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Review

Lipid dependencies, biogenesis and cytoplasmic micellar forms of integral membrane sugar transport proteins of the bacterial phosphotransferase system

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Permeases of the prokaryotic phosphoenolpyruvate–sugar phosphotransferase system (PTS) catalyse sugar transport coupled to sugar phosphorylation. The lipid composition of a membrane determines the activities of these enzyme/transporters as well as the degree of coupling of phosphorylation to transport. We have investigated mechanisms of PTS permease biogenesis and identified cytoplasmic (soluble) forms of these integral membrane proteins. We found that the catalytic activities of the soluble forms differ from those of the membrane-embedded forms. Transport via the latter is much more sensitive to lipid composition than to phosphorylation, and some of these enzymes are much more sensitive to the lipid environment than others. While the membrane-embedded PTS permeases are always dimeric, the cytoplasmic forms are micellar, either monomeric or dimeric. Scattered published evidence suggests that other integral membrane proteins also exist in cytoplasmic micellar forms. The possible functions of cytoplasmic PTS permeases in biogenesis, intracellular sugar phosphorylation and permease storage are discussed.

Introduction

About one-third of all proteins in a cell are embedded in membranes, and about one-third of these are transport proteins. Ubiquitous membrane phenomena, essentially the same in bacteria and vertebrates, include membrane biogenesis, fluidity determination, lipid biosynthesis, protein insertion, and molecular transport. An understanding of integral membrane function depends on understanding membrane protein structure and the biosynthetic rules that govern the overall biogenesis, folding and assembly of native and mutant membrane proteins as well as the principles governing transient interactions of ‘soluble’ proteins or proteins ‘in transit’ with membranes of different lipid compositions.

Integral membrane transport proteins play crucial roles in all processes associated with life. They catalyse nutrient uptake, metabolite excretion, drug and toxin efflux, the establishment of electrochemical gradients, macromolecular export and intercellular communication (Busch & Saier, 2002; Chan *et al.*, 2010; Lam *et al.*, 2011; Natale *et al.*, 2008; Rettner & Saier, 2010). All of these activities are dependent on lipid–protein interactions. However, their effectiveness can be utilized in ways that are detrimental to humans and other organisms. This is exemplified by multi-drug resistant pathogenic microbial strains, arising partially because of the excessive use of antibiotics in medicine and the corporate meat industry (Yen *et al.*, 2010). It is therefore crucial to understand the nature of the lipid dependences of integral membrane proteins.

Characterizing transporters can pave the way for computational modes of drug discovery, which would allow the more effective targeting of various multi-drug resistant pathogens and diseases (Gatti *et al.*, 2011; Slavic *et al.*, 2011). The importance of transport proteins, constituting roughly 10 % of the proteome of an organism, cannot be overstated (Paulsen *et al.*, 2000). The current view is that these proteins exist only in a membrane-integrated state. Limited evidence, however, suggests that these proteins

Abbreviations: II^{Glc}, glucose-specific enzyme II complex; II^{Man}, mannose-specific enzyme II complex; II^{Mtl}, mannitol-specific enzyme II complex; CL, cardiolipin; DAG, diacylglycerol; EI, enzyme I; HPr, heat-stable protein; MBP, maltose binding protein; MtlA, mannitol-specific enzyme II complex; PC, phosphatidyl choline; PE, phosphatidyl ethanolamine; PEP, phosphoenolpyruvate; PG, phosphatidyl glycerol; PhoA, alkaline phosphatase; PgsA, phosphatidyl glycerol-phosphate synthase; PS, phosphatidyl serine; PssA, phosphatidyl serine synthase; PTS, phosphotransferase system; SRP, signal recognition particle; TMS, transmembrane α -helical segment; TP, transphosphorylation.

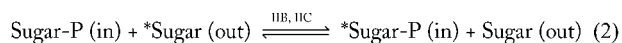
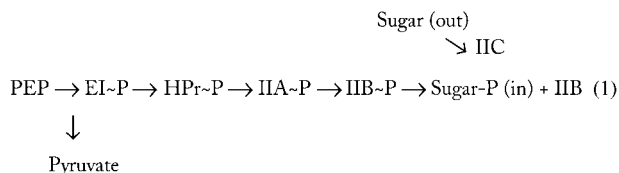
can exist in properly folded, near-native 'soluble' states (Aboulwafa & Saier, 2003; Ito & Akiyama, 1991; Roepe & Kaback, 1989, 1990). Experimental evidence concerning the nature of such soluble states has only recently become available (Aboulwafa & Saier, 2011).

The bacterial phosphotransferase system (PTS)

The prokaryotic phosphoenolpyruvate (PEP)–sugar phosphotransferase system (PTS) is an unusual system for taking up sugars as it couples sugar uptake to phosphorylation (Kaback, 1968, 1970; Lengeler & Jahreis, 2009; Postma *et al.*, 1993; Simoni *et al.*, 1967). It serves as an enzyme-coupled transport system that modifies its substrate sugars by phosphorylation concomitant with transport (Meadow *et al.*, 1990). It also mediates catabolite repression, regulates cytoplasmic inducer levels and coordinates nitrogen metabolism with carbon metabolism (Deutscher *et al.*, 2006; Pflüger-Grau & Görke, 2010; Saier, 1989; Saier *et al.*, 1996). This system uses an elaborate signal transduction pathway and can sense and respond to external stimuli as well as the internal metabolic status of the cell. Thus, the PTS can be considered to be the 'nervous system' of the bacterium.

The PTS consists of a phosphoryl transfer chain involving minimally four phosphocarrier proteins plus the integral membrane permease. The first PTS phosphocarrier, enzyme I (EI), transfers a phosphoryl group from the high-energy phosphoryl donor PEP to itself, generating a high-energy phosphoramidate in which the phosphoryl group is covalently linked to a nitrogen atom in the imidazole ring of a histidyl residue in the enzyme. EI~P then transfers its phosphoryl group to a nitrogen atom in the imidazole ring of a histidyl residue in a small heat-stable protein (HPr). These two proteins comprise the general energy-coupling proteins of the PTS, required for the transport and phosphorylation of all PTS sugar substrates. HPr~P then releases its phosphoryl group sequentially to a histidyl residue in enzyme IIA, a cysteyle residue in enzyme IIB and then sugar. This last reaction is dependent on the integral membrane permease, enzyme IIC, and is the only phosphoryl transfer reaction in the chain that is physiologically irreversible. In one type of PTS permease system, the mannose-specific enzyme II complex (II^{Man}), the IIB constituent is phosphorylated on a histidyl residue rather than a cysteyle residue, and sugar phosphorylation depends on two, rather than one, integral membrane proteins, IIC and an auxiliary protein, IID. The constituents of these mannose-type systems are non-homologous to the majority of PTS permeases, showing that the PTS is a mosaic system, having arisen from a variety of dissimilar protein sources (Hvorup *et al.*, 2003; Saier *et al.*, 2005). In fact, sugar-specific IIC proteins appear to have evolved at least four times independently. These systems can have 0, 6, 10 or 12 transmembrane α -helical segments (TMSs) per polypeptide chain, depending on the protein family (Hvorup *et al.*, 2003; Saier *et al.*, 2005).

The proteins of the PTS catalyse two vectorial reactions, one unidirectional and irreversible (reaction 1) and one bidirectional and fully reversible (reaction 2) (Lengeler & Jahreis, 2009; Saier *et al.*, 1977a, b).



In reaction 2, the sugar moiety of a cytoplasmic sugar-phosphate is expelled while an extracellular sugar (*Sugar) is taken up and phosphorylated (Rephaeli & Saier, 1978; Saier & Newman, 1976; Saier & Schmidt, 1981). This reaction depends only on the IIB and IIC constituents of a PTS enzyme II complex, but the PEP-dependent process depends on the entire phosphoryl transfer chain of the PTS (Postma *et al.*, 1993). There are many sugar-transporting enzyme II complexes in the *E. coli* PTS, and most have been shown to catalyse both of these reactions (Aboulwafa & Saier, 2003; Saier *et al.*, 1977a, b). The transphosphorylation (TP) reaction (reaction 2) is a reflection of the mechanism of phosphoryl transfer from enzyme IIB~P to sugar, catalysed by IIC, the last of the five phosphoryl transfer reactions shown in reaction 1. It is thus an essential 'partial reaction' of the overall process whereby sugar is phosphorylated using PEP.

Sugar phosphorylation by the PTS

Unlike almost all other transport systems, the tight coupling of PTS-mediated sugar transport and phosphorylation allows assay of the PTS permeases both *in vivo*, using a sugar uptake assay, and *in vitro*, in the absence of intact cells or membrane vesicles, using a sugar phosphorylation assay. The *in vitro* assay can utilize either the PEP-dependent reaction (reaction 1) or the sugar-P-dependent reaction (reaction 2).

Previous studies have suggested that both reactions 1 and 2 can usually be catalysed by monomeric and dimeric IIB,C. However, when monomeric enzyme IIB,C catalyses TP, it is not subject to substrate inhibition, while when the dimeric form catalyses this reaction, it is subject to strong substrate inhibition, easily measured at high concentrations of the sugar-phosphate substrate (Aboulwafa & Saier, 2003; Rephaeli & Saier, 1978; Saier *et al.*, 1977a, b). Interestingly, and in contrast to most PTS enzymes II, the dimer of the mannitol-specific enzyme II complex (II^{Mtl}) is much more efficient at catalysing TP than the monomer (Leonard & Saier, 1983; Robillard & Blaauw, 1987; Stephan & Jacobson, 1986).

Phospholipid dependencies of enzymes II

The three principal phospholipids in *E. coli* membranes are phosphatidyl ethanolamine (PE), phosphatidyl glycerol (PG), and cardiolipin (CL). The ratios of these

phospholipids are about 75:20:5, depending on growth conditions, stage of growth and genetic background (Dowhan & Bogdanov, 2009). PE synthesis is dependent on phosphatidyl serine (PS) synthase (PssA) while both PG and CL synthesis relies on phosphatidyl glycerol-phosphate synthase (PgsA). PE is a zwitterionic lipid while PG and CL are anionic lipids with characteristics very different from those of PE (Dowhan & Bogdanov, 2009). *E. coli* membranes also contain small amounts of PS, diacylglycerol (DAG) and lysophospholipids.

In an early paper, Milner & Kaback (1970) used phospholipase D to provide evidence for a specific role of PG in vectorial sugar phosphorylation catalysed by the PTS. Kundig & Roseman (1971) then examined the lipid dependency of enzyme II activities *in vitro* using PEP as the phosphoryl donor, and glucose, mannose or fructose as the phosphoryl acceptor. They extracted the enzyme II fraction in a 'lipid-free' state, and then added back various *E. coli* phospholipids plus a divalent cation, Ca^{2+} or Mg^{2+} . They concluded that a single lipid, PG, was the active lipid factor required for the PEP-dependent sugar phosphorylating activity of the enzyme II complex.

Almost 30 years later, Meijberg *et al.* (1998) examined II^{Mtl} and showed that this enzyme was active in a pure dimyristoyl phosphatidyl choline (PC) bilayer. This observation was surprising since this lipid is not native to *E. coli*. PC is lacking in *E. coli* membranes, and the principal *E. coli* phospholipids are palmitoylated, not myristoylated. The conclusion of these investigations disagreed with the claim by Milner & Kaback (1970) and Kundig & Roseman (1971) that PG was the active lipid factor. However, while Meijberg *et al.* (1998) examined mannitol phosphorylation catalysed by II^{Mtl} , Kundig & Roseman (1971) used glucose, mannose and fructose, which we now know are phosphorylated by three other enzyme II complexes, II^{Glc} , II^{Man} and II^{Fru} (Tchieu *et al.*, 2001). As we shall see, II^{Mtl} is the least sensitive to lipid perturbation of those that have been examined.

We have considered the possibility that PG is specifically required for the *in vitro* activities of the hexose- and hexitol-phosphorylating enzymes II of the *E. coli* PTS by measuring the properties of a null *pgsA* mutant lacking appreciable PG (Aboulwafa & Saier, 2002; Dowhan & Bogdanov, 2009, 2012). The mutant showed lower *in vitro* phosphorylation activities towards several sugars when both PEP-dependent and sugar-phosphate-dependent [^{14}C]sugar phosphorylation reactions were measured. The order of dependency on PG for the different enzymes II was: mannose > glucose > fructose > mannitol. Non-sedimentable (220 000 g for 2 h) enzymes II exhibited a greater dependency on PG than pelletable enzymes II (Aboulwafa & Saier, 2003, 2004). This observation provided the first evidence that an enzyme II complex might exist in more than one physical state (Aboulwafa & Saier, 2011).

Western blot analyses showed that the native glucose enzyme II in this *pgsA* mutant was present in normal

amounts (Aboulwafa & Saier, 2002). Consequently, it was concluded that a PG deficiency primarily affected the activity, rather than the biogenesis of the protein. *In vivo* transport and fermentation measurements revealed substantially diminished activities for all enzymes II. Thermal stability of these enzymes except II^{Mtl} was significantly decreased in the *pgsA* mutant compared to the isogenic wild-type strain, and sensitivity to detergent treatments was enhanced (Table 1). Sugar transport proved to be the most sensitive indicator of proper enzyme II-phospholipid association, being inhibited by the loss of PG much more than the *in vitro* sugar phosphorylation activities. These results showed that PG stimulates but is not required for enzyme II function in *E. coli* (Aboulwafa & Saier, 2002).

An isogenic pair of *E. coli* strains lacking (*pssA*) and possessing (wild-type) the enzyme PssA was used to estimate the effects of the total loss of PE, the major phospholipid in *E. coli* membranes, on the activities of several enzymes II of the PTS (Aboulwafa *et al.*, 2004). This *pssA* mutant exhibits greatly elevated levels of PG, the lipid that had been reported to stimulate the *in vitro* activities of several PTS permeases (Kundig & Roseman, 1971; Milner & Kaback, 1970). As for the *pgsA* mutant described above, the activities, thermal stabilities and detergent sensitivities of three PTS permeases, the glucose-specific enzyme II complex (II^{Glc}), II^{Man} and II^{Mtl} , were characterized (see Table 1). Western blot analyses revealed that the protein levels of II^{Glc} were not appreciably altered by the loss of PE.

In the *pssA* mutant, II^{Glc} and II^{Man} activities were depressed both *in vivo* and *in vitro*, with the *in vivo* transport activities being depressed much more than the *in vitro* phosphorylation activities. II^{Mtl} also exhibited depressed transport activity *in vivo* but showed normal phosphorylation activities *in vitro* (Table 1). II^{Man} and II^{Glc} exhibited greater thermal lability in the *pssA* mutant membranes than in the wild-type membranes, but unexpectedly, II^{Mtl} showed enhanced thermal stability. All three enzymes were activated by exposure to Triton X-100 (0.4 %) or deoxycholate (0.2 %) and inhibited by SDS (0.1 %), but II^{Mtl} was the least affected. II^{Man} and, to a lesser degree, II^{Glc} were more sensitive to detergent treatments in the *pssA* mutant membranes than in the wild-type membranes, while II^{Mtl} showed little differential effect. The results suggested that all three PTS permeases exhibit strong phospholipid dependencies for transport activity *in vivo* but much weaker and differential dependencies for phosphorylation activities *in vitro*, with II^{Man} exhibiting the greatest and II^{Mtl} the least dependency (Aboulwafa *et al.*, 2004).

The effects of lipid composition on thermal sensitivities and detergent activation responses paralleled the effects on *in vitro* phosphorylation activities (Table 1). These results suggested that while the *in vivo* transport activities of PTS enzymes II require an appropriate anionic to zwitterionic phospholipid balance, the *in vitro* phosphorylation activities of these same enzymes show much weaker and

Table 1. Properties of three integral membrane PTS permeases in *pgsA* and *pssA* mutants of *E. coli* compared to the corresponding isogenic wild-type strain, and comparison of their monomeric cytoplasmic forms with their membrane-integrated forms

Data were taken from Aboulwafa & Saier (2002) and Aboulwafa *et al.* (2004). Vertical arrows indicate a direction and relative magnitude of the effect on the indicated property: ↓, decrease; ↑, increase; –, no appreciable effect. For the monomeric and dimeric forms of EII ++, + and – indicate strong, weak and no activity, respectively.

Property		II ^{Man}	II ^{Glc}	II ^{Mtl}
<i>pgsA</i> (–PG and CL)*	<i>In vivo</i> transport	↓↓↓	↓↓	↓
	<i>In vitro</i> phosphorylation	↓	↓	–
	Thermal stability	↓	↓	–
	Detergent sensitivity	↑↑↑	↑↑	↑
<i>pssA</i> (–PE)	<i>In vivo</i> transport	↓↓↓	↓↓	↓
	<i>In vitro</i> phosphorylation	↓	↓	–
	Thermal stability	↓	↓	↑
	Detergent sensitivity	↑↑↑	↑↑	↑
Monomeric EII	<i>In vitro</i> PEP-dependent activity	++	++	++
	<i>In vitro</i> sugar-P-dependent activity	++	+	–
Dimeric EII	<i>In vitro</i> PEP-dependent activity	++	++	++
	<i>In vitro</i> sugar-P-dependent activity	++	++	++

*Small amounts of PG and CL have been detected in this mutant.

differential dependencies (Aboulwafa & Saier, 2002; Aboulwafa *et al.*, 2004). Alteration of the phospholipid composition of the membrane thus allows functional dissection of transport from the phosphorylation activities of PTS enzyme II complexes (Aboulwafa & Saier, 2002; Aboulwafa *et al.*, 2004). Perhaps wild-type cells also exhibit partially uncoupled activities, but this possibility has not been tested. If so, a population of integral membrane enzyme II complexes might catalyse both vectorial and nonvectorial sugar phosphorylations. It is even possible that a single enzyme II can catalyse both reactions. These possibilities must be rigorously examined in future experiments in order to obtain definitive information concerning these issues.

Novel mechanistic features of PTS enzyme II biogenesis

Like mitochondrial targeting sequences, PTS permeases, almost without exception, possess N-terminal amphipathic α -helical sequences terminated by a helix breaker, a prolyl or a pair of adjacent glycyl residues. The involvement of this helix in II^{Mtl} membrane insertion was studied by Yamada *et al.* (1991). Elimination of this helix resulted in inactivation of both *in vivo* mannitol transport and *in vitro* sugar phosphorylation activities using either the PEP or mannitol-1-P-dependent reaction. Alkaline phosphatase (PhoA) fusions, in which II^{Mtl} N-terminal sequences of varying lengths replaced the normal hydrophobic leader sequence of PhoA, revealed that with a short II^{Mtl} amphipathic sequence, PhoA was poorly, but significantly, targeted and translocated across the membrane. However with a longer sequence that included the full II^{Mtl} amphipathic helix as well as the first TMS, PhoA was

efficiently targeted to the external surface of the cytoplasmic membrane (Yamada *et al.*, 1991). Eight different point mutations in the N-terminal amphipathic helix of II^{Mtl} inhibited transmembrane translocation of the fused PhoA and active II^{Mtl} [mannitol-specific enzyme II complex (MtlA)] membrane insertion in parallel when these mutations were present in these two types of constructs. Evidence that the N-terminal amphipathic sequence plays a role in II^{Mtl} biogenesis was compelling (Yamada *et al.*, 1991), and its involvement was later confirmed when II^{Glc} was examined (Aboulwafa *et al.*, 2003). Both *in vivo* and *in vitro* protein-synthesis-coupled insertion experiments with II^{Mtl} provided evidence for a co-translational insertion process mediated by the general secretory SecYEG protein secretion/insertion complex (but not SecA or SecB) apparatus (Werner *et al.*, 1992).

Subsequent studies showed that the signal recognition particle (SRP) complex plays an essential role in II^{Mtl} insertion (Beck *et al.*, 2001; Koch *et al.*, 1999), and that SecY, required for secretion of periplasmic proteins across the membrane, was not required for SRP to target II^{Mtl} to the SecYE translocon complex (Koch & Müller, 2000; Neumann-Haefelin *et al.*, 2000). In all of these studies, II^{Mtl} appears to be co-translationally inserted into the membrane in a process dependent on the SRP-SecYE-YidC complex where each of these three constituents plays a different role in the insertion process (Beck *et al.*, 2001). However, targeting of polytopic membrane proteins to either SecYEG or YidC by SRP has been observed to exhibit promiscuity for II^{Mtl} insertion (Welte *et al.*, 2012).

More recent studies of integral membrane protein insertion in *E. coli* using various membrane proteins have confirmed and extended these observations. For example, Raine *et al.*

(2003) showed that archaeal bacteriorhodopsin and the human melanocortin 4 receptor co-translationally interact sequentially with SRP, SecY and YidC as they move through the translocon. All these complexes are also required for optimal assembly and stability of the *E. coli* maltose permease (MalFGK₂) (Wagner *et al.*, 2008). Yi *et al.* (2004) examined the three dissimilar membrane-embedded subunits of the *E. coli* F-type ATPase: a, b and c. Insertion of all three subunits required YidC, but only the more hydrophilic a and b subunits depended on the SRP/Sec proteins. YidC, which alone can insert the strongly hydrophobic c subunit (Yi *et al.*, 2004), seems to function in the integration of protein hydrophobic domains (Dalbey & Chen, 2004; Luirink *et al.*, 2005). The small mechanosensitive channel (MscL) protein is targeted by SRP in the absence of the Sec translocon to the YidC insertase, which assembles the pentameric channel complex (Francetic *et al.*, 2007; Pop *et al.*, 2009). It is therefore clear that different factors are required for insertion of different integral membrane proteins (Bibi, 2011; Driessen & Nouwen, 2008; Nannenga & Baneyx, 2011; van Bloois *et al.*, 2007).

Cytoplasmic forms of PTS enzyme II complexes

Non-sedimentable enzyme II activity has been observed by many investigators over the years, but this was considered to be due to the presence of small membrane fragments and was not studied further. Even when our studies with *E. coli* membranes of altered lipid composition revealed the presence of substantial amounts of non-sedimentable enzyme II activity, it was not clear that this was of a novel unanticipated nature (Aboulwafa & Saier, 2002).

When the non-sedimentable fraction, the high-speed centrifugation supernatant (Mohammadi *et al.*, 2011), was passed through a gel filtration column, two activity peaks resulted: one in the void volume exhibiting high PEP-dependent and TP activities, and a second including a peak with high PEP-dependent sugar phosphorylating activities and high II^{Man}, moderate II^{Glc} and negligible II^{Mtl} TP activities (Fig. 1 and data not shown; Aboulwafa & Saier, 2003). Both log and early stationary phase cells exhibited comparable relative amounts of pelletable and soluble enzyme II activities, but extended exposure of cells to chloramphenicol, a protein synthesis inhibitor, resulted in selective loss of the soluble fraction with retention of most of the pelleted activity (Aboulwafa & Saier, 2003). Fairly extensive protein degradation was observed, suggesting, but not proving, that loss of the soluble fraction resulted from proteolysis. It is also possible that the soluble form disappeared because it integrated slowly into the membrane in the absence of further protein synthesis but the continuance of lipid synthesis.

Short-term exposure of cells to chloramphenicol resulted in increased enzyme II activities in both fractions, possibly because of increased lipid synthesis in the absence of protein synthesis. Percentagewise, there was more activation of the soluble fraction than the pelleted fraction (Aboulwafa

& Saier, 2003). These observations were interpreted to mean that the increased lipid content of the cells gave rise to enzyme II activation, and that the cytoplasmic forms of the enzymes were lipid-deficient compared to the membrane-integrated forms.

Western blot analyses showed that the soluble II^{Glc} exhibited a subunit size of about 45 kDa, the size of a monomer. Two other soluble enzymes II eluted from the gel filtration column with apparent molecular masses of 40–50 kDa. It was therefore proposed that enzymes II of the PTS can exist in at least two physically distinct forms in the *E. coli* cell, one tightly integrated into the membrane, and one either soluble or loosely associated with the membrane. The membrane-integrated enzymes II are largely dimeric, whereas the soluble enzymes II, retarded during passage through a gel filtration column, are largely monomeric (Aboulwafa & Saier, 2011; van Montfort *et al.*, 2001, 2002).

Construction of plasmid-encoded His-tagged II^{Glc} proved useful in confirming and extending the conclusion that II^{Glc} exists in at least two physically distinct forms, a membrane-integrated dimeric form and a cytoplasmic monomeric form. Western blot analyses using anti-His-tag monoclonal antibodies revealed that although II^{Glc} from the two gel filtration peaks noted above migrated similarly during SDS gel electrophoresis, the two fractions migrated differently in native gels, both before and after Triton X-100 treatment. Peak 1 II^{Glc} migrated much more slowly than peak 2 II^{Glc}. Both preparations exhibited both

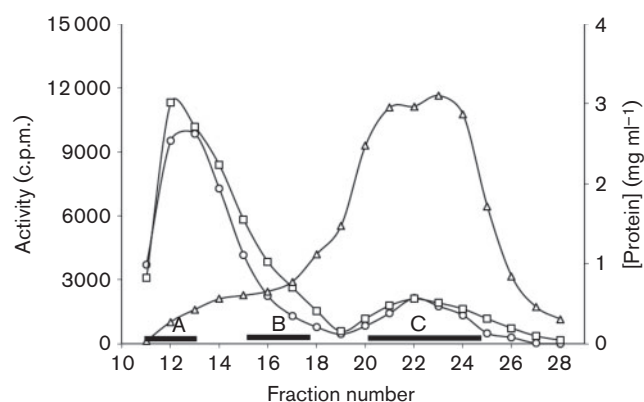


Fig. 1. Profiles of PEP-dependent activity (○), TP activity (□) and protein concentration (△) following gel filtration of a 2 h high-speed centrifugation supernatant (HSS) from an extract of *E. coli* strain ZSC112L(pJFGH11). [¹⁴C]methyl- α -glucoside (20 μ M for the PEP reaction; 100 μ M for the TP reaction) plus either PEP (10 mM) and a 4 h HSS or glucose-6-P (10 mM) were used for assay under standard conditions (Aboulwafa & Saier, 2003, 2011). Horizontal black bars represent: A, dimeric bilayer; B, dimeric micelles; C, monomeric micelles. Modified with permission from Fig. 1 in Aboulwafa & Saier (2004).

PEP-dependent sugar phosphorylation activity and sugar-phosphate-dependent sugar TP activity (Aboulwafa & Saier, 2003, 2004).

The kinetics of the TP reaction catalysed by the two II^{Glc} fractions differed: peak 1 activity was subject to substrate inhibition, while peak 2 activity was not (Aboulwafa & Saier, 2003, 2004, 2011) (Fig. 2). Moreover, the pH optima for the PEP-dependent activities differed for the two fractions. The results provided direct evidence that the two forms of II^{Glc} differ with respect to their physical states and their catalytic activities. These general conclusions appeared to be applicable to II^{Man} as well (Aboulwafa & Saier, 2003). Thus, both PEP-dependent PTS enzymes exist in soluble and membrane-integrated forms that exhibit dissimilar physical and kinetic properties (Aboulwafa *et al.*, 2004).

The work described above showed that integral membrane sugar permeases of the PTS exist in two forms. But can they be interconverted *in vitro*? In a later publication (Aboulwafa & Saier, 2007), the two forms of the His-tagged *E. coli* II^{Glc} were shown reversibly to interconvert by *in vitro* manipulation. Conversion of the membrane-integrated form to the soluble form was promoted by (1) low protein concentration, (2) detergents, (3) high pH, and (4) phospholipase A treatment. Conversion of the soluble form to the membrane-integrated form was promoted by: (1) high protein concentrations, (2) adherence to and elution of a His-tagged II^{Glc} from a Ni^{2+} column, (3) neutral pH, and (4) incorporation into phospholipid liposomes (Aboulwafa & Saier, 2007). This was confirmed with a maltose binding protein (MBP)- II^{Glc} fusion protein which could be purified to a greater degree than the His-tagged protein (Aboulwafa & Saier, 2008). These results confirmed the conclusions that the two forms were

attributable to a single enzyme II complex without permanent covalent modification, and that this interconversion process was fully reversible.

'Soluble' forms of non-PTS integral membrane proteins

Are the results described above for the glucose-specific PTS enzyme II specific to these enzyme permeases, or are both cytoplasmic and membrane-integrated forms of other membrane proteins known? 'Soluble' forms of the β - and α -galactoside permeases, lactose permease (LacY) and melibiose permease (MelB) of *E. coli*, have been reported to exist in 'metastable' soluble forms after overproduction (Ito & Akiyama, 1991; Roepe & Kaback, 1989, 1990; Zen *et al.*, 1995). In these early studies, it was not clear if these states represented 'natural' states, present in the cell cytoplasm, or artefacts of protein preparation, but it now seems clear that they are not artefactual (Bogdanov & Dowhan, 1998; Dowhan & Bogdanov, 2012). Other integral membrane proteins that appear capable of existing in soluble forms include SecY (van der Does *et al.*, 1998; M. Bogdanov, personal communication), the bacterial signal recognition particle receptor, FtsY, a glycerol-phosphate acyltransferase (PlsB) (Zhang & Rock, 2008) and PS decarboxylase (Psd) (Tyhach *et al.*, 1979). Thus, the existence of non-membrane-integrated α -helical trans-membrane proteins may not be uncommon. Further studies will be required to evaluate the relevance of our PTS permease studies to those of other integral membrane proteins.

The occurrence of a cytoplasmic form of integral membrane transporters was unexpected, but potentially similar forms had previously been described for two secondary carriers as noted briefly above. Roepe & Kaback (1989, 1990) and Ito & Akiyama (1991) had characterized 'metastable soluble forms' of LacY and MelB of *E. coli* after overexpression of the permease-encoding *lacY* and *melB* genes, respectively. They suggested that the soluble forms arose when the membrane was 'saturated' with the carrier. The 'soluble' form of either one of these two permeases was believed to have existed in the cell, but possibly to have dissociated from the membrane after *in vitro* treatment with 5 M urea. After urea removal, the protein remained in a soluble, apparently monomeric form. It was shown that these soluble permeases could be reconstituted into proteoliposomes and exhibited transport activity (Roepe & Kaback, 1989, 1990). The authors concluded that the soluble form existed in its native α -helical state. They considered the possibility that it was an artefact of the *in vitro* preparation procedure. However, Bogdanov & Dowhan (1998) have since provided evidence that the cytoplasmic form is not an artefact of preparation. It is not known if this protein fraction is micellar. Moreover, Ito & Akiyama (1991) showed that other integral membrane proteins can exist in 'soluble' forms.

In our hands, 5 M urea, and even 8 M urea (an excellent chaotrope), is effective in stripping peripheral membrane

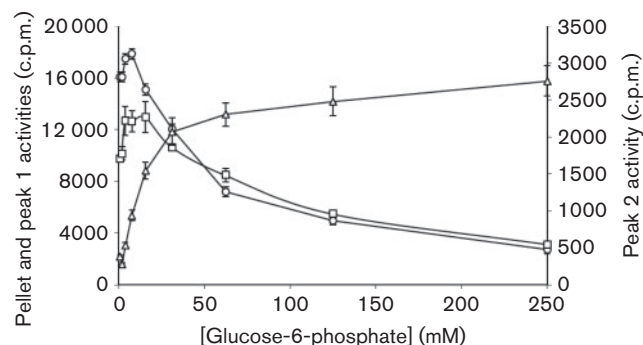


Fig. 2. TP activities of pellet (○), peak 1 (□) and peak 2 (△) from a gel filtration column conducted as shown in Fig. 1. Peak 1 includes fractions A and B indicated by the first two bars in Fig. 1. Purified MBP- II^{Glc} was prepared from *E. coli* strain BW25113 $\Delta\text{ptsG}\Delta\text{malE}::\text{km}(\text{pMALE-ptsG})$. The radioactive substrate was 100 μM [^{14}C]methyl α -glucoside, used with different concentrations of glucose-6-phosphate as indicated. Modified with permission from Fig. 15 in Aboulwafa & Saier (2004).

proteins from biological membranes, but it does not solubilize PTS integral membrane proteins (Rephaeli & Saier, 1978; Saier *et al.*, 1977b). Further, our results led to the conclusion that cytoplasmic II^{Glc} exists in the cell and is not just an artefact of cell rupture or protein purification (Aboulwafa & Saier, 2003, 2011). As a result, a more detailed study of the nature of the protein was conducted.

Physical characterization of the soluble II^{Glc}

To determine the characteristics of the cytoplasmic forms of the PTS II^{Glc} , a combined biophysical/biochemical approach was taken. Six approaches were used to evaluate protein–protein and protein–lipid interactions [see Table 2 and Aboulwafa & Saier (2011)]. Fluorescence resonance energy transfer (FRET) using MBP- II^{Glc} -YFP (yellow fluorescent protein) and MBP- II^{Glc} -CFP (cyan fluorescent protein) revealed that the homodimeric enzyme II complex in cell membranes is stable but can be dissociated and reassociated to the heterodimer in the presence of a detergent such as Triton X-100. By contrast, the soluble monomeric species could form a heterodimeric species by incubation and purification without detergent exposure.

Formaldehyde cross-linking studies, conducted *in vitro*, revealed that the dimeric MBP- II^{Glc} activity decreased dramatically with increasing formaldehyde concentrations, due to both aggregation and activity loss, but that the monomeric MBP- II^{Glc} retained activity more effectively in response to the same formaldehyde treatments. *In vivo*, the differential effect of formaldehyde was enhanced so that peak 2 activity was largely retained while peak 1 activity was reduced to near zero (Table 2, Fig. 3). These results provided further evidence that the membrane-integrated and cytoplasmic forms of II^{Glc} coexist in intact cells. Electron microscopy of MBP- II^{Glc} indicated that the dimeric form of the protein is larger than the monomeric form, as expected. In fact, dynamic light scattering

confirmed this conclusion and provided semiquantitative estimates of size (Aboulwafa & Saier, 2011).

NMR analyses suggested that the dimeric form is present primarily in a lipid bilayer while the monomeric form is present within micelles. This cytoplasmic form was estimated to have about 12 lipid molecules bound per II^{Glc} monomer. This was in contrast to the soluble LacY studied by Roepe & Kaback (1989), as these workers reported the presence of only three phospholipids per monomer. Later, the same laboratory reported seven phospholipids per LacY monomer (Guan *et al.*, 2006). In this case, the preparation had been exposed to cholate, which is known to strip phospholipids from proteins. These measurements should be considered approximations, possibly explaining some of the different values reported.

Evidence for a minor dimeric micellar species of II^{Glc} , possibly an intermediate between the monomeric micellar and the dimeric bilayer forms, was presented (see Fig. 4) (Aboulwafa & Saier, 2011). The dimeric micelle was found in the trailing edge of peak 1 obtained by gel filtration (bar B in Fig. 1). These results provide convincing evidence for at least three physical forms of II^{Glc} and identified the ‘soluble’ forms as probable micellar structures (Aboulwafa & Saier, 2011). Lipid analyses of the different fractions revealed that the three dominant lipid species in *E. coli* (PE, PG and CL) are present in all fractions, but the monomeric micellar structure contains a higher percentage of anionic lipids (PG and CL), while the dimeric bilayer form has a higher percentage of zwitterion lipids (PE).

Future perspectives on functional, biogenic and interactive themes

A cytoplasmic micellar transporter cannot catalyse a vectorial reaction since there is no membrane barrier to create separate compartments. However, all PTS enzymes II also catalyse a chemical reaction: sugar phosphorylation.

Table 2. Biophysical and biochemical approaches used to characterize the different physical forms of the *E. coli* glucose permease

Peak 1 is primarily the dimeric membrane-bilayer-associated form of II^{Glc} obtained from a gel filtration column (Aboulwafa & Saier, 2003, 2004); peak 2 is the monomeric micellar form of II^{Glc} obtained from the same column.

Technique	Nature of information obtained
Fluorescence resonance energy transfer (FRET)	Protein–protein interactions; peak 2 can associate to peak 1 without detergent; peak 1 cannot dissociate to peak 2 without detergent
Formaldehyde reactivity and cross-linking	Protein–protein interactions; particularly <i>in vivo</i> , peak 1 becomes insoluble and inactivated with formaldehyde exposure, but peak 2 does not
Enzyme activity	Dependency of substrate inhibition on monomeric versus dimeric form: identification of dimeric micellar II^{Glc}
Cryo-electron microscopy (C-EM) and dynamic light scattering (DLS)	Purified French pressed membrane vesicles are heterogeneous; sizes: pellet >peak 1 >peak 2
Nuclear magnetic resonance (NMR)	Pellet is only in a bilayer form; peak 1, leading edge: only in bilayer form; peak 1, central part: a mixture of bilayer form and dimeric micelles; peak 1 trailing edge: dimeric micelles; peak 2 monomeric micelles

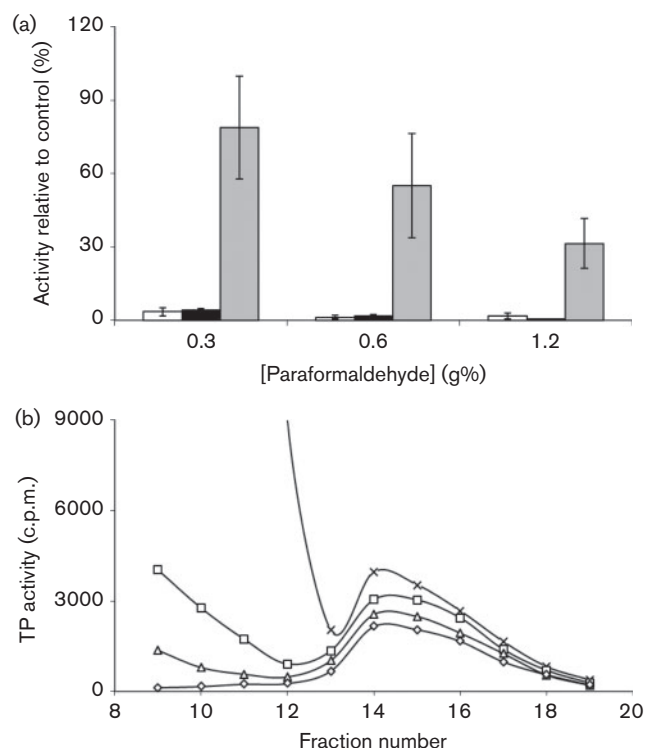


Fig. 3. Effect of *in vivo* cross-linking with different concentrations of paraformaldehyde (PFA) on the TP activities of different preparations obtained from a cell lysate of *E. coli* strain BW25113Δ*ptsG*Δ*malE::km*(pMALE-*ptsG*). (a) Low-speed pellets, white bars; high-speed pellets, black bars; high-speed supernatants, grey bars. Error was less than 1 SD ($n=3$) (b) TP activities of gel filtration fractions of the high-speed supernatants (x, control; □, 0.3% PFA; △, 0.6% PFA; ◇, 1.2% PFA). (a) Reproduced with permission from Fig. 8 in Aboulwafa & Saier (2011); (b) modified from Fig. 9B in Aboulwafa & Saier (2011).

Using non-PTS transporters, many sugars enter the cell via facilitated diffusion, or by a primary or secondary active transport process that does not involve concomitant sugar phosphorylation. Further, disaccharides and oligosaccharides are taken up and hydrolysed to free monosaccharides that must be phosphorylated (Busch & Saier, 2002; Lorca *et al.*, 2007). These sugars in general are phosphorylated in the cytoplasm. In *E. coli*, the ATP-dependent kinase activities for several sugars (e.g. glucose, fructose, mannose) are too low to support normal rates of growth with these sugars as the sole carbon source, and many PTS substrates in *E. coli* cannot be phosphorylated by a non-PTS mechanism (e.g. mannitol, glucitol, galactitol and possibly *N*-acetyl glucosamine and *L*-ascorbate) (Kaup *et al.*, 2003; Saier *et al.*, 1971, 1973; Zhang *et al.*, 2003). There is consequently a need for intracellular phosphorylation (Saier & Schmidt, 1981). In a reconstituted system, Mukhija & Erni (1996) showed that the nonvectorial sugar phosphorylation reaction exhibits about 12× lower affinity for the sugar substrate than the vectorial process,

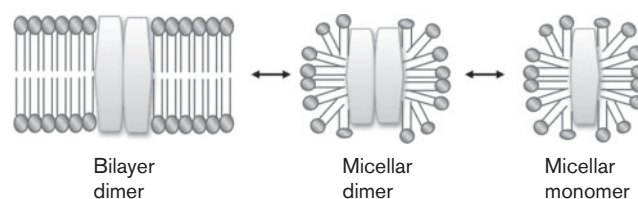


Fig. 4. Schematic illustration of the three documented forms of II^{Glc} of *E. coli*. Left, dimeric II^{Glc} in the lipid bilayer of the plasma membrane. Middle, dimeric II^{Glc} in a micellar structure with lipid coating the hydrophobic surfaces of the protein. Right, monomeric II^{Glc} in a micellar structure with lipid coating the hydrophobic surfaces of the protein. If these species are in equilibrium as indicated by the double-headed arrows, the stabilities of these structures, and the activation energies for their interconversions, must be very substantial, accounting for the low rates of these interconversions both *in vivo* and *in vitro*.

but that the maximal reaction rate for the former process is $2.5 \times$ greater than for the latter. Thus, while the cytoplasmic forms of PTS enzymes II clearly can provide the function of nonvectorial phosphorylation, it is also possible that the membrane-integrated forms can catalyse this process.

If other transporters, such as LacY and MelB of *E. coli*, exist in cytoplasmic micellar forms, such a proposed function, analogous to that suggested for the soluble enzymes II, would seem less likely, as no chemical process is catalysed by a secondary active transporter. The presence of micellar forms could instead (or in addition to) be a consequence of biogenesis, of a bilayer–micellar equilibrium or of a membrane saturation phenomenon (Dowhan & Bogdanov, 2009; Ito & Akiyama, 1991; Roepe & Kaback, 1989).

The possibility that an enzyme II can actually be synthesized on the ribosome in a micellar form, and then be inserted into the membrane, had not previously been considered. It has been found that without the minor *E. coli* lipid DAG, II^{Mtl} can insert into phospholipid vesicles *in vitro* via a spontaneous unassisted integration process (Nishiyama *et al.*, 2006). However DAG addition provides a kind of ‘seal’ so that II^{Mtl} insertion is entirely dependent on the SRP and SecYEG complexes (Kawashima *et al.*, 2008). If DAG is present in sufficient amounts in native *E. coli* membranes, then the experiments reported by Kawashima *et al.* (2008) would argue against the spontaneous insertion of a micellar form of II^{Mtl} into the membrane. However, these experiments do not address three questions. (1) Is there sufficient DAG in *E. coli* membranes to provide the ‘seal’ discussed above? (2) Are SecY and other proteins involved in integral membrane insertion (e.g. YidC and the SRP complex) involved in micellar enzyme II complex biogenesis? (3) Might there be an enzyme system that can catalyse the reversible interconversion of the bilayer and micellar forms of various permease proteins? Demonstration of the interconversion

Table 3. Unanswered questions concerning cytoplasmic micellar forms of integral membrane proteins

(I) Questions concerning vectorial versus nonvectorial phosphorylation
Do the cytoplasmic enzymes II function to phosphorylate intracellular free sugars, and if so, what fraction of the total intracellular activity derives from this form of the enzyme?
What are the molecular bases for the coupling of phosphorylation to transport, and what are the conditions, in addition to abnormal lipid ratios, that promote uncoupling?
Do single enzymes II catalyse both vectorial and nonvectorial sugar phosphorylation, or do the two reactions depend on different forms within a population of these enzymes?
(II) Questions concerning the functional significance of protein–lipid interactions
What are the close lipid associations of the different PTS permeases and how do these influence coupled versus uncoupled enzyme II transport/phosphorylation activities?
Why is the <i>in vivo</i> transport activity of a PTS enzyme II complex much more sensitive to lipid composition than is its <i>in vitro</i> phosphorylation activity?
Why is lipid dependency so much greater for some PTS permeases than for others?
(III) Questions concerning the source(s) of cytoplasmic membrane proteins
Is the monomeric micellar form of a PTS permease in slow equilibrium with the dimeric micellar and membrane-integrated forms <i>in vivo</i> , or do they represent stable species?
Do other multispreading integral membrane proteins form micellar complexes comparable to those characterized for PTS permeases? If so, do they behave similarly?
Why do the micellar forms of enzymes II disappear during long-term stationary phase incubation with chloramphenicol when the membrane-integrated forms remain relatively stable? Do the micellar forms represent ‘storage forms’ of permeases?
(IV) Questions concerning II^{Glc} biogenesis
Can the cytoplasmic II ^{Glc} be produced by lipid associations during synthesis, or is co-translational membrane insertion the only route by which active PTS permeases are produced?
During biogenesis of integral membrane proteins, is there a ‘saturation point’ beyond which the proteins go into micellar forms, and if so, do other integral membrane proteins compete for these sites or are they protein specific? What determines this ‘saturation point’?
Are SecYEG, YidC and/or the SRP complex also present in micellar forms, and if so, do some or all of these complexes function to incorporate PTS and other integral membrane proteins into micelles?

of the bilayer and micellar forms of II^{Glc} *in vitro* does not prove that this occurs at an appreciable rate *in vivo*. Interestingly, the presence of ‘soluble’ SecY and FtsY has been demonstrated (Ito & Akiyama, 1991; M. Bogdanov, personal communication). Is it possible that micellar SecY might facilitate the incorporation of a protein such as II^{Glc} into micelles?

DAG is certainly present in *E. coli* membranes (Dillon *et al.*, 1996), and it serves a function in eukaryotes since it is essential for protein trafficking through the Golgi complex in yeast (Kearns *et al.*, 1997). However, its levels and potential functions in *E. coli* represent unknowns. It may be that the cytoplasmic micellar forms of these integral membrane proteins do not play a significant functional or biogenic role. Even so, their study would be most valuable for a detailed mechanistic understanding of micellar–bilayer interconversions *in vivo*.

Unanswered questions concerning cytoplasmic PTS enzymes II are summarized in Table 3. We have shown that the cytoplasmic and membrane-integrated forms of II^{Glc} are not in rapid equilibrium, but a precursor–product relationship has not been investigated. In this regard, three

possibilities must be considered. First, the micellar form could appear during biogenesis, perhaps as a minor species. If so, it could serve as a biogenic precursor of part of the mature membrane-integrated enzyme II complex. Second, the two forms could be in slow equilibrium so that the primary source of the micellar enzyme is the membrane (Fig. 4). Third, the micellar form could appear only when the membrane is ‘saturated’ with the enzyme or deficient for the SecYEG translocon. While we consider this third possibility to be reasonable, there is little published evidence for a saturation point for protein content. It should be noted that these three possibilities are not mutually exclusive.

It might be possible to distinguish these alternative explanations for the occurrence of the cytoplasmic forms of the PTS enzymes II by conducting pulse-chase experiments. Moreover, such studies might reveal whether the dimeric micellar II^{Glc} species is an intermediate between the other two forms. Partially inverted conformations of PTS enzymes II may occur, similar to those that have been documented for secondary carriers (Dowhan & Bogdanov, 2009, 2012). It will therefore be important to know if most

of the micellar forms derive from the plasma membrane, intracellular membranes, both or vice versa. If it is derived from a membrane, under what conditions does this occur? The detailed interactions of these proteins with lipids also pose a number of additional questions (see Table 3). Further studies are likely to reveal novel aspects of protein–lipid interactions as well as the mechanisms of *in vivo* micelle–bilayer interconversions. Our knowledge of membrane protein folding and quaternary complex formation could benefit from such studies. It seems clear that our understanding of these phenomena is still in its infancy.

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