, the large complexity amongst enzymes II, and other PTS and phosphoryl transfer proteins suggests a complex process of evolution for these proteins, involving domain shuffling and convergent and divergent modes of evolution (Saier and Reizer, 1994; Saier and Reizer, 1996).

Considering the importance of enzymes II in bacteria, and their wide variety, it is not surprising that these proteins have been the subject of intense investigation. Perhaps
Table 1.1 Enzymes II of the different PTSs from various bacteria (Lengeler et al., 1994)

<table>
<thead>
<tr>
<th>Number</th>
<th>Substrate</th>
<th>Organism(s)*</th>
<th>Domainsb</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. The glucose-sucrose family</td>
<td>Glucose</td>
<td>Enteric bacteria</td>
<td>IICB&lt;sup&gt;Gl&lt;/sup&gt;, IIA&lt;sup&gt;Gl&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Glucose</td>
<td><em>Bacillus subtilis</em></td>
<td>IICB&lt;sup&gt;Gl&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Maltose</td>
<td><em>Escherichia coli</em></td>
<td>IICB&lt;sup&gt;mal&lt;/sup&gt;, IIA&lt;sup&gt;mal&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>N-Acetyl-glucosamine</td>
<td>Enteric bacteria</td>
<td>IIBC&lt;sup&gt;ag&lt;/sup&gt;, IIA&lt;sup&gt;ag&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Sucrose</td>
<td>Enteric bacteria</td>
<td>IIBC&lt;sup&gt;Sc&lt;/sup&gt;, IIA&lt;sup&gt;Sc&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Sucrose</td>
<td><em>Streptococcus mutans</em></td>
<td>IIBC&lt;sup&gt;sr&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>β-Glucosides</td>
<td><em>Escherichia coli</em></td>
<td>IIBC&lt;sup&gt;Bi&lt;/sup&gt;</td>
</tr>
<tr>
<td>2. The mannitol-fructose family</td>
<td>Mannitol</td>
<td><em>Escherichia coli</em></td>
<td>IICB&lt;sup&gt;mi&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Mannitol</td>
<td><em>Staphylococcus carnosus</em></td>
<td>IICB&lt;sup&gt;mi&lt;/sup&gt;, IIA&lt;sup&gt;mi&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Fructose</td>
<td>Enteric bacteria</td>
<td>FPr, IIIB&lt;sup&gt;Bi&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Fructose</td>
<td><em>Rhodobacter capsulatus</em></td>
<td>MTP, IIIB&lt;sup&gt;Bi&lt;/sup&gt;</td>
</tr>
<tr>
<td>3. The lactose-cellobiose family</td>
<td>Cellobiose</td>
<td><em>Escherichia coli</em></td>
<td>IIBC&lt;sup&gt;Cl&lt;/sup&gt;, IIC&lt;sup&gt;Cl&lt;/sup&gt;, IIA&lt;sup&gt;Cl&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Lactose</td>
<td><em>Lactobacillus lactis</em></td>
<td>IICB&lt;sup&gt;lac&lt;/sup&gt;, IIA&lt;sup&gt;lac&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Lactose</td>
<td><em>Staphylococcus aureus</em></td>
<td>IICB&lt;sup&gt;lac&lt;/sup&gt;, IIA&lt;sup&gt;lac&lt;/sup&gt;</td>
</tr>
<tr>
<td>4. The mannose family</td>
<td>Mannose</td>
<td><em>Escherichia coli</em></td>
<td>IIAB&lt;sup&gt;man&lt;/sup&gt;, IIC&lt;sup&gt;man&lt;/sup&gt;, IID&lt;sup&gt;man&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>L-Sorbose</td>
<td>Enteric bacteria</td>
<td>IIA&lt;sup&gt;sm&lt;/sup&gt;, IIIB&lt;sup&gt;sm&lt;/sup&gt;, IIC&lt;sup&gt;sm&lt;/sup&gt;, IID&lt;sup&gt;sm&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Fructose</td>
<td><em>Bacillus subtilis</em></td>
<td>IIA&lt;sup&gt;lev&lt;/sup&gt;, IIIB&lt;sup&gt;lev&lt;/sup&gt;, IIC&lt;sup&gt;lev&lt;/sup&gt;, IID&lt;sup&gt;lev&lt;/sup&gt;</td>
</tr>
<tr>
<td>5. Separate classification</td>
<td>Glucitol</td>
<td><em>Escherichia coli</em></td>
<td>IIC&lt;sup&gt;Bi&lt;/sup&gt;, IIA&lt;sup&gt;Bi&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

* Enteric bacteria: *Escherichia coli*, *Salmonella typhimurium*, *Klebsiella pneumoniae*.

b FPr comprises IIA<sup>fru</sup>, a receiver domain, and an HPr-like domain. MTP, multiphasphoryl transfer protein, contains IIA<sup>fru</sup>, and HPr-like domain and an Enzyme I-like domain.
the best understood of the enzymes II are II^{\text{IIIC}} of *E. coli* and *B. subtilis*, which will be described here in further detail.

### 1.2.2 Enzyme II^{\text{IIIC}}

Enzyme II^{\text{IIIC}} is probably the most important enzyme II *in vivo*, and *E. coli* II^{\text{IIIC}} is also probably the most studied of all enzymes II. To reiterate, it is comprised of a soluble IIA subunit and a membrane bound IICB subunit, and each component has been investigated separately in detail.

#### 1.2.2.1 *E. coli* IIA^{\text{IIIC}} - structure and function

The highly homologous *E. coli* and *S. typhimurium crr* genes, found in the PTS operon, encode the IIA^{\text{IIIC}} proteins with an approximate mass of 18,250 Da (Meadow *et al.*, 1982; De Reuse and Danchin, 1988). The enzymes from these two species can be used interchangeably and will not be distinguished in this discussion. IIA^{\text{IIIC}} is phosphorylated by HPr at the active site His90 residue (Dorschug *et al.*, 1984). Residue His75 of IIA^{\text{IIIC}} is also important for enzymatic activity as the His75Gln mutant was found to accept a phosphoryl group from HPr, but was not able to phosphorylate IICB^{\text{IIIC}} (Presper *et al.*, 1989). The amino terminus of IIA^{\text{IIIC}} also appears to be important for full activity. During purification of IIA^{\text{IIIC}}, another form of the enzyme appears on sodium dodecyl sulfate (SDS) polyacrylamide gels which migrates faster than the native form. This variant was called IIA^{\text{IIIC fast}}, due to its electrophoretic mobility, as opposed to the native form, IIA^{\text{IIIC slow}} (Meadow and Roseman, 1982). IIA^{\text{IIIC fast}} was able to accept a phosphoryl group from HPr at a similar rate to IIA^{\text{IIIC slow}}, but catalyzed the phosphorylation of IICB^{\text{IIIC}} at a greatly diminished rate. It was discovered that conversion of IIA^{\text{IIIC slow}} to IIA^{\text{IIIC fast}} occurred by the cleavage of the seven N-terminal amino acids of the protein by an unidentified membrane associated protease during cell breakage, any potential physiological significance of which remains unknown (Meadow *et al.*, 1986). Using
sugar phosphorylation assays, the HPr $K_m$ for IIAslc was determined to be 0.3 µM for the *E. coli* proteins (Reizer *et al.*, 1992). Rate and equilibrium constants for the *E. coli* IIAslc:HPr interaction have also been obtained using rapid reaction quenching techniques (Meadow and Roseman, 1996), and the rate of phosphorylation is near that of a diffusion-controlled process.

The structure of *E. coli* IIAslc has been solved to 2.1 Å resolution by X-ray crystallography (Worthylake *et al.*, 1991), and the structure is in agreement with previous functional characterization. The protein consists mainly of β-strands, forming an antiparallel β-sandwich. The residues His75 and His90 are located in a depression on one side of the sandwich, only 3.3 Å apart, and are surrounded by hydrophobic residues. The structures of phosphorylated and unphosphorylated IIAslc were also compared using two- and three-dimensional NMR techniques (Pelton *et al.*, 1992; Pelton *et al.*, 1993), and revealed little change in secondary or tertiary structure upon phosphorylation, except minor changes at the active site. The markedly different properties of phosphorylated and unphosphorylated IIAslc in a variety of protein:protein interactions must be mediated by changes in charge distribution and hydrophobicity.

Further structural studies have been undertaken in order to investigate the role of IIAslc in protein:protein interactions. The interaction of IIAslc with one of its targets of allosteric inhibition, glycerol kinase, has been studied extensively, culminating with several structures of the complex, each solved by X-ray crystallography, in the presence of glycerol kinase substrates and/or products (Hurley *et al.*, 1993; Feese *et al.*, 1994). IIAslc binds distant from the active site of glycerol kinase, indicating that long-range structural transitions would be required to inhibit glycerol kinase. The active site histidine of IIAslc is buried within a mainly hydrophobic region of the interaction, such that phosphorylation would directly inhibit binding. The target proteins for binding with IIAslc are diverse, and the inclusion of nonspecific hydrophobic interactions and a few specific
salt bridges in these heterologous binding sites may permit IIA^{ele} to bind to this wide variety of proteins (Feese et al., 1997).

However, other researchers have more recently attempted to deduce consensus binding sequences on target proteins that bind IIA^{ele}. Sondej et al. (1999) used site-directed mutagenesis of the IIA^{ele} target protein lactose permease to determine which residues of the permease were responsible for the interaction with IIA^{ele}. Two interacting sequences on lactose permease were discovered and compared with interacting residues on other interacting proteins. From this analysis, two regions of consensus sequence necessary for target proteins to bind IIA^{ele} were deduced. Other potential target proteins containing these interaction sequences were discovered from a database search of the E. coli genome.

Not surprisingly, no tertiary structure of the integral membrane lactose permease protein, either native or in complex with IIA^{ele}, is available for investigators. As well, no tertiary structure of IIA^{ele} in complex with its interacting proteins of the PTS, namely HPr and IICB^{ele}, has been determined. Several researchers have investigated the HPr:IIA^{ele} interaction using experiments with B. subtilis proteins, but a description of the structure of B. subtilis IIA^{ele} protein is needed first.

1.2.2.2 B. subtilis IIA^{ele} – structure and function

The B. subtilis enzyme II^{ele} exists as part of a single IICBA polypeptide (Zagorec et al., 1992). The soluble IIA^{ele} domain, consisting of 162 amino acid residues, has been separately purified and its structure solved, independently by both by X-ray crystallography (Liao et al., 1991) and NMR (Fairbrother et al., 1992). The protein has an antiparallel β-barrel structure, with the main eight-stranded β-sheet structure forming a modified “Greek key” or “jellyroll” topology. The two conserved histidine residues, His83 and His68, analogous to the E. coli residues His90 and His75, respectively, are close to one another in a pocket surrounded by hydrophobic residues. The N^{ε2} atom of
His83, the site of phosphorylation, is partially surrounded by solvent and is only 3.2 Å from the Nδ atom of His68. The active site of *E. coli* IIA^Gal^ has a remarkably similar arrangement of residues, despite differences in overall topology, and there is approximately 42% sequence identity between the two proteins (Chen *et al.*, 1993a). The interaction of each IIA^Gal^ with its respective HPr would likely be very similar.

Herzberg (1992) proposed a model for the *B. subtilis* HPr:IIA^Gal^ interaction and phosphotransfer reaction, with proteins from *B. subtilis*, using high resolution crystal structures of each protein linked together with a phosphate ‘tether’ with trigonal bipyramidal geometry. The interaction site was more conclusively determined by Chen *et al.* (1993b) with NMR chemical shift experiments using ^15^N-labeled IIA^Gal^ and unlabeled HPr. An NMR spectrum of IIA^Gal^ alone in solution was taken, and then another spectrum was taken after the addition of HPr. The investigators compared the spectra and found several regions in IIA^Gal^ which underwent backbone amide ^15^N and ^1H shift changes after addition of HPr, which indicate the IIA^Gal^ residues involved in the interaction. These residues included several from the eight-stranded β-sheet and a segment of the Ω-loop which is close to the active site. Surprisingly, the active site histidines of IIA^Gal^, residues 68 and 83, did not show an NMR chemical shift after addition of HPr.

Several laboratories have attempted to solve the structure of IIA^Gal^ and HPr, using proteins from different bacterial species, different phosphorylation states, and mutant proteins, but so far these attempts have been unsuccessful. The interaction between HPr and IIA^Gal^ including interacting residues of HPr and kinetics, will be further discussed in the Introduction section regarding HPr (Section 1.3).

### 1.2.2.3 *E. coli* IICB^Gal^ – structure and function

The *E. coli* IICB^Gal^ protein is 477 residues in length, encoded by the *ptsG* gene (Erni and Zanolari, 1986). This protein mediates the final steps of the glucose PTS, namely the uptake and concomitant phosphorylation of glucose. The active form of the
IICB\textsuperscript{\textgreek{g}lc} transporter is a homodimer (Meins et al., 1988). The protein is phosphorylated by P-IIA\textsuperscript{\textgreek{g}lc} on the Cys421 residue of the IIB domain (Peri et al., 1984; Meins et al., 1993). IICB\textsuperscript{\textgreek{g}lc} is able to catalyze the phosphoryl exchange between P-IIA\textsuperscript{\textgreek{g}lc} and IIA\textsuperscript{\textgreek{g}lc}, as well as the phosphoryl exchange between glucose-6-phosphate and glucose (Rephaeli and Saier, 1978), again indicative of a stable phosphorylated IIB intermediate during catalysis. The transport and phosphorylation of glucose can also be uncoupled, in that mutants have been isolated which are unable to transport glucose but are able to catalyze the phosphorylation of intracellular glucose at wild type levels (Buhr et al., 1992; Ruijter et al., 1992). More recently, a comprehensive study of site-directed mutants of IICB\textsuperscript{\textgreek{g}lc} has been published (Lanz and Erni, 1998), and complementation between two inactive mutants at separate sites of the protein suggested the formation of active heterodimers.

Other research has been devoted to the study of the separated domains, IIB and IIC, of the glucose transporter. Buhr et al. (1994) were able to separately overexpress and purify the hydrophilic IIB domain and the transmembrane IIC domain. The structure of this protein, residues 386 - 477 of IICB\textsuperscript{\textgreek{g}lc}, has been solved by NMR (Eberstadt et al., 1996). The protein consists of a four stranded \(\beta\)-sheet with three \(\alpha\)-helices arranged on one side of the sheet. On the other side of the \(\beta\)-sheet is the active site Cys421. These residues are on a convex surface of the protein, highly exposed to solvent. Gemmecker et al. (1997) characterized the structure of the phosphorylated form of the IIB protein, and elucidated the site of interaction between the IIB protein and II\textsuperscript{\textgreek{g}lc}\textsuperscript{\textgreek{g}lc}. The IIB binding site on II\textsuperscript{\textgreek{g}lc} roughly coincides with the HPr binding site identified on II\textsuperscript{\textgreek{g}lc}\textsuperscript{\textgreek{g}lc} from \textit{B. subtilis} (Chen et al., 1993b), displaying similar shape complementarity, indicating that II\textsuperscript{\textgreek{g}lc} likely possesses overlapping active sites for binding and for phosphoryl transfer between HPr and IIB\textsuperscript{\textgreek{g}lc}.

Little direct structural information regarding the IIC portion of the IICB\textsuperscript{\textgreek{g}lc} protein is available to date. Considerable structural evidence can be gained from functional analysis of the transporter, as previously described. In addition, using alkaline phosphatase
(PhoA) and β-galactosidase (LacZ) fusion proteins of IICB\textsuperscript{elc}, Buhr and Erni (1993) were able to generate a membrane topological map of the protein. Corroborating all other functional evidence, the model indicated that the amino terminus of the protein faced the cytoplasm, followed by eight transmembrane spanning helices, including three cytoplasmic and four periplasmic loops, followed by the hydrophilic, cytoplasmic IIB domain.

1.2.3 Structures of other enzymes II

In terms of structure and function, the glucose enzymes II from \textit{E. coli} and \textit{B. subtilis} have undoubtedly been the most well characterized of all enzymes II. However, several other structures of other sugar-specific enzymes II are available for analysis (Robillard and Broos, 1999).

1.2.3.1 Structures of other IIA proteins

The glucose transporter of \textit{Mycoplasma capricolum} has also been investigated. The IIA\textsuperscript{elc} protein, 154 residues, is encoded by the \textit{crr} gene in a unique dicistronic operon of \textit{ptsI-crr} (Zhu \textit{et al.}, 1994). IIA\textsuperscript{elc} was purified and its structure was solved by X-ray crystallography to 2.5 Å resolution (Huang \textit{et al.}, 1998). Its interactions with \textit{M. capricolum} HPr and IIB\textsuperscript{elc} in transition state complexes were also modeled. The interactions were mostly hydrophobic with little specific electrostatic interactions, which are similar to those observed with binding studies of IIA\textsuperscript{elc} from other species.

The structures of two other sugar-specific IIA proteins from \textit{E. coli} have been determined. Crystallographic methods were used to determine the structure of the \textit{E. coli} mannitol-specific IIA domain, IIA\textsuperscript{mnl} (van Montfort \textit{et al.}, 1998). The enzyme has a novel fold, unlike folds of other sugar-specific IIA proteins discovered so far, with a central five stranded β-sheet surrounded by mostly α-helical structures. The IIA domain of the soluble \textit{E. coli} IIB\textsubscript{mannose} protein was also solved by X-ray crystallography (Nunn \textit{et al.}, 1996).
Superficially it appears to be topologically similar to IIA^{ml} with a central β-sheet surrounded by α-helices. Its site of phosphorylation is His10, with no other catalytically important active site histidine, unlike other IIA proteins.

A structure of IIA^{lactose} from *Lactococcus lactis* has been obtained by X-ray crystallography (Sliz et al., 1997). It is a unique IIA protein, which exists as a trimer in the unphosphorylated state. Each monomer consists of three helices which pack together to form a nine-helix bundle in the trimer. Phosphorylation at the active site His78 likely leads to disruption of the bundle and dissociation of the subunits.

1.2.3.2 Structures of other IIB proteins

The IIB subunits of a *B. subtilis* fructose transporter, IIIB\textsuperscript{lev}, and an *E. coli* cellobiose transporter, IIIB\textsuperscript{cell}, have been solved by X-ray crystallography (Schnauder et al., 1998). Superficially they appear to be similar in structure, with a central β-sheet surrounded by a few α-helices.

1.3 The general PTS protein HPr

HPr, the histidine containing phosphocarrier protein, is the most well characterized of all the proteins of the PTS. It was the first protein initially investigated when the PTS was discovered (Kundig et al., 1964). The HPr proteins in *E. coli* and *S. typhimurium* are identical, with 85 amino acid residues and a molecular weight of 9109 Da. This small protein is readily soluble in aqueous solutions, which makes it amenable to a wide variety of *in vitro* investigations, including structural determination by NMR and X-ray crystallography.

In the PTS, HPr is phosphorylated at the N\textsuperscript{ε1} position of the conserved His15 by the PEP-dependent protein kinase enzyme I. Phospho-HPr then serves as a phosphoryl donor for the IIA domain of a sugar-specific enzyme II. Gram-positive bacteria also have
an ATP-dependent regulatory HPr kinase which phosphorylates HPr at the Ser46 residue, and this has been previously discussed (Section 1.1.5.2).

1.3.1 Comparison of HPrs from various organisms

The gene encoding HPr, *ptsH*, has been identified in numerous Gram-negative and Gram-positive bacteria through genome sequencing or direct investigation. HPr-like domains are also found in other PTS proteins, namely FPr of enteric bacteria, and the multiprophosphoryl transfer protein of *Rh. capsulatus*. All told, more than twenty sequences of HPrs and HPr-like proteins have been identified in prokaryotes to date.

An alignment of several HPrs and HPr-like proteins (Koch *et al.*, 1996) reveals several conserved residues. The catalytic histidine, His15 in all HPrs, is by definition strictly conserved, along with the active site residues Gly13, Arg17, and Pro18. Residue 14 is commonly a leucine or isoleucine, and residue 16 is commonly threonine in Gram-negative HPrs and valine in Gram-positive HPrs. Ser46 is a strictly conserved residue, and residues surrounding Ser46 are also well conserved. However, phosphorylation of Ser46 only occurs in Gram-positive bacteria.

1.3.2 Structural and functional analysis of HPr

The structure of HPr has been very well characterized by both NMR and X-ray crystallography (Waygood, 1998). In 1986, *E. coli* HPr was the largest protein to be described by two-dimensional NMR approaches (Klevit and Waygood, 1986). The structure of the protein consisted of a mixed α + β structure folded together to form an open-faced β-sandwich composed of three α-helices and a four-stranded β-sheet. *E. coli* HPr was subsequently crystallized and investigated by X-ray diffraction (El-Kabbani *et al.*, 1987). This structure differed substantially from the one determined by NMR. This difference was resolved when the crystal structure was redetermined (Jia *et al.*, 1993). This structure (Figure 1.7) was essentially the same as the one seen by NMR, although
Figure 1.7  Ribbon diagram of the structure of *E. coli* HPr, taken from the crystal structure of HPr in complex with the Jel42 antibody fragment (Prasad *et al.*, 1998), PDB accession code 2JEL. The side chains of the active site residues His15 and Arg17 are shown in red and blue, respectively. Diagram was produced with SETOR (Evans, 1992).
some side chain resonance assignments in the NMR structure were not in agreement. This HPr structure was also confirmed by site-directed mutagenesis and purification of mutant HPr proteins followed by "epitope mapping" using anti-HPr antibodies (Sharma et al., 1991), and later by the determination of the crystal structure of HPr with one of those antibodies (Prasad et al., 1998). X-ray crystallography was also used to obtain the structures of HPrs from *B. subtilis* (Herzberg et al., 1992), *Streptococcus faecalis* (Jia et al., 1994b), and *M. capricolum* (Pieper et al., 1995). The structure of HPr from *Staphylococcus aureus* has also been determined by two-dimensional NMR (Kalbitzer and Hengstenberg, 1993). When these structures are compared (Jia et al., 1994a; Waygood, 1998), they show considerable homology, and all of these structures display the similar fold of three α-helices on one face of a four-stranded β-sheet.

In the structure of HPr, the active site His-15 is at the amino terminal end of the first helix – ‘A’. The effect of the helix dipole, in combination with influences from other amino acid side chains in the microenvironment of the active site, results in a relatively low pKₐ, 5.6, at the N⁶¹ position of His15 of *E. coli* HPr (Kalbitzer et al., 1982; Anderson et al., 1993). After phosphorylation of HPr, the pKₐ of His15, at the N² position, increases to 7.8 (Kalbitzer et al., 1982; Anderson et al., 1993). These values indicate that at physiological pH, unphosphorylated HPr is mostly unprotonated at His15, which leaves a free lone pair of electrons available for the phosphorylation reaction. Phosphorylated HPr would be mostly protonated at His15, which would increase the potential of phosphotransfer to a IIA^{17} (Anderson, 1994). Phosphorylation of His15 also results in small changes in the orientations of side chains at the active site (van Nuland et al., 1993), which is also important for phosphotransfer activity (Waygood, 1998).

A detailed examination of the active centre of HPr (Anderson, 1994) reveals the importance of the conserved His15 and Arg17, as well as residues 12 and 16, which are not strictly conserved. A kinetic study of Arg17 mutants of *E. coli* HPr (Anderson et al., 1993) helped elucidate the role of the residue. With all mutants tested, both Kₘ and Vₘₐₓ
values were affected for the kinetic interaction with enzyme I, and in general the catalytic efficiencies of the mutants were reduced by about 100-fold. The kinetic interactions of Arg17 mutants of HPr with enzymes II$^{\text{nag}}$, II$^{\text{man}}$, and II$^{\text{mtl}}$ were more complicated, with each IIA protein showing different changes to $V_{\text{max}}$ and $K_m$. These differences indicate that the enzymes II do not employ the identical catalytic mechanism, and Arg17 may or may not play a role in these interactions. Anderson et al. (1991) also investigated the role of the C-terminal Glu85 residue in the active site of E. coli HPr, but it appears that only the carboxyl end of the main peptide chain may play a minor role, if any, in the phosphotransfer capability of HPr. Indeed, the C-terminal region of HPrs from various microorganisms is poorly conserved.

Surprisingly, it has recently been demonstrated that the presence of a histidine at position 15 of E. coli HPr is not essential for activity. Napper et al. (1999) showed that His15Asp HPr displayed phosphotransfer activity during interactions with both enzyme I and IIA$^{E_{coli}}$, albeit at a greatly reduced efficiency. No other His15 mutant of HPr, including Glu, Ser, Thr, and Cys, had phosphotransfer activity. The position of the carboxyl oxygen in aspartate is similar to that of the $N^\text{\delta1}$ position of a histidine side chain, so phosphorylation of the two residues would be similar. By the same token, the carboxyl oxygen of glutamate can be compared to the $N^\text{\alpha2}$ position of histidine. Thus by analogy, the His189Glu mutant of E. coli enzyme I and the His90Glu mutant of E. coli IIA$^{E_{coli}}$ could potentially be phosphorylated (Napper, 1999), but this has yet to be tested. It was also observed (Napper et al., 1999) that phosphorylation of His15Asp HPr led to the formation of a protein species with a higher isoelectric point, which in turn underwent hydrolysis to reform unphosphorylated His15Asp HPr. It was proposed that the higher $pI$ form of the protein was due to the formation of a cyclic isomide structure at residue 15.

The importance of Asn12 (not a conserved residue) in the activity of E. coli HPr was also investigated in this report (Napper et al., 1999). Various mutants at the 12 position were created and their kinetic interactions with enzyme I were determined. All
mutants tested produced only mild decreases to $V_{\text{max}}$ and $K_m$ for the enzyme I interaction. This is not surprising because even though structural evidence indicates that residue 12 may participate in a hydrogen bond with His15, this interaction is likely weak and of only moderate physiological significance (Waygood, 1998; Napper et al., 1999).

1.3.3 Deamidation of *E. coli* HPr

During the initial purification and characterization of *E. coli* HPr (Anderson et al., 1971), it was observed that two other protein species which are more negatively charged than HPr can appear over time in the preparation. These two species, named HPr-1 and HPr-2, and were found to arise from the loss of one and two amide groups, respectively, from HPr. HPr-1 has kinetic and phosphohydrolysis parameters similar to HPr, while HPr-2 is impaired in both properties (Waygood et al., 1985). It was later revealed that Asn38 deamidates to Asp or *iso*-aspartic acid to form HPr-1 and HPr-1 in turn can further deamidate at Asn12 producing Asp or *iso*-Asp to form HPr-2 (Sharma et al., 1993). Unlike the phospho-Asp15 cyclization product, this deamidation proceeds via a previously characterized process involving succinimide ring formation (Geiger and Clarke, 1987). Deamidation of HPr is accelerated by increases in pH (buffers above 8.0), and temperature (Waygood et al., 1985), and these factors must be monitored during any *in vitro* experiments involving HPr. The deamidation of Asn12 results in the reduced activity of HPr-2, which gave the first indication of the potential importance of residue 12 in the active site.

1.3.4 Protein:protein interactions involving HPr

In the PTS, HPr interacts with enzyme I as well as the IIA domains of a number of sugar-specific enzymes II. Gram-positive bacteria also have a regulatory HPr kinase, and Ser46-phosphorylated Gram-positive HPr is involved in complex protein:protein interactions with a number of regulatory targets (Section 1.1.5.2). Binding sites on HPr
for these proteins have been investigated by a number of methods. Anti-HPr antibodies have also been developed, and HPr used as a model protein to investigate antibody interactions with protein antigens (Anderson, 1995).

1.3.4.1 Enzyme I binding site on HPr

Two main methods have been utilized in order to determine the amino acid residues of HPr which interact with enzyme I. These methods are structural characterization, primarily by NMR, and kinetic analysis by enzyme assays.

1.3.4.1.1 Characterization of HPr:enzyme I interaction by NMR

Van Nuland et al. (1995) reported a high resolution NMR structure of phosphorylated E. coli HPr. The enzyme I binding site on HPr was also determined in this report by plotting the HPr residues that underwent $^{15}$N and $^1$H NMR chemical shift displacements when the spectrum of HPr alone in solution was compared with a spectrum of HPr in complex with enzyme I. Most HPr residues that exhibited NMR shifts were on the surface of the protein, and included the active site region (residues 12 – 17), Gln 21, Lys24, and residues 47 – 55, which form a loop containing a small helix.

More recently, the structure of E. coli HPr in complex with a soluble 30 kDa N-terminal domain of enzyme I (EIN) has been solved by NMR (Garrett et al., 1999). The binding of the two proteins involves many hydrophobic interactions. Key HPr residues, which made three or more contacts with EIN were Thr16, Arg17, Ala20, Leu47, Phe48, and Thr52. Several electrostatic interactions are also in the binding interaction, involving Arg17, Lys24, Lys27, Lys45 and Lys49 of HPr. These residues are almost the same as those previously identified (van Nuland et al., 1995), and are found on helices 1 and 2 of HPr. This structure was determined in the absence of phosphorylation of HPr and EIN, and differences in the HPr:EIN complex during phosphotransfer cannot be ruled out.
1.3.4.1.2 Kinetic characterization of HPr:enzyme I interaction

Numerous mutagenesis studies have revealed E. coli HPr residues which may be important for the interaction with enzyme I. These residues are those that, when mutated, give enzyme I kinetic parameters different than wild type HPr when assayed as a limiting substrate for enzyme I. Mutations that affect primarily $K_m$ but not $V_{max}$ for enzyme I indicate that these residues are involved in binding and not catalysis. Catalytically important residues, affecting both $K_m$ and $V_{max}$, identified to date include Asn12 (Sharma et al., 1993; Napper et al., 1999), His15 (site of phosphorylation), and Arg17 (Anderson et al., 1993). Residues which, when mutated, affect primarily $K_m$ include Ser46 (Napper et al., 1996) and Gln51 (Sharma et al., 1993). A more comprehensive mutagenic and kinetic study to elucidate other HPr residues important for binding enzyme I is presented in this thesis.

1.3.4.2 HPr:IIA<sup>utfr</sup> interactions

In contrast to the interaction of enzyme I with HPr, less is known about the residues on HPr which interact with various A domains of enzymes II of the PTS. The structures of several sugar-specific IIA domains from various bacteria have been solved (Robillard and Broos, 1999) and in several cases a model of their interaction with HPr was also presented. However, to date no structure of HPr in complex with a IIA protein has been solved. NMR chemical shift experiments have been used to determine residues on HPr which interact with IIA<sup>utfr</sup> using proteins from E. coli (van Nuland et al., 1993) and B. subtilis (Chen et al., 1993b). The HPr residues that underwent an NMR chemical shift after addition of IIA<sup>utfr</sup>, delineating the IIA<sup>utfr</sup> binding site on HPr, were similar in each study. Key interacting residues of HPr included the active site (residues 15 – 21) and residues 47 – 56. Kinetic studies involving mutant HPrs and various enzymes II have been undertaken and reveal the direct importance of Arg17 (Anderson et al., 1993) and Ser46 (Napper et al., 1996), and the indirect importance of Asp69 and Glu70 (Koch et al.,
1996) for interaction with enzymes II. The enzymes II are a heterologous group of proteins, and their sites of interaction on HPr, and kinetic mechanisms, may not consistent.

1.3.4.3 HPr:antibody interactions

Four monoclonal antibodies specific to *E. coli* HPr have been isolated and characterized (Waygood *et al.*, 1987). The epitopes of three of the antibodies, Jel42, Jel44, and Jel323, were mapped by measuring their relative binding to a large number of site-directed mutants of HPr (Sharma *et al.*, 1991; Sharma, 1992). Binding constants were later directly determined by fluorescence polarization (Smallshaw *et al.*, 1998). The structure of a complex of the Jel42 Fab fragment and HPr has been determined by X-ray crystallography (Prasad *et al.*, 1998), which is in agreement with previous conclusions about the Jel42 binding site on HPr.

A very recent communication (Wang *et al.*, 2000) has helped to condense the information regarding protein:protein interactions involving HPr. Using NMR chemical shift experiments, the authors delineated binding sites on *E. coli* HPr for its interacting proteins, namely the N-terminal domain of enzyme I, enzyme IIA\textsuperscript{ßC}, and glycogen phosphorylase. All proteins occupy essentially the same binding site on HPr.

1.3.5 HPr kinetics

In kinetic terms, HPr may be considered a substrate for phosphodonation by enzyme I and a substrate for phosphoacceptance by a sugar-specific IIA protein. In both reactions, normal, hyperbolic Michaelis-Menten kinetics can be observed. However, even though HPr can most accurately be described as a substrate, or “phosphocarrier protein”, the protein also contains characteristics of an enzyme, as it has an active site, and can contribute to the catalysis of a phosphotransfer reaction (Anderson, 1994).
1.3.5.1 HPr/Enzyme I kinetics

The phosphorylation of HPr by enzyme I, at the expense of the conversion of PEP to pyruvate, proceeds via a ping-pong bi-bi mechanism (Waygood and Steeves, 1980; Weigel et al., 1982a; Waygood, 1986). The latter part of the reaction mechanism, concentrating on the kinetic interaction with HPr, is shown in equation 1.2.

\[
\text{enzyme I-P + HPr} \xrightleftharpoons[k_2]{k_1} \text{enzyme I-P-HPr} \xrightarrow{k_3} \text{enzyme I + P-HPr} \quad (1.2)
\]

The effects of site-directed mutants of HPr in EI kinetics can be kinetically analyzed, and, as stated earlier, mutants in which both \(K_m\) and \(V_{max}\) of enzyme I are affected result from a decrease in catalysis, whereas HPr mutants in which only the enzyme I \(K_m\) is affected result from a decrease in binding potential, not catalysis.

1.3.5.2 HPr/Enzyme II kinetics

The kinetics describing the reaction of a sugar-specific enzyme II with HPr are more complicated. The complete reaction proceeds via the following equation:

\[
(P)\text{HPr + IIABC} \leftrightarrow \text{HPr + II(P)ABC} \leftrightarrow \text{IIA(P)BC + sugar}_{(\text{out})} \leftrightarrow \text{IIABC + (P)sugar}_{(\text{in})} \quad (1.3)
\]

Each step of this reaction mechanism has rate constants in either direction. The interpretation of data from enzyme assays of wild type and mutant HPr substrates in the presence of saturating concentrations of sugar is difficult. It is not known which is the rate limiting step of the above mechanism under different conditions or in the presence of various HPr mutants. In addition, the domain structure, binding interactions, and catalytic mechanisms of the sugar-specific enzymes II vary considerably (Section 1.2), which
makes it difficult to apply results gained from the assay of one enzyme II to the mechanism of another.

1.4 The general PTS protein enzyme I

Enzyme I (enzyme I) catalyzes the initial step of the PTS, which is the phosphorylation of the N\textsuperscript{\text{\#1}} nitrogen of His15 of HPr at the expense of the conversion of one molecule of phosphoenolpyruvate (PEP) to pyruvate. This reaction proceeds via a stable phospho-enzyme I intermediate, with the protein phosphorylated at the N\textsuperscript{\text{\#2}} position of His189. Enzyme I plays an important role as the first enzyme in the PEP:sugar phosphotransferase system, and as such it has been postulated as the major enzyme of regulation of the PTS in Gram-negative bacteria, which would indicate that it may be important for the regulation of carbon metabolism as well (Chauvin et al., 1996a).

1.4.1 Comparison of enzymes I and homologs

Enzymes I have been identified in numerous prokaryotic microorganisms, either through direct investigation or genome sequencing projects. Enzymes I have considerable homology, and possess areas of high conservation (Reizer et al., 1993) which include the active site region surrounding the histidine residue of phosphorylation. Enzyme I sequences from E. coli (De Reuse and Danchin, 1988) and S. typhimurium (LiCalsi et al., 1991) show the most homology, each with 575 amino acid residues which are 97% identical. These two enzymes are functionally interchangeable; their HPr substrates are identical.

In addition, two other enzymes with sequence and functional similarity to enzyme I are known (Reizer et al., 1993). The best studied of the two homologs is pyruvate phosphate dikinase (PPDK), which catalyzes the interconversion of PEP and pyruvate along with phosphate (P\textsubscript{i}) and pyrophosphate (PP\textsubscript{i}):
Pyruvate + Pi + ATP $\leftrightarrow$ PEP + PPi + AMP \hfill (1.4)

PPDK genes have been identified in bacteria such as *Clostridium symbiosum* (Pocalyko et al., 1990), several protists such as *Trypanosoma brucei* (Bringaud et al., 1998), and a wide variety of plants, where the enzyme is most important for the C4 cycle of carbon metabolism (Matsuoka, 1995). PPDK employs a reaction mechanism which is similar to that of enzyme I, and involves an N\(^{\epsilon2}\)-phosphorylated histidine. This residue is His455 in *C. symbiosum* PPDK (Goss and Wood, 1982), which is the most characterized of all PPDKs.

The other homolog of enzyme I is phosphoenolpyruvate synthetase. This enzyme has been poorly studied in comparison to enzyme I and PPDK. It catalyzes the conversion of pyruvate to PEP at the expense of the conversion of ATP to AMP + Pi:

pyruvate + ATP $\leftrightarrow$ PEP + AMP + Pi \hfill (1.5)

This reaction also proceeds via a stable N\(^{\epsilon2}\)-phosphohistidine intermediate (Narindrasorasak and Bridger, 1977).

An alignment of several representative enzymes I and homologs (Figure 1.8) reveals important information. Several areas of sequence conservation are highlighted, the most important of which is the region surrounding the active site histidine (residue 256 in the alignment numbering). Other areas of conservation are evident, but these regions lie only on the C-terminal side of the active site histidine. The sequence alignment reveals no significant conservation in the area between the N-terminus of the proteins and the active site region. Indeed, all the sequences align poorly in this area, and the alignment varies depending on the program and parameters used. The program used for the alignment in Figure 1.8 is Match-box, which places heavy emphasis on areas of local similarity, and little penalty on the inclusion of gaps in the alignment (Depiereux et al., 1997). Other sequence alignments (Reizer et al., 1993; Pocalyko et al., 1990) may be different.
Figure 1.8  Multiple sequence alignment of representative enzymes I and related sequences. The alignment program used was Match-Box (Depiereux et al., 1997). Sequences are: Ec EI, E. coli enzyme I (De Reuse and Danchin, 1988); St EI, S. typhimurium enzyme I (LiCalsi et al., 1991); Bs EI, B. subtilis enzyme I (Reizer et al., 1993); Sc EI, Staphylococcus carnosus enzyme I (Kohlbrecher et al., 1991); Cs PPDK, Clostridium symbiosum pyruvate, phosphate dikinase (Pocalyko et al., 1990); Zm PPDK, Zea mays pyruvate, phosphate dikinase (Matsuoka et al., 1988); Ec PPS, E. coli phosphoenolpyruvate synthetase (Niersbach et al., 1992). Residues in the alignment highlighted in black correspond to residues conserved in at least five of the seven sequences. The numbers to the left of each sequence indicate the first amino acid in the row. The numbers above the alignment correspond to the consensus sequence and not to any particular amino acid sequence. The consensus sequence is presented below the alignment; residues in capital letters are conserved throughout all seven sequences, those in lower case letters are conserved in five or six out of seven sequences. The conserved active site histidine, residue 256 in the alignment numbering, is marked with an asterisk above the alignment.
1.4.2 Dimerization of enzyme I

*E. coli* and *S. typhimurium* enzymes I are functionally interchangeable in vitro and in vivo, and will be discussed interchangeably in this and ensuing sections. Enzyme I has been purified from both *E. coli* (Robillard *et al.*, 1979; Waygood and Steeves, 1980) and *S. typhimurium* (Weigel *et al.*, 1982b). Numerous studies have shown that the active form of enzyme I is a homodimer of 63 kDa subunits, which dissociates at low temperature (Waygood and Steeves, 1980; Missel *et al.*, 1980; Kukuruzinska *et al.*, 1982; Kukuruzinska *et al.*, 1986). This active form of the enzyme has two sites of phosphorylation per dimer (Waygood, 1986).

*E. coli* enzyme I has been specifically labeled at its C-terminal cysteine residue with a fluorescent pyrene tag (Han *et al.*, 1990). This labeled enzyme was found to be functionally identical to the unlabeled enzyme. The labeled enzyme was studied by fluorescence anisotropy in order to characterize the monomer/dimer equilibrium (Chauvin *et al.*, 1994a) and monomer/dimer kinetics (Chauvin *et al.*, 1994b). Ratio of dimer to monomer increased as a function of temperature and presence of the ligands PEP and Mg$^{2+}$, but conversely decreased slightly if the subunits were phosphorylated. In a range of conditions, the association constant $K_{eq}$ ranged from $0.5 \times 10^5$ M$^{-1}$ to $240 \times 10^5$ M$^{-1}$ (Chauvin *et al.*, 1994a). The kinetics of association is slow, with a measured $k_A$ of $3.4 \times 10^3$ M$^{-1}$s$^{-1}$, which is two to three orders of magnitude slower than that measured for most other proteins. This association was again influenced by degree of phosphorylation and presence of ligands. Dissociation kinetics were similarly slow (Chauvin *et al.*, 1994b).

The quaternary structure of enzyme I from the Gram-positive bacterium *Staphylococcus carnosus* has also been studied (Hübner *et al.*, 1995), and it was found that addition of both Mg$^{2+}$ and PEP favours dimerization. In the absence of those ligands the enzyme was primarily in the monomeric state, even at room temperature with high concentrations of enzyme I (3 mg/mL). However, Reizer *et al.* (1992) report that enzyme I from another Gram-positive bacterium, *B. subtilis*, elutes from a gel filtration column as
a monomer, independent of temperature, and only a small amount of dimer was observed at room temperature after inclusion of PEP during gel filtration chromatography. Conversely, purification of enzymes I from the Gram-positive bacteria *Streptococcus salivarius* (Vadeboncoeur *et al.*, 1983), *Streptococcus mutans* (Thibault and Vadeboncoeur, 1985), and *Streptococcus faecalis* (Alpert *et al.*, 1985) has also been reported, and in each case the protein eluted as a dimer from gel filtration columns at 4°C.

Despite the apparent contradictions amongst studies of enzymes I from Gram-positive bacteria, the quaternary structures of enzymes I from Gram-negative *E. coli* and *S. typhimurium* are well characterized. The slow association kinetics are consistent with the hysteretic behaviour of the enzyme, where the room temperature activity of enzyme I preincubated at 4°C slowly increases over time, as a function of the slow association of the subunits (Waygood and Steeves, 1980; Misset *et al.*, 1980). The slow monomer/dimer transition has been proposed as a target of regulation of enzyme I, and thus in vivo regulation of the PTS (Waygood *et al.*, 1977; Chauvin *et al.*, 1996a). However, besides PEP and Mg²⁺, no other significant physiological effectors of enzyme I activity or dimerization have been found (Saier *et al.*, 1980; Waygood and Steeves, 1980; Misset and Robillard, 1982; Weigel *et al.*, 1982). Still, enzyme I remains a candidate for a target of regulation, and cellular PEP levels, and/or PEP:pyruvate ratio, may in fact play a primary role in the regulation of enzyme I activity (Weigel *et al.*, 1982).

### 1.4.3 Domain structure of enzyme I

Proteolytic cleavage studies of *S. typhimurium* enzyme I resulted in the discovery of a 30 kDa fragment of the protein that remained resistant to digestion (LiCalsi *et al.*, 1991). This fragment was identified as the N-terminal fragment of enzyme I, EIN, which contained the active site His189 residue. EIN was able to accept a phosphoryl group form phospho-HPr, but not phosphoenolpyruvate. Thermodynamic studies indicated that the N-terminal domain of enzyme I was structurally and thermodynamically distinct from the
more conformationally unstable C-terminal domain, and that the two domains are likely joined by a flexible linker (LiCalsi et al., 1991). With the further utilization of molecular biology techniques in the mid-1990's, several labs sought to separately subclone and overexpress EIN. In 1996, two laboratories published reports where the cloning and characterization of EIN had been accomplished (Seok et al., 1996a; Chauvin et al., 1996b). In both cases the cloned fragment had similar characteristics to those identified by LiCalsi et al. (1991). Chauvin et al. (1996b) also demonstrated that purified EIN did not dimerize, regardless of temperature or inclusion of PEP or Mg$^{2+}$. Putative functions to the N- and C-terminal domains of enzyme I were then assigned. It was evident that the N-terminal domain contained the HPr binding site and the phosphotransferase catalytic site, whereas the C-terminal domain contained sites for binding PEP and subunit interaction. The sequence alignment of enzymes I with other PEP-utilizing enzymes (Figure 1.8) also lends evidence to these functional assignments. The N-terminal domain of enzyme I does not align well with PPDK or PEP synthetase (PPS), which would indicate that this region may contain the specific HPr-binding sequences. Conversely, the C-terminal domain of enzyme I does have homology to PPDK and PPS, and both of these enzymes bind PEP and readily form oligomers (Narindrasorasak and Bridger, 1977; Herzberg et al., 1996). Thus it is likely that the C-terminal domain of enzyme I contains PEP binding and oligomerization activities.

The EIN protein has been further characterized in more recent studies. It was observed, through differential scanning calorimetry, that phosphorylation of EIN results in a decrease in conformational stability, which may promote phosphotransfer to HPr (Nosworthy et al., 1998). Fomenkov et al. (1998) were able to demonstrate complementation of EIN by the cloned C-terminal domain of enzyme I (EIC), in that a mixture of the two proteins had complete enzymatic activity. This complementation was shown by in vitro phosphorylation of proteins followed by autoradiography, and by fermentation of PTS sugars by ptsI bacterial strains harboring EIN and EIC
overexpression plasmids. The authors were not able to efficiently overexpress and purify EIC from the plasmid construct, citing lethality to the bacteria and sensitivity to protease digestion as possible reasons. Other researchers were able to purify a similar *M. capricolum* EIC protein, and it was able to complement EIN using *in vitro* enzyme assays (Zhu et al., 1999). However, this activity was on the order of 10^6-fold lower than wild type enzyme I activity.

1.4.4 Tertiary structure of enzyme I

A wealth of information regarding the function and putative structure of enzyme I has been gained from studies of purified proteins and protein fragments. Unfortunately, to date no laboratory has been able to obtain crystals of enzyme I suitable for X-ray diffraction. Furthermore, enzyme I, at ~60 kDa, is too large to be studied by modern NMR techniques. As a result, no tertiary structure of intact enzyme I is available. However, the smaller size (~30 kDa), high solubility, and conformational stability of EIN make it more amenable to study by NMR and X-ray crystallography, and the structure of this domain has been solved.

1.4.4.1 Structure of enzyme I N-terminal domain

The structure of the 259 residue N-terminal domain of *E. coli* enzyme I has been determined by both multidimensional NMR (Garrett et al., 1997a) and X-ray crystallography (Liao et al., 1996). The two structures are very similar, and in each case the protein is elliptical in shape, and has two subdomains: an \( \alpha/\beta \) subdomain, consisting of residues 1-20 and 148-230 which is composed of six \( \beta \)-strands and three \( \alpha \)-helices, and a second all \( \alpha \) subdomain, residues 33-143, consisting of four helices (Figure 1.9). The four \( \alpha \)-helices of the \( \alpha \)-subdomain are arranged as two hairpins in a claw-like conformation. The active site His189 is located on the \( \alpha \)-subdomain, and faces a cleft between the two subdomains. The \( N^2 \) atom of His189 is a hydrogen bond acceptor with
Figure 1.9 X-ray crystal structure of *E. coli* enzyme I N-terminal domain, PDB accession code 1ZYM, with the α-subdomain shown on the bottom and the α/β-subdomain shown on the top. The large number of acidic residues in the active site (red) are labeled, as are the catalytic His189 (green) and its hydrogen bonding partner Thr168 (yellow). Figure displayed in similar orientation to that shown in Liao *et al.* (1996). Diagram was produced with SETOR (Evans, 1992).
the hydroxyl hydrogen of Thr168. The pKₐ of His189 was determined to be 6.5 (Garrett et al., 1997).

The α/β-subdomain of EIN, containing the active site histidine, has sequence similarity to C. symbiosum PPDK (Reizer et al., 1993; Figure 1.8). The structure of C. symbiosum PPDK has been determined by X-ray crystallography (Herzberg et al., 1996), and its phosphohistidine domain contains a topological fold which is very similar to that found in the α/β-subdomain of EIN. Thus in the intact enzyme I, the α/β-subdomain is responsible for autophosphorylation, whereas the α-subdomain of enzyme I, not found in the structure of PPDK, is responsible for binding and phosphotransfer to HPr (Liao et al., 1996).

This postulate was subsequently proven by a number of methods. The α-helical subdomain of intact M. capricolum enzyme I was deleted and the resulting protein [enzyme I(ΔHD)] was subsequently isolated. This protein was not capable of phosphotransfer to HPr, but phosphotransfer activity was reconstituted after the addition of another separate protein; the isolated α-subdomain of enzyme I (Zhu et al., 1999). A binding site for HPr on EIN was also delineated by NMR chemical shift experiments (Garrett et al., 1997b), and EIN residues which underwent chemical shift perturbations upon addition of HPr were restricted to the α-subdomain. Certainly, the most conclusive determination of the interaction site between EIN and HPr appeared when the solution structure of the ~40 kDa complex of EIN and HPr was reported (Garrett et al., 1999). It is necessary to describe this structure in greater detail.

The EIN binding site on HPr has been discussed previously (Section 1.3.4.1.1), and is found on helices 1 and 2 of HPr, and the residues of EIN involved in the interaction will be described here. Three of the four helices of the α-subdomain of EIN form contacts with HPr, and thus the interaction involves only helical structures. The conformations of each protein change very little upon complexation. Residues 71 to 85 and 111 to 126 are the most important residues of EIN involved in the interaction. This interaction is mainly
hydrophobic, but several key residues of EIN are involved in electrostatic interactions; these are Glu67, Glu68, Glu74, Asp82, and Glu84. There are only three side chain–side chain hydrogen bonds, with Glu84, Arg126, and Asp129 the participating residues of EIN.

Garrett et al. (1999) also modeled a transition state of the phosphotransfer complex. The hydrogen bond between Thr168 and His189 of EIN was removed, and the side chain of His189 was allowed to rotate in order to be linked via a phosphate ‘tether’ to His15 of HPr. In this model, EIN is more conformationally unstable than in the absence of phosphorylation, which may promote phosphotransfer to HPr. Unfortunately, the phosphorylated state of the EIN/HPr complex is too short lived to be directly investigated by NMR, and neither has the structure of phospho-EIN been determined. Therefore, a different interaction between EIN, or for that matter intact enzyme I, and HPr during phosphotransfer cannot be ruled out.

1.4.4.2 Structure of enzyme I C-terminal domain

The C-terminal domain of enzyme I, approximately 30 kDa in size, has an unknown tertiary structure. Thermodynamic unfolding and protease digestion experiments (LiCalsi et al., 1991) and study of the reactivity of the thiol groups of its four cysteine residues (Han et al., 1990) indicate that the C-terminal domain of enzyme I is conformationally unstable, which makes the structure difficult to determine. However, a protein with sequence and functional homology, and thus likely structural homology, to enzyme I has been structurally characterized by X-ray crystallography. This structure of C. symbiosum pyruvate phosphate dikinase can be used as a model for the structure of the C-terminal domain of enzyme I.
1.4.4.2.1 Structure of *C. symbiosum* PPDK

X-ray crystallography was used to obtain the three-dimensional structure of the dimer of *C. symbiosum* pyruvate phosphate dikinase (Herzberg *et al.*, 1996). The protein is quite large, with a monomer molecular mass of 96 kDa. The protein (Figure 1.10) has three structurally and functionally distinct domains, connected by two flexible linkers (Carroll *et al.*, 1994). The N-terminal domain, 38 kDa, contains the ATP and P\textsubscript{i} binding sites (McGuire *et al.*, 1996; McGuire *et al.*, 1998). The middle domain, 18 kDa, is the catalytic phosphotransfer domain containing the active site histidine-455, and the final 35 kDa C-terminal domain contains the pyruvate/PEP binding site (Xu *et al.*, 1995a). The dimer of the protein forms a 'butterfly' conformation (Figure 1.10) with the C-terminal domain forming the site of interaction and the other two domains of each subunit facing away from each other. The structure of the dimer indicates that the active sites of each subunit work independently, and that phosphotransfer between subunits cannot occur. The three domains of the monomer are joined by two flexible partly helical linkers. These linkers interact with each other to form a pivot which allows the central catalytic domain to 'swivel' between the N- and C-terminal domains in order for phosphotransfer to occur between the remote reaction sites (Herzberg *et al.*, 1996). A similar domain-swiveling mechanism can be envisioned for enzyme I.

The C-terminal domain of PPDK forms an eight-stranded \(\alpha/\beta\)-barrel, which is a structural motif found in PEP/pyruvate binding domains of other proteins such as pyruvate kinase (Larsen *et al.*, 1994). Structural modeling and comparison to pyruvate kinase indicates that in PPDK, phosphoenolpyruvate would be bound in the centre of the barrel (Herzberg *et al.*, 1996). The C-terminal domain also contains the subunit interaction site. Through a sequence alignment (Figure 1.8), residues in the C-terminal domain of PPDK which are conserved between enzyme I and PEP synthetase are identified; these residues are likely important for binding PEP and/or subunit interaction. Using the structure of PPDK, combined with sequence comparison, a putative structure of
Figure 1.10  Space filling diagram of the crystal structure of *C. symbiosum* PPDK, PDB accession code 1DIK, shown in “butterfly” arrangement. The N-terminal ATP/P\(_i\) binding domains (residues 1-400) are shown in red, the central phosphohistidine domains (residues 401-504) are shown in blue, and the C-terminal PEP binding/dimerization domains (residues 505-874) are shown in two shades of yellow. Diagram was produced with SETOR (Evans, 1992).
the enzyme I C-terminal domain can be fashioned, and when connected to the structure of the enzyme I N-terminal domain, a working model of intact enzyme I can be created (Zhu et al., 1999). Further structural and functional investigations are needed in order to determine the validity of this model.

1.4.5 Enzyme I kinetics

As previously discussed, enzyme I participates in a covalent catalysis type of mechanism, which can be characterized by ping-pong bi-bi kinetics. However, during kinetic studies of the enzyme from E. coli or S. typhimurium it is important to take into account the monomer:dimer hysteresis. In most kinetic studies, the enzyme is first preincubated at room temperature in a buffer containing PEP and Mg\(^{2+}\). This converts the enzyme to an active, phosphorylated dimer, which is then added to the assay mixture (Waygood and Steeves, 1980; Weigel et al., 1982). However, Misset and Robillard (1982) assayed the enzyme after preincubation in a dilute state at 4°C, and under these conditions the assay data did not fit the classical ping-pong bi-bi mechanism. This may be attributed to the slow increase in enzyme activity due to the hysteretic behaviour of the enzyme. Thus, the enzyme must be preincubated with PEP and Mg\(^{2+}\) at room temperature in order to have the enzyme in an active dimeric form with two sites of phosphorylation per dimer (Waygood, 1986). With these assay procedures, reliable kinetic data can be obtained. E. coli enzyme I has a PEP \(K_m\) of 0.18 mM and an HPr \(K_m\) of 9 \(\mu\)M (Waygood and Steeves, 1980), while S. typhimurium enzyme I has similar characteristics with \(K_m\)'s of 0.2 mM and 5.4 \(\mu\)M for PEP and HPr, respectively (Weigel et al., 1982b).

1.4.6 Catalytically important residues of enzyme I

In contrast to the extensive site-directed mutagenesis work done in order to characterize the structure and function of HPr (Anderson, 1994; Waygood, 1998), comparably little is known about the residues of enzyme I which are crucial for catalytic
function. Seok et al. (1996b) described the importance of the Gly338 residue of *E. coli* enzyme I. Mutants at that residue displayed substantially lower autophosphorylation activity, although phosphotransfer to HPr was apparently unaffected. That study also described a His189Gly mutant of enzyme I, which was able to bind PEP but was inactive in phosphotransfer, as expected. No other biochemically characterized enzyme I mutants are available; however, some information can be gained from work on *C. symbiosum* pyruvate phosphate dikinase. The importance of residues Cys83 (Xu et al., 1995b) and Arg561 (Yankie et al., 1995) for the PEP/pyruvate partial reaction has been described. Both of these residues are conserved amongst all enzyme I, PPDK, and PEP synthetase genes sequenced to date (Figure 1.8), so they almost certainly have importance for enzyme I function as well.

### 1.5 Aims and objectives

Modern experimental analysis of HPr, involving the creation of a large number of site-directed mutants and complex investigation of their properties, has led to a highly detailed picture of the structure and function of the small phosphocarrier protein. However, comparably less is known about the structure and function of the protein responsible for phosphorylating HPr, namely enzyme I. Enzyme I undergoes complex interactions with its chemical ligands, phosphoenolpyruvate and Mg$^{2+}$, as well as protein:protein interactions during the formation of homodimers and also phosphotransfer with HPr. At the start of this research project, no study regarding single amino acid mutants of enzyme I or the tertiary structure of enzyme I had been published. Considering the refinement of modern molecular biological and biochemical techniques, as well as the high solubility and established purification procedures of enzyme I, it was decided to take the opportunity to study the structure and function of enzyme I in detail.

Some time ago two "leaky" mutants of enzyme I, which had low activity and were not able to form dimers, and one "tight" mutant of enzyme I which had no activity but was
able to dimerize, were briefly investigated using cell extracts and partially purified proteins. This project describes the cloning, sequencing, purification, and analysis of these enzyme I mutants, as well as other mutants created for this project. The importance of the identified amino acid residues for various aspects of enzyme I activity will be investigated. Also, the intragenic (or interallelic) complementation of the original mutants identified during initial studies will be further characterized. These enzyme I proteins will be used as a tool to investigate the complex function of enzyme I, in particular the potential role of the monomer:dimer transition of enzyme I, in the in vivo regulation of the PTS.

In addition, the interaction of enzyme I with its substrate protein of the PTS, HPr, will be investigated. The kinetic interactions of enzyme I with the large number of HPr mutants already available, as well as other mutants created specifically for this project, will be investigated separately in detail. Mutations to HPr residues that affect $K_m$ but not $V_{max}$ of enzyme I indicate that these residues are involved primarily in binding and not catalysis. Using this kinetic data, a map of the functional interactions of the binding site between enzyme I and HPr will be created.
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

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<td>Agarose, electrophoresis grade</td>
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<tr>
<td>Agarose, low melting point (LMP)</td>
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**Microbiological supplies**

| Agar | BDH |
| Ampicillin | Sigma |
| Bacto-agar | Difco |
| Bacto-trypotone | Difco |
| Bacto-yeast extract | Difco |
| Kanamycin | Roche |
| MacConkey agar | Difco |
| Tetracycline | Sigma |

**Enzymes/proteins**

| Calf intestinal alkaline phosphatase (CIP) | Promega |
| Deep Vent DNA Polymerase | New England Biolabs |
| Dpn I endonuclease | Stratagene/New England Biolabs |
| Pfu DNA polymerase | Stratagene |
| Lactate dehydrogenase (rabbit muscle) | Sigma |
| Restriction endonucleases | New England Biolabs/Promega/Amersham-Pharmacia Biotech |
| T4 DNA ligase | New England Biolabs/Promega/Novagen |
| T4 Polynucleotide kinase | New England Biolabs/Novagen |
| T7 DNA polymerase | Amersham-Pharmacia Biotech |
Table 2.2 Name and address of suppliers

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<td>Whatman</td>
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2.2 Non-commercial chemicals and proteins

Purified *E. coli* phosphoenolpyruvate carboxykinase was provided by Dr. Hughes Goldie, Department of Microbiology, University of Saskatchewan. The Jel45 antibody was provided by Dr. Jeremy Lee, Department of Biochemistry, University of Saskatchewan. Many purified mutant HPr proteins were prepared in the Waygood laboratory by Drs. Bill Anderson, Scott Napper, and Sadhana Sharma. Phosphoenolpyruvate (monocyclohexylammonium salt) was prepared by the method of Clark and Kirby (1963).
2.3 Bacterial Strains

The bacterial strains utilized in this work are listed with their genotypes in Table 2.3. The *E. coli* strain ESK108, possessing no detectable HPr activity, was used for the overexpression of mutant HPr proteins. ESK108 was produced by converting the *E. coli* strain CSH4 to *ptsH* by N-methyl-N'-nitro-N-nitrosoguanidine mutagenesis (Waygood *et al.*, 1987). This strain (ESK120) was made *recA* by P1 phage transduction using a *recA56* mutation from strain JC10240 (Csonka and Clark, 1980) to produce ESK108. The *E. coli* strain TP2811, carrying a deletion through the entire *pts* operon (Levy *et al.*, 1990), was also used for overexpression of HPr and other PTS proteins.

The *E. coli* strain ESK238, containing no detectable enzyme I activity or protein, was used for overexpression of mutant enzyme I proteins (Brokx *et al.*, 2000). ESK238 is a spontaneous *ptsl* mutant of strain BL21 (Studier *et al.*, 1990) selected by streptozotocin resistance (Lengeler, 1980). This strain allows for the IPTG inducible expression of enzyme I from a plasmid containing the transcriptional start site for T7 RNA polymerase, such as pT7-7 (Tabor and Richardson, 1985). This strain was prepared by James Talbot.

Several *S. typhimurium* strains were also used in this work. The strains SB1476, SB1681, and SB1690 were the strains from which mutant *ptsl* genes were cloned. Strains SB1476 and SB1681 are *ptsl* mutants in wild type *S. typhimurium* LT2 (Saier and Roseman, 1972) while SB1690 is a *ptsl* derivative of strain SB3507, which contains *trp223* (Cordaro and Roseman, 1972). All *S. typhimurium* mutant strains were kindly provided by Drs. Norman Meadow and Saul Roseman, Johns Hopkins University, Baltimore, Maryland.
Table 2.3  List of bacterial strains, genotypes, and references

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Escherichia coli</em> strains</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DH5α F'</td>
<td>F' endA1 hsdR17 (ρK, mK⁺) supE44 thi-1 recA1 gyrA (Nal') relA1 Δ(lacIZYA-ArgF)U169 deoR (Φ80dlacΔ(lacZ)M15)</td>
<td>Woodcock <em>et al.</em>, 1989</td>
</tr>
<tr>
<td>Epicurian</td>
<td>recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F proAB lacIΔ(M15]</td>
<td>Stratagene</td>
</tr>
<tr>
<td>Coli XL1-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blue</td>
<td>Tn10(Tet')</td>
<td></td>
</tr>
<tr>
<td>ESK108</td>
<td>F' trp thi rpsL ptsH465 recA56</td>
<td>Waygood <em>et al.</em>, 1987</td>
</tr>
<tr>
<td>ESK238</td>
<td>F- ompT gal [dcm] [lon] hsdSb (ρb⁺ m6') ptsl</td>
<td>Brox <em>et al.</em>, 2000</td>
</tr>
<tr>
<td>NovaBlue</td>
<td>endA1 hsdR17 (ρK, mK⁺) supE44 thi-1 recA1 gyrA96 relA1 lac [F proA⁺B⁺ lacIΔ(M15::Tn10(Tc₉))]</td>
<td>Novagen</td>
</tr>
<tr>
<td>TP2811</td>
<td>F- xyl arg HI ΔlacX74 aroB ilvA ΔptsH ptsI crr KanR</td>
<td>Levy <em>et al.</em>, 1990</td>
</tr>
<tr>
<td><em>Salmonella typhimurium</em> strains</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SB1476</td>
<td>ptsI17</td>
<td>Saier and Roseman, 1972</td>
</tr>
<tr>
<td>SB1681</td>
<td>ptsI16</td>
<td>Saier and Roseman, 1972</td>
</tr>
<tr>
<td>SB1690</td>
<td>ptsI34 trpB223</td>
<td>Cordaro and Roseman, 1972</td>
</tr>
<tr>
<td>SB3507</td>
<td>trpB223</td>
<td>Cordaro and Roseman, 1972</td>
</tr>
<tr>
<td>SB2950</td>
<td>trpB223 Δ(cysK-ptsH crr)</td>
<td>Cordaro <em>et al.</em>, 1972</td>
</tr>
</tbody>
</table>
2.4 Plasmid Vectors

Plasmid vectors are listed in Table 2.4.

Table 2.4 List of plasmid vectors

<table>
<thead>
<tr>
<th>Name</th>
<th>Source</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pET11dd</td>
<td>Novagen</td>
<td>Dubendorff and Studier, 1991</td>
</tr>
<tr>
<td>pT7-7</td>
<td>Amersham-Pharmacia Biotech</td>
<td>Tabor and Richardson, 1985</td>
</tr>
<tr>
<td>pT7Blue</td>
<td>Novagen</td>
<td>Novagen</td>
</tr>
<tr>
<td>pT7Blue-3</td>
<td>Novagen</td>
<td>Novagen</td>
</tr>
<tr>
<td>pUC19</td>
<td>Amersham-Pharmacia Biotech</td>
<td>Yanisch-Perron et al., 1985</td>
</tr>
</tbody>
</table>

2.5 Bacterial growth media

Media preparation was as described by Sambrook et al. (1989). Liquid Luria-Bertani (LB) media was composed of 10 g/L tryptone, 5 g/L yeast extract, and 10 g/L NaCl in H₂O. Solutions were autoclaved at 121°C and 18 psi. Sterile filtered (0.22 μm filter) ampicillin was added to the media (100 μg/mL) after the liquid had cooled to 50 – 60°C. For solid agar media, 15 g/L of agar was added to the liquid media prior to autoclaving. Liquid Terrific Broth (TB) contained 12 g/L tryptone, 24 g/L yeast extract, and 4 mL/L glycerol, as well as sterile potassium phosphate buffer, pH 7.3, which was added to the sterile broth to a final concentration of 90 mM after autoclaving. One litre of minimal salts A media contained 4.5 g KH₂PO₄, 10.5 g K₂HPO₄, 1 g (NH₄)₂SO₄ and 0.5 g sodium citrate. These ingredients were mixed in deionized water with 10 mM MgCl₂ and autoclaved. MacConkey agar media (50 g/L) was dissolved in deionized water and autoclaved at 121°C and 18 psi. Ampicillin (50 μg/mL) and sugars (5 g/L) were added to the cooled media prior to pouring into petri dishes.
2.6 Hazardous Materials

2.6.1 Ethidium Bromide

Ethidium bromide is a potent mutagen if ingested, inhaled, or allowed to come in contact with skin. Latex gloves were worn at all times when working with ethidium bromide to limit exposure. Ethidium bromide (10 mg/mL) was dissolved in deionized water and stored in a brown glass bottle. Solutions containing ethidium bromide and agarose gels stained with ethidium bromide were disposed of in a dedicated waste container and decontaminated (Quillardet and Hofnung, 1988).

2.6.2 Phenol

Phenol is toxic and corrosive if ingested or allowed to come in contact with skin. Phenol was prepared by five equilibrations with 1 volume of 0.1 M Tris-HCl buffer, (pH 8.0) removing the top aqueous layer after each equilibration. The phenol was always used in a well-ventilated area and latex gloves were used when handling solutions containing phenol. Liquids containing phenol were collected in a glass bottle and disposed of as chemical waste.

2.6.3 Chloroform:isoamyl alcohol

Chloroform is volatile and toxic when inhaled or ingested. Chloroform was always handled in a well ventilated area while wearing latex gloves. Chloroform solutions used in DNA plasmid purifications were prepared by mixing 24 volumes of chloroform and one volume of isoamyl alcohol. Samples containing chloroform were collected in a glass bottle and disposed of as chemical waste.
2.6.4 Acrylamide

Acrylamide, when in the unpolymerized form, is a potent neurotoxin and dangerous if inhaled, swallowed, or allowed to come in contact with skin. Acrylamide solutions were prepared by dissolving 28.2 g acrylamide and 0.8 g bis-acrylamide in 100 mL deionized water with vigorous mixing and heat. Acrylamide powders were always handled in a fumehood and while wearing latex gloves and appropriate face mask to minimize exposure. Acrylamide solutions were handled while wearing latex gloves. Any unused acrylamide solutions were polymerized by the addition of ammonium persulfate and TEMED in order to reduce toxicity, and disposed of in regular waste.

2.6.5 Sodium Azide

Sodium azide is hazardous when inhaled or ingested. It also forms explosive compounds when combined with heavy metals. Aqueous solutions containing sodium azide (0.2% or 0.02% w/v) were prepared while wearing gloves and face mask to minimize exposure. Any solutions containing sodium azide were collected in storage bottles and disposed of as chemical waste.

2.7 Mutagenic and PCR primers

Most mutagenic and PCR primers were synthesized by Dr. F. Georges, Plant Biotechnology Institute, National Research Council, Saskatoon, Saskatchewan.

2.7.1 Mutagenic and PCR primer design

Mutagenic primers were designed to introduce a specific mutation at a specific location of the DNA template. The sequence of the primer depended on the nature of the mutation and the surrounding template, but in general mutagenic primers were designed with certain characteristics in mind. Each primer was approximately twenty to twenty five nucleotides in length, with the mismatch located at or near the middle of the primer.
Preference was given for primers with high proportions of the bases G and C. The annealing temperature \((T_m)\) of each primer was calculated using the formula:

\[
T_m (^{\circ}C) = 81.5 + 0.41(\%GC) - 675/N - (\% \text{mismatch})
\]  

(2.1)

Where \(N\) is the number of nucleotides of the primer.

Primers used for amplifying \(ptsl\), the gene encoding enzyme I, by polymerase chain reaction (PCR) were also designed with the above characteristics in mind, although not all primers contained mismatches. The primers used for cloning \(S.\ typhimurium\ ptsl\) contained mutations necessary to incorporate \(Nde\ I\) and \(BamH\ I\) restriction sites. All primers utilized in this thesis are listed in Table 2.4.

**Table 2.4** Sequences of primers used for mutagenesis and PCR

**Primers used for PCR cloning and subcloning:**

\(S.\ typhimurium\ ptsl\) cloning:

\(ptsl.1\)  
5' - AAGTAAGCCTTGGCATATGATTTCT - 3'

\(ptsl.2\)  
5' - CAGTAATTGGATCCGCGTCT - 3'

\(E.\ coli\ ptsl\) cloning:

\(E15'\)  
5' - GTAAGGTTAGGCATATGATTTGCAGGC - 3'

\(E13'\)  
5' - CGCATCTCGTGGATTAGCAG - 3'

EIC37 subcloning:

\(M239*+\)  
5' - CGAAGTTATTGATCATATGCGGCGCTG - 3'

\(M239*-\)  
5' - CAGCAGCGCATATGATCAATAACTTG - 3'

**Primers used for \(E.\ coli\) enzyme I site directed mutagenesis:**

\(R126C+\)  
5' - CCTGGAAAGAATGTGCCGCTGACG - 3'

\(R126C-\)  
5' - CGTCAGCCGCACACATTCTTCAGG - 3'

\(G356S+\)  
5' - CCTGATCTTCTCAGCTGGCCGCGCTATC - 3'

\(G356S-\)  
5' - GATAGCGCCGAGCTGAGGAACGGG - 3'

\(R375C+\)  
5' - CGATCAGCTCTGCGGCTATCGG - 3'
Primers used for HPr site directed mutagenesis:

R375C- 5'- CAGGATAGCAGGCTGATCG -3'
R296L+ 5'- GGTCGATCTCTACTGAATTC -3'
R296L- 5'- GGAATTCAAGATACAGACC -3'
N352A+ 5'- CCGAAAGAAGAGGAGCCGCTCCCTCGGC -3'
N352A- 5'- GCCGAGGAAACGGCGCTCTTCTTCCGG -3'
N352E+ 5'- CCGAAAGAAGAGGAGCCGCTCCCTCGGC -3'
N352E+ 5'- GCCGAGGAAACGGCGCTCTTCTTCCGG -3'
L355A+ 5'- GAGAAACCCCGCTCGAAGGCTGGGCGCGC -3'
L355A- 5'- GAGAAACCCCGCTCGAAGGCTGGGCGCGC -3'
L355E+ 5'- GAGAAACCCCGCTCGAAGGCTGGGCGCGC -3'
L355E- 5'- GAGAAACCCCGCTCGAAGGCTGGGCGCGC -3'
H189D+ 5'- CCGTACCTCCGACACCTATCATGG -3'
H189D- 5'- CCATGATAGAGGTGTCGGAAGTACGG -3'
H189E+ 5'- CCATGATAGAGGTGTCGGAAGTACGG -3'
H189E- 5'- CCATGATAGAGGTGTCGGAAGTACGG -3'

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>T16A+</td>
<td>5’- CGGTCTGCACGCGCGCCTGC -3’</td>
</tr>
<tr>
<td>T16A-</td>
<td>5’- GCAGGCGCGGCGGTACGAGCCG -3’</td>
</tr>
<tr>
<td>A20T+</td>
<td>5’- CCGCCCTGCTACCCAGTTGTAACAGG -3’</td>
</tr>
<tr>
<td>A20T-</td>
<td>5’- CTTTTACAAACTGGGTAGCAGGGGCGG -3’</td>
</tr>
<tr>
<td>Q21A+</td>
<td>5’- GCCCTGGCTGCGGGCTTCTTCAAGG -3’</td>
</tr>
<tr>
<td>Q21A-</td>
<td>5’- CTTTTACAAACTGGGTAGCAGGGGCGG -3’</td>
</tr>
<tr>
<td>K24A+</td>
<td>5’- CCAAGTTTCTAGCAGAAAGCTAAGGCGG -3’</td>
</tr>
<tr>
<td>K24A-</td>
<td>5’- GCCCTGGCTGCGGGCTTCTTCAAGG -3’</td>
</tr>
<tr>
<td>K27A+</td>
<td>5’- GTAAAGAAGCTGGGCGGCTCTGACTCC -3’</td>
</tr>
<tr>
<td>K27A-</td>
<td>5’- GAAGTGAAGCCCGCAGCTCTTCTTCTTAC -3’</td>
</tr>
<tr>
<td>L47A+</td>
<td>5’- AGCGCGGCGAACAGCGGGCTTCTTCTTCTTCT -3’</td>
</tr>
<tr>
<td>L47A-</td>
<td>5’- AGCGCGGCGAACAGCGGGCTTCTTCTTCTTCT -3’</td>
</tr>
<tr>
<td>Q51A+</td>
<td>5’- TGCTTTTACAACCTGGCGGACTCTGGG -3’</td>
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<tr>
<td>Q51A-</td>
<td>5’- TGCTTTTACAACCTGGCGGACTCTGGG -3’</td>
</tr>
<tr>
<td>L55A+</td>
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<tr>
<td>L55A-</td>
<td>5’- CAGACGCTGGCCTGACCTAAGGCTACC -3’</td>
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<td>T56A+</td>
<td>5’- TGTTGGCTGCTCAAGGCTACC -3’</td>
</tr>
<tr>
<td>T56A-</td>
<td>5’- TGTTGGCTGCTCAAGGCTACC -3’</td>
</tr>
</tbody>
</table>
2.8 General cloning procedures

Throughout this thesis work, protocols regarding the manipulation of plasmid DNA have been carried out according to standard procedures (Sambrook et al., 1989). Several important and often used techniques will be described in this section.

2.8.1 Isolation of plasmid DNA

2.8.1.1 Small-scale isolation of plasmid DNA ("mini-prep")

One colony of the desired cells were transferred from an LB-antibiotic plate to 5 mL LB broth containing the appropriate antibiotic, which in almost all cases was ampicillin (100 μg/mL). The broth in a 14 mL round bottom tube, loosely capped, was incubated at 37°C with shaking at 300 rpm for 10 - 16 h. A 1.5 mL microfuge tube was filled with the culture and this was centrifuged at 12,000 g for 1 min. The pelleted cells were resuspended in 100 μL of a solution containing 25 mM Tris-HCl buffer (pH 8.0), 50 mM glucose, and 10 mM EDTA and incubated on ice for 5 min on ice. Then 200 μl of a freshly prepared solution of 0.2 N NaOH and 0.1% SDS was added, mixed by inversion, and incubated for 5 - 10 min on ice. After incubation, 150 μL of cold (4°C) 3M potassium acetate (pH 5.2) was added and the well mixed solution was incubated on ice for 10 min.

The microfuge tube was then centrifuged at 12,000 g for 10 min at 4°C. The resulting supernatant was transferred to another microfuge tube and 200 μL each of buffer equilibrated phenol and 24:1 chloroform:isoamyl alcohol were added. The solution was mixed for 2-5 min and centrifuged for 1 min at 12,000 g to separate the phases. The upper aqueous layer was transferred to a new microfuge tube and 1 mL ethanol was
added. After incubation at -20°C for 10 min, the precipitated RNA and plasmid DNA were pelleted by centrifugation at 12000 g for 15 min. The pellet was washed twice with 1 mL ice cold 70% ethanol, and dried in a vacuum dessicator. The pellet was then dissolved in 30 µL TE buffer (10 mM Tris-HCl buffer, 1 mM EDTA, pH 8.0), and 1 µL of DNase-free Ribonuclease A (1 mg/mL) was added, and the solution was left at room temperature for 30 min to digest the RNA, before long-term storage at 4°C.

2.8.1.2 Medium scale plasmid isolation

One colony of the desired cells was suspended in 5 mL LB broth containing 100 µg/mL ampicillin. This culture was incubated at 37°C with shaking at 300 rpm until late log phase (approximately 8 h). Then 1 mL of this culture was transferred to 50 mL LB broth + ampicillin in a 250 mL Erlenmeyer flask, and the culture was grown overnight (16 - 18 h) at 37°C with shaking. Forty mL of this culture was transferred to a polypropylene Oak Ridge tube, and the cells were centrifuged for 10 min at 4000 g with a JA-20 rotor in a J2-HS centrifuge (Beckman-Coulter Inc.). The cell pellet was resuspended in 2.5 mL of a solution containing 25 mM Tris-HCl (pH 8.0), 50 mM glucose and 10 mM EDTA, and incubated on ice for 10 min. Then 5 mL of a freshly prepared solution of 0.2 N NaOH and 1% SDS was added, mixed thoroughly, and incubated for 5 min on ice. Then 3.75 mL of 3 M potassium acetate (pH 5.2) was added and the tube was incubated for a further 10 min on ice. The tube was then centrifuged at 16,000 g for 15 min at 4°C, and the supernatant was decanted into a fresh Oak Ridge tube, to which 5 mL of buffer saturated phenol and 5 mL of chloroform:isoamyl alcohol (24:1) were added. The tube was mixed by inversion for 5 min and then centrifuged for 5 min at 4000 g to separate the phases. The upper aqueous phase was transferred to another Oak Ridge tube, and 20 mL of ice cold 95% ethanol was added. The solution was then incubated at -20°C for 1 h. The tube was centrifuged at 16,000 g for 10 min to pellet the precipitated DNA. The pellet was washed twice with 10 mL ice cold 70% ethanol, and dried in a vacuum dessicator. The
pellet was then dissolved in 300 μL TE buffer, and 2 μL DNAse-free RNAse (10 mg/mL) was added. The solution was transferred to a microfuge tube and incubated for 30 min at room temperature, before long term storage at 4°C. This solution of DNA was sometimes pure enough for certain uses, but most of the time was further purified by precipitation with polyethylene glycol.

The volume of the DNA solution was adjusted to 500 μL by the addition of 200 μL TE buffer. Then the solution was extracted with a combination of 250 μL phenol and 250 μL chloroform:isoamyl alcohol (24:1). The microfuge tube was then centrifuged for 2 min at 12,000 g to separate the phases and the upper aqueous phase was transferred to a new microfuge tube on ice. Then 500 μL of a solution containing 1.6 M NaCl and 13% (w/v) polyethylene glycol, 8000 average molecular mass (PEG-8000) was added and the solution was incubated on ice for 1 h. The solution was then centrifuged at 12,000 g for 15 min to pellet the DNA. The pellet was thoroughly washed three times with ice cold 70% ethanol, and dried in a vacuum dessicator. The pellet was then dissolved in 300 μL of 0.1× TE buffer (1 mM Tris-HCl buffer, 0.1 mM EDTA, pH 8.0) and stored at 4°C.

2.8.2 Restriction endonuclease digests

Plasmid DNA was routinely digested with various restriction endonucleases, in order to determine the nature of the plasmid, or in order to cleave circular DNA into linear pieces to ligate together into a different arrangement. The procedure is well established (Sambrook et al., 1989), and involves the suspension of ~1 μg DNA in a total reaction volume of 10 - 50 μL. Also included in this restriction digest was the correct buffer used for the particular enzyme(s), and 10 - 20 U of the restriction enzyme(s). For double digests, a buffer was chosen which gave reasonable activity for each enzyme. Most reactions were carried out at 37°C, although some heat stable or heat labile enzymes required a different temperature of incubation. Reaction times ranged from 30 min to 2 h.
Restriction fragments were then routinely separated and analyzed by agarose gel electrophoresis, followed by visualization of ethidium bromide stained DNA.

2.8.3 Agarose gel electrophoresis of DNA

To each sample DNA solution, whether plasmid DNA, polymerase chain reaction (PCR), or restriction digest, gel-loading buffer was added to a final concentration of 8% (w/v) sucrose and 0.05% (w/v) bromophenol blue. An agarose gel was previously prepared by sealing the ends of a Pharmacia gel casting tray with labeling tape. Regular or low melting point (LMP) agarose gels of 1% (w/v) were prepared by the suspension of 0.5 g agarose in 50 mL TAE buffer (40 mM Tris-acetate buffer, 1 mM EDTA, pH 8.0) in an 125 mL Erlenmeyer flask. This solution was microwaved until boiling (1 - 2 min), and the solution was swirled in order to dissolve the agarose. The solution was left to cool to ~55°C, and then optionally 1 µL of a 10 mg/mL solution of ethidium bromide was added to the agarose before pouring into the gel casting tray with sample application comb. Once the gel was solidified, the comb was removed and the prepared samples were applied. The gel was then electrophoresed at 100 V for 45 to 60 min in an electrophoresis cell containing TAE buffer. The gels which did not yet contain ethidium bromide were then stained in a weak solution (~0.5 µg/mL) of ethidium bromide in TAE buffer for 30 min. The DNA in the gel was then visualized by exposure to ultraviolet light (254 nm) in a transilluminator. A picture of the gel was often taken, using a Polaroid GelCam with hood, with Polaroid Polapan 665PN film, 5-10 s exposure. Small blocks of agarose containing the DNA bands of interest were excised from the gel, to be recovered.

2.8.4 Recovery of DNA from agarose gels

Several procedures for the isolation of DNA from agarose gel slices have been used in this thesis work. Originally, DNA was isolated from Low Melting Point (LMP) agarose gels. The gel slice was then added to a microfuge tube containing 300 µL TE
buffer, and the agarose was melted by incubation at 60°C for 10 min. The DNA was then purified from this solution, by one of two methods. One method (Sambrook et al., 1989) involved phenol:chloroform extraction of the solution, followed by precipitation of the DNA with ethanol, as in plasmid purification procedures. In, the other method (Qian and Wilkenson, 1991), the dissolved gel was placed at -70°C for 10 min, and the frozen gel was then slowly melted. The solution was mixed vigorously, and the disrupted agarose was removed by centrifugation at 12,000 g for 2 min. The solution containing the DNA was then removed, and concentrated by ethanol precipitation if necessary.

DNA was most recently purified from regular agarose gels. The Agarose Gel DNA Extraction kit (Roche/Boehringer-Mannheim) was used. The gel slice was placed in a microfuge tube, and 300 μL of agarose gel solubilization buffer plus 10 μL of silica matrix was added. The gel was incubated at 60°C with occasional vortexing, and then the solution was centrifuged at 12,000 g for 30 s. The supernatant was discarded and 500 μL of nucleic acid binding buffer was added to the silica pellet, and the solution was mixed for 1 min. before centrifuging at 12,000 g for 30 s. The pellet was then washed twice with washing buffer, containing 70% ethanol, and the pellet was dried at room temperature. The DNA was eluted from the matrix by application of 30 μL TE buffer, incubation at 60°C for 10 min, followed by centrifugation and transfer of the supernatant to a new microfuge tube. This DNA solution was of sufficient purity and concentration to use directly in ligation reactions.

2.8.5 Ligation of fragments into plasmid vectors

Ligations were commonly done as described (Sambrook et al., 1989). Plasmid DNA and insert DNA were linearized with the appropriate restriction enzymes, these reactions were electrophoresed on an agarose gel, and then the correct DNA bands were excised from the gel and purified. Purified insert DNA was combined with vector DNA at a molar ratio of approximately 3:1, along with with the addition of commercial ligase
buffer (commonly to a final concentration of 50 mM Tris-HCl buffer, pH 7.6, 10 mM MgCl₂, 10 mM DTT, and 1 mM ATP) and 5U T4 DNA ligase, in a total volume of 10-20 µL. Ligations were incubated at 16°C or 22°C for 1-4 h. Appropriate controls were always performed to assess the success of a ligation. The ligations were most commonly directly transformed into competent E. coli cell strains after the reaction was complete.

2.8.6 Transformation of vector DNA

Plasmid DNA was used to transform cells made competent by treatment with cold CaCl₂ solutions (Sambrook et al., 1989). In this protocol to prepare competent cells, 50 mL of LB broth in a 250 mL Erlenmeyer flask was inoculated with 2-5 mL of an overnight culture. The cells were then grown at 37°C with shaking at 300 rpm until mid-log phase was attained, approximately 2 h. The culture was harvested by centrifugation at 5000 g for 10 min at 4°C. The cell pellet was resuspended in 20 mL ice cold sterile 0.1 M CaCl₂, and the cells were left on ice for 20-40 min. The cells were pelleted once more by centrifugation, and the pellet was resuspended in 1-2 mL of 0.1 M CaCl₂. These cells were incubated on ice for a minimum of 1 h, up to a maximum of 48 h, before use in a transformation. The cells were also commonly stored in aliquots at -70°C after the addition of 15% (v/v) glycerol.

For a transformation, 100 µL of a suspension of competent cells, either freshly prepared or taken from frozen stocks, was combined with vector DNA. For transformation of purified plasmid, about 0.2 µg plasmid was used; for transformation of ligation reactions, 5-10 µL of the reaction was added to the competent cells. This mixture was incubated for 20-40 min on ice, followed by a 2 min heat shock at 42°C and a further 2 min incubation on ice. In most cases this suspension was then directly spread on to an LB agar plate containing 100 µg/mL ampicillin. The plates were incubated overnight (12 to 16 h), and ampicillin resistant colonies of cells containing the desired plasmid were then visible.
2.9 Cloning of *ptsI* genes

Polymerase chain reaction (PCR) was used to amplify the *ptsI* genes from the *S. typhimurium* strains SB1476, SB1681, SB1690, and SB3507, containing *ptsIl7*, *ptsIl6*, and *ptsI34*, and wild type *ptsI*, respectively. PCR was also used to amplify the wild type *E. coli* *ptsI* gene. The amplified fragments were subsequently cloned into the pT7Blue vector, and further transferred to the plasmid pT7-7.

2.9.1 Isolation of *ptsI* from *S. typhimurium*

A crude preparation of bacterial chromosomal DNA was obtained by suspension of one colony from an LB-agar plate into 0.05 mL H₂O, and adding 1 µL of this suspension into a PCR reaction tube. The DNA was denatured by a 5 min incubation at 95°C, followed by rapid cooling on ice. Reagents were then added to give: 20 mM Tris-HCl buffer (pH 8.8), 10 mM KCl, 10 mM (NH₄)₂SO₄, 2 mM MgSO₄, 0.1% Triton X-100, 0.2 mM each dNTP, 10 pmol of each primer *ptsI.1* and *ptsI.2*, and 0.5 U Deep Vent DNA polymerase in a total volume of 25 µL. The PCR program used was 30 cycles of 30 s at 95°C (denaturation), 30 s at 52°C (annealing), and 90 s at 72°C (primer extension). The PCR reaction product, 1750 bp, was separated on a 1% agarose gel and purified by the Agarose Gel DNA Extraction Kit (Roche/Boehringer-Mannheim). This isolated DNA was then cloned by blunt end ligation into pT7Blue using the Perfectly Blunt Cloning Kit (Novagen). The resulting plasmids were then digested with Nde I and BamH I restriction endonucleases and the 1750 bp *ptsI* gene fragment was then ligated into the plasmid pT7-7 using standard techniques (Section 2.8). This gave the plasmids pT7-7(EIst), pT7-7(EIstI16), pT7-7(EIstI17), and pT7-7(EIstI34).

2.9.2 Isolation of *E. coli* *ptsI*

The *E. coli* *ptsI* gene was first amplified from the plasmid pTSHIC9 which contains the *E. coli ptsH1crr* operon (Mao *et al.*, 1996). The PCR reaction contained, in a
total volume of 50 μL: 10 ng pTSHIC9, 10 pmol of each primer E15’ and E13’, and other reagents as described previously (Section 2.8.1). The 1750 bp DNA fragment was then cloned into pT7Blue and subcloned into pT7-7 as described for the *S. typhimurium* gene cloning, to give pT7-7(Elec).

2.10 Construction of enzyme I fragments

Several fragments of *E. coli* enzyme I were created in this thesis work in order to separately investigate the function of the N- and C-terminal domains.

2.10.1 Construction of C-terminal enzyme I fragments

In *E. coli* enzyme I, residue 334 is a methionine and part of an *Nco* I site in *ptsI*. The *Nco* I – *BamH* I fragment digested from pT7-7(Elec) was ligated into the vector pET11d to give pET11(ElecC334), which encoded the C-terminal 27 kDa (residues 334 - 575) of enzyme I.

Residue 239 of *E. coli* enzyme I is also a methionine, and site directed mutagenesis (Section 2.11) was used to change the DNA sequence in order to create an *Nde* I site at that position. The 1020 bp *Nde* I – *BamH* I fragment from the resulting plasmid was excised and ligated into pT7-7. This gave the plasmid pT7-7(ElecC239), which encoded the C-terminal 37 kDa (residues 239 - 575) of enzyme I.

2.10.2 Construction of N-terminal enzyme I fragments

The 5’-end fragments of *E. coli ptsI* necessary for the production of N-terminal fragments of enzyme I were amplified from pTSHIC9 by PCR in a manner similar to that previously described (Section 2.9.2), using two 3’ end primers that provided for a *BamH* I restriction site and termination at residues 220 and 250. The PCR products were ligated into pT7-7 using the *Nde* I and *BamH* I restriction sites. The two plasmids, pT7-7(ElecN219) and pT7-7(ElecN249), produced enzyme I N-terminal fragments from...
residues 1-219 and 1-249, respectively. The construction of these plasmids was performed by James Talbot.

2.11 Site-directed mutagenesis

Construction of site-directed mutants of HPr has over the years been done by several different procedures (Kunkel et al., 1987; Landt et al., 1990), but most recently has been done by the QuikChange site-directed mutagenesis protocol from Stratagene. All site-directed mutants of HPr and enzyme I in this thesis have been prepared by the QuikChange methodology.

Site-directed mutagenesis by the QuikChange protocol involves the synthesis of a set of two mutagenic primers, complementary to each other, to mutate each strand of the template plasmid. Primer extension, and thus synthesis of new mutant plasmid, is carried out through thermal cycling reactions, much like polymerase chain reaction (PCR). Each mutagenesis reaction contained, in a total volume of 50 μL in a 0.2 mL microfuge tube: 20 mM Tris-HCl buffer (pH 8.8), 10 mM KCl, 10 mM (NH₄)₂SO₄, 2 mM MgSO₄, 0.1% (v/v) Triton X-100, 0.1 mg/mL nuclease-free bovine serum albumin (BSA), 0.4 mM of each dNTP, 20 ng plasmid DNA template, 125 ng of each mutagenic primer, and 2.5 U Pfu DNA polymerase. This polymerase is thermostable and is not able to displace the mutagenic primer after complete extension of the primer around the plasmid. The mutagenesis reaction consisted of sixteen cycles of 30 s at 95°C (denaturation), 60 s at 55°C (annealing), and 60 s per kilobase of template at 68°C (primer extension).

Following this reaction, 10 U of Dpn I restriction enzyme was added, and the solution was incubated at 37°C for 1 h. The restriction enzyme Dpn I is able to digest the parental, methylated, template DNA but not the newly synthesized unmethylated mutant DNA. Following this incubation, one μL of the mixture was added to 50 μL prechilled (4°C) Epicurian Coli supercompetent cells in a Falcon 2059 polypropylene tube. The cells were incubated on ice for 30 min, followed by a 42°C heat shock for 45 s, followed by a 2
min incubation on ice. Then 0.5 mL of prewarmed (42°C) LB broth was added to the cells and the tube was incubated at 37°C, with shaking at 300 rpm, for 30 min. Following this incubation, 0.2 mL of cells were plated onto an LB-agar plate containing 100 μg/mL ampicillin. The plates were incubated for 12 - 18 h at 37°C, and resulting colonies contained the desired mutant plasmid DNA with a typical success rate of 80 - 90%.

2.12 DNA Sequencing

Plasmids created by site-directed mutagenesis or ligation reactions had to be confirmed by identification of the DNA sequence. All sequencing procedures used the dideoxy termination method initially devised by Sanger et al. (1977). Some sequencing was done by the method described in Section 2.12.1. Most sequencing was done as described in Section 2.12.2, and in all cases the entire gene or plasmid insert was sequenced to be assured that no unwanted mutations occurred during PCR or mutagenesis reactions.

2.12.1 Sequencing with radioisotope labeling

2.12.1.1 Sequencing reactions

In this laboratory, sequencing reactions were performed using the T7 Sequenase kit (Amersham-Pharmacia) using a modified protocol. In a total volume of 7 μL, 1-2 pmol double stranded DNA template and 5 pmol of the appropriate primer were combined. Then 1 μL of dimethyl sulfoxide (DMSO) was added, and the solution was incubated at 95°C for 3 min to separate the DNA strands. This solution was then frozen quickly by plunging into a -80°C ethanol bath, and thawed at room temperature. Then 2 μL of annealing buffer was added, followed by a room temperature incubation of 5 min. Then 7.5 μL of labeling mix (1 μL of 0.1 M DTT, 1 μL DMSO, 3 μL labeling mix 'A', 1 μL of [α-35S]dATP (500 Ci/mmol), 2 U T7 DNA polymerase, and water to 7.5 μL) was added.
Following a 5 min room temperature incubation, 4.3 µL of this reaction mix was dispensed into each of four prewarmed (37°C) termination tubes containing the sequencing termination mixes (T, C, G, A), and allowed to incubate for 2 - 3 min. 'T' mix contained 840 µM each of dATP, dCTP, and dGTP, 93.5 µM dTTP, 14 µM ddTTP, 40 mM Tris-HCl (pH 7.6), 50 mM NaCl, and similarly for the other termination mixes 'C', 'G', and 'A'. Reaction mixtures were terminated by the addition of 5 µL stop buffer, which contained 0.3% (w/v) each of bromophenol blue and xylene cyanol, 10 mM EDTA (pH 7.5), and 97.5% deionized formamide to each tube. The reactions were immediately subjected to electrophoresis or frozen for later use.

2.12.1.2 Urea-polyacrylamide gels

DNA sequencing reaction mixtures were analyzed by urea-polyacrylamide gel electrophoresis. A glass sequencing plate was cleaned using 95% ethanol and lint-free kimwipes, and assembled into the pouring tray for use with the Sequi-Gen GT DNA Electrophoresis cell (Bio-Rad). A mixture containing TBE buffer (45 mM Tris-borate buffer, pH 8.0, 1mM EDTA), 6% acrylamide (acrylamide:bisacrylamide, 19:1), and 7.7 M urea in a total volume of 100 mL was prepared. Polymerization was started by the addition of 200 µL 25% (w/v) ammonium persulfate and 100 µL N,N,N',N'-tetramethylethylenediamine (TEMED). This solution was injected into the space between the glass plate and the tray with a 120 mL syringe, and a sharktooth or sawtooth comb was immediately applied. Polymerization was allowed to take place for 30 min, and then the gel was used immediately or sealed with plastic wrap and stored at room temperature for later use.

2.12.1.3 Electrophoresis and autoradiography

The acrylamide gel was assembled into the Bio-Rad apparatus according to manufacturer's instructions. The upper and lower chambers were filled with TBE buffer,
and the gel was warmed with an electrophoresis pre-run of approximately 30 min with constant power (80 W). During this time, the DNA sequencing reaction samples were incubated in a 95°C water bath for 3 min and placed on ice. The samples were loaded into the preformed wells of the sawtooth comb, or in the wells formed by the sharktooth comb and the gel. Once applied, the gel was electrophoresed until the bromophenol blue dye reached the end of the gel, about 3 h. The sequencing apparatus was then disassembled and the glass plate was pried apart from the electrophoresis assembly. The exposed gel was then transferred to a Whatman 3M filter paper. This was placed onto a Savant gel drier, and covered with plastic wrap. The gel was dried under vacuum for 2 h at 80°C. The gel was then placed into a film cassette with Kodak X-Omat AR X-ray film, and exposed for 16 - 24 h at room temperature. The film was developed using an AFP Imaging MiniMed/90 automatic X-ray film processor.

2.12.2 Automated DNA sequencing

Automated DNA sequencing, using fluorescent labeled reactions, was performed by the DNA Services Unit of the Plant Biotechnology Institute, National Research Council of Canada, Saskatoon SK. An Applied Biosystems 373 (Stretch) DNA sequencer was used.

2.13 Protein purifications

Several types of protein were purified during this thesis work, namely HPr, IIA\textsuperscript{glc} enzyme I, and enzyme I C- and N-terminal fragments. Purification of each protein will be discussed separately.

2.13.1 Purification of HPr proteins

Wild type and mutant HPr proteins were purified by established procedures (Waygood and Steeves, 1980), with modifications (Brokx \textit{et al.}, 1999). Either of the \textit{E.}
coli strains ESK108 or TP2811 were transformed by pUC(HPr). The pUC(HPr) plasmid contains the open reading frame of ptsH, the gene encoding HPr, as well as the 5' promoter region, inserted into the Cla I and Sma I sites of pUC19. Cells harboring this high copy number pUC(HPr) plasmid constitutively express large amounts of HPr protein. A colony of transformed cells was used to inoculate 5 mL of LB broth containing 100 µg/mL ampicillin, and this was grown overnight at 37°C with shaking at 300 rpm. This culture was used to inoculate 150 mL LB-ampicillin broth in a 500 mL Erlenmeyer flask, which was incubated with shaking until mid-log phase. This culture was used to inoculate four 6 L flasks containing 1.5 L each of Terrific Broth containing ampicillin. These flasks were grown for 16 - 20 h at 37°C with shaking at 300 rpm. The cells were harvested by centrifugation with a GS-3 rotor in a Sorvall RC2-B refrigerated centrifuge. The cell pellets were combined into one centrifuge bottle and washed with 0.85% (w/v) NaCl and recentrifuged. The cells were stored at -20°C until needed for crude extract preparation.

The cells (20-30 g wet weight) were resuspended in 0.01 M Tris-HCl buffer (pH 8.0) buffer at a ratio of 3.0 mL buffer to 1.0 g cells. This suspension was passed through a cooled French press cell (Aminco) at a pressure of 1000 psi for two to three times. Unbroken cells and cell debris were removed by initial centrifugation at 10,000 g for 10 min at 4°C. This supernatant was then centrifuged at 200,000 g in a Beckman Ti60 rotor for 1.5 h at 4°C in order to remove cell membranes.

This supernatant (~100 mL) was loaded onto an AcA54 Ultrigel gel filtration column (10 cm x 100 cm) equilibrated with 10 mM Tris-HCl buffer (pH 8.0). Buffer was pumped through the column at a rate of ~300 mL/h, and ~450 mL fractions were collected. Isoelectric focusing gels (described in proceeding text) were used to determine which fractions contained HPr. HPr, at a molecular mass of 9100 Da, is a small protein which is relatively easy to separate by this method. As a result, the fraction(s) containing HPr were often of sufficient purity to be used without further purification. In some cases, however,
the fractions were pooled and loaded onto a Q-Sepharose anion exchange column (150 mL) equilibrated with 10 mM Tris-HCl buffer (pH 8.0). HPr was eluted by using a gradient of 0 to 0.3 M NaCl in the same buffer (1L each). The pure HPr fractions obtained from either chromatography step were pooled and dialyzed (Spectrapor tubing, pore size <3,500 Da) against three changes of double distilled water. Samples were then frozen and lyophilized. Lyophilized protein samples were stored airtight at -20°C.

Purification of Phe2Cys HPr was carried out as described, except 0.2 mM dithiothreitol (DTT) was included in all buffers in order to keep the protein as a monomer. The protein was stored at -20°C in a solution containing 10 mM Tris-HCl buffer (pH 7.5) and 1 mM DTT instead of lyophilized.

2.13.2 Purification of enzyme I

Enzyme I has been overexpressed from two different sources for the purposes of purification for this thesis work. Enzyme I, along with HPr and IIAεG, was originally overexpressed from TP2811 cells containing the plasmid pTSHIC9. More recently, and for the bulk of wild type and all mutant protein purifications, enzyme I has been purified from ESK238 cells containing pT7-7(EI) overexpression plasmids. Large scale growth and overexpression protocols were similar. One colony was used to inoculate 5 mL LB broth with 100 μg/mL ampicillin, and this was grown to mid-log phase (8-10 h). This culture was then used to inoculate 100 mL LB-ampicillin broth in a 500 mL Erlenmeyer flask, which was grown to stationary phase (14-18 h). Four 6 L Erlenmeyer flasks containing 1.5 L terrific broth with ampicillin were each inoculated with 25 mL of overnight culture. These cultures were grown to late log phase (OD<sub>600nm</sub> = 0.8 - 1), and protein overexpression was induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 0.5 mM. The cultures were grown for a further 4 h to allow for protein overexpression. The cells were then harvested by centrifugation with a GS-3 rotor in a Sorvall RC2-B refrigerated centrifuge.
The cell pellets were combined into one centrifuge bottle and washed with 0.85% (w/v) NaCl and recentrifuged. The cells were stored at -20°C until needed for crude extract preparation.

Crude extract preparation and purification of enzyme I was carried out as described (Brokx et al., 2000). The cells (15 - 25 g wet weight) were suspended in 3 mL buffer A (10 mM potassium phosphate buffer, pH 7.5, 1mM EDTA, 0.2 mM DTT) per gram of cells. The cells were then broken by two passages through the French pressure cell at 1000 psi. Unbroken cells and cell debris were removed by initial centrifugation at 10,000 g for 10 min at 4°C. This supernatant was then centrifuged at 200,000 g in a Beckman Ti60 rotor for 1.5 h at 4°C in order to remove cell membranes.

The resulting supernatant was adjusted to the pH and conductivity of buffer A by the addition of 1 N NaOH and distilled water. The extract was then loaded onto a 150 mL Q-Sepharose anion exchange column, a 1 L + 1L gradient of 0 to 0.5 M KCl was applied, and fractions were collected. Enzyme I adhered strongly to the resin and eluted at ~0.4 M KCl. Purity of fractions was analyzed by sodium dodecyl sulfate polyacrylamide electrophoresis (SDS-PAGE; see proceeding text), and fractions containing essentially homogeneous enzyme I were pooled. This pool was phosphorylated by the addition of phosphoenolpyruvate (PEP) to 2 mM and MgCl₂ to 5 mM and incubation at 37°C for 10 min. The enzyme I preparation was then concentrated by passage through a PM-10 membrane using an Amicon ultrafiltration cell at 4°C. Part of the preparation was often dialyzed against three changes of buffer A and used directly. The rest of the preparation was dialyzed against three changes of double distilled water, frozen, lyophilized, and stored airtight at -20°C.

2.13.3 Purification of enzyme I N-terminal domain proteins

Protein overexpression of 24 kDa or 27 kDa E. coli enzyme I N-terminal proteins from plasmids pT7-7(ElecN219) and pT7-7(ElecN249), respectively, and preparation of
crude extract was as described (Section 2.13.3). The crude extract was applied to a 150 mL Q-Sepharose column and a 0 - 0.5 M KCl gradient was applied. Fractions containing the N-terminal protein, either EIN24 or EIN27, were identified by SDS-PAGE. The fractions were pooled and concentrated to ~20 mL by passage through a PM-10 membrane using the Amicon concentrator. This solution was then loaded onto an AcA44 Ultragel column (5 × 100 cm) equilibrated with buffer A plus 0.1 M KCl, 5 mM MgCl₂, and 20 mM ε-amino-caproic acid (a protease inhibitor). Fractions which contained the desired protein were again identified by SDS-PAGE, concentrated by Amicon, and dialyzed against buffer A or lyophilized, as described previously (Section 2.13.2).

2.13.4 Purification of enzyme I C-terminal domain proteins

The 27 kDa C-terminal protein was expressed from ESK238 cells carrying the plasmid pET11(EIecC334) by IPTG induction, and the cells were harvested and broken by passage through the French press as described previously. However, the protein was found in the pellet of the initial low speed (10,000 g) centrifugation, indicating that the protein was expressed but formed insoluble inclusion bodies. This protein aggregate was solubilized in buffer A containing 3 M urea, and cell debris was removed by centrifugation at 10,000 g. This protein solution was then dialyzed against three changes of fresh buffer A without urea, and the EIC27 protein remained soluble. The protein was further purified by passage through a Q-Sepharose column, but the protein did not adhere to the column. This preparation was judged to be about 50 - 75% pure by SDS-PAGE.

The 37 kDa C-terminal enzyme I protein (EIC37), was expressed from ESK238 cells containing the plasmid pT7-7(EIecC239). This protein remained soluble in the cell extract, prepared as described (Section 2.13.2) and remained in the supernatant after the centrifugation at 200,000 g. The supernatant was applied to a 150 mL Q-Sepharose column and a gradient of 0 - 0.4 M KCl was applied. The fractions which contained the EIC37 protein were eluted with ~0.2 M KCl, as judged by SDS-PAGE. The relevant
fractions were pooled, concentrated by Amicon filtration, and applied to an AcA44 gel filtration column (5 × 100 cm) equilibrated with buffer A with the additions of 0.1 M KCl, 5 mM MgCl₂, and 20 mM e-amino-caproic acid. Fractions which contained the EIC37 protein were pooled, concentrated by Amicon filtration, dialyzed against three changes of buffer A, and stored at -20°C. This protein formed a precipitate when dialyzed against water, so lyophilization was not performed.

2.13.5 Purification of IIA^{elc}

Enzyme IIA^{elc} was originally overexpressed in TP2811 cells transformed with the plasmid pTSH1C9, and more recently in ESK108 cells containing pT7-7.crr, a plasmid with crr, the gene encoding IIA^{elc}, inserted into the vector pT7-7. In either case, large scale overexpression of IIA^{elc} and preparation of crude extracts was as described (Section 2.13.2). The supernatant from the high speed centrifugation was applied to a 200 mL Q-Sepharose column equilibrated with 10 mM potassium phosphate buffer (pH 7.5), 1 mM EDTA, and 0.2 mM DTT. A gradient of 0 to 0.5 M KCl, in the same buffer, was applied, and fractions containing IIA^{elc} as identified by SDS-PAGE were collected. The pool was concentrated by Amicon ultrafiltration using a PM10 membrane, and applied to an AcA44 gel filtration column (5 × 100 cm) equilibrated with the same buffer with the addition of 0.1 M KCl. Fractions containing homogeneously pure IIA^{elc} were pooled, concentrated, and either dialyzed against the same buffer and stored at -20°C, or lyophilized.

2.14 SDS-PAGE

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed with gels made as described by Laemmli (1970), and run in a discontinuous buffer system as given in Sambrook et al. (1989). Gels were 80 mm × 100 mm × 0.75 mm with a final acrylamide concentration of 10 - 15%, depending on the nature of the experiment. The stacking gel always contained 5% acrylamide. A Bio-Rad Mini-Protean
II electrophoresis apparatus was used for the electrophoresis. Most gels were electrophoresed at 200 V for approximately 60 min at room temperature. For analysis of protein phosphorylation reactions, electrophoresis was carried out at 150 V for 90 min at 4°C due to the heat lability of phosphohistidines.

Gels were stained for 15 min in SDS-PAGE stain (0.25% [w/v] Coomassie Brilliant Blue R250, 45% [v/v] methanol, and 10% [v/v] acetic acid), and then destained in a solution containing 25% (v/v) methanol and 7.5% (v/v) acetic acid for 1 - 18 h, with several changes of the destaining solution. With proteins phosphorylated with [32P]PEP, the gels were not stained, but rather the gel, while adhered to one glass plate, was covered with plastic wrap and exposed with Kodak X-Omat X-ray film in an autoradiography cassette at -80°C.

2.15 Isoelectric focusing (IEF) gels

Isoelectric focusing gels were constructed using a Bio-Rad capillary tray as described by Mattoo et al. (1984). A cleaned 10 cm × 10 cm glass plate was placed on the tray one the side with the 0.8 mm thick spacers. A solution containing 1.1 g sucrose, 4.8 mL deionized water, and 5.5 mL acrylamide solution (24.25 g acrylamide and 0.75 g bis-acrylamide in 250 mL deionized water) was prepared in a 125 mL sidearm flask. This solution was degassed for 1 min, and then 0.75 ml ampholyte mixture (1:2:2 of pH 3.5-10.0: pH 3.5-5.0: pH 5.0-7.0) was added. Polymerization was initiated by the addition of 25 µL 10% (w/v) ammonium persulfate, 25 µL riboflavin-5-phosphate (2.5 mg/mL), and 5 µL TEMED. This solution, containing 6.0% acrylamide, was poured between the glass plate and the casting tray, and allowed to polymerize for 30 min under a fluorescent lamp.

Electrode wicks, soaked in 1 N NaOH (cathode) and 1 N phosphoric acid (anode) were added, one to each end of the polymerized gel after it was removed from the casting tray. A plastic sample applicator was placed near the cathode wick, and samples, generally ~5-10 µg protein in ~20 µL, were applied. The gel was electrophoresed on a
pre-cooled (4°C) flat bed Pharmacia IEF gel apparatus for 2 h at 8 W with a limit of 1000 V.

Gels were fixed and stained for 30 min as described by Crowle and Cline (1977) in a solution containing 5.0 g CuSO₄, 0.4 g Coomassie Brilliant Blue R250, and 0.5 g Crocecin Scarlet dissolved in 270 mL isopropanol, 100 mL acetic acid, and 630 mL distilled water. Gels were destained overnight in several changes of a solution containing 140 mL acetic acid, 240 mL isopropanol dissolved in 1620 mL distilled water.

2.16 Protein determinations

HPr protein concentrations were determined by the spectrophotometric method of Waddell (1956) where \(1A(A_{215\text{nm}}-A_{225\text{nm}}) = 0.144 \text{ mg/mL}\). However, the most accurate HPr concentrations were determined by the lactate dehydrogenase depletion assay (Waygood et al., 1979). These assays were performed using a Gilford 2400 recording spectrophotometer at 340 nm. A cuvette with 1.0 mL of a solution containing 0.1 M N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid (Hepes) buffer, pH 7.0, 0.15 mM NADH, 10 mM PEP, 5 mM MgCl₂, 35 U lactate dehydrogenase, and an unknown amount of HPr was prepared. A basal activity was recorded, and then 5.0 \(\mu\text{L (~5 \mu g)}\) enzyme I was added. The reaction was allowed to proceed until all HPr in the solution was phosphorylated, about 5 min.

Enzyme I protein concentrations were determined by the method of Waddell (1956), and the extinction coefficient of \(A_{280\text{nm}} \text{ (10 mg/mL)} = 4.4\) (Waygood and Steeves, 1980). Concentrations of all other proteins used in this thesis were determined by the method of Waddell (1956), and in some cases the Bradford protein assay (Bradford, 1976) was used.
2.17 Assays for enzymatic activity

A number of enzyme assays have been used to measure the enzymatic activity of enzyme I and the phosphocarrier activity of HPr. These assays have been used to monitor purification, as well as to determine characteristics of wild type and mutant proteins under different conditions.

2.17.1 LDH coupled assay

A spectrophotometric assay to measure the interaction between enzyme I and HPr, coupled to the reaction catalyzed by rabbit muscle lactate dehydrogenase (LDH) has been used extensively in this work. As enzyme I catalyzes the phosphorylation of HPr, PEP is converted to pyruvate. This pyruvate is converted by LDH to lactate, with the concomitant conversion of NADH to NAD. If LDH is in large excess in the reaction, the amount of NADH converted to NAD, monitored spectrophotometrically by a decrease in absorbance at 340 nm, is directly proportional to the amount of HPr converted to P-HPr. In this assay, HPr is used as a substrate for enzyme I, and $V_{max}$ and $K_m$ values for this interaction can be obtained. However, large amounts of HPr protein are needed, so reaction solutions are often pooled together and the HPr repurified. In addition, if a large excess of HPr is used, the amount PEP may be varied, and an enzyme I $K_m$ for PEP can then be obtained.

Assays were carried out in 1 mL reaction volumes in quartz cuvettes, and a Gilford 2400 recording spectrophotometer at 340 nm was used to monitor activity. In general, reactions contained 0.1 M N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid (Hepes) buffer (pH 7.0), 0.15 mM NADH, 5 mM MgCl$_2$, 35 U lactate dehydrogenase, with varying concentrations of PEP and/or HPr, depending on the nature of the experiment. All reagents and proteins were filtered through syringe filters (maximum 0.5 µm pore size) in order to minimize noise in the assay. Commonly, a basal level of PEP conversion was monitored, and then a small volume (5 µL) of enzyme I, of a concentration to allow ease
of monitoring in the assay, is added, and activity is measured. The enzyme I is usually
diluted to an appropriate concentration at room temperature in a buffer containing 10 mM
potassium phosphate (pH 7.5), 1 mM EDTA, 0.2 mM DTT, 5 mM MgCl₂, and 2 mM
PEP and left at room temperature before addition to the assay. This filtered enzyme
preparation is stable for several days at room temperature.

2.17.2 Sugar phosphorylation assays

Sugar phosphorylation assays were carried out as described by Waygood et al. (1979). Cell membranes from S. typhimurium strain SB2950 were first isolated as a
source of enzyme II₃ to be used in the assay. The cells were grown at 37°C with
shaking at 300 rpm until early stationary phase (~18 h) in 1.5 L minimal salts A media
with 0.2% (w/v) glucose in a 6 L Erlenmeyer flask. The cells were harvested by
centrifugation at 8000 g with a GSA rotor in a Sorvall RC2-B refrigerated centrifuge. The
cells were then washed with 0.85% (w/v) NaCl and the cell pellet collected. The cells
were resuspended in 3 mL of a buffer containing 10 mM Hepes buffer (pH 7.5), 1 mM
EDTA, and 0.2 mM DTT per gram of cell paste. This suspension of cells was frozen until
needed, or used immediately. Approximately 10 mL of the suspension was passed twice
through a French press cell at 1000 psi. Unbroken cells and cell debris were removed by
low-speed centrifugation at ~10,000 g for 10 min. The membranes were then collected by
centrifugation at 200,000 g in a Beckman Ti60 rotor for 1.5 h at 4°C. This pellet was
resuspended in 5 mL of the same buffer as the cells were originally suspended. The
membranes were homogenized using a tissue homogenizer with a Teflon pestle. This
homogenate was recentrifuged for 1.5 h at 200,000 g, and the pellet was again
homogenized in 1-2 mL of the same buffer. This membrane preparation containing
enzyme II₃, to be used in assays of HPr and enzyme I, was stable for approximately two
weeks when stored on ice. The optimal amount of SB2950 membranes to be used was
determined in a sugar phosphorylation assay (see next section) with varying amounts of membranes, but with both HPr and enzyme I present in excess.

2.17.2.1 Sugar phosphorylation assay of HPr

Sugar phosphorylation assays were performed as described by Waygood et al. (1979). Assays were performed in a total volume of 0.05 mL in microtitre wells containing 0.05 M potassium phosphate (pH 7.5), 10 mM [U-14C]glucose (10^5 cpm/mol), 2.0 mM DTT, 12.5 mM KF, 10 mM PEP, 5 mM MgCl₂, 10 U of enzyme I, and 0.4 to 1 U of enzyme II^E. From SB2950 membranes, as well as a varying amount of HPr. When monitoring HPr purifications, 10 - 20 μL of a chromatography fraction was commonly used. The assays were incubated for 30 min at 37°C, and stopped by the addition of 0.1 mL ice-cold water. The mixtures were loaded onto 1.5 mL columns containing AG 1X2 (50-100 mesh, Cl^- form) anion exchange resin equilibrated with water. The columns were washed twice with 5 mL distilled water, and then the phosphorylated sugar was eluted with two washes of 1.5 mL of 1 M LiCl, into scintillation vials fitted with 10 mL Nalgene filmware bags. Six mL of liquid scintillation cocktail was added, and the bags were sealed, capped, shaken, and counted in a liquid scintillation counter.

2.17.2.2 Sugar phosphorylation assay of enzyme I

Assays were carried out as described for the measurement of HPr by the sugar phosphorylation assay, except for the following modifications. The amount of HPr was kept constant at 5 μg per assay. The amount of enzyme I, either in a cell extract or a chromatography fraction, was varied depending on the assay, typically in a volume of 5 - 20 μL. If a cell extract was investigated in the assay, 10 mM [U-14C]α-methyl-D-glucose (10^5 cpm/mol) was used in place of [U-14C]glucose, because the glucose-6-phosphate would be further metabolized by other enzymes in the extract.
2.17.3 Phosphorylation of PTS proteins using $^{[32P]}$PEP

PTS proteins were phosphorylated with $^{[32P]}$PEP and analyzed by SDS-PAGE and autoradiography in several experiments. In a total volume of 20 µL, a solution was prepared with 50 mM Hepes buffer (pH 7.5) and 5 mM MgCl$_2$, with enzyme I and/or other PTS proteins (~1-20 µg) and 2 µL of $^{[32P]}$PEP (prepared as described in Section 2.18). The reaction was incubated at 37°C for 5 min, and then stopped by the addition of 20 µL SDS loading buffer. The proteins were then separated by SDS-PAGE (Section 2.14) using a 12% acrylamide gel. The unstained gel, attached to one glass plate, was covered in plastic wrap and exposed for 12 - 24 h at -80°C with Kodak X-Omat AR film.

2.18 Preparation of $^{[32P]}$PEP

The preparation of $^{[32P]}$PEP was carried out essentially as described (Mattoo and Waygood, 1983). In a total volume of 250 µL, a solution containing 50 mM Hepes (pH 7.5), 1 mM oxaloacetate, 5 mM MgCl$_2$, 0.2 mCi of $[^{32}P]$ATP (3000 Ci/mmol), 12.5 mM KF, 1 mM ATP, and 20 µg E. coli phosphoenolpyruvate carboxykinase was prepared. The mixture was incubated for 10 min at 37°C. This preparation was used without further purification, and was stored at -20°C.

2.19 Western blotting

The mouse monoclonal antibody Je145, specific for E. coli enzyme I purified from E. coli strain P650 (Waygood and Steeves, 1980) was used for Western blots. Protein samples were separated on an SDS-PAGE gel using a Bio-Rad minigel apparatus as described (Section 2.14). The proteins were transferred to a nitrocellulose filter with a Bio-Rad transblot apparatus using a buffer containing 25 mM Tris base, 190 mM glycine, 20% (v/v) methanol, and 0.1% (w/v) SDS for 2 h at 4°C. The membrane was placed in a glass petri dish and blocked with 10 mL 4% (w/v) nonfat skim milk powder (Bio-Rad) in TBST buffer (50 mM Tris-HCl buffer, pH 8.0, 0.15 M NaCl, 0.02% [v/v] Tween-20) for
1 h at room temperature with shaking. Then 1-2 μL of Jel45 antibody was added, and the mixture incubated for a further 1 h. The membrane was then washed with three changes of TBST buffer without skim milk powder. A solution of goat anti-mouse immunoglobulin G conjugated with horseradish peroxidase (Bio-Rad) in TBST buffer containing 1% (w/v) skim milk powder was added, and the mixture was incubated for 1 h at room temperature with shaking. The membrane was then washed with two changes of TBST buffer, and once with TBS buffer (50 mM Tris-HCl buffer, pH 8.0, 0.15 M NaCl). Then a suitable amount (1-2 mL) of chemiluminescent blotting substrate (Roche) was added, and the bands were detected by exposure to Kodak X-Omat AR autoradiography film at room temperature for ~10 s to 5 min.

2.20 Gel filtration chromatography

Gel filtration chromatography was used analytically to estimate the molecular weight of enzyme I constructs under various conditions. For room temperature molecular weight determinations, a Superose-12 column was used, attached to a Pharmacia GradiFrac apparatus. The column was equilibrated with a buffer containing 0.05 M potassium phosphate buffer (pH 7.5), 0.1 M KCl, 1 mM EDTA and 0.02% (w/v) sodium azide. Samples (0.2 mL) were applied with a syringe using a loading loop. The column was first calibrated with molecular weight standards (1 mg/mL each) of blue dextran, ~2 × 10^6 Da; enzyme I, 127,000 Da; ovalbumin, 45,000 Da; soy bean trypsin inhibitor, 21,000 Da. The column was run at 0.2 mL/min and protein elution was detected at 280 nm. Enzyme I preparations (0.2 mL of 1-2 mg/mL) were applied, and 0.5 mL fractions were collected when necessary. For molecular weight determinations at 4°C, a Sephadex-75 column was used with the identical conditions, except the buffer did not contain sodium azide, and the enzyme I standard eluted at a position corresponding to 63,000 Da. For molecular weight determinations of enzyme I C-terminal domain proteins, conditions were identical to those used for enzyme I. However, for molecular weight determinations of the
N-terminal domain proteins, the Superdex-75 column was used at both room temperature and 4°C.

2.21 Fluorescein labeling of Phe2Cys HPr

Fluorescein-5-maleimide reacts specifically with thiol groups (Majima et al., 1995). This fluorescein derivative is non-fluorescent prior to reacting with thiols, and thus labeling was monitored by changes in fluorescence polarization and intensity (Smallshaw, 1997). The optimized labeling reaction contained 2-5 mg Phe2Cys HPr in 1 mL of a solution containing 50 mM Tris-HCl (pH 7.5) and 0.2 mM DTT. Then fluorescein-5-maleimide was added to a final concentration of 0.45 mM, and the solution was incubated for 30 min in the dark at 0°C. The labeled HPr was separated from other reaction components using a 1 cm x 30 cm Sephadex G25 desalting column eluted with 50 mM Tris-HCl (pH 7.5). A small amount of unlabeled HPr, ~5-10% of total HPr, was often present in the final preparation, but did not interfere with binding determinations. Total HPr concentration of this HPr preparation was determined by the LDH assay.

2.22 Determination of binding of HPr with IIA_{glc} and enzyme I

Binding determinations of fluorescein labeled Phe2Cys HPr with IIA_{glc} and enzyme I were similar to those described for binding determinations of HPr-specific antibodies and antibody fragments (Smallshaw, 1997). Assays contained 50 mM potassium phosphate buffer (pH 7.5), 100 mM NaCl, ~2 nM labeled Phe2Cys HPr, and varying IIA_{glc}/enzyme I concentrations in a total volume of 1 mL, using 12 x 75 mm glass tubes. The tubes were analyzed on a BEACON Fluorescence Polarization System (PanVera Corp.).
3.0 RESULTS

3.1 Binding interactions with Phe2Cys HPr and PTS proteins

An attempt was made to develop an assay to determine binding constants for the HPr:enzyme I interaction and the HPr:IIA^Sle interaction. The technique of fluorescence polarization was employed, using the BEACON Fluorescence Polarization analyzer. This technique is capable of detecting the difference in size resulting when a small protein, labeled with a fluorescent tag, is bound by a larger unlabeled protein. Plane polarized monochromatic light (488 nm wavelength in this case) is directed at the sample and excites the fluorescein tag. A very short time later (4 ns), the fluorescein emits a photon at a different wavelength (520 nm). During this time, the fluorescein-labeled protein molecule has rotated, or tumbled, in solution. Larger molecules tend to tumble more slowly than smaller ones. The intensity of the emitted light is measured by a detector at two positions, each at a 68.5° angle from the beam of excitation light, with one position in the same plane as the plane polarized light, the other in a perpendicular plane. A larger proportion of light reaching the perpendicular plane would indicate a faster tumbling rate, and thus a smaller molecule or complex. Fluorescence polarization (P) is defined by the equation:

\[ P = \frac{I_{\text{parallel}} - I_{\text{perpendicular}}}{I_{\text{parallel}} + I_{\text{perpendicular}}} \]
where $I_{\text{parallel}}$ is the intensity of the emission light parallel to the excitation light plane and $I_{\text{perpendicular}}$ is the intensity of the emission light perpendicular to the excitation light plane. $P$ has no units and is commonly expressed as a millipolarization (mP) value. In the absence of any binding protein, labeled HPr would have a relatively low mP number, and upon addition of an HPr-binding protein, the mP of the solution would increase, and thus a binding constant for the interaction can be obtained.

### 3.1.1 Rationale for choice of Phe2Cys HPr

Wild type *E. coli* HPr has no cysteine residues, so the mutation of one residue to a cysteine would result in a single specific location for attachment of a thiol-specific tag, in this case fluorescein-5-maleimide. HPr cysteine mutants already available in the laboratory included Phe2Cys and Arg17Cys. Arg17 is in the active site of HPr, and its mutation to cysteine severely affects kinetic parameters, and thus binding interactions, for enzyme I and several enzymes II (Anderson et al., 1993). Residue 17 was thus not considered as a suitable site for fluorescein labeling. However, kinetic and structural data indicated that Phe2 was a surface residue, but not involved in the interaction of HPr with other PTS proteins. The Phe2Cys HPr mutant was therefore used for labeling with the thiol-specific fluorescein-5-maleimide.

### 3.1.2 Purification of Phe2Cys HPr

The Phe2Cys pUCHPr plasmid was transformed into ESK108 cells and overexpression and purification of Phe2Cys HPr were performed as described in Materials and Methods. All buffers used during extract preparation and protein
purification contained 0.2 mM DTT as a reducing agent, in order to ensure that the protein existed as a single monomeric species. In the absence of DTT, Phe2Cys HPr readily formed disulfide linked dimers or oligomers with lower isoelectric points than the wild type protein (Figure 3.1), which hindered purification by both gel filtration and ion exchange chromatography.

3.1.3 Fluorescein labeling of Phe2Cys HPr

The labeling of Phe2Cys HPr with fluorescein-5-maleimide was performed as described in Materials and Methods. Figure 3.2 illustrates the results of a typical labeling reaction. This labeled HPr was then purified from excess DTT-reacted fluorescent label, which would interfere with the polarization assay, by Sephadex G-25 gel filtration ("desalting") chromatography. This labeled HPr preparation was used in fluorescence polarization assays.

3.1.4 Interaction of labeled HPr with HPr binding proteins

The labeled Phe2Cys HPr was used in titrations with Jel323, an anti-HPr monoclonal antibody, and the PTS proteins enzyme I and enzyme IIa<sup>IEC</sup> (Figure 3.3). The Jel323 antibody bound HPr with a high affinity, with a Kd of approximately 5 nM, and a complete binding curve was obtained with the titration. This labeled Phe2Cys HPr was also used to further examine the binding of Jel323 antibody and its Fab fragment to HPr (Smallshaw, 1997; Smallshaw et al., 1998). Both enzyme I and IIa<sup>IEC</sup> bound the labeled HPr with much lower affinities. Complete titrations were not accomplished since the very high concentrations of enzyme I or IIa<sup>IEC</sup> that would be necessary for
Figure 3.1 Isoelectric focusing gel showing disulfide-linked oligomer formation of Phe2Cys HPr. Lane 1: Wild type HPr standard (20 µg). Lane 2: Purified Phe2Cys HPr (10 µg) in the presence of 0.2 mM DTT. Lane 3: The same Phe2Cys HPr preparation (10 µg) in the absence of DTT. The gel is depicted with the cathode at the top.

Figure 3.2 Isoelectric focusing gel of Phe2Cys labeling reaction with fluorescein-5-maleimide. Lane 1: Wild type HPr standard (20 µg). Lane 2: Unlabeled Phe2Cys HPr (10 µg). Lane 3: Phe2Cys HPr (10 µg) after 30 min. reaction with fluorescein-5-maleimide. The gel is depicted with the cathode at the top.
Figure 3.3 Titration of fluorescein labeled Phe2Cys HPr with HPr binding proteins. Labeled Phe2Cys HPr (2 nM) was titrated with increasing amounts of either Jel323 antibody, enzyme I, or IIAGlc in separate experiments. ○: Titration with Jel323 antibody (concentration of Jel323 given in nM). Δ: Titration with IIAGlc (concentration of IIAGlc given in μM). □: Titration with enzyme I (concentration of enzyme I given in μM).
further data points to complete the titration were to difficult to achieve. Nevertheless, an approximate binding constant of 10 - 100 µM was estimated for both the HPr:enzyme I interaction and the HPr:IIA^slc interaction, with the interaction with enzyme I likely of slightly higher affinity. The very low affinity of binding interactions with HPr and its partner PTS proteins has been reported by other laboratories (Chauvin et al., 1996; Wang et al., 2000), which severely limits attempts to quantify the interaction. It was originally hoped to develop a competition assay, whereby unlabeled mutant HPrs would be added to a complex of labeled HPr with enzyme I or IIA^slc, in order to determine which residues were important for the interactions, but this assay was determined to be unfeasible.

3.2 Enzyme I binding site on HPr

The interaction site between enzyme I and HPr has been determined with NMR chemical shift experiments (van Nuland et al., 1995; Wang et al., 2000). In these studies, NMR spectra were taken with ^15N labeled HPr alone in solution, and then again after the addition of excess enzyme I. The spectra were compared, and HPr residues which underwent a chemical shift upon complexation with enzyme I were identified. These residues represented an enzyme I binding site on HPr. The HPr:enzyme I interaction was determined more conclusively with the NMR solution structure of the complex of EIN with HPr (Garrett et al., 1999). However, this structure did not utilize intact enzyme I, nor did it involve phosphorylation of either protein. It was decided to further determine the residues on HPr which interact with enzyme I using an assay more similar to physiological conditions – namely a kinetic assay. A large number of HPr
mutants were available for this study, and other mutants were created and purified especially for this investigation. Each HPr mutant was assayed as a substrate for enzyme I, and the kinetic parameters $V_{\text{max}}$ and $K_m$ were determined. Mr. Ahmad Mirza prepared and assayed several of the HPr mutants as part of an undergraduate research project in the Waygood laboratory. HPr mutants which affect both kinetic parameters for enzyme I indicate catalytically important residues. HPr mutants which affect only the $K_m$ for enzyme I indicate residues important for binding enzyme I, but not catalysis. Thus these residues delineate an enzyme I binding site on HPr.

3.2.1 Reliability of kinetic data

The kinetic data indicate that some of the HPr mutations do not result in a dramatic change in $K_m$ for enzyme I. Thus the concentration of each HPr preparation must be determined with a large degree of certainty. The method of Waddell (1956) is a convenient method for determination of protein concentration. The lactate dehydrogenase coupled depletion assay (Waygood et al., 1979) was also always used for determination of HPr concentration. This determination is specific for the amount of HPr in a preparation that can be phosphorylated. However, both the quantitation of HPr, as well as the kinetic assay itself, have inherent errors (Anderson et al., 1991). Such errors may be minimized by many measurements of both HPr concentration as well as HPr kinetic data, but that would require excessive amounts of each HPr preparation to be used. Thus all $K_m$ and $V_{\text{max}}$ values during kinetic measurements of HPr mutants have been rounded to the nearest whole number for $K_m$ values, and to the nearest 5% for $V_{\text{max}}$ measurements. In the kinetic assay the $K_m$ determinations were reproducible with
acceptable variation (plus or minus 10%), and >2-fold changes indicate significant residues in the enzyme I binding site.

For these assays, a commercially available rabbit muscle lactate dehydrogenase was used, which was supplied as a suspension in a concentrated ammonium sulfate solution. This solution was dialyzed against water before use, because sulfate ion is a competitive inhibitor for HPr. In the presence of 16 mM sulfate, as would be the case if undialyzed LDH was used, the enzyme I $K_m$ for HPr increases to ~12 µM, as opposed to 6 µM in the absence of sulfate (E. B. Waygood, unpublished observations). The $V_{max}$ for enzyme I also varied with each preparation, so the assay conditions were standardized with wild type HPr for each set of kinetic assays of HPr mutants. The enzyme I was phosphorylated by the addition of 2 mM PEP and 5 mM Mg$^{2+}$ and incubation at room temperature. If this enzyme I preparation was sterilized using a 0.22 µm syringe filter, it was stable for several days if left at room temperature.

3.2.2 Enzyme I kinetic parameters of HPr mutants

The lactate dehydrogenase coupled enzyme I assay was used to examine the effects of mutation of many HPr residues. Typical assay results are shown in Figure 3.4. The results of the entire kinetic study, including results from previous publications for ease of reference, are summarized in Table 3.1. Through this study residues which are important for the kinetic interaction of HPr with enzyme I were identified. These residues are compared to the HPr residues that were concluded to be involved in the interaction by examination of the NMR structure of the complex of HPr with the enzyme.
Figure 3.4 Examples of Lineweaver-Burk plots of kinetic data of enzyme I with mutant HPrs as substrates, using the lactate dehydrogenase coupled assay. (a): Ala20Thr, with the same kinetic parameters as wild type HPr. (b): Leu47Ala, with a higher $K_m$, but an identical $V_{max}$, as wild type.
Table 3.1 Kinetic parameters of *E.coli* enzyme I with HPr mutants.

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<sup>a</sup>Site of phosphorylation

<sup>b</sup>Implicated in catalysis

<sup>c</sup>Hydrogen-bond Asp129, Hydrophobic Ile72, His76

<sup>d</sup>Hydrophobic Ala71, His76

<sup>e</sup>Salt bridges Glu67, Glu68
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<td>100</td>
<td>This study</td>
</tr>
<tr>
<td>Thr56Gln</td>
<td>6</td>
<td>100</td>
<td>This study</td>
</tr>
</tbody>
</table>

---

a. Mutations at several other HPr positions had no effect on kinetic parameters. The mutants were: Pro11Ser, Ser31Ala, Asn38Asp, Gln57Glu, Glu70Ala, Glu70Lys, Gln71Glu, Glu83Ala, Glu85Gln, Glu85Lys, Deletion Glu85, and Glu83Asp, Gln and Lys in Deletion Glu85.

b. Kinetic parameters have been rounded as previously described (Anderson et al., 1991).

c. The residues given are in enzyme I. The residue and the type of interaction is taken from Garrett et al., 1999.

d. The following His15 mutants are also inactive: alanine, asparagine, cysteine, serine, threonine, and tyrosine.
I N-terminal domain (Garrett et al., 1999). That report identified residues in the interaction site on the basis of intermolecular nuclear Overhauser effects (NOEs).

Mutations to the two key catalytic residues, His15 and Arg17, produce significant changes to $V_{\text{max}}$. Modest perturbations to enzyme I $V_{\text{max}}$ were also observed with mutations to Asn12, Thr16, Lys24, and Gln51, indicating their potential minor importance for catalysis. Although Asn12 was not identified as an interacting residue in the HPr-enzyme I domain complex (Garrett et al., 1999), it is believed to form a hydrogen bond with the N$^\text{ε2}$ atom of His15 (van Nuland et al., 1995; Prasad et al., 1998). Kinetic analyses of Asn12 mutants do indicate that it plays a minor role in catalysis (Sharma et al., 1993; Napper et al., 1999). The HPr mutants Ser46Asp and Ser46Glu both gave an extremely high $K_m$ for enzyme I, and these $K_m$ values must be considered estimates since it was impossible to assay the mutant HPr in concentrations above the $K_m$ values. The mutant HPr Val23Ala was purified but contained a small contaminating "NADH oxidase" activity which compromised kinetic results. However, kinetic assays indicated that this mutant HPr did not have any significant defect in either $K_m$ or $V_{\text{max}}$ for the enzyme I interaction.

3.2.3 Description of enzyme I binding site on HPr

The altered residues of HPr mutants which have an affected $K_m$ but not $V_{\text{max}}$ for enzyme I are concentrated on one face of the HPr structure (Figure 3.5). Arg12, His15, and Arg17, the active site residues, are clustered on one side of the enzyme I binding site on HPr. Some mutations seemingly within the enzyme I binding site did not produce significant changes to enzyme I $K_m$ for HPr. These residues include Gln21, Val23,
Figure 3.5 Enzyme I binding site on *E. coli* HPr. The spacefilling representation is of the structure of *E. coli* HPr from the Jel42 Fab-HPr complex (Prasad *et al.*, 1998). The active site residues Asn12, His15, and Arg17 are coloured red. Residues that, when mutated, led to an altered HPr $K_m$ for enzyme I are blue. Residues which gave no change in HPr $K_m$ for enzyme I are green. Residues that were previously identified as making contacts with the enzyme I N-terminal domain (Garrett *et al.*, 1999) and have not been mutated in this study are coloured yellow. Numbers identify the residues.
Thr52, and Leu55. This might be explained in two ways; either the residues are not important for interaction, or the mutations that were made were not severe enough to alter the interaction. In the NMR structure of the HPr:enzyme I N-terminal domain complex (Garrett et al., 1999), several other residues were identified through NOEs as important for the interaction with enzyme I. For various reasons these residues were not mutated here. For instance, the interaction with Leu14 is with the α-carbon, and residue 54 is a highly conserved glycine. In addition, Ala19, Phe22, and Leu50 have single intermolecular NOEs and are buried within the HPr molecule, so these residues were also not mutated.

3.3 Functional characterization of enzyme I

The study of HPr mutants, most of which are site-directed mutants, has proven essential to the characterization of the structure and function of the protein. However, even though there has been extensive research involving the structural and biophysical characteristics of enzyme I, there has been very little study on single amino acid mutants of enzyme I. This section of the thesis will provide some key information about the structure, function, and in vivo role of enzyme I through some of the initial studies of enzyme I mutants.

3.3.1 Original S. typhimurium ptsI mutant strains

Several mutants of ptsI from S. typhimurium have been ordered by deletion mapping (Cordaro and Roseman, 1972). Two of the mutants, strains SB1476 (allele number ptsI17) and SB1681 (ptsI16), produced “leaky” low level enzyme I activity
relative to wild type. The physiological consequences of the \textit{ptsI7} mutation have been described (Saier and Roseman, 1972). Briefly, this mutant is more sensitive to catabolite repression than wild type, because in the presence of even low amounts of PTS-substrate sugars, enzyme \textit{IIA\textsuperscript{gle}} is dephosphorylated more rapidly than it is phosphorylated, leading to a loss of the binding and activation of adenylate cyclase by P-\textit{IIA\textsuperscript{gle}}. It was later determined that the \textit{I16} and \textit{I17} mutant enzymes did not form dimers at room temperature, unlike wild type enzyme I, as judged by gel filtration chromatography of crude extracts. In addition, it was discovered that the low enzyme I activity of these mutants could be complemented by the addition of enzyme I from crude extracts of either \textit{S. typhimurium} strain SB1690 (\textit{ptsI34}) or SB2227 (\textit{ptsI39}). These two strains by themselves had no detectable enzyme I activity, but did dimerize as determined by room temperature gel filtration chromatography (E. B. Waygood, J. C. Cordaro, and S. Roseman, unpublished results). In this present work, these \textit{S. typhimurium} strains were resurrected from agar stabs and the crude extracts of these strains were assayed for enzyme I activity. Similar results to those originally determined were observed. It was then decided to further study these enzyme I mutants, beginning with the isolation of the mutant \textit{ptsI} genes.

3.3.2 PCR cloning of \textit{ptsI} genes

The \textit{ptsI} genes were isolated and cloned from \textit{S. typhimurium} strains SB1476, SB1681, SB1690 and the wild type strain SB3507 by amplification of \textit{ptsI} from chromosomal DNA using the polymerase chain reaction as described in Materials and Methods. The attempts to clone the mutant gene \textit{ptsI39} from strain SB2227 have so far
been unsuccessful. Both strands of the cloned genes were sequenced, and single point mutations were identified in each of the mutant *ptsI* genes (Table 3.2). In addition, the sequence of wild type *ptsI* (Li Calsi et al., 1991) was confirmed.

<table>
<thead>
<tr>
<th>enzyme I strain, allele</th>
<th>DNA sequence change</th>
<th>protein sequence change</th>
</tr>
</thead>
<tbody>
<tr>
<td>SB1681, <em>ptsI</em>16</td>
<td>residue 1066G→A</td>
<td>Gly356→Ser</td>
</tr>
<tr>
<td>SB1476, <em>ptsI</em>17</td>
<td>residue 1123C→T</td>
<td>Arg375→Cys</td>
</tr>
<tr>
<td>SB1690, <em>ptsI</em>34</td>
<td>residue 376C→T</td>
<td>Arg126→Cys</td>
</tr>
</tbody>
</table>

Wild type *E. coli ptsI* was also isolated by polymerase chain reaction, using the plasmid pTSHIC9 as a source of template DNA, as described in Materials and Methods.

### 3.3.3 Overexpression and purification of enzymes I

The wild type and mutant *ptsI* genes were subcloned into the vector pT7-7 for use in protein overexpression. The *E. coli* host strain for the overexpression of these mutant enzyme I proteins was ESK238, which is a derivative of the common strain BL21 with the production of wild type enzyme I eliminated (Section 2.3). This strain is a spontaneous *ptsI* mutant selected by streptozotocin resistance. Each pT7-7.EI plasmid was transformed into ESK238 and a three litre culture of bacteria was prepared. Protein was overexpressed after the addition of 0.5 mM isopropylthiogalactoside (IPTG), as described in Materials and Methods. The protein overexpressed well, as judged by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE; Figure 3.6).
Figure 3.6  Time course of overexpression of *S. typhimurium* Gly356Ser enzyme I. Cells of strain ESK238 were transformed with the Gly356Ser pT7-7.EI plasmid and grown in culture to late log phase (OD$_{600nm}$ = 0.8). Then isopropylthiogalactoside (IPTG) was added to the culture to a final concentration of 0.5 mM. Aliquots of the culture were taken at set times after addition of IPTG and analyzed by SDS-PAGE. Lane 1, 0 h after IPTG addition; lane 2, 1 h; lane 3, 2 h; lane 4, 3 h; and lane 5, 4 h after IPTG addition. Lane labeled ‘M’ contains protein molecular weight markers: 66 kDa, bovine serum albumin; 45 kDa, ovalbumin; 31 kDa, carbonic anhydrase; 21 kDa, soybean trypsin inhibitor; 12 kDa, cytochrome c. The protein band corresponding with enzyme I is indicated with an arrow.
A high-speed centrifugation supernatant crude extract of each enzyme I overexpression was prepared, and the proteins were purified by Q-Sepharose anion exchange chromatography. This chromatography step was in most cases sufficient to obtain a preparation of enzyme I that was essentially homogeneous (Figure 3.7). In some cases an extra AcA44 gel filtration step was needed to produce a sufficiently pure preparation. These enzyme I preparations were used for subsequent kinetic and physical characterizations.

3.3.4 Overexpression and purification of enzyme I fragments

Overexpression and crude extract preparation of enzyme I N-terminal and C-terminal fragments were as described in Materials and Methods. The N-terminal fragments of 24 kDa and 27 kDa, referred to as EIN24 and EIN27, respectively, were relatively facile to purify by Q-Sepharose chromatography using identical conditions as described for enzyme I. The N-terminal fragments adhered strongly to the resin and were eluted with ~0.4 M KCl, well after the elution of most contaminating proteins. Further purification by AcA44 gel filtration chromatography was still necessary.

The C-terminal fragments were more difficult to purify. The smaller 27 kDa fragment (EIC27) had moderate levels of overexpression and formed insoluble inclusion bodies. The inclusion bodies were solubilized by the addition of urea, and after dialysis against buffer without urea a Q-Sepharose chromatography step provided some level of purification. This preparation was acceptable for the work that was needed. The 37 kDa fragment (EIC37) provided very good levels of overexpression, and did not appear to be sensitive to proteolytic digestion during crude extract preparation, in contrast to the
Figure 3.7 Purification of *S. typhimurium* Arg126Cys enzyme I. Lane 1, crude extract before ultracentrifugation (20 µg); lane 2, crude extract after ultracentrifugation (20 µg); lane 3, enzyme I pool after Q-Sepharose purification (10 µg); lane 4, enzyme I pool after further purification by AcA44 gel filtration chromatography (5 µg). Lane labeled ‘M’ contains protein molecular weight markers, as described in Figure 3.6.
characteristics reported for a similar C-terminal construct (Fomenkov et al., 1998). However, the protein did not adhere strongly to the Q-Sepharose resin, as it eluted at \( \sim 0.2 \text{ M KCl} \) along with many other protein impurities, so complete purification needed the addition of an AcA44 gel filtration chromatography step. Indeed, it appears that the relative ease of purification of full size enzyme I is due to the strong overexpression of the protein, and the strong adherence of the protein to Q-Sepharose and other anion exchange resins, which is a characteristic of the N-terminal domain.

A Western blot of enzyme I mutants and fragments was performed (Figure 3.8), using the monoclonal antibody Jel45, which was raised against \textit{E. coli} enzyme I. Both \textit{E. coli} and \textit{S. typhimurium} enzyme I react with Jel45, again indicating the large degree of similarity between the two proteins. The Jel45 epitope is apparently on the C-terminal domain, as both EIC27 and EIC37 react with Jel45, while the N-terminal fragments do not (not shown).

3.3.5 Gel filtration chromatography and temperature dependent dimerization

Preliminary experiments with crude extracts of \textit{S. typhimurium ptsI} mutants indicated that the enzymes I encoded by the mutant alleles \textit{ptsI16} and \textit{ptsI17} failed to form dimers. Similar gel filtration experiments with purified proteins of wild type and mutant forms of enzyme I were performed in this study in order to determine the molecular weight and association of the subunits. All enzyme I proteins consisted of a single protein species which migrated at \( M_r \approx 63 \text{ kDa} \) on a denaturing SDS polyacrylamide gel. Furthermore, all wild type and mutants of enzyme I eluted at a position corresponding to \( \approx 70 \text{ kDa} \) from a nondenaturing gel filtration column at 4°C.
Figure 3.8 Enzyme I immunoreactivity. Enzyme I wild type, enzyme I mutants, and the C-terminal fragments were separated by SDS-PAGE, transferred onto a nitrocellulose filter, and reacted with the enzyme I-specific monoclonal antibody Je145. Lane 1, *E. coli* enzyme I; lane 2, 27 kDa C-terminal fragment from *E. coli* enzyme I; lane 3, 37 kDa *E. coli* C-terminal fragment; lane 4, *S. typhimurium* Gly356Ser; lane 5, *S. typhimurium* Arg375Cys; lane 6, *S. typhimurium* Arg126Cys; lane 7, *S. typhimurium* wild type enzyme I. The amount of protein in each lane was 50 ng. This particular Western blot was performed by James Talbot.
Gel filtration chromatography at room temperature showed that Arg126Cys enzyme I formed dimers in a manner similar to wild type (Figure 3.9). Gly356Ser enzyme I had impaired dimerization and eluted in a single peak corresponding to a molecular weight of ~80-90 kDa. Arg375Cys enzyme I was susceptible to proteolytic degradation and aggregation at room temperature, but contained a major broad peak eluting at a molecular weight of 70 – 85 kDa (not shown). Addition of 10 mM PEP and 5 mM MgCl₂ to the column buffer did not significantly change the elution profile of any enzyme I protein. The separately purified 37 kDa C-terminal domain eluted at a position corresponding to a dimer (75 kDa). Thus the C-terminal domain contains the subunit interaction site, and it alone is necessary and sufficient for dimerization at room temperature. However, the smaller 27 kDa C-terminal domain protein did not dimerize as judged by Superose-12 chromatography (not shown), as this protein does not contain all sequences necessary for binding PEP and dimerization.

The apparent molecular masses of the N-terminal enzyme I fragments were estimated by gel filtration chromatography to be 43 kDa and 44 kDa for the EIN24 fragment, and 36 kDa and 40 kDa for the EIN27 fragment at 4°C and 22°C, respectively (not shown). The structure of the N-terminal domain is an elongated ellipsoid (Liao et al., 1996, Garrett et al., 1997a; Figure 1.9), which explains the apparent high molecular weight determined by gel filtration chromatography. These results indicate that the N-terminal domain by itself cannot form dimers. A similar aberrant molecular weight, 35kDa, and the lack of dimerization, has been determined for the N-terminal fragment of 269 residues (Chauvin et al., 1996b).
Figure 3.9 Gel filtration chromatography at room temperature. Proteins were eluted from a Sepharose-12 column calibrated with standards of 127, 45, and 21 kDa as indicated. Chromatographs are traces of $A_{280 \text{nm}}$, which show that: (a) Arg126Cys enzyme I eluted at $\sim 127$ kDa; (b) Gly356Ser enzyme I eluted at $\sim 80-90$ kDa; (c) EIC37 C-terminal fragment eluted at 75 kDa, the position expected for a dimer. Detection was at 280 nm; peak height maximum was $\sim 0.1$ A for (a) and (b), and $\sim 0.05$ A for (c).
3.3.6 Kinetic characterization of enzymes I

The enzyme I assay in which pyruvate production was coupled to lactate dehydrogenase (LDH) was extensively used to characterize wild type and mutant enzyme I species, not only to determine their kinetic parameters of $V_{max}$ and $K_m$, but also to determine the role of the enzyme I monomer:dimer equilibrium in the activity of these enzymes.

3.3.6.1 PEP and HPr kinetic parameters

The enzyme I catalyzed reaction, $HPr + PEP \leftrightarrow HPr-P + \text{pyruvate}$, was measured by the LDH-coupled spectrophotometric assay. Enzyme I preparations were kept phosphorylated and at room temperature, conditions which lead to maximal activity (Waygood et al., 1979; Weigel et al., 1982a; Waygood, 1986), and kinetic properties of the enzymes were obtained (Table 3.3). It is important to note that in these assays, as well as all subsequent assays, undialyzed LDH was used, so $16 \text{mM}$ sulfate was present in the assay. Under these conditions, wild type enzyme I has an HPr $K_m$ of $12 \mu\text{M}$ (see Section 3.2.1). Thus all enzyme I mutants have an HPr $K_m$ identical to wild type ($12 \mu\text{M}$). However, PEP $K_m$s differed, with Gly356Ser and Arg375Cys mutants showing a similar increase of approximately 30-fold from wild type PEP $K_m$ to $3.0 \text{mM}$ and $3.1 \text{mM}$, respectively. Thus, PEP-binding is affected by these mutations. Arg126Cys enzyme I had a PEP $K_m$ substantially lower than wild type, but this difference was likely due to the extremely low activity of the enzyme. Arg126Cys had a very low $V_{max}$ of $0.04\%$ of wild type, which is consistent with the lack of measurable activity in the crude extract of the original SB1690 $S. \text{typhimurium}$ strain. The activity of Gly356Ser and
Table 3.3 Kinetic properties of enzyme I mutants.

<table>
<thead>
<tr>
<th>enzyme I</th>
<th>HPr $K_m$ (µM)</th>
<th>PEP $K_m$ (mM)</th>
<th>$V_{max}$ (µmol·min$^{-1}$·mg$^{-1}$)</th>
<th>$V_{max}$ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>wild type</td>
<td>12</td>
<td>0.10</td>
<td>159</td>
<td>100</td>
</tr>
<tr>
<td>Arg126Cys</td>
<td>11</td>
<td>0.01</td>
<td>0.08</td>
<td>0.04</td>
</tr>
<tr>
<td>Gly356Ser</td>
<td>12</td>
<td>3.0</td>
<td>7.0</td>
<td>4.4</td>
</tr>
<tr>
<td>Arg375Cys</td>
<td>12</td>
<td>3.1</td>
<td>3.0</td>
<td>1.9</td>
</tr>
<tr>
<td>Gly356Ser + Arg126Cys</td>
<td>12</td>
<td>&lt;0.01$^a$</td>
<td>40</td>
<td>25</td>
</tr>
<tr>
<td>Arg375Cys + Arg126Cys</td>
<td>12</td>
<td>&lt;0.01$^a$</td>
<td>5</td>
<td>3</td>
</tr>
<tr>
<td>Gly356Ser + EIC37$^{b,c}$</td>
<td>12</td>
<td>&lt;0.01$^a$</td>
<td>34</td>
<td>21</td>
</tr>
</tbody>
</table>

$^a$ These are estimates. Activity at lower PEP concentrations was dependent on association of subunits in the heterodimer (see Section 3.3.6.3). $b$ The protein molar ratio during the assays of the complementation mixtures was 1:2, with either Gly356Ser or Arg375Cys at 1. $c$ EIC37 is the 37 kDa C-terminal fragment.
Arg375Cys enzymes I, with \( V_{\text{max}} \) values of 4% and 2% of wild type, are as expected for "leaky" mutants.

3.3.6.2 \textit{In vitro} intragenic complementation

Activity complementation between purified proteins of two different enzyme I mutants, presumably through heterodimer formation, was investigated by the LDH-coupled spectrophotometric assay. The assay conditions used included 30 \( \mu \text{M} \) HPr and 20 mM PEP, which would give near maximal activities for all of the enzyme I mutants. In order to demonstrate effective complementation between Arg126Cys and either Gly356Ser or Arg375Cys purified proteins, the enzymes had to be combined together while on ice. This low temperature leads to monomer formation. The mixtures were then diluted to appropriate concentrations using room temperature buffer containing 10 mM potassium phosphate buffer, pH 7.5, 1 mM EDTA, 0.2 mM DTT, 5 mM MgCl\(_2\), and 2 mM PEP, and preincubated at room temperature for 5 min. before addition to the assay. These conditions would lead to the formation of dimers, presumably either composed of the same or different subunits. The \textit{in vitro} intragenic complementation between Arg126Cys enzyme I and either Gly356Ser or Arg375Cys enzyme I is shown in Figure 3.10. If the two enzyme I mutants were preincubated separately at room temperature, leading to the formation of homodimers of at least the Arg126Cys enzyme I, and after this preincubation the enzymes were combined, effective activity complementation was not demonstrated (data not shown). This indicates that heterodimers form optimally when two enzymes are first preincubated separately under conditions which favour monomers, and conditions are changed to those favouring
Figure 3.10  *In vitro* complementation of Gly356Ser or Arg375Cys enzymes I with Arg126Cys enzyme I. Gly356Ser or Arg375Cys enzyme I (50 μg/mL) was preincubated at room temperature for 5 min with increasing concentrations of Arg126Cys enzyme I in separate tubes. Then 5 μL of each preincubation mixture was measured by the LDH assay with 30 μM HPr and 20 mM PEP, so thus 250 ng of either Gly356Ser or Arg375Cys enzyme I was assayed with increasing amounts of Arg126Cys enzyme I. The amounts of Arg126Cys enzyme I added to the assay did not yield any measurable activity if assayed alone. □: Assay of complementation of Gly356Ser enzyme I. Δ: Assay of complementation of Arg375Cys enzyme I.
dimers only after the two mutants are combined. Furthermore, if the assay displaying poor complementation was allowed to proceed for as long as 30 min, still no activity increase was observed. This suggests that at saturating PEP concentrations no dissociation of the subunits occurs during enzyme turnover, which could lead to heterodimer formation. No complementation was observed between Gly356Ser and Arg375Cys enzymes I, as is expected since both mutants have impaired dimerization (not shown).

The extent of complementation for Gly356Ser enzyme I is much greater than Arg375Cys enzyme I, likely either because Gly356Ser is more active in a heterodimer, or because Gly356Ser is more able to form heterodimers. In both cases, not only was there an increase in $V_{\text{max}}$ but also a decrease in PEP $K_{m}$ for the active Gly356Ser or Arg375Cys subunit of the heterodimer (Table 3.3). However, the PEP $K_{m}$ for the heterodimers must be considered an estimate, because of the instability of the heterodimers in the assay (see Section 3.3.6.3 for explanation).

3.3.6.3 Mutant enzyme I heterodimer instability

The activity of the heterodimers was influenced by the degree of association of the subunits in the assay. At saturating PEP concentrations (20 mM), the enzyme I activity of the complementing mixtures was stable, and similar in this respect to wild type enzyme I (Figure 3.11a and b). However, at a concentration of 1 mM PEP, the activity of the heterodimer decreased over time (c), much more than could be accounted for by simple substrate utilization. This rapid decline in activity was even more pronounced at lower PEP concentrations, so much so that an accurate determination of
Figure 3.11 Enzyme I activity in an assay, demonstrating heterodimer instability. The recording chart traces of the assay are shown. Each enzyme I was measured with the standard assay, which included preincubation for 5 min at room temperature in buffer containing 2 mM PEP and 5 mM MgCl₂, and measurement in the assay with 30 μM HPr and 20 mM PEP. The enzymes assayed were: 50 ng wild type enzyme I (a), 2 μg Gly356Ser enzyme I (b), or the Gly356Ser/Arg126Cys heterodimer, 250 ng of each subunit (c). The amount of each enzyme used was chosen in order to give roughly the same rate for all of the measurements.
the initial velocity could not be obtained. Thus the value of <10 μM for the PEP $K_m$ for the heterodimer mixtures of either Gly356Ser or Arg375Cys with Arg126Cys must be considered an estimate. Nevertheless, there is a dramatic improvement in PEP $K_m$ for the Gly356Ser and Arg375Cys subunits from the original values of 3 mM.

It was still unclear as to whether the instability of the heterodimers was due to the dissociation of subunits during the enzyme I catalytic cycle and phosphorylation of HPr, or due to the dissociation of the subunits following the dilution of the heterodimers from the preincubation mixture to the assay mixture. A simple experiment was performed to investigate these two potential answers. A heterodimer mixture was added to an assay cuvette containing 20 mM PEP but no HPr. Then, after a 10 min. incubation at room temperature, HPr was added to start the reaction (Figure 3.12b). The activity observed was nearly as high as when the heterodimer mixture was added to a cuvette which already contained HPr (a). If the identical experiment was performed, but in both cases 1 mM PEP was used instead of 20 mM PEP, the results were different. In the assay cuvette which was preincubated for 10 min. without HPr, no activity was initially detected after addition of HPr, although after a short time a constant low activity was observed (d). If the heterodimer was added to a cuvette with 1 mM PEP and HPr present, there was strong initial activity which decreased over time (c), as previously observed. Together these results indicate that dilution of the enzyme into the assay conditions causes subunit dissociation, and that this dissociation is dependent on PEP concentrations. Turnover of the enzyme does not appear to cause significant dissociation.
Figure 3.12 Recording chart traces of enzyme I activity in an assay, demonstrating the effect of dilution. The Gly356Ser/Arg126Cys heterodimer (250 ng each subunit) was measured with 20 mM PEP either immediately (a) or after 10 min. in the assay without HPr, which was then added to start the reaction (b). Similarly, the heterodimer was measured with 1 mM PEP either immediately (c) or after 10 min. without HPr (d).
3.3.6.4 Kinetic study of mutant enzyme I homodimerization

It remained to be determined if the Gly356Ser and Arg375Cys enzyme I mutants, when assayed alone, were active as monomers or dimers. The effects of dilution and temperature change on enzyme I activity are shown in Figure 3.13. If wild type enzyme I was preincubated alone at room temperature with 2 mM PEP and 5 mM MgCl₂ before addition to the assay, a stable dimeric species with high activity was obtained (a). However, if wild type enzyme I was preincubated in a cold dilute condition before addition to the assay, very low activity was observed which slowly increased over time (b). This hysteretic behaviour of the enzyme is due to the slow association of subunits to form active dimers in the assay, after transfer from the initial cold incubation which favoured monomer formation. When Gly356Ser enzyme I was subjected to the same warm and cold preincubation treatments, the measured activity showed no dependence on preincubation condition (c and d). No hysteretic behaviour of slow association of subunits was observed. Thus the activity of Gly356Ser enzyme I, with a PEP \( K_m \) of 3 mM and a \( V_{\text{max}} \) of 4% of wild type, can be attributed to the monomeric form of enzyme I. Similar experiments showed that Arg375Cys enzyme I did not exhibit hysteresis, and was active as a monomer (not shown), whereas all indications are that Arg126Cys enzyme I is active as a dimer, although the activity of the protein was too low to test the dependence on preincubation conditions.

3.3.7 Chromatographic isolation of enzyme I heterodimers

A Gly356Ser/Arg126Cys heterodimer mixture was prepared and analyzed by gel filtration chromatography. However, results were different from expected (Figure 3.14).
Figure 3.13 Recording chart traces of enzyme I activity in an assay, demonstrating the effect of temperature of preincubation. Wild type enzyme I (50 ng) was assayed after preincubation at room temperature (a) or on ice (b). Gly356Ser enzyme I (2 μg) was assayed after preincubation at room temperature (c) or on ice (d). Arg375Cys enzyme I had similar behaviour to Gly356Ser.
Figure 3.14 Room temperature gel filtration chromatography of heterodimer mixtures. Chromatographs are traces of protein elution as detected by absorbance at 280 nm. Different ratios of Arg126Cys and Gly356Ser enzymes I were combined on ice, incubated at room temperature for 5 min. with 2 mM PEP and 5 mM MgCl₂, and then loaded and eluted on the room temperature Superose-12 column. Arg126Cys:Gly356Ser ratios were (a) 1:1; (b) 4:1; (c) 7:1; all with the column equilibrated with normal buffer (see Section 2.20); and (d) 4:1 with column equilibrated with buffer containing 10 mM PEP and 5 mM MgCl₂. Maximum peak heights were ~0.1 A (280 nm) except for (d) which was ~0.15 A. In all cases, the first peak (left) and second peak (right) correspond to an estimated apparent molecular weight of ~127 kDa and ~70 kDa, respectively.
It appears that there is a dynamic monomer:dimer equilibrium between the two enzyme I species as the proteins are eluting from the column. The net effect of this equilibrium was that more of the Arg126Cys enzyme I protein eluted at a lower molecular weight than the expected 127 kDa, through interaction with the Gly356Ser subunits. This indicates that heterodimers do form. The assay results (Section 3.3.6) indicate that the heterodimer was more stable in the presence of high concentrations of PEP. The gel filtration column was equilibrated with a buffer containing 10 mM PEP and 5 mM MgCl₂, and the heterodimer mixture was applied. Under these conditions, more protein eluted at 127 kDa (Figure 3.14d). Thus the chromatography results also show that the heterodimer stability is dependent on PEP concentration.

It was also attempted to examine the chromatographic separation of the Gly356Ser/Arg126Cys heterodimer by analysis of enzyme I activity in the collected fractions (Figure 3.15). The position of the heterodimer, indicated by the original peak of enzyme I activity without any added mutant enzyme I subunit, is at a position intermediate between monomer and dimer. However, the majority of Gly356Ser subunits, indicated by the enzyme I activity profile with 250 ng Arg126Cys enzyme added to the assay, are more near the monomeric position. Similarly, the majority of Arg126Cys subunits, detected with the addition of extra Gly356Ser enzyme I to the assay, are at the dimeric position. The position of the ‘heterodimer’ activity could be simply explained as the position during the elution where the trailing edge of the Arg126Cys enzyme I peak and the leading edge of the Gly356Cys enzyme I mix, and that heterodimers really do not form on the column. However, the previous chromatographic separations with different ratios of the subunits had already shown that
Figure 3.15  Enzyme I activity of Gly356Ser/Arg126Cys heterodimer separated by Superose-12 chromatography, using buffer with no added PEP or MgCl₂. A 4:1 mixture of Arg126Cys:Gly356Ser enzyme I was incubated at room temperature for 5 min. with 2 mM PEP and 5 mM MgCl₂, and then 100 µL (0.05 mg total protein) was loaded onto the column and 1 mL fractions were collected. The protein elution profile is not shown, but is similar to that shown in Fig. 3.16b, in that the size of the monomer and dimer peaks at ~70 kDa and at ~130 kDa are approximately equal. The approximate positions of those peaks are labeled ‘M’ and ‘D’, respectively. The enzyme I activity of 5 µL of each fraction was determined by the enzyme I PTS assay, with no additions (□) or with 250 ng Arg126Cys enzyme I added (Δ), or with 125 ng Gly356Ser enzyme I added (●). The background activity of the added enzymes was subtracted.
there is a dynamic formation and dissociation of heterodimers occurring. The enzyme I activity of a similar separation of heterodimer, but in this case with 2 mM PEP and 5mM MgCl₂ added to the column buffer, indicated that the heterodimer was still at an intermediate position between monomer and dimer (not shown).

3.3.8 Examination of the structure of PPDK

The structure of the C-terminal domain of *C. symbiosum* PPDK was used as a model to help explain the effects of the Gly356Ser and Arg375Cys mutations in enzyme I. Figure 3.16 shows a direct amino acid sequence alignment between enzyme I and *C. symbiosum* PPDK. When this alignment is compared to the structure of the C-terminal domain of PPDK (Herzberg *et al.*, 1996), the sequence segments which are deleted in enzyme I (deletions A to E) correspond for the most part to exposed loops in the structure of PPDK. In this study, the interaction site between the subunits of PPDK was determined from the tertiary structure using a standard method (Sheriff *et al.*, 1987). The residues involved in subunit interaction are described in detail in Table 3.4. This information is summarized in Table 3.5. The residues involved in subunit interaction form a set of five sequences, numbered 1 to 5 in Tables 3.4 and 3.5, which are fairly well conserved between enzyme I and PPDK (Figure 3.16). In these sequences, several residues interact with the same residue in the other subunit (called “self interacting” in Table 3.5).

After determination of the subunit interaction site of PPDK, the sequence segments that are missing in the sequence alignment of enzyme I and PPDK (deletions
Figure 3.16 PEP-binding domain sequence alignment. The sequence deletions, DelA-E in this alignment, are deleted in the model of the structure of the enzyme I C-terminal domain (Figure 3.17). The enzyme I sequences from *E. coli* and *S. typhimurium* differ at five residues and are indicated, for example, as M/L, respectively. Residues conserved between PPDK and enzyme I are underlined. Bolded residues are those identified as involved in PEP-binding (Herzberg et al., 1996). Residues in square brackets are those involved in the subunit interaction site of PPDK. I16 and I17 identify the enzyme I residues Gly356 and Arg375, respectively. The number 338 identifies Gly338, the residue which when mutated, impairs dimerization (Seok et al., 1996b).
Table 3.4 Pyruvate phosphate dikinase residues in contact at the subunit interaction site.

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b. Total number of H bonds = 15  
c. Total number of ion pairs = 3.  
d. There are five sequences in which interacting residues are found.  
e. Residues in parentheses are the next in the sequence, but were not aligned.  
f. Bolded residues are conserved between C. symbiosum pyruvate phosphate dikinase and enzyme I from both E. coli and S. typhimurium.  
g. Italicized residues interact with the same residue in the other subunit.  
h. Pyruvate phosphate dikinase residues 784-794 do not align well with enzyme I.
Table 3.5 Summary of the subunit site of interaction in pyruvate phosphate dikinase

<table>
<thead>
<tr>
<th>Description</th>
<th>Sequence Grouping</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Residues in PPDK</td>
<td>654-672</td>
</tr>
<tr>
<td>Residues in enzyme I</td>
<td>347-365</td>
</tr>
<tr>
<td>Number of residues making contacts:</td>
<td>13</td>
</tr>
<tr>
<td>Conserved residuesb</td>
<td>7</td>
</tr>
<tr>
<td>Self-interacting residuesc</td>
<td>Phe658</td>
</tr>
<tr>
<td>Interacting sequence numberd</td>
<td>Seq. 1 &amp; 4</td>
</tr>
<tr>
<td>Number of:</td>
<td></td>
</tr>
<tr>
<td>vdw</td>
<td>98 (10)</td>
</tr>
<tr>
<td>H-bonds</td>
<td>12 (1)</td>
</tr>
<tr>
<td>Ion pairs</td>
<td>3</td>
</tr>
</tbody>
</table>

a. The alignment of enzyme I with C. symbiosum pyruvate phosphate dikinase is uncertain after residues ~470 and 784 respectively. The sequences of all the enzymes I show a deletion in this part of the sequence.

b. Residues conserved between C. symbiosum pyruvate phosphate dikinase and enzyme I in the interaction sequences.

c. Residues in each subunit which interact with the identical residue.

d. Does not include "self-interacting" residues.

e. Except for the bonds involving the "self-interacting" residues, each bond reported is given twice, once in each sequence. The numbers in parentheses are those involving the "self-interacting" residues. The total number of interactions: van der Waals, 154; H-bonds, 15; ion pairs 3.
A to E in Figure 3.16) were deleted from the PPDK structure. Then energy minimization was performed using the X-PLOR structural analysis program (Brünger, 1993) to create a model of the structure of the C-terminal domain of enzyme I. The resulting structure (Figure 3.17) was similar to the original PPDK structure, and resulted in only one significant structural change to the subunit interaction site. Deletion E, residues 794-803, led to the convergence of subunit interaction sequences 3 and 4. This resulted in both movement and local disruption of structures on either side of the deletion, which would affect the subunit binding site. The PPDK subunit interaction sequences are shown in yellow, with residues conserved with enzyme I in amber, on the structure of the model of the enzyme I C-terminal domain (Figure 3.17). These sequences do not overlap with the residues of the putative PEP-binding site of PPDK (Herzberg et al., 1996; residues in purple in Figure 3.17), but they are clearly very close. The deletions in this analysis did not significantly affect the orientation of the conserved residues of the PEP-binding site.

Gly663 of PPDK, which is Gly356 in enzyme I, is within interaction sequence 1 (Table 3.5) which is at the axis of two-fold symmetry between subunits of the PPDK crystal structure (Herzberg et al., 1996). The conserved PPDK residue analogous to Arg375 of enzyme I is Arg682. This residue is not in a subunit interaction sequence in PPDK and is located at some distance away from both the subunit interaction site and the PEP-binding site. Both the Arg and Gly residues occupy approximately the same location and orientation in the model of the structure of the C-terminal domain of enzyme I (Figure 3.17). From the position of Gly663/Gly356, it is readily apparent why
**Figure 3.17** The structure of the model of the C-terminal domain of enzyme I, originating from the structure of *C. symbiosum* PPDK, beginning at residue 505. The residues colour coded in this structure are identified in the sequence alignment in Figure 3.16. Purple, conserved residues that form the PEP-binding site of PPDK (Herzberg *et al.*, 1996); yellow, sequences involved in subunit interaction with amber those conserved between enzyme I and PPDK; pale blue, other residues that are conserved between the two enzymes; black, the residues of characterized mutants – Gly is Gly356, Arg is Arg375, and Leu is Gly338 in enzyme I.
mutation of this residue may cause disruptions of subunit interaction. However, it is less obvious why mutation of Arg682/Arg375 would result in impaired dimerization.

The mutations Gly663Ser and Arg682Cys were made to the original structure of PPDK, followed by energy minimization. Neither mutation resulted in a significant disruption of the structure or the subunit interaction site. Thus after the structural comparisons presented in this thesis it still remains relatively unclear why the Gly356Ser and Arg375Cys mutations in enzyme I produce the observed effects of impaired dimerization. Further in vitro work was then performed to further investigate the subunit interactions of enzyme I.

3.3.9 Enzyme I fragments and activity complementation

Using the LDH-coupled spectrophotometric assay, activity complementation between the cloned 37 kDa C-terminal domain and Gly356Ser or Arg126Cys enzyme I has surprisingly been demonstrated. Kinetic parameters for the Gly356Ser/EIC37 heterodimer were obtained (Table 3.3), and are similar to those of the Gly356Ser/Arg126Cys heterodimer. The fact that the EIC37 protein is capable of complementation conclusively demonstrates that the only active subunit in the heterodimer is the Gly356Ser subunit. This complementation also shows that the dimerization activity of enzyme I resides within the C-terminal domain, and that this domain alone is necessary and sufficient for dimerization. The EIC27 protein, lacking potentially important PEP-binding and subunit interaction sequences, is understandably not able to complement Gly356Ser enzyme I.
Using the *in vitro* LDH-coupled enzyme I assay, no activity complementation between separately cloned and purified N-terminal and C-terminal enzyme I fragments could be demonstrated, even with relatively large amounts of protein. However, Fomenkov *et al.* (1998) demonstrated activity complementation between the cloned N-terminal and C-terminal domains of enzyme I, by *in vivo* PTS-sugar fermentation properties of cells transformed with overexpression plasmids, and by phosphorylation of proteins with $^{32}$PPEP. Later, Zhu *et al.* (1999) also showed activity complementation between cloned N-terminal and C-terminal domains, albeit at a severely reduced rate compared to intact enzyme I, using the *in vitro* sugar phosphorylation assay. It was then decided to try to show activity complementation between the 27 kDa N-terminal and 37 kDa C-terminal constructs in this work, using the more sensitive *in vitro* phosphorylation of proteins with $^{32}$PPEP.

Figure 3.18 illustrates the results of the phosphorylation experiment. It was conclusively shown (lane 5) in that there is a phosphoryl transfer between full length enzyme I and the 27 kDa N-terminal enzyme I fragment (EIN27). More importantly, the phosphorylation of the EIN27 fragment was also shown in the presence of the 37 kDa C-terminal fragment of enzyme I (lane 4). Thus complementation between separate domains can be demonstrated, if enough protein and a sensitive method of detection are used. Not surprisingly, phosphorylated EIN27 is capable of phosphorylating HPr (lane 7), as had previously been shown by several laboratories.
Figure 3.18 *In vitro* phosphorylation of PTS proteins with [$^{32}$P]PEP. Phosphorylation reactions were prepared and separated by SDS-PAGE as described in Materials and Methods. Lane 1, 0.5 µg wild type *E. coli* enzyme I (EI); lane 2, 2.5 µg EIN27 + 5 µg EIC37; lane 3, 2.5 µg EIN27 + 0.5 µg EI; lane 4, 0.5 µg EI + 1 µg HPr; lane 5, 2.5 µg EIN27 + 5 µg EIC37 + 1 µg HPr. There is no phosphorylation of EIN27 when assayed alone (not shown).
3.3.10 Characterization of other enzyme I mutants

A great deal of information regarding the structure and function of enzyme I has been gained from the study of the three *S. typhimurium* enzyme I mutants Arg126Cys, Gly356Ser, and Arg375Cys. In order to progress further in the understanding of enzyme I, several site-directed mutants of *E. coli* enzyme I were created and studied. Firstly, the same three mutants as just described for *S. typhimurium* were created in *E. coli* enzyme I. Although the proteins were not purified, similar reductions in $V_{\text{max}}$ compared to wild-type, and activity complementation between mutants was found with assay of crude extracts (not shown). This work was carried out by Mr. Alastair MacFadden as an undergraduate research project. Subsequently, other site-directed mutants of *E. coli* enzyme I were created. The rationale for creation of each of these mutants will be discussed.

3.3.10.1 Asn352 mutants

Enzyme I mutants of Asn352 were created in order to investigate the potential importance of this residue in dimerization, and in order to potentially create a stable monomeric form of enzyme I. Asn352 is conserved in all enzymes I, *E. coli* PEP synthetase, and all pyruvate phosphate dikinases identified to date, 41 sequences in total (Pfam website <http://pfam.wustl.edu/cgi-bin/getdesc?name=PPDK_N_term>; Reizer *et al.*, 1993; Figure 1.8). Asn352 is conserved as Asn659 in *C. symbiosum* PPDK, and using the structure of this protein (Herzberg *et al.*, 1996), detailed analysis shows that Asn659 makes several contacts with residues of the other subunit of the dimer, including
the other Asn659 residue (Table 3.4). Thus it was decided to mutate Asn352 of *E. coli* enzyme I to Glu and Ala and determine the effects on the monomer:dimer equilibrium.

The Asn352Ala and Asn352Glu mutants were created by site-directed mutagenesis, and dimerization and kinetic parameters of the mutants were investigated. When subjected to gel filtration chromatography at room temperature, both mutants exhibit an impaired dimerization equilibrium compared to wild type (Figure 3.19), but appeared to have a slightly higher dimerization potential than Gly356Ser enzyme I. Both mutants also exhibited a significant decrease in \( V_{\text{max}} \) and a significant increase in PEP \( K_m \) compared to wild type enzyme I (Table 3.6). These characteristics are similar to those found for Gly356Ser and Arg375Cys enzymes I. However, the Asn352Glu mutant, although more active than Asn352Ala with \( V_{\text{max}} \) values of 8.3% and 1.3% of wildtype, respectively, has a much higher \( K_m \) for PEP than Asn352Ala, with values of 8.4 mM and 2.2 mM, respectively.

The activity of Asn352Ala and Asn352Glu enzyme I mutants while combined with Arg126Cys enzyme I in an activity complementation assay was then investigated. Asn352Glu enzyme I is capable of activity complementation with Arg126Cys enzyme I (Figure 3.20). However, the complementation of Asn352Ala by Arg126Cys enzyme I was not nearly as high, with only a ~2-fold stimulation compared to a ~6-fold stimulation of Asn352Glu enzyme I. Kinetic analysis was performed on the Asn352Glu/Arg126Cys complementation mixture in order to estimate a PEP \( K_m \) for the heterodimer. The activity of the heterodimer was very unstable at low PEP concentrations, perhaps even more so than that seen with the Gly356Ser/Arg126Cys heterodimer. The PEP \( K_m \) did not appear to be as low as 0.01 mM as had been
Figure 3.19 Analysis of wild type and mutant enzymes I by Superose-12 chromatography at room temperature. In all separations, ovalbumin (45 kDa) was used as an internal standard. The first peak in each chromatograph corresponds to the elution position of the enzyme I protein, labeled with a number signifying the apparent molecular weight of the enzyme I species. The proteins analyzed were (a) wild type enzyme I; (b) Gly356Ser enzyme I; (c) Asn352Glu enzyme I; (d) Leu355Ala enzyme I. Asn352Ala enzyme I eluted at a similar position to Asn352Glu, and similarly with Leu355Ala and Leu355Glu.
Table 3.6 Kinetic analysis of mutants of enzyme I.

<table>
<thead>
<tr>
<th>EI species</th>
<th>PEP $K_m$ (mM)</th>
<th>$V_{max}$ (μmol·min⁻¹·mg⁻¹)</th>
<th>$V_{max}$ (% WT)</th>
</tr>
</thead>
<tbody>
<tr>
<td>wild type</td>
<td>0.12</td>
<td>155</td>
<td>100</td>
</tr>
<tr>
<td>Asn352Ala</td>
<td>2.2</td>
<td>2.1</td>
<td>1.3</td>
</tr>
<tr>
<td>Asn352Glu</td>
<td>8.4</td>
<td>13.3</td>
<td>8.3</td>
</tr>
<tr>
<td>Leu355Ala</td>
<td>1.6</td>
<td>12.3</td>
<td>7.7</td>
</tr>
<tr>
<td>Leu355Glu</td>
<td>1.8</td>
<td>5.7</td>
<td>3.6</td>
</tr>
<tr>
<td>Arg296Leu</td>
<td>0.05</td>
<td>0.06</td>
<td>0.04</td>
</tr>
</tbody>
</table>

Kinetic parameters were obtained using the LDH coupled spectrophotometric assay.
Figure 3.20 *In vitro* complementation of Asn352Ala or Asn352Glu enzyme I with Arg126Cys enzyme I. Asn352Ala or Asn352Glu enzyme I (50 μg/mL) was preincubated for 5 min with increasing concentrations of Arg126Cys enzyme I. Then 250 ng Asn352Ala (○) or Asn352Glu (□) enzyme I with increasing amounts of Arg126Cys enzyme I, corresponding to 5 μL of each preincubation mixture, was assayed with 30 μM HPr and 20 mM PEP. The amounts of Arg126Cys enzyme I added to the assay did not yield any measurable activity if assayed alone.
determined for the Gly356Ser/Arg126Cys heterodimer, and was estimated to be 0.5 – 1 mM, although accurate kinetic data were difficult to obtain (results not shown). The differences in properties of the Asn35Ala and Asn352Glu mutants, namely kinetic parameters and *in vitro* complementation, are important to note in that they provide some information as to the role of the Asn352 residue in wild type enzyme I.

### 3.3.10.2 Leu355 mutants

Leu355 fairly well conserved between all enzymes I and other enzymes of this PEP-utilizing class (Reizer *et al.*, 1993; Figure 1.8). However in *C. symbiosum* PPDK, the residue analogous to Leu355 is Met662. Met662 in PPDK is part of a residue segment that is important for subunit interaction, and, like Asn659, is a ‘self interacting’ residue, in that both Met662 residues form contacts with each other in the dimer (Table 3.4). Also, Leu355 in enzyme I is obviously very close to the Gly356 residue previously identified as important in dimerization. Thus the enzyme I mutants Leu355Ala and Leu355Glu were created to investigate the importance of Leu355 in enzyme I dimerization, and perhaps create a stable monomeric form of enzyme I.

The chromatographic analysis and kinetic parameters of Leu355Ala and Leu355Glu enzymes I have previously been presented in Figure 3.19 and Table 3.6, respectively. Again, both mutants exhibit impaired dimerization, and have kinetic parameters characteristic of ‘leaky’ mutants with a $V_{\text{max}}$ of 6-12% of wild type, and a PEP $K_m$ of 1.6-1.8 mM, compared to a wild type $K_m$ of 0.1 mM. Both mutants were also capable of activity complementation when assayed in combination with Arg126Cys enzyme I, but the degree of complementation was smaller than that seen for other
heterodimers, roughly a two- to four-fold increase in activity (Figure 3.21). Also, the complementation activity did not reach a plateau level at approximately a one- to two-fold excess of Arg126Ser enzyme I, as had been seen with other complementation studies. Rather, the slight increase in complementing activity increased, up to at least an eight fold excess of Arg126Cys enzyme I, even when the very slight activity of the Arg126Cys enzyme I when measured alone was subtracted (not shown). Thus Leu355 enzyme I certainly appears to be important for dimerization of enzyme I, but the Leu355 mutants have characteristics which are slightly different than the characteristics of the Gly356Ser mutant of S. typhimurium enzyme I. However, it does not appear that the Leu355 mutants which have been created are stable monomers.

3.3.10.3 Arg296Leu mutant

The residue Arg561 in C. symbiosum PPDK has been identified as important for PEP catalysis, in that the Arg561Leu mutant was incapable of the interconversion of PEP and pyruvate (Yankie et al., 1995). PPDK Arg561 is conserved in the PEP-utilizing family (Reizer et al., 1993; Fig. 1.8), and the analogous residue in E. coli enzyme I is Arg296. The analogous mutant Arg296Leu in E. coli enzyme I was created in this study in order to investigate the importance of the Arg296 residue in interaction with PEP, and also perhaps to further clarify the role of PEP binding in the monomer:dimer equilibrium of enzyme I.

The Arg296Leu mutant enzyme I protein was analyzed by room-temperature Superose-12 chromatography, but the protein, although >95% pure as judged by SDS-PAGE, eluted from the column in several peaks. However, the major protein species
Figure 3.21 *In vitro* complementation of Leu355Ala or Leu355Glu enzyme I with Arg126Cys enzyme I. Leu355Ala or Leu355Glu enzyme I (50 µg/mL) was preincubated for 5 min at room temperature with increasing concentrations of Arg126Cys enzyme I. Then 250 ng Leu355Ala (□) or Leu355Glu (●) enzyme I with increasing amounts of Arg126Cys enzyme I, corresponding to 5 µL of each preincubation mixture, was assayed with 30 µM HPr and 20 mM PEP. The amounts of Arg126Cys enzyme I added to the assay did not yield any measurable activity if assayed alone.
eluted at ~90 kDa, with several lower molecular weight peaks (not shown). Thus the Arg296Leu mutant appeared to have an impaired monomer:dimer equilibrium, relative to wild type. The Arg561Leu mutant of PPDK showed a susceptibility to proteolytic fragmentation during storage (Yankie et al., 1995), so perhaps the Arg296Leu enzyme I mutant was also susceptible to proteolytic degradation during room temperature chromatographic separation.

The Arg296Leu enzyme I mutant was also analyzed by enzyme assay, and kinetic parameters were obtained (Table 3.6). The mutant had a very low activity, 0.04% of wild type, as expected, although the Arg561Leu PPDK mutant did not have any detectable activity (Yankie et al, 1995). However, the PEP $K_m$ for the mutant was in fact similar to that of wild type (0.05 mM vs. 0.1 mM, respectively).

Gel filtration chromatography indicated that Arg296Leu enzyme I eluted at a position intermediate between monomer and dimer, and had impaired dimerization. In vitro complementation enzyme assays were then performed using Arg296Leu enzyme I (Figure 3.22). Arg296Leu enzyme I appears to behave similarly to Arg126Cys enzyme I, in that both have impaired catalytic activity but are capable of complementation of other C-terminal enzyme I mutants. Furthermore, there is no direct complementation between the two enzymes. It appears then that Arg296Leu enzyme I is capable of forming heterodimers with other enzyme I mutants, although the stability of the heterodimers is likely very low.
Figure 3.22  *In vitro* activity complementation involving Arg296Leu enzyme I with other enzyme I mutants. *S. typhimurium* Gly356Ser or *E. coli* Asn352Glu enzyme I (50 μg/mL) was preincubated for 5 min at room temperature with increasing concentrations of Arg296Leu enzyme I. Then 250 ng Gly356Ser (Δ) or Leu355Glu (□) enzyme I with increasing amounts of Arg296Leu enzyme I, corresponding to 5 μL of each preincubation mixture, was assayed with 30 μM HPr and 20 mM PEP. Note: an assay of Arg296Leu enzyme I in combination with either *S. typhimurium* Arg126Cys, *E. coli* Asn352Ala, Leu355Ala, or Leu355Glu enzymes I shows little or no activity complementation.
3.3.10.4 His189 mutants

HPr is phosphorylated at the N² atom of the His15 residue. It has been shown that the His15Asp mutant of HPr is still capable of phosphocarrier activity, albeit at a greatly reduced activity (Napper et al., 1999). Enzyme I is phosphorylated at the N⁵ position of histidine 189, and as previously discussed in the Introduction, by analogy to HPr the His189Glu mutant of enzyme I may also have some catalytic activity. To investigate this possibility, His189 mutants of enzyme I were created. To date, the enzyme I mutants His189Asp and His189Glu have been made, and investigated for catalytic activity by in vitro phosphorylation with [³²P]PEP (Figure 3.23). Other His189 mutants are presently being created by Dr. Napper. His189Glu enzyme I is indeed phosphorylated after the addition of [³²P]PEP (lane 3), whereas phosphorylated His189Asp enzyme I is not visible under the same conditions (lane 2). However, the degree of autophosphorylation of His189Glu enzyme I in this experiment is about ten-fold lower than wild type, since ten times as much His189Glu enzyme I was loaded onto the SDS polyacrylamide gel in order to get a band of similar intensity of wild type on the autoradiogram. Phosphorylated His189Glu enzyme I is also capable of phosphotransfer to HPr (lane 6), again at a greatly reduced efficiency compared to wild type. Unexpectedly, phosphorylated HPr also appears when incubated with [³²P]PEP and His189Asp enzyme I (lane 5). This experiment has been repeated with the same results (not shown). However, at no time was a band visible indicating phosphorylated His189Asp enzyme I, perhaps due to very weak autophosphorylation activity and/or poor stability of the acyl phosphate during the experiment. Acyl phosphates in proteins are unstable at both acid and alkaline pH (Stock et al., 1989), and the half lives of such
Figure 3.23 Phosphorylation of mutant enzymes I and phosphotransfer to HPr. Proteins were incubated with $[^{32}\text{P}]$PEP, electrophoresed, and subjected to autoradiography as previously described (Section 2.20). Lane 1, 0.5 wild-type enzyme I (EI); lane 2, 5 µg His189Asp EI; lane 3, 5 µg His189Glu EI; lane 4, 0.5 µg wild-type EI + 1 µg HPr; lane 5, 5 µg His189Asp EI + 1 µg HPr; lane 6, 5 µg His189Glu EI + 1 µg HPr.
acetyl phosphates vary widely, ranging from ~4 min. for the P-Asp of NtrC (Keener et al., 1988) to a few seconds for the P-Asp of CheY (Hess et al., 1988) or the P-Glu residues of prothymosin α (Trumbore et al., 1997).

The phosphotransfer activity of the His189Asp mutant is surprising, since schematically the carboxyl oxygen of aspartate does not mimic the Nε2 position of histidine, but rather best mimics the Nε1 atom. However, this should be taken as a real result, since His189Asp enzyme I was purified from a ptsHicrr bacterial strain deficient in wild type enzyme I, while purified HPr is not phosphorylated in the absence of any added enzyme I, and this experiment was performed repeatedly in part to assure no phospho-HPr contamination or "spillover" into the sample lane containing His189Asp enzyme I.

It was attempted to assess the catalytic activity of His189Asp and His189Glu enzyme I mutants using the LDH-coupled spectrophotometric assay. However, even when relatively large amounts of protein were used, and under conditions which gave maximal activity for the wild type enzyme, no activity for either mutant was detected, and no kinetic parameters could be obtained. Both mutant enzymes were capable of activity complementation of Gly356Ser enzyme I, in a manner similar to the Arg126Cys active site mutant (not shown), as expected.
4.0 DISCUSSION

4.1 Binding constants of HPr with other PTS proteins

Phe2Cys HPr was purified from a cell extract most optimally when 0.2 mM DTT was included in all buffers, in order to limit dimerization of the protein. Other cysteine-containing mutants of HPr, namely Arg17Cys (Smallshaw, 1997; Smallshaw et al., 1998) and Glu83Cys (Liu et al., 1998) were also able to form disulfide-linked dimers, and 0.2 mM DTT was used in purifications of these proteins as well. During ion exchange chromatography it was observed that disulfide-linked dimers of Phe2Cys HPr had a lower apparent isoelectric point (pI) than the monomeric form, which was also observed by isoelectric focusing gel (Figure 3.1). A similar charge difference was also observed with monomeric and dimeric forms of Arg17Cys HPr (Smallshaw, 1997). Interestingly, Phe2Cys HPr dimers seemed to have two distinct forms, which was not the case with Arg17Cys HPr. A possible explanation is that some charged amino acid side chains would be masked in a dimer, due to interactions of each HPr molecule, and these interactions would differ depending on the arrangement and attachment of the two HPr proteins.

Purified cysteine-containing mutants of HPr have proven relatively easy to label with the thiol-specific fluorescein-5-maleimide. These labeled HPrs have been used effectively in fluorescence polarization binding studies involving anti-HPr antibodies.
(Smallshaw, 1997; Liu et al., 1998; Smallshaw et al., 1998). However, using this technique, only an incomplete binding curve of labeled Phe2Cys HPr with either enzyme I or IIA^{ Gle } was obtained (Figure 3.3). The interaction of HPr with its partner PTS proteins is of very low relative affinity. Very weak interactions between HPr and enzyme I or enzymes II are easily rationalized, since these interactions must be transient in order to sustain the flow of phosphoryl transfer reactions in the PTS. The physiological concentrations of these proteins in E. coli or S. typhimurium are relatively high: 2-5 μM for enzyme I (Misset et al., 1980; Mattoo et al., 1983), 20-100 μM for HPr (Waygood et al., 1979), and 25 – 50 μM for IIA^{ Gle } (Scholte et al., 1982) Conversely, antibody-antigen interactions are for the most part high affinity interactions. The fluorescence polarization technique did not prove completely useful in the study of the interactions of HPr with enzyme I and IIA^{ Gle }.

4.2 Enzyme I binding site on HPr

A kinetic assay of wild type and site-directed mutants of HPr as substrates for enzyme I was undertaken. This proved to be a useful method for delineating the enzyme I binding site on HPr, and complemented NMR chemical shift studies of the binding interaction (van Nuland et al., 1995; Wang et al., 2000) as well as the NMR solution structure of the complex of HPr with the N-terminal domain of enzyme I (Garrett et al., 1999). This approach is similar to the “epitope mapping” mutagenesis investigation of the interaction sites between HPr and a number of monoclonal antibodies (Sharma et al., 1991; Smallshaw et al., 1998).
This kinetic study identified the key catalytic residues of the active centre of HPr, His15 and Arg17, as expected (Table 3.1). These active centre residues of HPr are clustered on one side of the HPr molecule (Figure 3.5). Mutations to Ser46 also produced drastic effects on the $K_m$ and $V_{max}$ of enzyme I. Ser46 is the residue of phosphorylation by a regulatory kinase in Gram-positive bacteria such as B. subtilis, and this phosphorylation severely reduces the phosphoryl transfer activity of HPr. The structure of the Ser46Asp mutant of E. coli HPr indicates that the mutation produces significant alterations to the electrostatic surface potential of the molecule (Napper et al., 1996). In this kinetic study, other residues were identified which, when mutated, affected the $K_m$ but not $V_{max}$ for enzyme I. These residues form a distinct binding site for enzyme I on HPr (Figure 3.5). The Thr16Ala mutation produces a significant increase in $K_m$ of enzyme I, which is an important finding because Thr16 is not a conserved residue and many Gram-positive bacteria such as B. subtilis have an alanine at residue 16 (Reizer et al., 1993). Also, E. coli enzyme I is less effective with B. subtilis HPr (Reizer et al., 1992).

4.2.1 Comparison with the NMR structure of the HPr:EIN complex

Garrett et al. (1999) identified several residues of HPr which made one or more contacts with residues of the N-terminal domain of enzyme I (EIN), as observed by intermolecular nuclear Overhauser effects (NOEs). The key HPr residues, making three or more contacts with EIN, were identified as Thr16, Arg17, Ala20, Leu47, Phe48, and Thr52. In this kinetic study, the mutations Thr16Ala, Arg17Gly, and Leu47Ala produce significant changes in $K_m$, indicating a role for these residues in binding. However, little
or no change was observed with the mutations Ala20Thr, Phe48Met in a Lys49Gly background, and Thr52Ala. The other HPr residues identified by Garrett et al. (1999) which made one or two contacts with EIN are Leu14, Ala19, Gln21, Phe22, Val23, Lys27, Lys45, Ser46, Lys49, Leu50, Gln51, Leu53, Gly54, and Leu55. Of these, the mutations Lys27Ala, Ser46Ala, and Lys49Gly produced significant changes in enzyme I $K_m$. However, mutation of either Gln21, Gln51, or Leu55 to alanine produced little or no effect. The other residues in the list of residues making one or two contacts were not mutated, for the reasons previously described (see Results).

In the structure of the HPr:EIN complex, Ser46 of HPr participates in a hydrogen bond with Glu84 of EIN, which also helps to explain the drastic effect of mutation of Ser46 of HPr. Garrett et al. (1999) did not identify Lys40 of HPr as a residue in the enzyme I binding site. However, the Lys40Ala mutation resulted in a significant increase in $K_m$ for enzyme I (Table 3.1), and Lys40 is closely associated with other residues of the enzyme I binding site (Figure 3.5). This increase in $K_m$ may be indirect, in that mutation of the lysine to the smaller alanine may result in some structural freedom which allows for movement of nearby residues which are important for enzyme I binding. The Lys40Phe mutation produced no significant change in enzyme I binding, which indicates that lysine itself may not be essential at that position. In the structure of the HPr:EIN complex (Garrett et al., 1999) the side chain of Gln51 of HPr was shown to participate in a hydrogen bond with the side chain of Arg126 of enzyme I. In this kinetic study, the mutation of Gln51 to Glu produced significant changes in enzyme I kinetics, but the Gln51Ala mutation, which would eliminate any potential of hydrogen bonding, surprisingly had no effect. Other sections of this thesis postulate a role for
Arg126 of enzyme I in catalysis, and not necessarily binding of HPr. Thus, it must be considered that the Gln51-Arg126 interaction may not be significant under physiological conditions.

Indeed, there may be two reasons as to why the interaction site as identified by the solution structure of the complex of EIN and HPr (Garrett et al., 1999) may not totally agree with that found physiologically. Firstly, the structure of the complex was obtained in the absence of phosphorylation of either protein. The two proteins may be able to bind to each other in the absence of phosphorylation, but this complex is by definition inactive in the PTS. Secondly, the N-terminal domain of enzyme I, not the intact protein, was used in the structure of the complex. While it is obvious that the isolated N-terminal domain is perfectly able to interact with HPr, a slightly different binding site during the physiological interaction of HPr and intact (and phosphorylated) enzyme I cannot be ruled out. Indeed, previous biochemical work from the same laboratory that published the structure of the HPr:EIN complex emphasized the importance of the C-terminal domain of enzyme I of E. coli enzyme I for phosphoryl donor specificity to E. coli HPr, in that isolated phospho-EIN was capable of more general phosphotransfer to HPrs from several bacterial species (Seok et al., 1996).

4.3 Structure and function of enzyme I

The remainder of this Discussion will be dedicated to examining the role of several amino acid residues in the function of E. coli and S. typhimurium enzyme I. Determination of the roles of these residues will be aided by the examination of the structure of the N-terminal domain of enzyme I, as well as comparison to the structure of
C. symbiosum pyruvate phosphate dikinase (PPDK), which will serve as a model for the unknown structure of the C-terminal domain of enzyme I. The properties of these enzyme I mutants will also lead to further insight into the role of the monomer:dimer transition of enzyme I in enzyme I activity, and the potential importance that this transition plays in the in vivo regulation of the PTS and overall metabolism of E. coli and S. typhimurium.

4.4 Role of Arg126 in phosphotransfer

The Arg126Cys mutation of S. typhimurium enzyme I has a large effect on $V_{\text{max}}$, which is 0.04% of wild type (Table 3.2), but is no apparent detriment to HPr or PEP binding, in that the $K_m$ for each of these substrates was unchanged. The residue Arg126 is located in the N-terminal domain of enzyme I, across the active site cleft from His189, where phosphorylation occurs (Figure 4.1). Arg126 is conserved in all enzymes I, but not in PPDK sequences (Reizer et al., 1993; Figure 1.8). Thus Arg126 likely plays a specific role in catalysis and phosphotransfer to HPr.

In the solution structure of the complex of HPr with EIN (Garrett et al., 1999), it was found that Arg126 of enzyme I formed hydrogen bonds with the backbone carbonyl of Leu14 and the side chain of Gln51 of HPr. It is interesting to note that Arg126Cys enzyme I has a wild type $K_m$ for HPr, indicating that this mutation has no effect on binding. The Gln51Ala mutation to HPr also does not affect binding, as previously discussed. Arg126 may then have a different interaction in the presence of a P-histidine(s) than that observed in the structure of the complex. In the unphosphorylated form, His189 exists as an $N^6\text{H}$ tautomer, with the unprotonated $N^2$ atom
Figure 4.1 Crystal structure of enzyme I N-terminal domain active site, shown in the same orientation as Figure 1.9. Arg126 is identified in blue, acidic residues are red, and Thr168 is shown in amber. Glu167 is not labeled due to crowding.
participating in a hydrogen bond with Thr168. Upon phosphorylation (or protonation) of the N\textsuperscript{2} atom, the hydrogen bond to Thr168 is lost, and the side chain conformation of His189 is altered from a g\textsuperscript{-} conformer to a g\textsuperscript{+} conformer, such that the phosphoryl group is more solvent accessible (Garrett et al., 1998). Lys69, which is not conserved amongst all enzymes I, was proposed as the residue which would interact with the phosphorylated His189 (Garrett et al., 1998). It is proposed here that Arg126 may instead play this role in aiding catalysis and phosphotransfer by interaction with phosphorylated His189.

Dimerization is not affected in the Arg126Cys enzyme I mutant, which is expected from all evidence regarding the properties of isolated N-terminal and C-terminal fragments reported here and elsewhere, and from comparison to the structural homolog pyruvate phosphate dikinase.

4.5 Roles of Gly356 and Arg375

The structure of the C-terminal domain of enzyme I is not known. Instead, comparisons must be made to the sequence and known structure of the enzyme I homolog C. symbiosum PPDK (Herzberg et al., 1996). Using these comparisons (Figure 3.16 and Figure 3.17; Tables 3.4 and 3.5), it is easy to see why the Gly356Ser mutation, identified in this work, would affect dimerization. Gly663, the PPDK residue analogous to Gly356 of enzyme I, is an important residue in the subunit interaction site of PPDK. Addition of a serine side chain to Gly356 of enzyme I would result in a larger side chain, which could potentially compromise interactions with residues of the other subunit. Arg375 is conserved as Arg682 in C. symbiosum PPDK, and the location of this residue is indicated in Figure 3.17. However, Arg682 does not appear to be within either the
subunit interaction site or the PEP-binding site, so the reason for the impaired
dimerization of Arg375Cys enzyme I remains unclear. A three dimensional structure of
the C-terminal domain of enzyme I would be needed to further elucidate the possible
role of Arg375 in dimerization.

4.5.1 Gly356Ser and Arg375Cys kinetics and dimerization

The enzyme I mutants Gly356Ser and Arg375Cys behave similarly in that they
do not readily form dimers, and that they have an impaired PEP $K_m$ of 3 mM, ~30-fold
higher than wild type, and a 'leaky' enzyme activity of 2-5% of wild type. The question
that must be asked is, "is the activity of these mutants due to a small population of
highly active dimers, or a larger population of less active monomers?" Figure 3.13
demonstrates the hysteretic behaviour of wild type enzyme I, in that when enzyme I is
preincubated in a cold dilute solution before addition to the room temperature assay, the
activity is at first very low, but slowly increases. This is attributed to the slow
association of monomeric subunits into dimers. However, Gly356Ser and Arg375Cys
enzymes I do not show this dependence on preincubation conditions, in that they are
equally active whether they are preincubated at room temperature or on ice. The most
plausible explanation of this is that Gly356Ser and Arg375Cys enzymes I are in fact
active as monomers. All previous characterizations of enzyme I have assumed that the
dimer is the only active form of enzyme I, and that the monomer is inactive. It is
proposed here that the monomeric form of wild type enzyme I may have a low level of
activity, with kinetic parameters similar to Gly356Ser or Arg375Cys enzyme I.
4.6 Intragenic complementation

As shown in Section 3.3.6.2, activity complementation between Arg126Cys enzyme I and either Gly356Ser or Arg375Cys enzymes I was demonstrated with purified proteins. However, this complementation only occurred under conditions which favoured heterodimer formation, which included mixing of the two enzymes on ice which would result in a large proportion of monomers, before incubation at room temperature which would favour dimer formation. Physical and structural characterizations of enzyme I, along with comparison of the structure of C. symbiosum PPDK (Herzberg et al., 1996), indicate that both subunits of the enzyme I dimer act independently, and there is no phosphoryl transfer across subunits. This is an important consideration when determining the mechanism of in vitro activity complementation. The active site mutant Arg126Cys would be essentially inactive in the heterodimer, so the only active subunit in the heterodimer would be the Gly356Ser or Arg375Cys subunit (Figure 4.2). Further proof of this is the fact that the isolated 37 kDa C-terminal domain of enzyme I is by itself sufficient to complement the activity of Gly356Ser or Arg126Cys enzyme I. It is proposed that the monomeric Gly356Ser and Arg375Cys enzymes I are poorly active in part because they do not have the optimal conformation of the PEP binding site which would result from subunit association. When this subunit is in the heterodimer form, interaction with the Arg126Cys subunit results in conformational changes of the Gly356Ser or Arg375Cys subunit, so that it is more able to bind PEP, and as a result has higher enzyme activity.

Kinetic data help to further prove this hypothesis. The Gly356Ser or Arg375Cys subunits not only have an increased $V_{\text{max}}$, but also the PEP $K_m$ is greatly decreased
Figure 4.2 Schematic representation of the mechanism of *in vitro* intragenic complementation of enzyme I mutants. In this diagram the N-terminal domain of enzyme I is represented by the closed circle, and the C-terminal domain is represented by the arc. Addition of the active site mutant Arg126Cys forces the Gly356Ser subunit into the more active dimeric form.
(Table 3.3). Results have shown that the Gly356Ser/Arg126Cys heterodimer is unstable, both kinetically when analyzed by enzyme assay, and physically when analyzed by gel filtration chromatography. This heterodimer instability is not dependent on catalytic turnover of the enzyme, but rather is dependent on the concentrations of both the heterodimer itself and PEP. The stability of the wild type homodimer of enzyme I is very likely dependent on the same conditions, although it has previously been proposed that interaction with and phosphorylation of HPr also caused the enzyme to dissociate (Chauvin et al., 1996a). All evidence presented here indicates that interaction with HPr does not cause the dimer of wild type enzyme I to dissociate. The activities of Gly356Ser and Arg375Cys enzymes I were also stable in the assay (Figure 3.11). In this case, this observation again leads to the conclusion that the low but stable activity measurements of these mutants are due to the monomer, instead of an unstable dimer.

4.7 Roles of other residues in the C-terminal domain of enzyme I

When compared to the structure and function of PPDK (Figures 3.16 and 3.17), potential key residues involved in dimerization and PEP-binding capability of enzyme I can be identified. Several mutants of key amino acid residues were created by site directed mutagenesis, and the effects of these mutations were determined. Thus direct information about the roles of these residues in the structure and function of enzyme I was obtained.
4.7.1 Role of Asn352

In C. symbiosum PPDK, Asn659 is the residue analogous to Asn352 of E. coli enzyme I (Figure 3.16). Asn659 is an important residue in the subunit interaction site in that the Asn659 residue from one subunit forms several van der Waals contacts with residues from the other subunit, including the other Asn659 residue, and in addition participates in hydrogen bonds with three residues from the other subunit (Table 3.4). The enzyme I mutants Asn352Ala and Asn352Glu were created, and room temperature gel filtration chromatography showed that both mutants had impaired monomer:dimer equilibrium compared to wild type, much like Gly356Ser enzyme I (Figure 3.17). Each mutant had a decreased $V_{max}$ relative to wild type, and a substantially larger PEP $K_m$ (Table 3.6), although each mutant showed significantly different kinetics. Thus Asn352 in enzyme I is required for dimerization, and may have at least indirect importance in PEP-binding and enzyme activity.

There is a surprising difference between the Asn352Ala mutant and the Asn352Glu mutant in the formation of an active heterodimer with Arg126Cys enzyme I (Figure 3.18). A mixture of Asn352Glu and Arg126Cys enzymes I displays effective activity complementation, whereas poor complementation between Asn352Ala and Arg126Cys enzymes I is demonstrated. Recall that in the subunit interaction of PPDK, the analogous residue Asn659 participates in hydrogen bonds with three residues of the second subunit (Table 3.4). However, the side chain of one Asn659 residue faces away from the side chain of Asn659 of the other subunit. It is then postulated that Asn352 of enzyme I also participates in hydrogen bonding during dimerization, and that mutation
of Asn352 to Glu conserves at least some of this hydrogen bonding potential. Modeling of the Asn659Glu mutation of the PPDK structure indicates that this is likely possible, as there is space available for rotation of the side chain to the correct orientation (result not shown). In this orientation, there would very likely be no charge repulsion of the two glutamate side chains. Mutation of PPDK Asn659 to Ala, and enzyme I Asn352 to Ala, would eliminate any potential of hydrogen bonding, and may also disrupt van der Waals interactions. The poor PEP $K_m$ of Asn352Glu enzyme I, even higher than that of Asn352Ala, remains poorly understood, as it is very unlikely that Asn352 directly participates in PEP-binding. Perhaps the addition of the negative charge is enough to produce indirect charge repulsion of the PEP.

One of the objectives of creating the Asn352 enzyme I mutants was the production of a stable monomeric form of enzyme I. The criteria for this are that the mutant would elute from a room temperature gel filtration column at the monomeric position of $\sim 65$ kDa, and would be incapable of activity complementation by Arg126Cys enzyme I. Thus neither Asn352 mutant is strictly a stable monomer. The potential that Asn352Ala is less able to form dimers than Asn352Glu will be investigated in the future by more rigorous physical techniques, such as dynamic light scattering.

### 4.7.2 Role of Leu355

The enzyme I residue Leu355 is not strictly conserved in PPDK, as the analogous residue is Met662 (Figure 3.16). However, in PPDK Met662 is an important residue in the subunit interaction site, as it forms many van der Waals contacts with other residues, including several side chain–side chain interactions, and is also a “self
interacting” residue. For this reason, and also because Leu355 is next to the Gly356 residue which has been shown to be important for dimerization, it was decided to investigate Leu355 by mutagenesis. The mutants Leu355Ala and Leu355Glu each exhibited impaired dimerization (Figure 3.17), and enzyme kinetics similar to those of the other mutants with impaired dimerization (Table 3.6). Both mutants also exhibit slight complementation when assayed in combination with Arg126Cys enzyme I (Figure 3.19), so again it appears that both mutants are not strictly stable monomers. Modeling of Met662Ala and Met662Glu mutations in the PPDK structure indicates that the Ala mutation may result in the loss of some van der Waals interactions, while the Glu mutation may result in charge repulsion in addition to disruption of van der Waals interactions.

It should be noted that mutation of Gly356 to Ser results in an increase in size of the side chain, whereas for the most part other mutations identified so far result in a smaller or similarly sized side chain. It is possible that an increase, rather than decrease, in the size of the side chain of a residue at the dimerization interface results in a greater disruption of van der Waals interactions which leads to a greater impairment of dimerization. However, the result of mutation of Arg375 to Cys (this work), as well as Gly338Asp (Seok et al, 1996a; Seok et al., 1998) and several other C-terminal mutations (Rizak et al., 2000) indicates that the C-terminal domain of enzyme I is extremely sensitive to mutations. Most mutations studied so far, even those apparently at some distance away from the dimerization site, have at least a small effect on dimerization and/or PEP-binding.
4.7.3 Role of Arg296

The enzyme I residue Arg296 is conserved in *C. symbiosum* PPDK as Arg561 (Figure 3.16). Through site-directed mutagenesis, Yankie *et al.* (1995) determined that Arg561 of PPDK was essential for the turnover of PEP. The analogous enzyme I mutant Arg296Leu was created, and it was found that this mutant had a very low enzyme activity, 0.04% of wild type, but the PEP $K_m$ of 0.05 mM was similar to the wild type PEP $K_m$ of 0.1 mM (Table 3.6). The significance of this apparently unaffected PEP $K_m$ remains unclear; perhaps Arg296 is not directly important for binding PEP, but for catalysis via PEP substrate strain and/or transition state stabilization. Other mutants of Arg296 may be created in the future in order to further elucidate the role of Arg296 in enzyme I activity. Room-temperature gel filtration chromatography shows that Arg296Leu enzyme I has impaired dimerization potential. However, Arg296Leu is still to some extent capable of formation of active heterodimers with other C-terminal enzyme I mutants, as shown by activity complementation (Figure 3.20). Thus it appears that Arg296 of enzyme I does not play a direct role in subunit interaction, but rather it is essential for interaction with PEP and catalysis. Only through impaired catalysis of PEP is the secondary effect of impaired dimerization realized. This is discussed further in Section 4.8.

Another enzyme I residue which is important for catalysis of PEP has been identified. Cys831 is in the PEP binding site of PPDK (Herzberg *et al.*, 1996) and is conserved in enzyme I as Cys502 (Figure 3.16). Mutation of Cys502 results in significant decrease in $V_{max}$, but little change to the PEP $K_m$, and only slight impairment
to dimerization (Rizak et al., 2000), similar to mutation of Arg296. Thus Cys502 is likely involved in catalysis. The α/β-barrel PEP-binding motif of *C. symbiosum* PPDK is shown in Figure 4.3. Arg561 and Cys831, along with other residues implicated in PEP-binding (Herzberg et al., 1996) are concentrated within the centre of the α/β-barrel.

4.8 Requirement for histidine at residue 189

Until recently, it was universally believed that enzyme I and HPr had an absolute requirement for histidine as the residue of phosphorylation. Unexpectedly, Napper et al. (1999) showed that the His15Asp mutant of HPr still had a low level of catalytic activity, whereas other His15 mutants did not. In this thesis, catalytic activity of both His189Asp and His189Glu mutants of enzyme I was demonstrated (Figure 3.21). Wild type enzyme I is phosphorylated at the N° atom of His189, and the structural comparison mentioned previously (Section 1.3.2; Napper, 1999) makes it surprising that the His189Asp mutant of enzyme I has phosphotransfer activity. In the structure of the N-terminal domain of enzyme I, the unmodified N° atom of His189 is the acceptor of a hydrogen bond from the side chain of Thr168. However, upon phosphorylation or protonation of the N° atom, the imidazole ring of His189 undergoes a ring flip conformational change. The ring flip upon protonation has to be fast because the neutral and protonated forms of His189 are in fast exchange on an NMR time scale (Garrett et al., 1998). Because of this apparent variability in the conformation of the His189 side chain, either during protonation or during phosphorylation and phosphotransfer, it is then more easily accepted that the Asp and Glu mutations may each have structural similarities to one or the other conformer of the His189 side chain. Thus, both mutants
Figure 4.3 The α/β-barrel PEP-binding motif of *C. symbiosum* PPDK, taken from the crystal structure. The residues necessary for binding PEP (Herzberg *et al.*, 1996) are labeled, including Arg561, analogous to Arg296 of enzyme I, and Cys831, analogous to Cys502 of enzyme I.
could then have some degree of catalytic activity. The in vitro phosphorylation study indicates that His189Glu enzyme I may be more capable of autophosphorylation than is His189Asp, whereas the reverse may be the case for phosphotransfer to HPr. Perhaps this is due to the fact that the side chain of each mutant mimics a different conformer of His189.

At any rate, the catalytic activity of an acyl phosphate in a PTS protein has once again been demonstrated. Much like Arg126Cys enzyme I, these His189 mutants are also capable of activity complementation of C-terminal enzyme I mutants, as expected. More precise characteristics of the His189Glu and His189Asp enzyme I, such as stability of the acyl phosphates, have yet to be determined. The potential catalytic activity of His90Asp and His90Glu mutants of enzyme I are also under investigation (S. Napper, personal communication).

4.9 Enzyme I mechanism and in vivo regulation

In this thesis, enzyme I mutants which are defective in dimerization, such as Gly356Ser, have been described, and these mutants have the secondary defect of poor PEP-binding. Conversely, Arg296Leu is an enzyme I mutant which cannot bind PEP, and as a secondary defect it has reduced dimerization potential, although not as poor as Gly356Ser. This and other evidence shows that PEP-binding and dimerization of enzyme I are cooperative events. It has also been demonstrated in this thesis that PEP concentrations can influence dimerization of enzyme I.

For the purposes of explanation, Figure 4.4 presents three possible mechanisms for PEP-binding and dimerization of enzyme I. Scheme I illustrates a concerted
Figure 4.4 A schematic diagram of possible mechanisms of dimerization and PEP-binding of enzyme I. 1: Concerted mechanism. 2: Dimerization induced conformational change. 3: PEP induced conformational change.
mechanism, whereby PEP-binding and dimerization occur simultaneously. Scheme 3 illustrates a mechanism whereby PEP-binding must occur first, and results in a conformational change which allows for dimerization to subsequently occur. Scheme 2, dimerization induced conformational change, best represents what is occurring with wild type enzyme I, although the exact mechanism is likely more complex. Several points will help explain why scheme 2 is the most accurate of the three representations. 1.) A monomer:dimer equilibrium exists with wild type enzyme I in the presence or absence of PEP, but addition of PEP favours dimerization. In effect, the presence of PEP "draws" a greater proportion of enzyme I subunits to the dimeric form. 2.) Mutants which have severely reduced dimerization, such as Gly356Ser, also have a high $K_m$ for PEP. However, addition of PEP does not significantly increase the dimerization of Gly356Ser subunits. Conversely, Arg296Leu enzyme I interacts poorly with PEP but has lost only some of its dimerization potential. Thus it appears that dimerization of enzyme I results in a conformational change which allows for effective PEP-binding. 3.) Addition of excess Arg126Cys enzyme I to a population of Gly356Ser subunits results in an active Arg126Cys/Gly356Ser heterodimer. In essence, the poor monomer:dimer equilibrium of the Gly356Ser subunits is overcome, forcing the Gly356Ser subunit into the active dimer conformation which is better able to bind PEP. However, this is a dynamic monomer:dimer equilibrium in that addition of even small amounts of Gly356Ser to Arg126Cys enzyme I may cause the Arg126Cys subunits to dissociate into monomers, as shown by gel filtration chromatography. Again, addition of PEP can help stabilize the heterodimer. 4.) Phosphotransfer to HPr does not cause enzyme I dimers to dissociate, so that the enzyme may still remain in the active dimeric form even in the
absence of phosphorylation or bound PEP. Enzyme I activity is highly dependent on PEP levels, not only because PEP is a substrate for enzyme I, but also because PEP concentrations strongly influence the monomer:dimer equilibrium.

The monomer:dimer equilibrium has been proposed as a target for regulation of enzyme I, and thus regulation of the PTS (Chauvin et al., 1996a). However, no effector molecule has been identified. The $K_a$ for subunit association of enzyme I is approximately $3 \times 10^6$ M$^{-1}$ (Chauvin et al., 1994a), and in *E. coli* the concentration of enzyme I is in the range of 2-5 $\mu$M (Misset et al., 1980; Mattoo and Waygood, 1983). This suggests that in the absence of PEP and phosphorylation, about 15-25% of enzyme I subunits would be monomers. In the presence of 2 mM PEP and 5 mM Mg$^{2+}$, $K_a$ increases on the order of one-hundred fold. Phosphorylation decreases $K_a$, and phosphorylated enzyme I in the absence of PEP would have an even greater proportion of subunits as monomers, up to 50% or more. In contrast, this study and others have kept enzyme I preparations phosphorylated during purification and storage as a “stabilizing” measure. Kinetic measurements, including measurement of *in vivo* enzyme I activity levels in crude extracts, have started with the addition of PEP and Mg$^{2+}$ to the enzyme before measurement, which would force the enzyme I into the active phosphorylated dimer form. These measurements would not be sensitive to the monomer:dimer equilibrium *in vivo*, and because of this the activity measurements would not accurately reflect the real *in vivo* level of enzyme I activity.

A closer examination of *in vivo* conditions is then necessary. The approximate physiological range of PEP concentrations in *E. coli* is 0.1 – 1 mM (Lowry et al., 1971). The work presented in this thesis has led to the proposal that wild type enzyme I may
exist in vivo either in a dimeric form with a PEP $K_m$ of 0.1 mM, or in a monomeric form with a PEP $K_m$ of approximately 3 mM. The fact that the two ranges of PEP concentrations are similar may be coincidence, or it may have importance which would lead to a better understanding of the mechanism and regulation of enzyme I.

Studies have been done to assess the flux determining step(s) of the PTS (Ruyter et al., 1991; van der Vlag et al., 1995), and these studies showed that an increase of the total in vivo level of enzyme IICB$^{elc}$ resulted in a significant increase in PTS-mediated glucose transport. In contrast, overexpression of either enzyme I, HPr, or IIA$^{elc}$ did not significantly increase overall PTS activity. Thus it was determined that the IICB$^{elc}$ mediated reaction is the flux determining step of the glucose PTS. However, the experiments were biased by preincubation of extracts with PEP and Mg$^{2+}$, leading to a fully active enzyme I, before assay. In the later study, the authors acknowledged that cellular PEP levels, and PEP/pyruvate ratio, may affect the flux through the PTS (van der Vlag et al., 1995), presumably through modulation of enzyme I activity. Indeed, in vivo overexpression of PEP synthetase, leading to an increased PEP/pyruvate ratio, resulted in an increase in glucose consumption which was thought to be due to an increase in glucose transport by the PTS (Patnaik et al., 1992). Modulation of enzyme I activity would result in a more global regulation of all PTS-mediated sugar transport, not only that of glucose.
5.0 SUMMARY AND PERSPECTIVE

The bacterial phosphoenolpyruvate:sugar phosphotransferase system (PTS) is an important system in many bacteria. The PTS is not only directly important for the transport of many sugars, but it also plays a key role in the regulation of many other processes of bacterial physiology and metabolism, including carbon metabolism, nitrogen metabolism, gene regulation, and chemotaxis. The first general, non sugar-specific, step of the PTS is catalyzed by enzyme I, and as such enzyme I has been viewed for many years as a potential regulatory target of the PTS.

The research presented in this thesis has led to a greater understanding of the function and structure of enzyme I of *Escherichia coli* and *Salmonella typhimurium*. Fluorescence polarization studies showed that the interactions of HPr with enzyme I, and also HPr with enzyme IIA’’, are relatively low affinity interactions which may be important for the dynamic phosphocarrier ability of HPr. A kinetic study, through the assay of mutant HPr proteins as substrates for enzyme I, has elucidated the actual binding site on HPr for enzyme I. This binding site agrees very well with the binding site identified by various NMR experiments, however this study identifies certain key residues that were previously overlooked, such as Lys40, and indicates that other residues, such as Gln51, may not be as important for the interaction as first thought. It
should be noted that this kinetic study best mimics the physiological interaction of HPr with enzyme I.

Most of this thesis has been spent studying single amino acid mutants of enzyme I. The catalytic activity of His\textsubscript{189}Glu and His\textsubscript{189}Asp mutants of enzyme I was demonstrated, and work is currently underway to more rigorously characterize these mutants. Arg\textsubscript{126} was identified as a residue important for the phosphotransfer ability of enzyme I. Gly\textsubscript{356} and Arg\textsubscript{375} were the first two residues shown to be necessary for the proper dimerization of enzyme I. The kinetic and physical study of these mutants, both alone and in combination using \textit{in vitro} intragenic complementation, has led to a greater understanding of the monomer:dimer equilibrium of enzyme I. Temperature, enzyme I concentration, and PEP concentration all influence this equilibrium, but phosphotransfer to HPr does not. These studies have led to the assertion that the \textit{in vivo} role of the monomeric form of enzyme I may be more important than first thought, in that monomers may exist in the cell in fairly high concentrations, and under certain conditions may have a small but significant activity. The monomer:dimer equilibrium of enzyme I is likely a key point of regulation of the enzyme, and thus of the PTS.

Although the tertiary structure of the C-terminal domain of enzyme I is unknown, the structure of the C-terminal domain of the homolog pyruvate phosphate dikinase (PPDK) from \textit{Clostridium symbiosum} was used as a model. From this model, other potentially important residues for the dimerization and PEP-binding capabilities were identified. Site-directed mutagenesis showed that Asn\textsubscript{352} and Leu\textsubscript{355} are both key residues for dimerization of enzyme I. Arg\textsubscript{296} is important for binding and catalysis of PEP. PEP-binding and dimerization are cooperative events, in that
dimerization induces conformational changes in the C-terminal domain of enzyme I which allows for better PEP binding, and an increase in PEP concentration results in an increased dimerization of enzyme I.

The exact physiological role of the monomer:dimer equilibrium of enzyme I is still not very well understood. More precise physical characterizations of wild type and mutant enzymes I, either full-length or the isolated C-terminal domain, using techniques such as dynamic light scattering will be attempted in the future. This will lead to a greater understanding of the actual binding interactions, and $K_s$ values, of wild-type and mutant forms of enzyme I under a variety of conditions. The more precise contributions of each amino acid residue in enzyme I, and of the factors of temperature and the concentrations of enzyme I, PEP, and HPr to the monomer:dimer equilibrium can be determined. Site-directed mutagenesis of other enzyme I residues is also currently being undertaken, which has shown that the monomer:dimer equilibrium of enzyme I is sensitive to perturbation by amino acid mutations in the C-terminal domain. In the near future, it is likely that the tertiary structure of the C-terminal domain of enzyme I may be known, which will also lead to more detailed knowledge of the importance of these identified residues. Mutagenesis studies are also underway to potentially create a stable monomeric form and a stable dimeric form of enzyme I. Investigations of these enzymes in vitro and in vivo, perhaps by gene replacement, will help further elucidate the potential physiological importance of the monomer:dimer equilibrium of enzyme I.
6.0 REFERENCES


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