Journal of Cellular Biochemistry 27:43–56 (1985)

Evidence for the Evolutionary Relatedness of the Proteins of the Bacterial Phosphoenolpyruvate:Sugar Phosphotransferase System

Milton H. Saier, Jr., Frank C. Grenier, Catherine A. Lee, and E. Bruce Waygood

Department of Biology, The John Muir College, University of California at San Diego, La Jolla, California 92093

The phosphoenolpyruvate:sugar phosphotransferase system (PTS) found in enteric bacteria is a complex enzyme system consisting of a non-sugar-specific phosphotransfer protein called Enzyme I, two small non-sugar-specific phosphocarrier substrates of Enzyme I, designated HPr and FPr, and at least 11 sugar-specific Enzymes II or Enzyme II-III pairs which are phosphorylated at the expense of phospho-HPr or phospho-FPr. In this communication, evidence is presented which suggests that these proteins share a common evolutionary origin and that a fructose-specific phosphotransferase may have been the primordial ancestor of them all. The evidence results from an evaluation of 1) PTS protein sequence data; 2) structural analysis of operons encoding proteins of the PTS; 3) genetic regulatory mechanisms controlling expression of these operons; 4) enzymatic characteristics of the PTS systems; 5) immunological cross reactivities of these proteins; 6) comparative studies of phosphotransferase systems from evolutionarily divergent bacteria; 7) the nature of the phosphorylated protein intermediates; 8) molecular weight comparisons among the different Enzymes II and Enzyme II-III pairs; and 9) interaction studies involving different PTS protein constituents. The evidence leads to a unifying theory concerning the evolutionary origin of the system, explains many structural, functional, and regulatory properties of the phosphotransferase system, and leads to specific predictions which should guide future research concerned with genetic, biochemical, and physiological aspects of the system.

Key words: phosphotransferase system, sugar transport regulation, bacteria, phosphoproteins, molecular evolution, glycolytic cycle

Dr. C.A. Lee's present address is Department of Microbiology, Harvard Medical School, 25 Shattuck Street, Boston, Massachusetts 02115.

Dr. E.B. Waygood's address is Department of Biochemistry, University of Saskachewan, Saskatoon, Saskachewan, Canada S7N OWO.

Received June 20, 1984; accepted July 26, 1984.

PROTEINS OF THE PHOSPHOTRANSFERASE SYSTEM IN E coli AND S typhimurium

The phosphoenolpyruvate:sugar phosphotransferase system (PTS) is found in a wide variety of bacterial species and is involved in the transport and phosphorylation of many sugars [Dills et al, 1980]. Figure 1 shows three of the sequences of phosphoryl transfer reactions which are responsible for sugar group translocation in Escherichia coli and Salmonella typhimurium. In these sequences, a high energy phosphoryl group is transferred first from phosphoenolpyruvate (the initial phosphoryl donor) to the N-3 position of a histidyl residue in Enzyme I and then from phospho-Enzyme I to the N-1 position of a histidyl residue in HPr. These two proteins are general (non-sugar-specific) cytoplasmic components of the PTS that are required for the transport of all sugar substrates of the system except for fructose (see below).

All additional proteins of the PTS exhibit sugar specificity. For example, glucose transport involves two glucose-specific proteins, a soluble (or peripheral membrane) Enzyme III^{glc} (III^{glc}) of 20,000 molecular weight, phosphorylated at the expense of phospho-HPr on the N-3 position of a histidyl residue in the protein as well as an integral membrane Enzyme II^{glc} of 40,000 to 50,000 molecular weight [Erni et al, 1982]. Enzyme II^{glc} is phosphorylated at the expense of phospho-III^{glc} [Begley et al, 1982; B. Erni, unpublished results] possibly on the N-1 position of a histidyl residue [Peri et al, 1984]. Finally, phospho-Enzyme II^{glc} donates its phosphoryl moiety to glucose. The sugar is concomitantly translocated and phosphorylated so that glucose 6-phosphate is the product released from the permease into the cytoplasm (Fig. 1).

Mannitol phosphorylation involves only a single sugar-specific enzyme, the integral membrane Enzyme II^{mtl}. The product of mannitol phosphorylation, cytoplasmic mannitol 1-phosphate, is oxidized to fructose 6-phosphate in a reaction catalyzed by mannitol 1-phosphate dehydrogenase (Fig. 1). The initiation of fructose metabolism, the third sugar whose transport-coupled phosphorylation is depicted in Figure 1, is more complex than that of either glucose or mannitol [Waygood et al, 1979; Waygood, 1980; Waygood et al, 1984]. Transfer of phosphate from phospho-Enzyme I probably can proceed either to HPr or to a fructose-inducible, HPr-like protein, designated FPr (MW = 8,000), which in the cell may be complexed with III^{fru} [E.B. Waygood and R. Mattoo, unpublished results; J. Deutscher, personal communication]. Phospho-HPr or phospho-FPr then probably catalyzes III^{fru} phosphorylation, and phospho-III^{fru} may phosphorylate Enzyme II^{fru} in the membrane. Whether or not phospho-HPr can donate its phosphoryl group to FPr or III^{fru} has not been determined. The presumed (but not proven) sequence of phosphoryl transfers is therefore: PEP → Enzyme I → HPr or FPr → III^{fru} → Enzyme II^{fru} → fructose.

In addition to glucose, mannitol, and fructose, the PTS phosphorylates several other sugars in E. coli. These sugars include hexoses of the gluco-configuration such as N-acetylglucosamine, phosphorylated by the Enzyme II^{nag}, β-glucosides, phosphorylated by the Enzyme II^{bgl}-III^{bgl} pair [E.B. Waygood, unpublished results], and mannose, phosphorylated by the Enzyme II^{man}-III^{man} pair [Waygood et al, 1984; B. Erni, personal communication]. This last enzyme complex shows low specificity for its sugar substrates, phosphorylating glucose, mannose, 2-deoxyglucose, N-acetylglucosamine, glucosamine, N-acetylmannosamine, mannosamine, fructose, and methyl α-glucoside with decreasing affinity in this order [Rephaeli and Saier, 1980a;

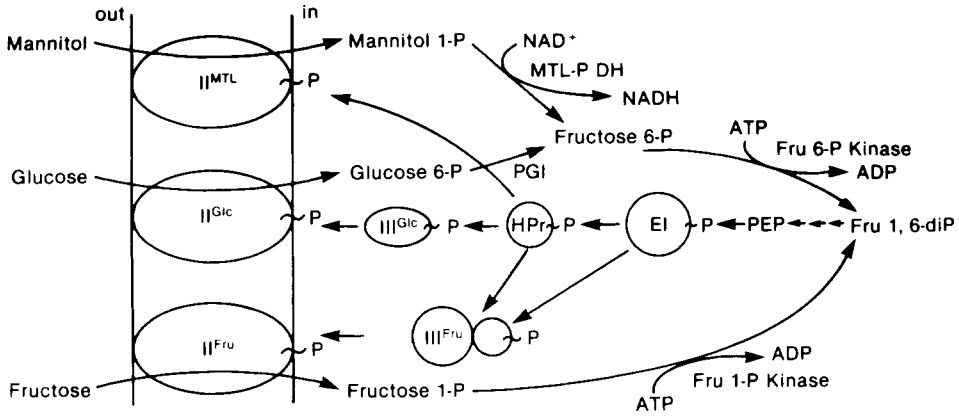

Fig. 1. Schematic depiction of the phosphoryl transfer chain of the bacterial phosphotransferase system showing the enzyme constituents responsible for the transport and phosphorylation of mannitol (mtl), glucose (glc), and fructose (fru). The enzymes involved in the conversion of the resultant cytoplasmic sugar phosphate to fructose 1,6-diphosphate are shown also. The abbreviations are as follows: PEP = phosphoenolpyruvate; EI = Enzyme I; HPr = heat stable phosphocarrier protein of the PTS; III^{glc} = the glucose-specific Enzyme III of the PTS; III^{fru} = the fructose-specific Enzyme III of the PTS; II^{mtl}, II^{glc}, and II^{fru} = the mannitol-specific, the glucose-specific, and the fructose-specific Enzymes II of the PTS, respectively. The Enzymes II are integral membrane proteins which function as the sugar permeases. The remaining enzymes of the PTS depicted are soluble or peripherally associated with the membrane. Each protein must be phosphorylated in sequence in order for group translocation of the sugar to occur. The phosphotransferase system is depicted as being integrated into the glycolytic pathway for the generation of phosphoenolpyruvate. Additional enzymes depicted are: mannitol 1-P dehydrogenase (MTL-P DH), fructose 6-P kinase, phosphoglucoisomerase (PGI), and fructose 1-P kinase. [Taken from M.H. Saier, "Mechanisms and Regulation of Carbohydrate Transport in Bacteria," Academic Press, NY, in press; reproduced with permission.]

Stock et al, 1982]. III^{man}, which is partially soluble and partially membrane bound, appears to have a molecular weight of about 33,000 [Waygood et al, 1984] whereas the Enzyme II^{man} has been reported to have a molecular weight of about 36,000 [Kundig and Roseman, 1971] or 25,000 [B. Erni, personal communication]. Also phosphorylated by distinct phosphotransferases are the hexitols, mannitol (phosphorylated by the Enzyme II^{mtl} [Lee et al, 1981]), glucitol (phosphorylated by the Enzyme II^{gut}-III^{gut} pair [Grenier et al, 1985; Waygood et al, 1984; Sarno et al, 1984] and galactitol (phosphorylated by the Enzyme II^{gat} [Lengeler, 1975a, 1977]). An Enzyme III specific for galactitol cannot be found as a phosphoprotein and may not exist [Dills et al, 1980; K. Peri and E.B. Waygood, unpublished results]. Extensive evidence has led to the conclusion that III^{mtl} is not present in E coli or S typhimurium [Jacobson et al, 1979; 1983b; Lee et al, 1981]. Thus, eight Enzymes II (glucose, mannose, N-acetylglucosamine, β-glucoside, fructose, mannitol, glucitol, and galactitol) and five Enzymes III (glucose, mannose, β-glucoside, fructose and glucitol) have been identified in E coli and S typhimurium to date. Table I summarizes the properties of these proteins.

TABLE I. Properties of Phosphoryl Transfer Proteins of the PTS in E coli*

Protein*	Molecular weight[a]	Purified to apparent homogeneity	Phosphorylation demonstrated	Position of phosphorylation
Enzyme I	60,000	+	+	N-3 his
HPr	9,000	+	+	N-1 his
FPr	8,000		+	N-1 his
III^{gut}	15,000	+	+	N-3 his
III^{glc}	20,000	+	+	N-3 his
III^{fru}	40,000		+	N-3
III^{man}	34,000		+	N-3 his
III^{bgl}	14,000		+	N-3 his
Enzyme II^{mtl}	68,000	+	+	N-3 his and N-1 his[b]
Enzyme II^{gut}	45,000		+	
Enzyme II^{gat}				
Enzyme II^{glc}	45,000	+	+	N-1 his[b]
Enzyme II^{fru}				
Enzyme II^{man}	25,000		+	
Enzyme II^{nag}	65,000		+	N-3 his and N-1 his[b]
Enzyme II^{bgl}			+	

*This list summarizes the best characterized proteins of the PTS in E coli. Extensive evidence argues against the existence of an Enzyme III^{mtl} in E coli. An Enzyme III specific for galactitol has not yet been identified, but the possibility of its existence has not been excluded. The abbreviations used are: glc = glucose; mtl = mannitol; gut = glucitol; gat = galactitol; fru = fructose; man = mannose; nag = N-acetylglucosamine; bgl = β-glucosides.

[a]Most of the molecular weights were determined by SDS polyacrylamide gel electrophoresis of the ^{32}P-phosphorylated proteins with the following exceptions: Enzyme I—sedimentation equilibrium and gel filtration under denaturing conditions [Weigel et al, 1982a]; HPr—amino acid sequence [Weigel et al, 1982b]; Enzyme II^{mtl}—amino acid sequence deduced from the gene sequence [Lee and Saier, 1983]. The molecular weight of Enzyme I estimated by SDS gel electrophoresis is 65,000 [Weigel et al, 1983], whereas that of the Enzyme II^{mtl} is 60,000. E coli III^{gut} has an apparent molecular weight of 16,000 [Grenier et al, 1985]; that of S typhimurium has an apparent molecular weight of 13,000 by SDS-polyacrylamide gel electrophoresis [Waygood et al, 1984]. The apparent difference in molecular weights of the III^{gut} proteins from these two bacterial species represents the only significant molecular weight difference so far observed for any one enzyme of the PTS. [F.C. Grenier, unpublished results; E.B. Waygood, unpublished results]. The molecular weight of II^{man} has been reported to be 36,000 by Kundig and Roseman [1971] but 25,000 by B. Erni [personal communication].

[b]The Enzyme II^{mtl} and Enzyme II^{nag} probably contain two phosphorylation sites, N-3 his and N-1 his [EB Waygood, unpublished results], whereas Enzyme II^{glc} probably contains a single phosphorylation site which is an N-1 histidyl residue [Peri et al, 1984].

PROPOSED EVOLUTIONARY ORIGIN OF THE COMPLEX PTS IN E coli

The possible evolutionary origin of complex phosphotransferase systems such as those found in E coli and S typhimurium has been considered in several earlier articles [Lengeler, 1977; Lengeler and Steinberger, 1978; Saier, 1977; Saier and Leonard, 1983]. As suggested in these articles, the PTS may have evolved from a much simpler system, possibly a phosphoenolpyruvate-dependent sugar kinase which

became membrane-associated in a fashion which allowed the active site to span the membrane. Thus, it became possible for the sugar substrate to bind to the enzyme surface localized to the external leaflet of the phospholipid bilayer, even though the sugar phosphate product was released into the cytoplasm. In this way, vectorial phosphorylation was accomplished. The anaerobic environment of early biotic earth and the evolution of glycolysis as the first carbohydrate catabolic pathway were responsible for the fact that phosphoenolpyruvate (the end product of glycolysis) instead of ATP served as the primary phosphoryl donor for sugar uptake. By virtue of the use of this phosphoryl donor for sugar uptake, the PTS plus the enzymes of glycolysis comprise a "glycolytic cycle" (Fig. 1).

Early events leading to a more complex phosphotransferase system may have involved intragenic duplications, gene duplications (possibly resulting from whole operon duplications), or even entire genome duplications [Riley and Anilionis, 1978]. Gene segmentation events, giving rise to a multigenic system encoding a number of distinct energy coupling and transport proteins, may have followed. The arguments in favor of this supposition have been detailed in earlier reviews [Saier, 1977; Saier and Leonard, 1983] and will not be discussed further here.

The proposed pathway for the evolution of the enzymes of the PTS is reproduced in Figure 2. It is suggested that the primordial PTS exhibited specificity toward fructose and that it was the duplication of the fructose phosphotransferase gene(s) which led to all of the present day genes encoding phosphotransferase proteins. These include the pts operon, encoding Enzyme I and HPr; the fructose regulon, encoding FPr, Enzyme II^{fru} and III^{fru}; the three hexitol phosphotransferases encoded within the three hexitol operons (mtl, gut and gat); and the five aldohexose phosphotransferases (Fig. 2). The central role of fructose in glycolysis, the presence of fructose-specific phosphotransferases in numerous Gram-negative bacteria, including primitive photosynthetic [Saier et al, 1971], nitrogen fixing [K. Basu and S. Ghosh, personal communication], and heterotrophic bacteria, [Sawyer et al, 1977; Durham and Phibbs, 1982] and the presence of a fructose-inducible HPr-like molecule, presumably encoded within the fru regulon but lacking in the other sugar-specific operons encoding PTS proteins [Saier et al, 1970; Waygood et al, 1979; Waygood, 1980; Waygood et al, 1984] all argue in favor of this postulate [Saier and Leonard, 1983].

New Evidence Concerning Evolution of the PTS

Comparative properties of the Enzyme II^{mtl} and the Enzyme $II^{gut}III^{gut}$ pair. Recently, information has become available which appears to substantiate the evolutionary relationships proposed above. The mannitol Enzyme II has been purified to homogeneity, and many of its physicochemical properties have been determined [Jacobson et al, 1979, 1983]. Moreover, the mtlA gene, encoding this protein, has been sequenced, and the primary aminoacyl sequence of the protein was deduced from the gene sequence [Lee and Saier, 1983]. The protein migrates on SDS gels as a protein of molecular weight equal to 60,000, whereas the molecular weight predicted from the gene sequence is 68,000. The protein posseses a hydrophobic amino-terminal half, presumably embedded in the membrane, and a hydrophilic carboxy-terminal half, localized to the cytoplasmic surface of the membrane [Jacobson et al, 1983a,b]. These and other properties of the enzyme are summarized in Table II and depicted in Figure 3.

Recent studies on the glucitol phosphotransferase revealed that this system consists of an Enzyme II^{gut}-III^{gut} pair as illustrated schematically in Figure 3 [Grenier

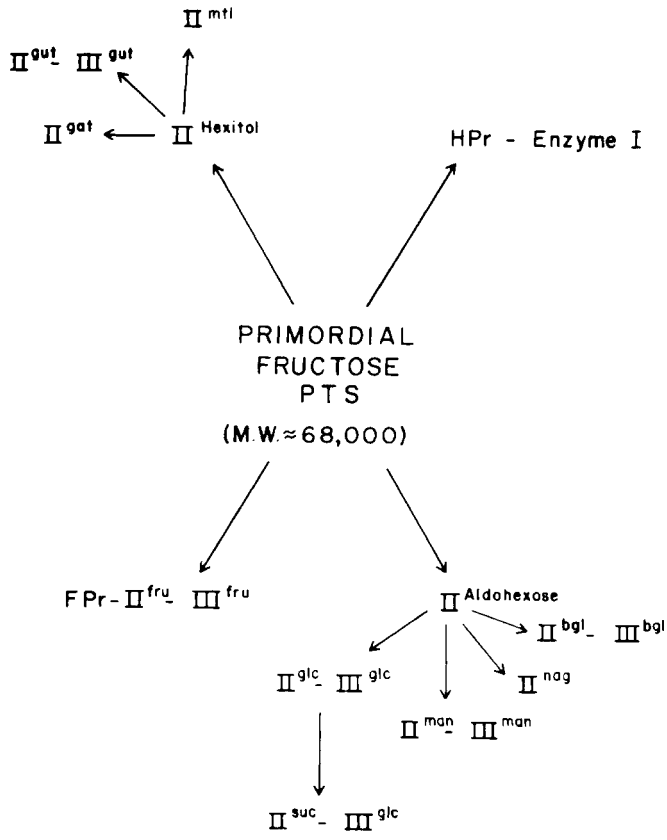

Fig. 2. Prosposed pathway for the evolution of the enzymatic constituents of the phosphotransfease system in E coli and S typhimurium. This scheme is based primarily on the apparent relatedness of the Enzymes II of the E coli PTS. The scheme indicates directionality in the evolutionary process with a fructose PTS (middle) as the primordial system giving rise to the present day fructose-specific system (bottom left), to the energy-coupling proteins of the PTS, Enzyme I and HPr (top right), to the hexose-specific systems (bottom right), and to the hexitol-specific systems (top left). MW = molecular weight. [Taken from M.H. Saier, "Mechanisms and Regulation of Carbohydrate Transport in Bacteria," Academic Press, NY, in press; reproduced with permission.]

et al, 1985; Sarno et al, 1984]. The properties of the Enzyme II^{gut} are strikingly similar to those of the Enzyme II^{mtl}, as revealed in Table II. In view of the previously proposed close evolutionary relationships between the mtl and gut operons [Lengeler, 1975a,b; 1977; Lengeler and Steinberger, 1978; Saier and Leonard, 1983] and the fact that only the A and D structural genes had been detected by earlier genetic analyses [Lengeler, 1975b], the discovery of an essential III^{gut} was surprising. A possible explanation for this difference resulted from the purification of the Enzyme $II^{gut}III^{gut}$ pair which revealed that whereas III^{gut} migrated in SDS gels with a molecular weight of 15,000, the apparent molecular weight of the Enzyme II^{gut} was about 45,000 [Grenier et al, 1985]. Thus, the sum of the molecular weights of the Enzyme II^{gut}-III^{gut} pair is equal to that of the Enzyme II^{mtl} (Table II). The two glucitol PTS

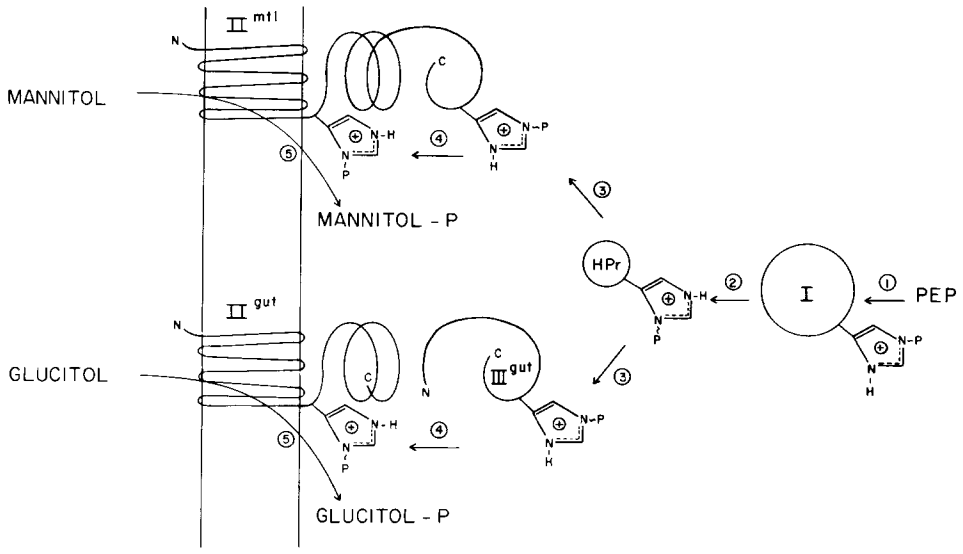

Fig. 3. Sequential protein phosphorylation reactions of the phosphoryl transfer chains for mannitol (mtl) and glucitol (gut). The figure shows five phosphoryl transfer reactions which result in the phosphorylation of four distinct protein sites. Phosphoenolpyruvate (PEP) phosphorylates Enzyme I on the N-3 positon of a histidyl residue; HPr is phosphorylated on the N-1 positon of a histidyl residue; the first phosphorylation site on the Enzyme II^{mtl} and the phosphorylation site on III^{gut} are N-3 histidyl residues, while the second phosphorylation site on the Enzyme II^{mtl} and the phosphorylation site on Enzyme II^{gut} are thought to be N-1 histidyl residues. The last step involves transfer of the phosphoryl moiety from the N-1 position of a histidyl residue in the Enzyme II to the incoming sugar. The Enzyme II^{gut}-III^{gut} pair is shown to be structurally and functionally equivalent to the Enzyme II^{mtl}. It is proposed that all Enzymes II and Enzyme II-III pairs of the PTS exhibit similar structural features, involve the same sequence of phosphoryl transfer reactions, and catalyze sugar transport by essentially the same mechanism as illustrated in the figure. These proposals result from their common evolutionary ancestry. [Taken from M.H. Saier, "Mechanisms and Regulation of Carbohydrate Transport in Bacteria," Academic Press, NY, in press; reproduced with permission.]

TABLE II. Comparative Aspects of the Mannitol- and Glucitol-Specific Enzymes of the Phosphotransferase System in S typhimurium

	Enzyme III^{gut}	Enzyme II^{gut}	Enzyme II^{mtl}
Molecular weight (PAGE)	15,000	45,000	60,000
K_m(gut): transport		12 μM	2500 μM
phosphorylation		44 μM	400 μM
K_m(mtl): transport		3300 μM	0.4 μM
phosphorylation		60 μM	3 μM
Induction by glucitol	+	+	±
Induction by mannitol	−	−	+
Solubilization by DOC/NaCl		+	+
Purification by hexylagarose		+	+
NEM sensitivity	−	+	+
DEPC sensitivity	+	+	+
Anti-Enzyme II^{mtl} antibody	±	−	+
Anti-Enzyme III^{gut} antibody	+	−	−

proteins might have arisen by the introduction of a nonsense mutation within a single primordial gene which has remained intact in the mtl operon. Consistent with this conclusion is the demonstration that the order of genes within the gut operon is probably gutCABD, where A codes for the Enzyme II^{gut}, B codes for III^{gut}, D codes for glucitol-6-P dehydrogenase, and C is a regulatory gene. The position of the B gene in the operon would suggest that III^{gut} is related structurally to part of the hydrophilic carboxyterminus of the Enzyme II^{mtl} [Sarno et al, 1984].

A separate line of evidence leading to the conclusion that III^{gut} is related structurally to the C-terminus of the Enzyme II^{mtl} resulted from studies with antibodies directed against the purified Enzyme II^{mtl}. This antibody was found to inhibit Enzyme II^{mtl} only from the cytoplasmic side of the membrane [Jacobson et al, 1983a]. It was found also to inhibit glucitol phosphorylation catalyzed by the Enzyme II^{gut}-III^{gut} pair [Jacobson et al, 1983a]; but, surprisingly, inhibition could be overcome by increasing the concentration of III^{gut} rather than that of Enzyme II^{gut} [F.C. Grenier, unpublished observation]. This result suggested that the principal inhibitory antibodies were directed against epitopes on the Enzyme II^{mtl} which were similar to sites on III^{gut}.

If the mtl and gut operons arose from a common ancestral operon, the genes encoding the two dehydrogenases might also share sequence homology which would be reflected in the properties of these enzymes. The mannitol-1-P and glucitol-6-P dehydrogenases therefore were purified to homogeneity, and their properties were compared [M.J. Novotny, J. Reizer, F. Esche, and M.H. Saier, Jr., unpublished results]. The two proteins have quite different subunit molecular weights as determined by sodium dodecyl sulfate gel electrophoresis (40,000 vs. 28,000), and their oligomeric structures differ. Whereas the former enzyme is a monomer in solution, the latter enzyme behaves like a tetramer. The two enzymes show absolute specificities for their two hexitol-phosphate substrates and exhibit normal Michaelis-Menten kinetics with absolute binding constants of 0.8mM and 0.2mM for mannitol-1-P and NAD^+, respectively, for the mannitol-1-P dehydrogenase and of 3mM and 0.2mM for glucitol-6-P and NAD^+, respectively, for the glucitol-6-P dehydrogenase. Of these two enzymes, only the glucitol-6-P dehydrogenase was inactivated by N-ethylmaleimide and diethylpyrocarbonate, reagents which respectively derivatize sulfhydryl and histidyl residues in proteins. Amino acid compositions and N-terminal aminoacyl sequences were also very different. These results show that the two dehydrogenases differ markedly with respect to many of their properties. The data do not provide evidence for a common ancestral origin for these two genes.

Combined molecular weights of the various Enzyme II-III pairs. As mentioned above, the molecular weight of Enzyme II^{mtl} is about equal to those of Enzyme II^{gut} and III^{gut} combined. Comparisons of the molecular weights of the Enzymes II and III for different sugars (Table III) led to a startling observation: The molecular weights of the sums of the four known Enzyme II-III pairs add up to about 68,000, the value of the mannitol Enzyme II molecular weight. Thus, the respective values reported for the glucose pair are 45,000 and 20,000 (sum = 65,000), [Erni et al, 1982; Meadow and Roseman, 1982]; those for the mannose pair are 36,000 and 33,000 (sum = 69,000) [Kundig and Roseman, 1971; Waygood et al, 1984] or 25,000 and 35,000 (sum = 60,000; [B. Erni, unpublished results]); and those for the lactose pair in Staphylococcus aureus are 55,000 and 12,000 [Hays et al, 1973; Schafter et al, 1981] (sum = 67,000). The N-acetylglucosamine Enzyme II, which functions

TABLE III. Molecular Weight Comparisons for Various Enzyme II-III Pairs of the PTS

Proteins[a] specific for	Molecular weight[b] ($\times 10^{-3}$)		
	Enzyme II	III	Sum
Mannitol	60	none	60
	(68)		(68)
Glucitol	45	15	60
Glucose	45	21	66
Mannose	25	35	60
	(36)	(33)	(69)
N-Acetylglucosamine	65	none	65
Dihydroxyacetone	65	none	65
Lactose	55	12	67

[a]All proteins are from E coli with the exception of the lactose Enzyme II-III pair which is from Staphylococcus aureus.

[b]Molecular weights usually were estimated by SDS-polyacrylamide gel electrophoresis. The value in parenthesis for the mannitol Enzyme II was deduced from the mtlA gene sequence [Lee and Saier, 1983]; the values for the mannose Enzyme II-III pair are those obtained by B. Erni [unpublished results]. Values in parentheses were reported by Kundig and Roseman [1971] and Waygood et al [1984] for Enzyme II^{man} and III^{man}, respectively.

without the participation of an Enzyme III, has a molecular weight of about 65,000 [Waygood et al, 1984]. Also, the dihydroxyacetone Enzyme II [Jin and Lin, 1984] has a molecular weight of 65,000, and no III^{dha} has been found [K. Peri and E.B. Waygood, unpublished results]. It is interesting to note that the sum of the molecular weights of HPr and Enzyme I, the protein products of the pts operon, is also about 68,000 [Weigel et al, 1982a,b]. These remarkable observations suggest a common evolutionary origin for the phosphotransferase proteins.

Nature of the Enzyme II-III interaction. If the Enzyme II-III pairs resulted from segmentation of a primordial Enzyme II structural gene, then the N-terminus of the Enzyme III would be expected to have been continuous with the C-terminus of the Enzyme II in the unsegmented, primordial protein. Assuming this to be true, the N-terminus of an Enzyme III might be expected to interact with the C-terminus of its Enzyme II in the Enzyme II-III complex. In other words, the N-terminus of an Enzyme III should function in binding to its homologous Enzyme II in the membrane, whereas the C-terminal region would be expected to interact with HPr. Recent preliminary evidence appears to substantiate this prediction. First, a proteolytically clipped III^{glc} protein in which the seven amino terminal aminoacyl residues had been removed was found to be fully active in phosphoryl transfer from phospho-HPr but deficient in its activity as a phosphoryl donor for methyl α-glucoside phosphorylation in the presence of Enzyme II^{glc} [Meadow and Roseman, 1982; Meadow et al, 1982]. Second, derivatization of the N-terminal glycyl residue in the E coli Enzyme III^{glc} with fluorescein gave a modified protein which was phosphorylated normally by phospho-HPr, but was only one tenth as active as native phospho-III^{glc} in phosphoryl transfer to Enzyme II^{glc} [Jablonski et al, 1983]. Third, a tryptic fragment of III^{lac} from Staphylococcus aureus, consisting of the first 38 residues at the amino terminal part of the protein, specifically inhibited the interaction between Enzyme II^{lac} and III^{lac} [Deutscher et al, 1982]. Finally, a mutant of S aureus has been isolated in which

III^{lac} showed a single amino acid substitution: The glycyl residue in position 18 was replaced by a glutamyl residue. This defect did not interfere with phosphoryl transfer from phospho-HPr to III^{lac}, but it abolished phosphoryl transfer from phospho-III^{lac} to Enzyme II^{lac} [H.M. Sobek, K. Stüber, K. Beyreuther, W. Hengstenberg and J. Deutscher, submitted for publication]. These observations, taken together, suggest that the N-termini of both III^{glc} of E coli and III^{lac} of S aureus function to bind the proteins to the Enzymes II at the cytoplasmic surface of the membrane. The C-terminal parts of these proteins presumably interact with phospho-HPr.

Cross antigenic properties of the Enzymes II of the PTS. An antibody preparation directed against the mannitol Enzyme II was tested for its inhibitory effect on other Enzymes II of the PTS. The relative inhibitory effects of low antibody concentrations on the activities of the different phosphotransferases were as follows [Jacobson et al, 1983]: MTL > GUT > FRU > MAN > GLC = NAG. The order of relatedness predicted from this result was as expected based on the evolutionary scheme shown in Figure 3.

Common properties of Enzymes II specific for hexoses of the gluco configuration. The proposed evolutionary scheme (Fig. 2) suggests that the different aldohexose-specific Enzymes II diverged relatively recently from one another. In connection with this suggestion, regulation of the genes encoding the glucose and mannose Enzymes II exhibits similar features [Rephaeli and Saier, 1980b]. Further, two novel Enzymes II have been characterized recently in Gram-negative bacteria which exhibit specificity for glucopyranosyl residues. One of these, a sucrose-specific Enzyme II, is plasmid encoded in S typhimurium and chromosomally encoded in Klebsiella pneumoniae. It phosphorylates the glucosyl moiety of sucrose [Lengeler et al, 1982]. The other Enzyme II from Vibrio parahaemolyticus is specific for trehalose. It phosphorylates one of the two symmetrical glucosyl residues in trehalose [Kubota et al, 1979]. A trehalose Enzyme II in E coli has been reported recently [Marechal, 1984]. In view of their substrate specificities, both of these Enzymes II might be expected to be related to the glucose Enzyme II. In fact, the sucrose Enzyme II, and possibly the trehalose Enzyme II as well, have been reported to depend on III^{glc} for activity [Lengeler et al, 1982]. It is therefore reasonable to propose that these Enzymes II diverged from the glucose specific Enzyme II during recent evolutionary history with the retention of the same Enzyme III.

Partial aminoacyl sequence homology between HPr and Enzyme II^{mtl}. If the different PTS enzymes did, in fact, evolve from a common ancestral protein, and if the fru regulon, which presumably encodes Enzyme II^{fru}, III^{fru}, and a fructose-inducible HPr-like protein (FPr) is related to the primordial gene, then the sequence of one PTS protein might be related to parts of the sequences of other PTS proteins. We have surveyed for possible sequence homology employing computer techniques and have discovered significant homology between half of Salmonella HPr (which is identical to E coli HPr [Weigel et al, 1982b]) and a part of the E coli Enzyme II^{mtl} [M.H. Saier, Jr. and R. Doolittle, unpublished observations]. The significance of the sequence homology observed between HPr and Enzyme II^{mtl} is emphasized by the fact that no other prokaryotic, eukaryotic, or viral protein screened showed significant homology with either HPr or Enzyme II^{mtl}. Part of this sequence homology is illustrated in Figure 4. Identical residues are boxed with solid lines, and near-identical residues (resulting from a single base substitution in the triplet code) are boxed with dashed lines. As can be seen, the degree of homology is striking. In the most

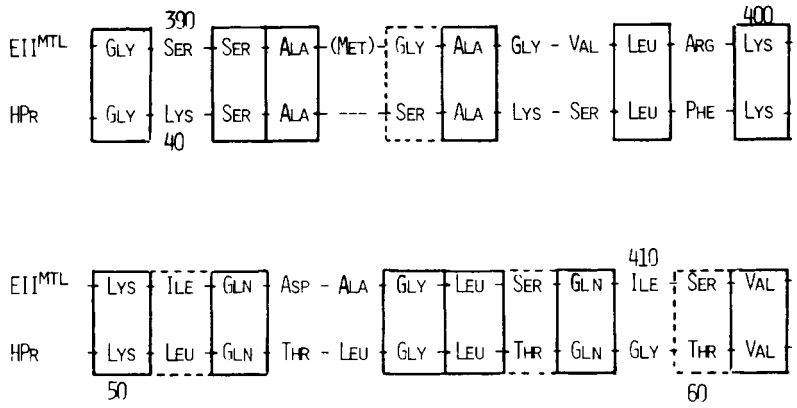

Fig. 4. Partial sequence homology between portions of HPr from S typhimurium and the Enzyme II^{mtl} of E coli. Those residues which are identical are boxed with solid lines. Those which differ with respect to a single base substitution are boxed with dashed lines. Fifty percent of the residues within this region are identities, and an additional 17% are near identities resulting from a single base substitution. The region of partial homology between these two proteins is more extensive than shown accounting for a total length of about 50 aminoacyl residues in each of the two proteins. [Taken from M.H. Saier, "Mechanisms and Regulation of Carbohydrate Transport in Bacteria," Academic Press, NY, in press; reproduced with permission.]

homologous region, the number of identities plus near-identities is about two thirds of the total amino acids. It is worthy of note that the region of partial homology is much more extensive than that shown in Figure 4. Interestingly, in the mannitol Enzyme II, a 15 aminoacyl insert of nonhomology is found within an extensive region of partial homology. This 15 aminoacyl residue segment carries a histidyl residue which is a prime candidate for one of the two proposed active site residues. The partially homologous segment of HPr is immediately adjacent to and to the left of the active histidyl residue in HPr (residue #15). These provocative observations provide preliminary evidence for the evolutionary scheme shown in Figure 2, in which a primordial operon encoding an HPr-like protein (as is suggested for the fru regulon) served as a precursor for the evolution of the pts genes, as well as for genes encoding sugar-specific proteins of the complex pts found in enteric bacteria. More extensive sequence analyses, particularly of the gut and the fru operons as well as secondary protein structural estimations deduced from the nucleotide sequences of the structural genes, may provide confirmation of the evolutionary ancestry of the PTS proteins proposed in this article.

SUMMARY OF EVIDENCE SUPPORTING THE PROPOSED SCHEME FOR THE EVOLUTION OF PTS PROTEINS

The phosphotransferase system in anaerobic and facultatively anaerobic bacteria initiates a pathway which results in the fermentation of sugars to phosphoenolpyruvate. Because bacteria appeared prior to the advent of molecular oxygen on earth, it is likely that glycolysis was the first carbohydrate degradative pathway to appear in living cells. The PTS may have initiated sugar metabolism by transporting the sugar

into the cell and phosphorylating it in a single, tightly coupled step. In this view, the PTS is an integral constituent of glycolysis. Further, because the end product of glycolysis (phosphoenolpyruvate) is the energy source for sugar uptake, the glycolytic pathway in anaerobic bacteria can be thought of as a cyclic process.

In this communication, extensive evidence is presented which suggests that the proteins of the PTS share a common evolutionary origin and that a fructose-specific phosphotransferase may have been the primordial ancestor of them all. The aldohexose-specific systems and the hexitol-specific systems are presumed to have diverged from the fructose system following two early genetic duplications. The following evidence supports this contention:

A) Fructose is the only sugar that feeds directly into glycolysis without conversion to another sugar. Many primitive photosynthetic, N_2-fixing and heterotrophic bacteria possess a fructose-specific phosphotransferase system [Saier et al, 1971; Sawyer et al, 1977; Durham and Phibbs, 1982; K. Basu and S. Ghosh, personal communication]. Further, only the fructose regulon in E coli encodes its own HPr-like protein (FPr) [Saier et al, 1970; Waygood et al, 1979; Waygood, 1980]. These facts suggest that the primordial PTS may have been specific for fructose.

B) Anti-Enzyme II antibody cross reactivities, catalytic properties of the Enzymes II (Table II), operon structures and transcriptional regulatory properties of these operons [Rephaeli and Saier, 1980; Lengeler, 1975b; 1977; Lengeler and Steinberger, 1978; Lengeler et al, 1982; Kubota et al, 1979] all suggest that the hexitol Enzymes II (those specific for mannitol, glucitol, and galactitol) comprise one closely related group, whereas the Enzymes II (or Enzyme II-III pairs) specific for the aldohexoses (glucose, mannose, N-acetylglucosamine, β-glucosides, sucrose, and trehalose) comprise a separate group of closely related enzymes. The fructose system appears to exhibit intermediate characteristics [Jacobson et al, 1983a]. These observations imply that two initial duplications of the primordial pts genes gave rise to the evolutionary precursor of a hexitol phosphotransferase on the one hand and an aldohexose phosphotransferase on the other hand. Presumably, these genes then duplicated and diverged to give the sugar-specific Enzymes II as we know them today.

C) HPr and FPr exhibit similar structural and catalytic properties [Saier et al, 1970; Waygood et al, 1979; Waygood, 1980; Waygood et al, 1984; J. Deutscher, personal communication], and both are phosphorylated at the expense of phospho-Enzyme I to give proteins phosphorylated on the N-1 positions of internal histidyl residues [Waygood et al, 1984 and unpublished observations]. Further, the size of the pts operon, encoding Enzyme I and HPr, is comparable to that of the Mt1A gene that encodes the mannitol Enzyme II. Both genetic units appear to encode two phosphorylation sites, an N-1 histidyl site and an N-3 histidyl site. It is therefore suggested that the pts operon, like the genetic units that encode the different Enzymes II or Enzyme II-III pairs, arose from a common operon encoding the primordial fructose-specific system.

D) All Enzymes III and those Enzymes II that do not function with an Enzyme III are probably phosphorylated on the N-3 position of a histidyl residue [Waygood et al, 1984]. A second phosphorylation site common to all Enzymes II appears to be an N-1 histidyl phosphorylation [Begley et al, 1982; Erni, personal communication; Peri et al, 1984; K. Peri, unpublished observations]. Thus, all phosphotransfer chains of the PTS appear to consist of five consecutive phosphoryl transfers and four protein

phosphorylation sites as illustrated in Figures 1 and 3. This scheme is consistent with the suggestion that all Enzyme II-III pairs arose from a common ancestral protein, and, as tentatively suggested above, it is possible that Enzyme I and HPr also arose from this protein (Fig. 2).

E) Sequence homology has been found between Salmonella HPr and an internal region of the E coli Enzyme II^{mtl} (Fig. 4). Such homology suggests that these two proteins share a common origin. Analyses of the fru regulon, presumably encoding FPr, Enzyme II^{fru} and III^{fru}, may provide further evidence regarding this possibility.

F) Many structural and catalytic similarities have been noted between the mannitol Enzyme II and the glucitol Enzyme II-III pair. In fact, all Enzyme II-III pairs examined to date exhibit similar net molecular weights. Further, the N-terminal regions of III^{glc} in S typhimurium and of III^{lac} in S aureus are involved in binding to their respective Enzymes II. These results suggest that each Enzyme II-III pair arose by segmentation of a precursor gene encoding an Enzyme II-like protein such as the mannitol Enzyme II. This mannitol Enzyme II-like precursor presumably served the catalytic functions of the Enzyme II-III pair and contained two phosphorylation sites.

Taken together, the observations summarized in this article provide compelling evidence for a coherent scheme accounting for many features of the proteins of the PTS. The evolutionary scheme proposed in Figure 2 explains a large body of otherwise unintelligible data. It also allows the prediction of many structural and functional features of the PTS. For example, we predict that *all* Enzyme II-III pairs will possess a hydrophobic N-terminal half and a hydrophilic C-terminal half; that phosphoryl transfer will *always* involve two sugar-specific phosphorylation events involving the N-3 and N-1 positions of two distinct histidyl residues; that the N-termini of *all* Enzymes III will bind to the C-termini of their homologous Enzymes II; and that the transport, phosphorylation, and chemoreceptive mechanisms for *all* Enzymes II will be fundamentally the same. Further sequence data, particularly of the gut operon and the fru regulon, as well as mechanistic studies concerned with Enzyme II-III function, will be required to establish these predictions.

ACKNOWLEDGMENTS

We thank the following colleagues for valuable discussion or for communicating unpublished results: K. Basu, K. Beyreuther, J. Deutscher, R. Doolittle, B. Erni, F. Esche, S. Ghosh, W. Hengstenberg, R. Mattoo, M.J. Novotny, K. Peri, J. Reizer, H.M. Sobek and K. Stüber. Protein sequence analyses were performed generously by Dr. R. Doolittle.

This work was supported by PHS Grant 1 ROI IA 14176-04 from the National Institute of Allergy and Infectious Diseases.

REFERENCES

1. Begley GS, Hansen DE, Jacobson GR, Knowles JR: Biochem 21:5552-5556, 1982.
2. Dills SS, Apperson A, Schmidt MR, Saier MH Jr: Microbiol Rev 44:385-418, 1980.
3. Deutscher J, Beyreuther K, Sobek HM, Stuber K, Hengstenberg W: Biochem 211:4867-4873, 1982.
4. Durham DR, Phibbs PV Jr: J Bacteriol 149:534-541, 1982.
5. Erni B, Trachsel H, Postma PW, Rosenbusch JP: J Biol Chem 257:13726-13730, 1982.
6. Grenier FC, Hayward I, Novotny MJ, Leonard JE, Saier MH Jr: J Bacteriol (in press).

7. Hays JB, Simoni RD, Roseman S: J Biol Chem 248:941–956, 1973.
8. Jablonski EG, Brand L, Roseman S: J Biol Chem 258:9690–9699, 1983.
9. Jacobson GR, Kelly DM, Finlay DR: J Biol Chem 258:2955–2959, 1983a.
10. Jacobson GR, Lee CA, Leonard JE, Saier MH Jr: J Biol Chem 258:10748–10756, 1983b.
11. Jacobson GR, Lee CA, Saier MH Jr: J Biol Chem 254:249–252, 1979.
12. Jin RZ, Lin ECC: J Gen Microbiol 130:83–88, 1984.
13. Kubota Y, Iuchi S, Fujisawa A, Tanaka S: Microbiol Immunol 23:131–146, 1979.
14. Kundig W, Roseman S: J Biol Chem 246:1407–1418, 1971.
15. Lee CA, Jacobson GR, Saier MH Jr: Proc Natl Acad Sci USA 78:7336–7340, 1981.
16. Lee CA, Saier MH Jr: J Biol Chem 258:10761–10767, 1983.
17. Lengeler J: J Bacteriol 124:39–47, 1975a.
18. Lengeler J: J Bacteriol 124:26–38, 1975b.
19. Lengeler J: Molec Gen Genet 152:83–91, 1977.
20. Lengeler JW, Mayer RJ, Schmid K: J Bacteriol 151:468–471, 1982.
21. Lengeler J, Steinberger H: Molec Gen Genet 167:75–82, 1978.
22. Maréchal LR: Arch Microbiol 137:70–73, 1984.
23. Meadow ND, Roseman S: J Biol Chem 257:14526–14537, 1982.
24. Meadow ND, Rosenberg JM, Pinkert HM, Roseman S: J Biol Chem 257:14538–14542, 1982.
25. Peri KG, Kornberg HL, Waygood EB: FEBS Lett 170:55–58, 1984.
26. Rephaeli AW, Saier MH Jr: J Biol Chem 255:8585–8591, 1980a.
27. Rephaeli AW, Saier MH Jr: J Bacteriol 141:658–663, 1980b.
28. Riley M, Anilionis A: Ann Rev Microbiol 32:519–560, 1978.
29. Saier MH Jr: Bacteriol Rev 41:856–871, 1977.
30. Saier MH Jr, Feucht BU, Roseman S: J Biol Chem 246:7819–7821, 1971.
31. Saier MH Jr, Leonard JE: In Kane JF (ed): "Multifunctional Proteins: Catalytic/Structural and Regulatory." Boca Raton, FL: CRC Press, 1983, pp 11–30.
32. Saier MH Jr, Simoni RD, Roseman S: J Biol Chem 245:5870–5873, 1970.
33. Sarno MV, Tenn LG, Desai A, Chin AM, Grenier FC, Saier MH Jr: J Bacteriol 157:953–955, 1984.
34. Sawyer MH, Baumann P, Baumann L, Berman SM, JL Canovas, Berman RH: Arch Microbiol 112:49–55, 1977.
35. Schäfer A, Schrecker O, Hengstenberg W: Eur J Biochem 113:289–294, 1981.
36. Stock JB, Waygood EB, Meadow ND, Postma PW, Roseman S: J Biol Chem 257:14543–14552, 1982.
37. Waygood EB: Can J Biochem 58:1144–1146, 1980.
38. Waygood EB, Mattoo RL, Peri KG: J Cell Biochem, 25:139–159, 1984.
39. Waygood EB, Meadow ND, Roseman S: Anal Biochem 95:293–304, 1979.
40. Weigel N, Waygood EB, Kukuruzinska MA, Nakazawa A, Roseman S: J Biol Chem 257:14461–14469, 1982a.
41. Weigel N, Powers DA, Roseman S: J Biol Chem 257:14499–14509, 1982b.