PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

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Structures and homologies of carbohydrate: phosphotransferase system (PTS) proteins

By J. W. Lengeler, F. Titgemeyer, A. P. Vogler and B. M. Wöhrl

Fachbereich Biologie/Chemie, Arbeitsgruppe Genetik, Universität Osnabrück, Postfach 4469, *D*-4500 *Osnabrück, F.R.G.*

The bacterial phosphotransferase system (PTS) is the major transport system for many carbohydrates that are phosphorylated concomitantly with the translocation step through the membrane (group translocation). It consists of two general proteins, enzyme I and histidine protein (HPr), and a series of more than 15 substrate-specific enzymes II (EII). The sequences of several of these derived from Gram-positive and Gram-negative bacteria were compared, which allowed the possible identification of the following functional domains: membrane-bound pore, substrate-binding site, linker domains, transphosphorylation domain and primary phosphorylation site. Several EIIs have been analysed in the meantime, also by topological tests, by sequential deletion of the corresponding structural genes, and by construction of intergenic hybrids between different domains of several EIIs. These data suggest evolutionary relationships between different EIIs; they also enable a general model to be constructed of EIIs as carbohydrate transport systems, phosphotransferases, chemoreceptors in chemotaxis and as part of a global regulatory network.

1. Introduction

Free-living unicellular microorganisms, such as the prokaryotes, must be able to detect the frequent and abrupt changes that occur in their environment and to adapt their metabolism rapidly to such fluctuations. Complex regulatory networks have evolved, which monitor the surroundings by means of a series of membrane bound receptors. In bacteria, the transport systems are of crucial importance in such cellular adaptation processes. It is by these molecules that the cells first detect attractants and repellants, which ultimately trigger a positive chemotaxis towards, or a negative chemotaxis away from, chemicals. Transport systems next translocate substrates through the cytoplasmic membrane. In general, these systems act as pacemakers for the peripheral catabolic pathways.

In enteric bacteria, more than 50 different transport systems have been described for carbohydrates and other substrates, but none of these is understood at the molecular level. A comparison of the DNA and amino acid sequences of some transport molecules, which have become available most recently, together with results of topological and mutagenesis studies, should provide new insights.

2. The carbohydrate: phosphotransferase system (PTS)

Many carbohydrates (mono- and disaccharides and polyhydric alcohols) are taken up and accumulated in bacteria not in the free, unchanged form, but as the corresponding phosphate ester, by the phosphoenolpyruvate (PEP) dependent carbohydrate: phosphotransferase system

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(PTS). It is the major transport system for many so-called PTS-carbohydrates that are phosphorylated concomitantly with the translocation of the substrate through the membrane (group translocation) (for recent reviews see Postma & Lengeler (1985); Saier (1985); Robillard & Lolkema (1988). The carbohydrate phosphate is the first intermediate in subsequent catabolism, thus providing a tight linkage between uptake and metabolism.

Figure 1 summarizes some of the components involved in the PTS and its essential biological functions. Most proteins have been purified and mutants for each component are available. Two general and soluble proteins called enzyme I (EI) and histidine protein (HPr) are involved in the first step, resulting in the sequential phosphorylation of first EI, then of HPr at the expense of PEP. In each case, the phosphoryl group is attached covalently to a histidine residue. Mutations in the structural genes *ptsH* and *pts*I, coding for HPr and EI respectively, cause a pleiotropic carbohydrate negative phenotype. This includes the inability of cells to respond in chemotaxis to any PTS carbohydrate, which, in wild type cells, is a good attractant. HPr has been shown recently to be the essential molecule of the PTS through which all chemotactic signals, emanating from this system, are transduced in an integrated form to the general tumble generator CheY. A 'phosphoryl chemotaxis protein' (PCP), itself phosphorylated/dephosphorylated at the expense of HPr, seems essential in this process (Lengeler & Vogler 1989).

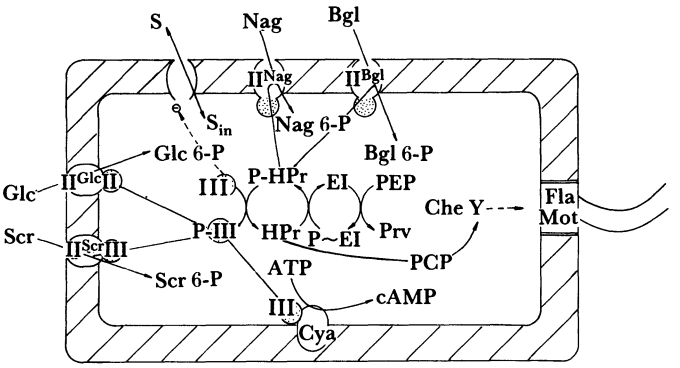

FIGURE 1. The PTS and its functions. Enzyme I (EI), the histidine protein (HPr) and four enzymes II (II) for sucrose (Scr), D-glucose (Glc), *N*-acetylglucosamine (Nag), and β-glucosides (Bgl) are shown together with enzyme III^{Glc} (III). P~EI, P~HPr, and P~III represent the corresponding phosphorylated forms; PCP, the phosphoryl-chemotaxis-protein, modulating the tumble regulator CheY and the flagellar motor switch (Fla, Mot); Cya, the adenylate cyclase activated by P~III, and S a substrate transported by a transport system (arrow) inhibited by III. PEP phosphoenolpyruvate, Prv pyruvate; the stippled parts of II^{Nag} and II^{Bgl} represent their III-like domain.

The phosphoryl group from phospho–HPr is transferred next to one of a series of substrate-specific and membrane-bound enzymes II (EII). This transfer is either direct (e.g. to EII^{Nag} in figure 1) or via a soluble enzyme III (EIII) that forms a complex with a specific EII (e.g. EII^{Glc} and $EIII^{Glc}$ in figure 1). About 15 different EIIs or EII/EIII pairs have been described thus far. Each of these seems to be phosphorylated twice, firstly by means of HPr, at a histidyl residue located either in EIII or in the C-terminal domain of EII and secondly on a residue located in a membrane associated domain of the EII. All large, EIII independent EIIs consist of a single polypeptide chain of 625 to 675 amino-acyl residues, almost exactly the sum of the small EIIs plus their corresponding EIII (see Saier *et al.* (1988) and table 1). The sequential phosphoryl group transfer from EIII to EII, coupled with a series of genetic arguments, have

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Table 1. The different PTS and their origins are given together with the numbers of subunits, amino-acid residues of the enzyme II (II) and enzyme III (III) subunits, the sum (Σ) of residues and the references for the gene sequences

PTS		origin	number of subunits	number of amino-acid residues II	III	Σ	reference
sucrose	Scr	pUR400	2	456	169	625	Ebner & Lengeler (1988)
sucrose	Scr	*K. pneumoniae*	2	456	169	625	F. Titgemeyer (unpublished results)
sucrose	Sac	*B. subtilis*	2	460	?	?	Fouet *et al.* (1987)
sucrose	Scr	*S. mutans*	1	664	—	664	Sato *et al.* (1989)
β-glucoside	Bgl	*E. coli*	1	625	—	625	Bramley & Kornberg (1987) Schnetz *et al.* (1987)
D-glucose	Glc	*E. coli*	2	477	169	646	De Reuse & Danchin (1988); Erni & Zanolari (1987); Nelson *et al.* (1984) Saffen *et al.* (1987)
N-acetyl-glucosamine	Nag	*E. coli*	1	648	—	648	Peri & Waygood (1988) Rogers *et al*, (1988)
Nag		*K. pneumoniae*	1	651	—	651	A. Vogler (unpublished results)
D-mannitol	Mtl	*E. coli*	1	637	—	637	Lee & Saier (1983)
Mtl		*St. carnosus*	?	?	140	?	Reiche *et al.* (1988)
D-glucitol	Gut	*E. coli*	2	506	123	629	Yamada & Saier (1987)
Lactose	Lac	*St. aureus*	2	572	103	675	Breidt *et al.* (1987)
Lactose	Lac	*L. casei*	?	?	103	?	Alpert & Chassy (1988)
D-fructose	Fru	*E. coli*	2	563	376	939	Prior & Kornberg (1988) Geerse *et al.* (1989)
D-mannose	Man	*E. coli*	3	266, 283	323	1421	Erni *et al.* (1987)
L-sorbose	Sor	*K. pneumoniae*	?	?	137 + 182	?	B. Wöhrl (unpublished results)

led to the hypothesis that these domains, and possibly all PTS molecules, share a common evolutionary origin (Lengeler & Steinberger 1978; Saier *et al.* 1985; further references in Postma & Lengeler (1985)).

According to present models of the evolution of genes and polypeptides, new PTS enzymes might have evolved by gene (fragment) duplications, followed by mutational diversification of the copies. If so, comparison of the sequences of the corresponding genes should allow the construction of a genealogical tree. More importantly, however, for our present analysis is that comparison of the amino-acid sequences, deduced from such DNA sequences, should indicate conserved domains of PTS enzymes, provided sequences with sufficient variable relations are available. As summarized in table 1, EII sequences, e.g. for sucrose, *N*-acetylglucosamine, D-mannitol and lactose, ranging from closely related species to sequences derived from Gram-positive and Gram-negative bacteria, can now be compared. Furthermore, similarities ranging from more than 95% to about 20% (close to the limit of statistical significance) are found if EII sequences, specific for the different substrates, are compared. For obvious reasons, the least conserved domains and sequences such as substrate binding sites can be found by comparing highly related molecules, whereas the most strongly conserved parts such as the pore domains or the phosphorylation sites may be detected by comparing the least related molecules.

DNA duplications and diversifications alone are not sufficient to explain the evolution of new PTS enzymes. During evolution, the molecules are split at the DNA level into the equivalent of functional enzyme domains. These gene fragments become part of a 'collective genome or chromosome', common to all prokaryotes, and are located in chromosomes, on plasmids and on transposable elements. They are constantly reshuffled while passing permanently through new organisms. Such intragenic rearrangements may render a meaningful computer analysis of sequence comparisons difficult. They are, however, a superb tool to reveal conserved functional domains.

Except for its functions as a transport system for group translocation and for chemoreception, the PTS also plays a major role in the regulation of the peripheral catabolic pathways. Central to this regulation is the phosphorylation state of one of its components, $EIII^{Glc}$ (see Postma & Lengeler (1985); Postma *et al.* (1988)). Non-phosphorylated $EIII^{Glc}$ interacts with certain active transport systems such as those for lactose, melibiose and maltose in enteric bacteria. When bound to them it inactivates these systems and is responsible for the phenomenon known as 'inducer exclusion'. This inhibition is eliminated in *crr*-mutants lacking $EIII^{Glc}$. It has been proposed furthermore that the phosphorylated $EIII^{Glc}$ is an activator of the enzyme adenylate cyclase. Crr^- mutants have low cyclase activity and become unable to grow on a variety of carbon sources. These include all substrates metabolized by enzymes encoded in operons that are dependent on cAMP (3′,5′-cyclic AMP).

3. Functional domains of PTS enzymes

(*a*) *Sequence comparisons*

In enteric bacteria, several PTS consist of an EII/EIII pair with a combined molecular mass almost exactly that of some large PTS, made up of a single polypeptide chain (e.g. EII^{Nag} and $EII^{Glc}/EIII^{Glc}$ or EII^{Bgl} and $EII^{Scr}/EIII^{Glc}$ in table 1). The primary structures of such EIIs and EII/EIII pairs have been recently deduced from DNA sequencing data (see references in table 1). These revealed the three large domains for the EIIs mentioned above and EII^{Mtl}

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

(Bramley & Kornberg 1987*a*; Fouet *et al.* 1987; Ebner & Lengeler 1988; Peri & Waygood 1988) (see also figure 7). The domains are.

1. A hydrophobic domain of about 350 to 380 amino acid residues, apparently the membrane bound part of the molecules;

2. A hydrophilic domain of about 80 to 100 amino acids in length. This domain, with the most highly conserved sequence, (see §6(*b*)), is located at the amino terminus (EII^{Scr} and EII^{Bgl}) or at the carboxy terminus (EII^{Glc} and its equivalent in EII^{Nag}). Its high conservation and location identify it clearly as an important and distinct domain, most likely involved in the phosphorylation of the substrate;

3. A second hydrophilic domain, about 170 amino acids in length. This is either the free EIII molecule encoded in a distinct gene (e.g. *crr* for EII^{Glc} and EII^{Scr}), or it is a domain covalently bound to the EII as for EII^{Nag} and EII^{Bgl}. As here a single gene (*nagE* and *bglS* respectively) encodes the three domains, and these show strong homologies at the amino-acid level, it is highly likely that large EIIs and EII/EIII pairs resulted from segmentation or fusion of ancestral genes.

(*b*) *Inter- and intramolecular complementation tests*

Homologies and genetic rearrangements alone are not sufficient to prove the existence of functional domains. In conjunction with Dr P. W. Postma (University of Amsterdam) we tested whether the large EIIs can substitute for the lack of the soluble $EIII^{Glc}$ in the transfer of phosphoryl groups from phospho-HPr to EII^{Glc} and EII^{Scr} (Vogler & Lengeler, 1988; Vogler *et al.* 1988). The results are presented schematically in figure 2.

A cloned $nagE^+$ gene on plasmid pAVL1 was transferred into an *E. coli crr* mutant (JLV86), lacking all other transport systems for glucose and its analogue methyl-α-glucopyranoside ($Me_\alpha Glc$). Growth on glucose, transport, and PEP-dependent *in vitro* phosphorylation of glucose and $Me_\alpha Glc$ were restored. This phosphorylation (e.g. of $Me_\alpha Glc$), which depends solely on EII^{Nag} and EII^{Glc} hybrid molecules, was inhibited by antiserum against $EIII^{Glc}$. When pAVL1 was introduced into a *glcA* mutant (otherwise isogenic to JLV86) lacking EII^{Glc}, growth and transport were not restored, thus excluding the possibility that EII^{Nag} is a glucose transporter itself. The large EII^{Bgl} could also substitute for $EIII^{Glc}$ and the large EIIs complemented the lack of $EIII^{Glc}$ in transport and phosphorylation of sucrose via EII^{Scr}, which normally depends on $EIII^{Glc}$ (Lengeler *et al.* 1982). From these results we concluded that the membrane-bound EIII-like domain of EII^{Nag} and EII^{Bgl} can substitute for $EIII^{Glc}$ in EII^{Glc}- and EII^{Scr}-dependent glucose and sucrose transport and phosphorylation.

To test whether truncated EII^{Nag} molecules lacking the carboxy terminal EIII-like domain could be complemented by the soluble $EIII^{Glc}$, a series of deletions were introduced into *nagE*. Clones lacking this terminal domain, in part or totally, were able to grow on *N*-acetylglucosamine (Nag), and to transport and phosphorylate this amino sugar when introduced into a crr^+ strain (lacking all other Nag transport systems), but not into a *crr* mutant, thus corroborating the above conclusions. Deletions extending beyond the EIII-like domain remained negative in both strains.

These results confirmed that the carboxy terminal domain of the large EIIs, which at the amino acid level show good homologies with the soluble $EIII^{Glc}$, was indeed the functional equivalent of this molecule. We therefore propose that, as has been shown for $EIII^{Glc}$ (Dörschug *et al.* 1984) the phosphoryl group from HPr is transferred to His569 in EII^{Nag} and to His547 in EII^{Bgl}, the structural equivalent of the phosphorylated His91 of $EIII^{Glc}$.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Furthermore, we propose that the phosphoryl group is transferred next, either in an intramolecular reaction to the amino-terminal part of the large EIIs, or in an intermolecular reaction to any EII able to interact with $EIII^{Glc}$ or EIII-like domains. For reasons given below, we propose finally that the domain required for this interaction is the second hydrophilic domain (§3(*a*)) and, more precisely, its highly conserved consensus sequence.

In the model proposed (figure 2) the two membrane-bound polypeptides EII^{Glc} and EII^{Nag} form a (transient) complex. This hypothesis was supported by an intramolecular complementation test in which truncated EII^{Nag} molecules were used together with another EII^{Nag} molecule, in which amino acids 204–281 of the hydrophobic domain were deleted. This molecule was unable to catalyse Nag transport and phosphorylation but was still able to substitute for $EIII^{Glc}$ in EII^{Glc}- and EII^{Scr}-dependent reactions and, especially, to complement the other class of truncated EII^{Nag} (A. Vogler, unpublished results).

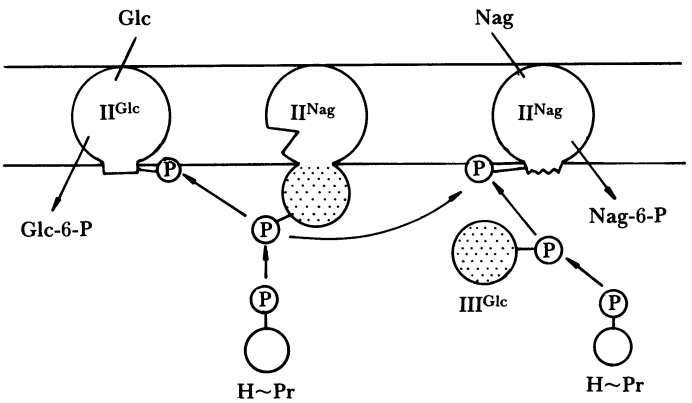

FIGURE 2. The phosphorylation steps during group translocation. The different phosphorylation steps from HPr to enzyme III^{Glc} (III^{Glc}) and to the III^{Glc}-like domain of the EII^{Nag}, thence to EII^{Glc}, or to truncated forms of EII^{Nag} lacking either the $EIII^{Glc}$-like domain, or carrying a large deletion in the membrane-bound domain (residue numbers 203 to 281), as deduced from complementation tests, are summarized.

The *crr* mutants were not able to react in chemotaxis tests to glucose, nor could the cells containing the truncated EII^{Nag} molecules react towards *N*-acetylglucosamine. However, chemotaxis was restored in all cells in which the transport and phosphorylation activities had been restored by the different inter- and intramolecular complementations described, (A. Vogler & J. W. Lengeler, unpublished results). We therefore conclude that both an intact substrate binding domain and the EIII(-like) domain are necessary to trigger a chemotactic response through the EIIs of the PTS.

Introduction of $nagE^+$ or $bglS^+$ genes into *crr* mutants, although restoring growth and transport of glucose in a $glcA^+$ strain, did not restore growth on the carbon sources depending on cAMP, whereas the addition of external cAMP did (Postma *et al.* 1988). It remains to be seen whether binding of an EIII-like domain to a membrane-bound protein is responsible for this apparent lack of adenylate cyclase activation, or whether the EIII part interacting with the cyclase is inactive in EII^{Nag} and EII^{Bgl}.

Crr^- mutants of *S. typhimurium* still show inducer exclusion, perhaps indicating that this function of membrane-bound EIII-like proteins is intact. Recently, the gene responsible for the transport of lactose into *Streptococcus thermophilus* was sequenced (Poolman *et al.* 1989). It codes for an active transport protein whose amino-terminal end is homologous to the melibiose

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

carrier of *E. coli*; its carboxy terminal end showed good homology with the different $EIII^{Glc}$-like domains. The melibiose carrier is one of the transport proteins from enteric bacteria that binds non-phosphorylated EIII and is then inactivated. It is tempting to speculate that in *Streptococcus*, in which an EIII-like domain is fused to EII^{Scr} (Sato *et al.* 1989), this part is also fused to the lactose carrier where it serves a regulatory function.

4. Domains in EII^{Mtl} as defined by a deletion analysis

The mannitol specific EII^{Mtl} is a large EII of 637 amino-acid residues and one of the best studied EIIs at the biochemical and genetic level. It catalyses, in addition to the general PEP-dependent vectorial phosphorylation, the following partial reaction in the absence of EI and HPr:

$$\text{mannitol 1-P} + [^{14}\text{C}]\text{mannitol} \leftrightarrow [^{14}\text{C}]\text{mannitol 1-P} + \text{mannitol}.$$

The topology of the purified and reconstituted mannitol permease in the cytoplasmic membrane has been analysed by Stephan & Jacobson (1986) who found that the molecule consists of a hydrophobic amino-terminal domain (residues 1–334) and of a hydrophilic carboxy-terminal domain (residues 335–637), exposed to the cytoplasmic surface of the inner membrane. When compared at the primary amino-acid level, EII^{Mtl} shows similarity to other EIIs, but only at a few characteristic places (Ebner & Lengeler 1988). The similarities are visible only if conserved amino acid exchanges are included. They indicate a general structure resembling EII^{Nag}.

We have undertaken, in collaboration with G. R. Jacobson (Boston University) (Grisafi *et al.* 1989) to study its putative functional domains by introducing a series of deletions into the cloned *mtlA* gene encoding EII^{Mtl} of *E. coli* K12. The deletions extended into the 3′-terminal end of the gene removing from a few residues to over 75% of the molecule. Functional analysis revealed at least three domains that correspond remarkably well to the domains deduced from the sequence similarities mentioned (Ebner & Lengeler 1988) and with domains postulated as a result of topological studies that used site-specific antibodies, proteases or inhibitors (Stephan & Jacobson 1986). There are three domains (see also figure 7).

1. A hydrophilic domain (residues 475–637) at the carboxy-terminus, the equivalent of an EIII. This domain shows homology with the soluble $EIII^{Mtl}$ of the two Gram-positive bacteria, *Staphylococcus aureus* and *S. carnosus*, (Reiche *et al.* 1988); it contains a highly conserved His554 residue. This residue is covalently phosphorylated by phospho-HPr (Kalbitzer *et al.* 1981; Reiche *et al.* 1988; Pas & Robillard 1988). The EIII-like domain also shows 45% homology with the amino-terminal part of FPr from *S. typhimurium*, a soluble protein of the fructose PTS that has $EIII^{Fru}$ activity (Geerse *et al.* 1989). All deletions extending to residue 519 lost transport and PEP-dependent vectorial phosphorylation activity, but retained a normal transphosphorylation activity. On SDS gels, many gave immunoblots of breakdown products of *ca.* 50 kDa. These end in a region with a characteristic enrichment (9 out of 14) of charged amino acids (residues 475–488) followed by a region of high β-turn probability (residues 487–492). We postulate that this region may be a 'hinge' connecting the EIII-like domain to the rest of the molecule and a site of preferential protease attack.

2. There is a second hydrophilic domain (residues 357–475). This corresponds to the domain of EII^{Bgl}, EII^{Scr}, EII^{Glc} and of EII^{Nag}, which contains the highly conserved consensus sequence (see §3(*a*)) and which flanks a cysteine residue. This Cys421 residue of EII^{Glc} has

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

been shown to be the oxidation sensitive residue involved in the formation of a disulphide bridge, and to be the only catalytically essential cysteine (Meins *et al.* 1988; Nuoffer *et al.* 1988). The EII^{Mtl}, although lacking pronounced similarity to other EIIs, also contains this cysteine residue (Cys384). As shown recently (Pas *et al.* 1988; Pas & Robillard 1988), Cys384 is phosphorylated during vectorial phosphorylation at the expense of His554.

In accordance with this result we found that carboxy-terminal deletions that removed His554 but not Cys384, inactivated transport and PEP-dependent phosphorylation without affecting mannitol–mannitol 1-P transphosphorylation (Grisafi *et al.* 1989). Unless highly unstable phospho-histidines occur as further intermediates in the hydrophobic domain of EII^{Mtl}, these observations imply that Cys384 is the immediate phosphoryl donor to mannitol, as was also suggested by the data of Pas & Robillard (1988). The phosphotransfer reactions of EII^{Mtl} would thus be from phospho-EI and phospho-HPr to His554 of the EIII-like domain, from phospho-His554 to Cys384 of the 'transphosphorylation' domain, and from phospho-Cys384 to the substrate mannitol.

All but one deletion of *mtlA* reaching to residue 391 gave only bands at 34 kDa in immunoblots instead of the predicted 41–55 kDa peptides. This 34 kDa fragment corresponds to the membrane-bound half of the permease left after mild trypsinolysis (Stephan & Jacobson 1986) and seems to be generated by splitting in a region of the protein strongly enriched for charged residues (12 out of 23) located at the border separating the hydrophobic and the hydrophilic parts of the permease (residues 334–357). Improper folding of truncated molecules may lead to this increased breakdown of the hydrophilic parts.

When intact mannitol-EII permease was phosphorylated by [^{32}P]HPr followed by tryptic digestion and separation of the fragments, only the hydrophilic part was phosphorylated. This phosphorylation of the carboxy-terminal part occurred even in the absence of the hydrophobic domain, but no transfer of the phosphoryl group to mannitol could be seen (G. R. Jacobson, personal communication). Phosphotransfer could also be shown between an NEM-inactivated intact permease lacking functional Cys384 and a truncated molecule lacking His554 but retaining Cys384.

These results, which are similar to the results on the inter- and intramolecular complementations (see § 3 (*a*)), imply that catalytically active phosphotransfer may occur from His554 in one subunit to Cys384 in another in a necessarily sequential way. In accordance with this was the observation that all deletions removing Cys384 also inactivated transphosphorylation activity.

3. There is an amino-terminal hydrophobic and membrane-bound domain (residues 1–334). It begins with an amphipathic helix (residues 1–19) and ends with the charged domain mentioned above. This half of the protein alone is sufficient to allow proper integration into the membrane, as deduced from deletions removing all of the hydrophilic parts of the permease. As these membrane-bound truncated fragments bound mannitol with the same high affinity as an intact permease, we conclude that they contain the mannitol binding site. No phosphorylation of these fragments could be observed, indicating that at least no stable phospho-intermediates were generated from phospho-His554 and phospho-Cys384 (Jacobson & Stephan 1988). No mannitol transport activity was observed in cells carrying a deletion of the *mtlA* gene in the chromosome and expressing the gene of a D-arabinitol dehydrogenase constitutively. This enzyme has previously been shown to catalyse the conversion of free mannitol to fructose and to allow a PTS-independent growth of strains of *E. coli* K12 on mannitol

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

when a fructokinase is also present in the cells. We have found that neither the small deletions preventing PEP-dependent transport and phosphorylation, nor the larger deletions removing up to residue 377, were able to restore growth on mannitol. Apparently, the amino-terminal half, even if integrated correctly into the membrane and able to bind its substrate with a normal affinity, was not able to translocate mannitol through the membrane in sufficient amounts to allow growth. A possible explanation is that the hydrophobic domain forms a closed pore or channel, perhaps by transient dimerization of subunits, which must be opened by the phosphorylated hydrophilic part (J. W. Lengeler *et al.*, unpublished results).

5. Other possible examples of PTS protein domains

Two, seemingly different, systems are the fructose and the mannose PTS. Both provide good evidence to support the hypothesis that all PTS genes and enzymes originated by domain rearrangements. The fructose PTS (H. L. Kornberg, this symposium) consists of an $EII^{Fru}/EIII^{Fru}$ pair. Its EII is made up of at least a hydrophilic (residues 1–230) and a hydrophobic domain (231–563) (Prior & Kornberg 1988), and the EIII of three domains, one (residues 1–155) showing homology to $EIII^{Mtl}$, the second (residues 156–280) of unknown function, and the third (residues 281–376) being the functional equivalent of HPr (Geerse *et al.* 1989). The mannose PTS, in contrast, consists of three proteins, two integral membrane proteins $EII\text{-}P^{Man}$ (266 residues) and $EII\text{-}M^{Man}$ (283 residues) both required for mannose transport–phosphorylation and, uniquely to this PTS, for infection of cells by phage λ (Williams *et al.* 1986; Erni *et al.* 1987). Their combined size exceeds the classic size of the membrane integral part of all other EIIs analysed thus far (about 350 residues) by about 100 residues, which, together with a 100 amino acids length hydrophilic stretch of $EII\text{-}M^{Man}$, might constitute the λ-specific part. Phosphorylation of the mannose PTS occurs only at the hydrophilic $EIII^{Man}$ molecule, but here twice. Hydrolysis of this complex yields two fragments of 13 and 20 kDa molecular mass, each still active in phosphorylation and able to be phosphorylated. (B. Erni, personal communication) showed that, *in vitro*, the amino-terminal 13 kDa fragment accepts the phosphoryl group from HPr and donates it to the 20 kDa fragment. This sequential phosphorylation is highly reminiscent of the His554 to Cys384 phosphotransfer described for EII^{Mtl}. Both fragments are also separated by a characteristic hinge, where splitting *in vitro* seems to occur. Another PTS, also made up of three proteins encoded in three different genes, is the L-sorbose specific PTS. In addition to the membrane-bound EII^{Sor}, it contains two soluble proteins, which correspond in size to the two $EIII^{Man}$ domains and show very good homology (40 % identical residues) to them. In cells deleted for the corresponding genes *sorF* and *sorB*, plasmids encoding *manX* and $EIII^{Man}$ restore the sorbose specific transport and phosphorylation activity (our unpublished results). This observation suggests (figure 3) that two domains, fused in the $EIII^{Man}$ molecule, are separated in the sorbose system and encoded by two independent genes. The putative hinge region, located between the two domains in $EIII^{Man}$, has its equivalent in the carboxy terminal part of gene *sorF*, itself located in front of gene *sorB*.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

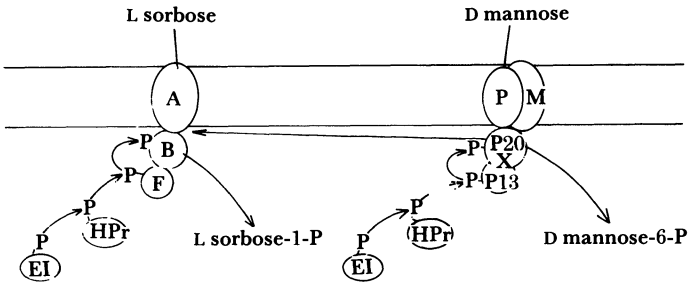

FIGURE 3. Comparison of the L-sorbose and the D-mannose PTS. The localization of EII^{Sor} (A) and the two soluble proteins (F, B) of the L-sorbose PTS (left) and of the two membrane-bound proteins (P, M) and the soluble enzyme III^{Man} with its two domains P13 and P20, of the D-mannose PTS (right) are represented, together with different intra- and intermolecular phosphorylation steps. Other symbols as in figure 1.

6. HOMOLOGIES AMONG PTS PROTEINS RELATED TO THEIR PUTATIVE STRUCTURE

Several computer-based secondary structure prediction methods are available to deduce hypothetical models for the structure of soluble polypeptides from primary sequence data. They may be used for membrane-integral parts of EII proteins only to provide comparisons of sequences of different EIIs, which are closely related at the primary sequence level; it is also essential to consider possible intramolecular rearrangements of functional domains.

The putative structural elements common to four sucrose PTS available are shown schematically in figure 4. The corresponding genes were derived from the chromosome of the Gram-negative *Klebsiella pneumoniae* (61% G+C) and from a sucrose plasmid (57% G+C) isolated from *Salmonella typhimurium*, as well as from *Bacillus subtilis* (42% G+C), *Streptococcus mutans* (43% G+C) and two Gram-positive bacteria (see references in table 1). As indicated, the general structure of the molecules and two-thirds of the sequences were highly conserved. These should contain the sucrose recognition site, the EIII-domain binding part and, perhaps, the pore domain. The latter might also be found in the sequences of the related EII^{Bgl} from *E. coli*, and the two EII^{Nag} from *E. coli* and *K. pneumoniae*, although their substrate binding domains should differ.

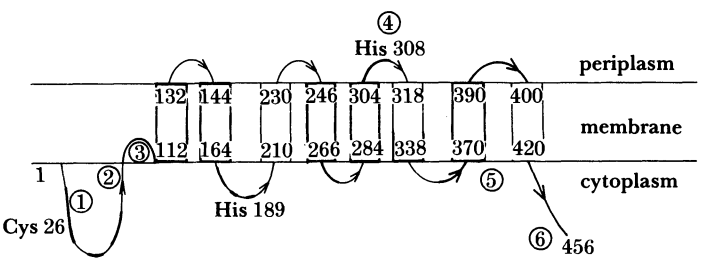

FIGURE 4. Hypothetical model of the sucrose-PTS. A hypothetical model based on the sequences of the sucrose-specific enzymes II from plasmid pUR400, *K. pneumoniae*, *B. subtilis* and *S. mutans* (references in table 1). (1) to (6) designate characteristic structures described in the text and in the legend to figure 7. The numbering of amino acid residues (1 to 456) is according to the first two sequences. Thin lines indicate non-conserved, thickened lines conserved sequences.

(*a*) *The* EIII *domain and the phospho-HPr acceptor histidine*

The $EIII^{Glc}$ domain, the product of the crr^+ gene, constitutes the soluble part of the $EII^{Glc}/EIII^{Glc}$ and $EII^{Scr}/EIII^{Glc}$ pairs in enteric bacteria, and has its functional equivalent in the large EII^{Bgl} and EII^{Nag} (§3(*b*)). It was also found as part of the EII^{Scr} from *S. mutans*

(Sato *et al.* 1989) and of the lactose carrier from *L. bulgaricus* and *S. thermophilus* (Poolman *et al.* 1989). From these sequences a 'consensus sequence' (structure 8 in figure 7) can be deduced, which, as summarized in figure 5, is best conserved around the equivalent of His91 of $EIII^{Glc}$. As this is the histidine phosphorylated by phospho-HPr, the consensus sequence probably contains the corresponding recognition site. It remains to be shown whether this includes the binding sites to the lactose and melibiose transport systems.

```
                    10        20        30        40        50
EIII^Glc    MGLFDKLKSLVSDDKKDTGTIEIVAPLSGEIVNIEDVPDVVFAEKIVKDGIAIKPTGNKM
Consensus                        :: P::G :V :  V D  F:   :  G:A: P:   :

                    70        80        90        100       110
EIII^Glc    VAPVDGTIGKIFEINHAFSIESDSGIELFVHFGIDTVELKGEGFKRIAEEGQRVKVGDTV
Consensus     P: G :  ::   HA: :::: G: :::H:G: TV : G  F   :     V  G  :

                    130       140          150       160
EIII^Glc    IEFDLPLLEEKAKSTLTPVVISNMDEIK---ELI-KLSGSVTVGETPVIRIKK
Consensus     :    :          :   N  ::                    :  :
```

FIGURE 5. Consensus sequence of enzyme III^{Glc} and EIII-like domains. The consensus sequence deduced from a comparison of the sequence from enzyme III^{Glc} ($EIII^{Glc}$) from *S. typhimurium* and the corresponding sequences from the two EII^{Nag}, EII^{Bgl}, and EII^{Scr} from *S. mutans* indicated in table 1 and the EIII-like domain of two lactose transport systems from *S. thermophilus* and *L. bulgaricus* (Poolman *et al.* 1989) are given. His91 is phosphorylated by HPr. A letter indicates a residue conserved in all sequences; (:) a conserved amino acid exchange in all sequences; a space, non-conserved residues.

No apparent similarity at the sequence level was detected between the sequences of $EIII^{Mtl}$ or the EIII-like domain of EII^{Mtl} and the above 'consensus sequence', although the mannitol-specific molecules showed good homology to each other and to FPr. Only the position of the phosphorylated histidine (78–83 residues from the carboxy terminus) seems strictly conserved. It remains to be shown which amino acids of these domains are recognized by phospho-HPr.

(*b*) *The EIII-binding domain*

Figure 6 summarizes and compares the transphosphorylation domains of 8 EIIs that interact with and are phosphorylated by $EIII^{Glc}$ or $EIII^{Glc}$-like domains. A clear consensus sequence can be seen that is centred around the cysteine residue marked with an asterisk (structure 1 in figure 4). This is Cys421 of EII^{Glc}, the only catalytically essential cysteine residue (Meins *et al.* 1988; Nuoffer *et al.* 1988). Because of its high conservation, second only to the consensus sequence of the EIII-domain itself, we postulate that it contains the amino acids interacting with $EIII^{Glc}$.

The sequence of the transphosphorylation domain of EII^{Mtl} contains Cys 384, the equivalent of Cys*, but no other similarity is seen. The $EIII^{Mtl}$ domain seems to require a completely different amino-acid sequence for interaction with its EII.

All transphosphorylation domains show a characteristic accumulation of charged residues at the carboxy-terminal end, whether fused to a hydrophobic domain (EII^{Scr}, EII^{Bgl}), a hydrophilic domain (EII^{Nag}) or remaining free (EII^{Glc}) (structure 2 in figure 4). In EII^{Nag} and EII^{Bgl}, this includes a Pro–Ala rich sequence also found in $EIII^{Man}$ (Erni *et al.* 1987), which has been postulated in other proteins to represent hinge regions between protein domains. This,

```
                          10        20        30        40        50        60        70
                                                  *
Consensus 1    MD. .  .. ..GGK.N....AHCATRLR... D.. ...  . .  .... .  GQ.Q...G ...  V.

ScrA:pU   1    MDF--I-C-LL-LLGGKENI-SAAHCATRLRLVL-DDS-ADQ--I-K-EGVKG-FR--GQMQIIFGTGVVNKVY
ScrA:Kp   1    MDF--I-R-LL-LLGGKENIASAAHCATRLRLVL-DDA-ADQ--I-KIDGVKG-FR--GQMQIMFGTGVVNKVY
SacA:Bs   1    MDY------LI-LLGGKENIISAAHCATRLRLVM-DESKIDQ--V--LDGVKG-F---GQYQIIFGTGLVNKVF
ScrA:Sm   1    MDY--V---VI-AVOGKDNLV-AAHCATRLRLVL-DDSKVDQ--L-K---VKG-FK--GQYQAIIG-G-VN-VY

BglS:Ec   1    MOO--L-R-IV--VGG-DNIVSL-HCATRLRF-L-DESKA----LKK--GI-------GQFQVVIG--VA--VF

NagE:Ec 384    EG-T-LA-NYIAAVGG-DNL--IDACITRLRLTVAD--RV--T--KRLGA-GVV-L-----QVIVG-K-E-I--
NagE:Kp        EG-T-LA-SYIAAVGG-DNL--IDACITRLRLTVGD--KV--A--KRLGA-GVV-------QVIVG-K-E-I--

GlcA:Ec 396    OG-S-MA--LVAAFGGKENI--LDACITRLRVSVAD-SKV-----KKLGA-GVV------OQAIFG-K-D-L--

Consensus 2     G .  A  ..AA.GG .N.  .DACITRLR..V.D  .V     K.LGA GVV       Q...G K . .
                                          *
```

FIGURE 6. Consensus sequence of the enzyme III^{Glc} binding domain. Consensus sequence 1 (residues 1 to 74) deduced from sucrose (upper 4 lines, from pUR400, *K. pneumoniae*, *B. subtilis* and *S. mutans*) and the β-glucoside PTS (5th line). Consensus sequence 2 deduced from the *N*-acetylglucosamine PTS from *E. coli* and *K. penumoniae* (lines 6 and 7, residues 384–458) and the D-glucose PTS (line 8, residues 396 to 470). A letter in the consensus sequences indicates residues conserved in all sequences of one group; the (·) a conserved residue, and an open space a non-conserved residue; (0) the lack of a residue at the corresponding place. The only non-conserved exchange in the closely related sucrose PTS (residue 8) from pUR400 and *K. pneumoniae* and in the Nag PTS (residue 43) are also given.

according to computer predictions, should allow maximal flexibility of the different domains. Such conformational changes seem necessary to allow sequential phosphorylation to occur (Robillard & Lolkema 1988).

(*c*) *The hydrophobic domain*

The hydrophobic domains of EII^{Scr}/EII^{Bgl} and of EII^{Glc}/EII^{Nag}, in contrast, do not seem to show pronounced similarities. However, a closer look reveals a few characteristic structures (labelled 3–6 in figure 7), which cannot be accidental (see also figure 4). Each starts with an amphipathic helix (structure 3) conserved in most, if not all EIIs analysed. This helix is followed by two conserved membrane spanning domains, separated by a hydrophilic linker of

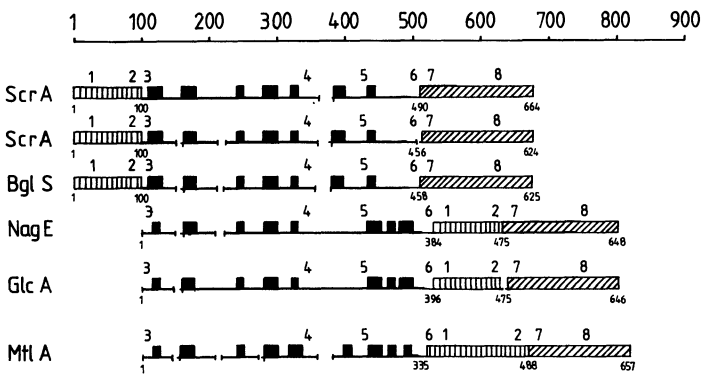

FIGURE 7. Structural domains common to several enzymes II. The structures of the PTS sequences indicated (see table 1) are summarized with the EIII(like) domain (diagonal hatching), the EIII-binding domain (vertical hatching), and the hydrophobic domain with strongly or less strongly membrane seeking structures indicated by wider or narrower black boxes. The scale above the sequences indicates from 1 to 900 residue length, given also for each sequence (numbers immediately below a sequence). Other numbers indicate highly conserved structures common to all EIIs listed: 1, phosphoryl-cysteine, acceptor from 8 and probable donator to the substrate; 2, 6 and 7, sequences enriched for charged amino acids, 6, with 'hinge' character; 3, amphipathic helix; 4, conserved histidyl-residue of unknown function; 5, GITE-motif; 8, phosphoryl-histidine acceptor from HPr and donator to 1.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

variable length. Both are found in a similar place in EII^{Nag}, EII^{Glc} and even EII^{Mtl}, although the amino acids lack any significant similarity. A large hydrophilic domain with conserved His189 Pro residues follows. The next 56 residues (numbers 190–246) constitute, together with residues 133–145, 310–330 and 400–455, the least conserved areas, all of which are located in hydrophilic loops. The remaining parts of the hydrophobic domain are well conserved, especially a histidine residue (His308 and structure 4 in figures 4 and 7) and a GITE motif (structure 5 in figures 4 and 7). Three domains (residues 210–230, 370–390 and 400–420 in figure 4) cannot be clearly assigned to the membrane, nor to hydrophilic areas. These regions may correspond to domains moving in and out of the membrane during the different translocation and phosphorylation steps. If located in a membrane bound domain, the negatively charged glutamate 372 of the GITE motif might play an essential catalytic role similar to other charged amino acids located in the membrane. The hydrophobic domain, whether remaining free or fused to another domain, invariably ends with a series of charged amino acids (structure 6 in figures 4 and 7).

The distribution of membrane-seeking domains between the closely related EII^{Nag} and EII^{Glc} (46% similarity) seems to be highly conserved and is similar to their location in the EII^{Scr} and EII^{Bgl} for the first 200 residues. This seems to apply even to EII^{Mtl} (figure 7). In the second half, they deviate, however, except for structure 5 in which the highly conserved GITE motif is found. It remains to be shown whether perhaps the amino-terminal membrane helices correspond to an essential pore domain, and the GITE motif with its surroundings to residues involved in the transfer of phosphoryl groups from Cys* to the corresponding substrates. Variable parts, which might be part of the substrate binding domains, are all in hydrophilic loops and, interestingly, the sequence surrounding His308, the only His conserved in all EIIs mentioned in figure 7 (except for the His of the EIII-domain). The sequences preceding His308 are conserved in all EII^{Scr} but not in EII^{Bgl}, whereas the sequence located afterwards is conserved in the EII^{Nag}/EII^{Glc} but not in the EII^{Scr}/EII^{Bgl}.

7. Concluding remarks

The PTS is the central regulatory system for many peripheral catabolic pathways. Its general molecules and (some) EIIIs coordinate the synthesis and the activity of the enzymes involved in these pathways. In contrast, membrane-bound EIIs both translocate substrates into the cell and act as stimulus sensors and chemoreceptors whose stimuli are transduced through HPr and phosphorylation/dephosphorylation processes to the tumble regulator of the cells. Such a system made up of many molecules readily permits its evolution by gene duplication, fragmentation and rearrangement to be studied. It also enables one to deduce structural enzyme domains from the analysis of such rearrangements, as has been attempted in this paper.

The scheme that emerges seems to be applicable both to pro- and in eukaryotic organisms. Recently, a model has been proposed for the transduction of environmental signals by two-component regulatory systems (references in Ronson *et al.* (1987)). According to this model, the membrane-bound part of sensors (analogous to the EIIs of the PTS) perceives environmental stimuli and transmits a signal to a conserved cytoplasmic domain (analogous to EIIIs). The activated carboxy-terminal domain of the chemosensors then interacts with and modifies an intracellular regulator protein (analogous to HPr, the adenylate cyclase or variable transport systems), which modulates variable functions such as transcriptional regulators or the

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

chemotactic tumble regulator. A similar model was proposed by us in 1981 for PTS-dependent chemotaxis (references in Lengeler & Vogler (1989)) and was supported by a recent computer-based search (Kofoid & Parkinson 1988). A series of eukaryotic regulatory systems containing membrane-bound hormone receptors as a sensor, also seem to be constructed according to the same principle. They confirm that the phylogenetically ancient PTS obviously has its eukaryotic counterparts. Its molecular analysis might yield further insight not only into the mechanism of signal transduction, but also into the evolution of such systems. Elucidation of the role of more extensive inter- and intragenic rearrangements in the evolution of seemingly non-related EIIs such as the ones for glucitol, mannose–sorbose, fructose, lactose or pentitols, could be the next step in such a programme.

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