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Properties of the Glucose Phosphotransferase System of *Clostridium acetobutylicum* NCIB 8052

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The glucose phosphotransferase system (PTS) of *Clostridium acetobutylicum* was studied by using cell extracts. The system exhibited a K_m for glucose of 34 μM, and glucose phosphorylation was inhibited competitively by mannose and 2-deoxyglucose. The analogs 3-*O*-methylglucoside and methyl α-glucoside did not inhibit glucose phosphorylation significantly. Activity showed no dependence on Mg^{2+} ions or on pH in the range 6.0 to 8.0. The PTS comprised both soluble and membrane-bound proteins, which interacted functionally with the PTSs of *Clostridium pasteurianum*, *Bacillus subtilis*, and *Escherichia coli*. In addition to a membrane-bound enzyme II^{Glc}, sugar phosphorylation assays in heterologous systems incorporating extracts of *pts* mutants of other organisms provided evidence for enzyme I, HPr, and III^{Glc} components. The HPr was found in the soluble fraction of *C. acetobutylicum* extracts, whereas enzyme I, and probably also III^{Glc}, was present in both the soluble and membrane fractions, suggesting a membrane location in the intact cell.

As a result of the fluctuating economics of the petrochemical industries, there has been a recent upsurge in interest in the biological production of fuels and chemicals. Acetone-butanol-ethanol fermentation by *Clostridium acetobutylicum* has been exploited on an industrial scale in the past, but for economic reasons the process has declined over the last 30 years. However, with an increased understanding of the biochemistry, physiology, and genetics of solvent formation and the potential for genetic manipulation of the organism, this fermentation is once again receiving attention (9). An economical process would be founded on the use of cheap, renewable substrates, ideally carbohydrate-based waste materials. However, despite its obvious importance in efficient conversion of substrate to metabolic end product, little is known about the mechanism and regulation of carbohydrate accumulation by *C. acetobutylicum* or the clostridia in general.

Bacteria exhibit several mechanisms of transport of nutrients into the cell. Many obligate and facultative anaerobes accumulate sugars via a phosphoenolpyruvate (PEP)-dependent phosphotransferase system (PTS) (13, 18). The PTS comprises several protein components which are involved in transfer of a phosphate group from PEP to the sugar, with the result that the sugar is phosphorylated as it crosses the membrane. This phosphorylation forms the basis of an assay for the system in vitro. Analysis of the system in several bacteria has shown that in most cases there are two soluble proteins, enzyme I and HPr, which are common to all the PTSs in the cell. Enzyme I catalyzes the PEP-dependent phosphorylation of HPr, which then donates the phosphate to a membrane-bound, sugar-specific enzyme II. In some systems, an additional sugar-specific protein, called III, mediates the transfer of phosphate from HPr to enzyme II. In addition to its direct function in sugar accumulation, the PTS in enteric bacteria is involved in the regulation of metabolism of sugars which are not substrates of the system (22, 24), has a role in chemotaxis (11), and has been implicated in the control of other aspects of metabolism (2, 32). There is evidence that in several gram-positive bacteria, a secondary phosphorylation of HPr by a protein kinase is an important feature of regulation of carbohydrate metabolism (3, 18, 19).

Several species of clostridia, including *C. acetobutylicum*, are known to possess a PTS (1, 8, 15, 17, 30). However, with the exception of the glucose PTS in *C. pasteurianum*, in which a HPr-like protein was identified (15), there have been no detailed descriptions of the system in this genus, and its potential role in metabolic regulation has not been assessed. The work reported here was therefore carried out to ascertain the general properties of the PTS in *C. acetobutylicum* and to investigate the relationship between this PTS and the system in other bacteria.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains used in this study are listed in Table 1. The clostridial strains were stored as spore suspensions in distilled water at 4°C. Samples of the suspensions were treated at 80°C for 10 min, inoculated into 20 ml of reinforced clostridial medium (RCM; Oxoid), and incubated for 24 h at 37°C. A sample of the culture was then inoculated into a larger volume (0.5 or 1 liter) of synthetic RCM containing glucose (0.5%, wt/vol) as the only fermentable carbon and energy source, and incubation continued for a further 18 h. All media were autoclaved and then cooled under N_2-H_2-CO_2 (80:10:10) in an anaerobic cabinet (Raven Scientific). Cultures were incubated within the cabinet or removed to an incubator after sealing with a Sub-a-Seal.

Strains of *Bacillus subtilis* and *Escherichia coli* were grown in an orbital incubator at 37°C in L broth, containing (per liter) the following: tryptone, 10 g; yeast extract, 5 g; NaCl, 0.5 g. Aliquots of an overnight culture were diluted into L broth containing 0.5% (wt/vol) glucose, and incubation continued until the culture was in the late exponential phase.

Preparation of cell extracts. All cultures were harvested in the late exponential phase of growth. Cells were centrifuged (8,000 × *g* for 10 min at 4°C), washed, and resuspended in 10 mM Tris-Cl buffer (pH 7.5) containing 5 mM $MgCl_2$ and 1 mM dithiothreitol (4 ml g [wet weight]$^{-1}$). Breakage of cells

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TABLE 1. Bacterial strains used in this study

Strain	PTS defect	Source	Reference
C. acetobutylicum NCIB 8052	None	NCIMB[a]	
C. pasteurianum NCIB 9486	None	NCIMB	
B. subtilis			
Marburg (NCIB 3610)	None	NCIMB	
PG554	*ptsI* (Enzyme I)	G. Rapoport	16
PG587	*ptsI* (Enzyme I)	G. Rapoport	16
E. coli			
7	None	I. R. Booth	5
HK929	*ptsH* (HPr)	H. Kornberg	
JLV86	*crr* (III^{Glc})	J. W. Lengeler	29
LM1	*crr* (III^{Glc})	I. R. Booth	11

[a] NCIMB, National Collection of Industrial and Marine Bacteria.

in a French press and preparation and fractionation of extracts by ultracentrifugation were as described previously (15), with the modification that Tris-Cl buffer replaced potassium phosphate buffer throughout. Protein concentrations in extracts were estimated by a microbiuret method, with bovine serum albumin as the standard (33). Soluble extracts were fractionated on a Sephadex G-100 column (2.5 by 90 cm) at 4°C at a flow rate of 14 ml h^{-1}. Fractions (5 ml) were collected and assayed for the presence of PTS components as described below. The protein concentration in active pools was measured by using the Bio-Rad protein assay kit (micro-assay) with bovine gamma globulin as standard.

Assay of PTS activity. PEP-dependent sugar phosphorylation was assayed by precipitation of radiolabeled sugar phosphate in ethanolic barium bromide as described previously (15). Standard assay mixtures contained, in a volume of 1.0 ml, 10 mM Tris-Cl (pH 7.5), 0.5 mM PEP [tri(cyclohexylammonium) salt], 2 mM dithiothreitol, 5 mM $MgCl_2$, 12 mM KF, 0.1 mM radiolabeled sugar (1.05 Ci mol^{-1}), and 25 mM potassium phosphate (pH 7.0), which was added to ensure adequate precipitation of sugar phosphate. At timed intervals, samples (0.15 ml) were taken and added to 2 ml of 1% (wt/vol) barium bromide in 80% (vol/vol) ethanol. Precipitates were removed by filtration on fiberglass discs (Whatman GF/F) and washed with 5 ml of 80% (vol/vol) ethanol. Discs were dried under a heat lamp, and radioactivity was determined by liquid scintillation counting in 4 ml of Scintillation Cocktail O (BDH Scintran).

In the assay of column fractions, the total volume was reduced to 0.25 ml and the reaction was terminated by addition of barium bromide to the assay tube. Samples were then treated in the normal way for estimation of sugar phosphate formed.

Materials. PEP [tri(cyclohexylammonium) salt], DL-dithiothreitol, and sugar analogs were purchased from Sigma; growth media were obtained from Oxoid. D-[U-^{14}C]glucose, 2-deoxy-D-[1-^{3}H]glucose, and methyl (α-D-[U-^{14}C]gluco)pyranoside were from Amersham. All other chemicals were of the highest purity available.

RESULTS

General properties of the glucose PTS. Extracts of *C. acetobutylicum* prepared from cells grown on synthetic RCM containing glucose exhibited PEP-dependent glucose phosphorylation. Separation of extracts into soluble and membrane fractions resulted in loss of activity, which was restored on recombining the two preparations. Thus, as for PTSs from other bacteria, the glucose PTS in *C. acetobutylicum* comprises both soluble and membrane-bound proteins.

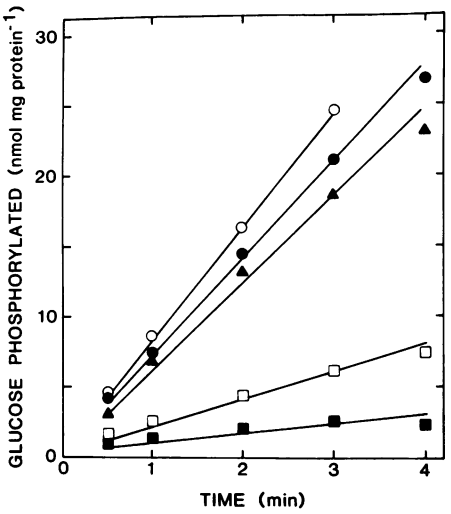

FIG. 1. Inhibition of glucose phosphorylation by analogs in cell extracts of *C. acetobutylicum*. Standard assay mixtures contained 1.1 mg of extract protein and the following additions: none (○), 10 mM mannose (■), 10 mM 2-deoxyglucose (□), 10 mM 3-*O*-methylglucoside (▲); and 10 mM methyl α-glucoside (●).

The K_m of the system for the substrate glucose was found to be 34 μM. Phosphorylation of glucose was abolished by a 100-fold excess concentration of mannose and was strongly inhibited by 2-deoxyglucose (Fig. 1). Kinetic analysis showed that inhibition by each of these sugars was competitive. Inhibition by 3-*O*-methylglucoside was much less severe, and virtually no inhibition was seen in the presence of methyl α-glucoside. In agreement with these results, extracts phosphorylated 2-deoxyglucose in a PEP-dependent manner, but no phosphorylation of methyl α-glucoside was observed. Therefore, the substrate range of the system is apparently restricted to glucose, mannose, and 2-deoxyglucose, with low affinity for 3-*O*-methylglucoside. These findings are very similar to the results of an earlier study of glucose uptake into whole cells of *C. acetobutylicum* ATCC 4259 (8).

The rate of phosphorylation of glucose varied little between pH 6.0 and 8.0. Addition of 10 mM EDTA to extracts had no inhibitory effect, and extensive dialysis of extracts against Mg^{2+}-free buffer containing 10 mM EDTA failed to inactivate the system. It is therefore concluded that activity is not dependent on Mg^{2+} or other divalent cations.

Interaction with other PTS systems. The functional relationship between the *C. acetobutylicum* PTS and the PTS from other bacteria was examined in heterologous sugar phosphorylation assays by using soluble and membrane fractions from different sources. The results of these experiments are illustrated in Fig. 2. The soluble fraction from *C. acetobutylicum* was capable of complementing membranes from *C. pasteurianum*, *B. subtilis*, and *E. coli* for phosphorylation of glucose or methyl α-glucoside, which is a substrate of the glucose PTS in these other organisms (Fig. 2A). Similarly, phosphorylation of glucose by *C. acetobutylicum* membranes was observed in the presence of the soluble fraction from each of the other organisms (Fig. 2B). In this

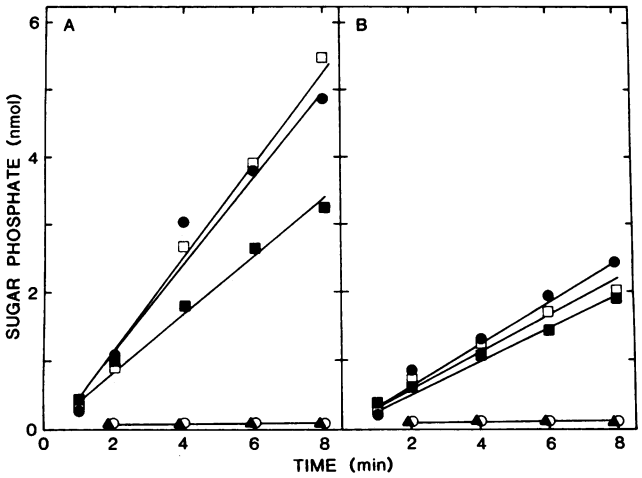

FIG. 2. Interaction between the glucose PTS of *C. acetobutylicum* and other bacteria. (A) Complementation of membranes by *C. acetobutylicum* soluble extract. The figure shows phosphorylation of methyl α-glucoside in standard assay mixtures containing *C. acetobutylicum* soluble extract (1.1 mg of protein) (○), *C. acetobutylicum* soluble extract and *C. pasteurianum* membranes (0.3 mg) (●), and *C. acetobutylicum* soluble extract and *B. subtilis* Marburg membranes (0.5 mg) (■). It also shows phosphorylation of glucose in standard assay mixtures containing *C. acetobutylicum* soluble extract (0.7 mg of protein) (▲) and *C. acetobutylicum* soluble extract and *E. coli* 7 membranes (0.7 mg) (□). Membrane fractions alone had no phosphorylation activity. (B) Complementation of soluble extracts by *C. acetobutylicum* membranes. The figure shows phosphorylation of glucose in standard assay mixtures containing *C. acetobutylicum* membranes (0.2 mg of protein) (○), *C. acetobutylicum* membranes (0.8 mg) (▲), *C. acetobutylicum* membranes (0.2 mg) and *C. pasteurianum* soluble extract (1.5 mg) (●), *C. acetobutylicum* membranes (0.2 mg) and *B. subtilis* Marburg soluble extract (1.7 mg) (■), and *C. acetobutylicum* membranes (0.8 mg) and *E. coli* 7 soluble extract (0.5 mg) (□). Soluble extracts alone had no phosphorylation activity.

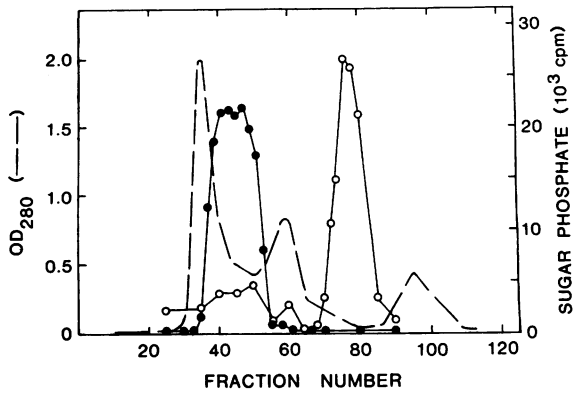

FIG. 3. Fractionation of *C. acetobutylicum* soluble extract by Sephadex G-100 gel filtration. A 136-mg portion of soluble protein in a volume of 10 ml was applied to the column, and samples (0.15 to 0.20 ml) of eluate fractions were assayed for sugar phosphorylation activity in a volume of 0.25 ml in the presence of *C. acetobutylicum* membranes (0.2 mg of protein; glucose as substrate) (○) and *B. subtilis* PG554 crude extract (1.1 mg of protein; methyl α-glucoside as substrate) (●). OD_{280}, optical density at 280 nm.

case, methyl α-glucoside was not phosphorylated, thus reflecting the substrate specificity of the *C. acetobutylicum* system. The PTS in *C. acetobutylicum* is therefore functionally compatible with other PTSs.

General PTS proteins. In *E. coli* and *B. subtilis*, two soluble proteins of the PTS, enzyme I and HPr, are required for uptake and phosphorylation of all substrates of the system. The above results (Fig. 2A) therefore suggest that both these proteins are present in the soluble fraction of *C. acetobutylicum*. The soluble fraction was subjected to gel filtration, which effectively separates the proteins owing to the large difference in their molecular weights. Assay of fractions in the presence of *C. acetobutylicum* membranes (Fig. 3) showed a low-molecular-weight activity peak in a position characteristic of HPr, as had been found previously for *C. pasteurianum* (15). The pool material was shown to stimulate phosphorylation of methyl α-glucoside by a crude extract of the *E. coli ptsH* mutant HK929, indicating the presence of HPr (data not shown).

The presence of enzyme I in the soluble fraction was demonstrated by complementation of a crude extract from the *B. subtilis ptsI* mutant PG554. When column fractions were assayed in the presence of this extract, a peak was found at a higher molecular weight, whereas the HPr-containing fractions did not give any activity. The pool material also complemented extracts prepared from the temperature-sensitive *ptsI* mutant PG587 when assayed at the nonpermissive temperature of 48°C (data not shown). Calibration of the column with bovine serum albumin (66,000), carbonic anhydrase (29,000), cytochrome *c* (12,400), and aprotinin (6,000) as markers allowed the estimation of the molecular weights of enzyme I and HPr as 64,000 and 12,500, respectively, values which are very similar to those of the corresponding proteins of *E. coli* and *B. subtilis* (4, 12, 20, 31). In fact, enzyme I and HPr activities in extracts of these two organisms eluted at the same positions as those from *C. acetobutylicum*.

Although enzyme I is present in the *C. acetobutylicum* soluble fraction, it is clearly not required for PTS function, since HPr alone is sufficient to complement membranes. The simplest explanation is that enzyme I is also present in the membranes. This was confirmed in two ways. First, HPr from *B. subtilis*, freed of enzyme I by fractionation of soluble extract on the Sephadex G-100 column, was sufficient to complement *C. acetobutylicum* membranes for phosphorylation of glucose (Fig. 4A). An enzyme I fraction from *B. subtilis*, on the other hand, did not stimulate phosphorylation significantly. Second, *C. acetobutylicum* membranes were shown to complement an extract of *B. subtilis* PG554 for phosphorylation of methyl α-glucoside (Fig. 4B). The rate of phosphorylation was low, although this may be explained by the separation of enzyme I and enzyme II in different membranes (only the *B. subtilis* enzyme II will phosphorylate methyl α-glucoside), an arrangement which could not be expected to be optimal for the transfer of phosphate from PEP to sugar. Thus, during preparation of extracts from *C. acetobutylicum*, enzyme I is distributed between the soluble and membrane fractions, while HPr is recovered only, or at least predominantly, in a soluble state.

Sugar-specific proteins. The properties of the membrane-bound enzyme II^{Glc} of *C. acetobutylicum* are reflected in the substrate specificity of sugar phosphorylation by the system (Fig. 1). The existence of a glucose-specific III protein was investigated by complementation of two *crr* mutants of *E. coli*, JLV86 and LM1, which are specifically defective for III^{Glc}. In each case, methyl α-glucoside was phosphorylated by a mixture of crude extracts from the mutant and *C.*

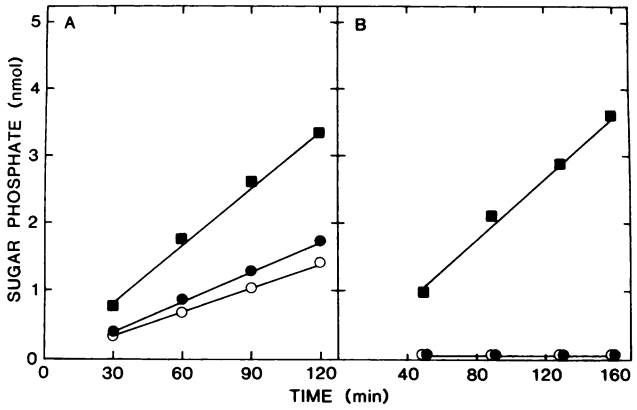

FIG. 4. Evidence for the presence of enzyme I in *C. acetobutylicum* membranes. (A) The figure shows phosphorylation of glucose by *C. acetobutylicum* membranes (0.4 mg of protein) supplemented with no addition (○), *B. subtilis* enzyme I (0.07 mg of protein) (●), and *B. subtilis* HPr (0.06 mg of protein) (■). The *B. subtilis* fractions alone had no activity. (B) The figure shows phosphorylation of methyl α-glucoside by *B. subtilis* PG554 crude extract (2.2 mg of protein) (●), *C. acetobutylicum* membranes (0.4 mg) (○), and *B. subtilis* PG554 crude extract and *C. acetobutylicum* membranes.

acetobutylicum (Fig. 5). Activity was retained in the *C. acetobutylicum* soluble fraction, and after gel filtration a small peak was observed with an apparent molecular weight of approximately 25,000, similar to the III^{Glc} protein in enteric bacteria (14, 20). A combination of peak fractions containing enzyme I and HPr did not stimulate methyl α-glucoside phosphorylation by the *crr* mutant extracts (data not shown). Once again, the fact that HPr is sufficient to complement *C. acetobutylicum* membranes is indicative of the presence of III^{Glc} in these membranes, but attempts to demonstrate this directly by complementation of extracts of *E. coli* JLV86 were unsuccessful.

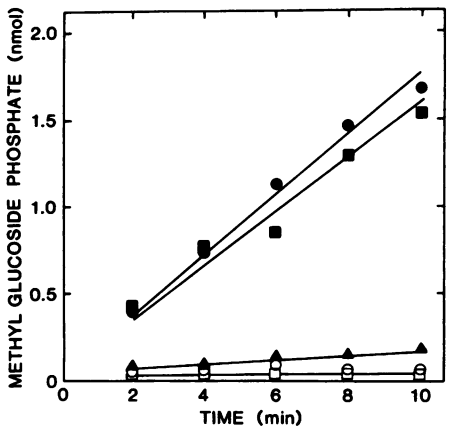

FIG. 5. Evidence for glucose-specific III protein in *C. acetobutylicum*. The figure shows phosphorylation of methyl α-glucoside in standard assay mixtures containing *E. coli* JLV86 crude extract (2.1 mg of protein) (○), *E. coli* LM1 crude extract (1.9 mg) (□), *C. acetobutylicum* crude extract (2.2 mg) (▲), *E. coli* JLV86 and *C. acetobutylicum* crude extracts (●), and *E. coli* LM1 and *C. acetobutylicum* crude extracts (■).

DISCUSSION

The glucose PTS of *C. acetobutylicum* exhibits affinity for mannose and the analog 2-deoxyglucose and thus belongs to a family of PTSs in gram-positive bacteria, particularly among the streptococci, which have a similar substrate specificity (7, 28). The system in extracts has been shown to consist of both soluble and membrane-bound components, which are functionally similar to the PTS proteins of other bacteria, whereas the enzyme I, HPr, and III^{Glc} components are of similar size to the corresponding proteins of other systems. Thus, at least superficially, the *C. acetobutylicum* PTS appears to have the same molecular architecture as that of previously characterized PTSs. As an increasing number of PTS gene and protein sequences have been established, it has become clear that some components from different bacteria, both gram positive and gram negative, show considerable homology (6, 13, 26), and this has led to the proposal that the systems recognized today arose from a single system present in a primordial ancestor of these two groups of organisms (23). This is reflected in the ability of proteins from different bacteria to interact functionally in vitro and also immunologically. There are, however, significant differences between proteins in gram-positive and gram-negative systems, and although the components in *E. coli* and *S. typhimurium* are fully cross-reactive and several examples of interaction among gram-positive bacteria have been reported (3, 6, 10), interactions between PTS components from gram-positive and gram-negative bacteria are generally poor (10, 27). Nevertheless, the interaction in vivo has proved efficient enough to enable the *pts* genes of *B. subtilis* to be cloned in *E. coli* by functional complementation of *pts* mutants (4). The fact that in vitro interactions have been demonstrated between the PTS components of *C. acetobutylicum* and *E. coli* suggests that a similar strategy will be successful for cloning of the clostridial *pts* genes.

In an earlier study of the PTS in *C. pasteurianum* (15), a soluble, low-molecular-weight HPr-like protein was shown to be sufficient to reconstitute PTS activity in the presence of membranes, and it was proposed that enzyme I, if present, was in the membrane fraction. Here we have provided direct evidence for an enzyme I in *C. acetobutylicum* by complementation of a *ptsI* mutant of *B. subtilis*. The experiment whose results are illustrated in Fig. 3 has been repeated by using a soluble extract of *C. pasteurianum*, with identical results. Thus, in each of the two clostridia, enzyme I is present in both the soluble and membrane fractions. The distribution of proteins between soluble and membrane fractions in cell extracts is recognized to be a consequence of the methods used in extract preparation, and the fact that a protein is found in the soluble (cytoplasmic) fraction need not necessarily mean that it exists free in the cytoplasm in the intact cell. Although the PTS of several bacteria has been shown to fractionate into soluble and membrane-bound proteins, evidence has been presented that all the components may exist together as a membrane-bound complex in vivo (21), and in at least one case all PTS components are found predominantly in the membrane fraction (25). In *C. acetobutylicum*, the distribution of enzyme I and apparently also of III^{Glc} between soluble and membrane fractions may indicate that the PTS proteins are normally associated with the membrane in the cell. Furthermore, some membrane preparations do appear to contain HPr also, since they support a low rate of glucose phosphorylation (Fig. 4A), lending further support to the notion of a membrane-associated PTS complex. The HPr is removed by washing the

membranes with 50 mM KCl, yielding membranes which are stimulated in the normal way by the HPr-containing gel filtration pool. Further extraction of membranes with up to 0.5 M KCl did not remove any more PTS components, suggesting that these are much more tightly associated with the membrane.

In terms of the number of identified substrates, the PTS represents a major route of carbohydrate accumulation in the saccharolytic clostridia (1, 17, 30; unpublished results), and it is now clear that the clostridial PTS is related both structurally and functionally to the PTS in other bacteria. Further characterization of the system and of its interaction with other cellular activities is required for an evaluation of its potential role in the regulation of carbohydrate metabolism.

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