Journal of Cellular Biochemistry 18:231-238 (1982)

Evidence for the Functional Association of Enzyme I and HPr of the Phosphoenolpyruvate-Sugar Phosphotransferase System With the Membrane in Sealed Vesicles of Escherichia coli

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Several independent assay procedures were used to estimate the activities of the enzyme constituents of the phosphoenolpyruvate-sugar phosphotransferase system (PTS) in osmotically shocked bacterial membrane vesicles. The soluble enzymes of the system were found to be in association with the membrane by several criteria. Phosphoenolpyruvate-dependent sugar phosphorylation was catalyzed by this membrane-bound enzyme system far more efficiently than by a mixture of the individual enzymes at corresponding concentrations. By contrast, the rates of the phosphoryl exchange reactions catalyzed by enzyme I and the enzyme II complexes were essentially the same for the associated and dissociated forms of the system. Functional association of the PTS-enzyme complex was stabilized by Mg^{++} and phosphoenolpyruvate and could be destroyed by detergent treatment, sonication, or by passage of the vesicle preparation through a French pressure cell. These results lead to the possibility that in the intact bacterial cell the soluble enzymes of the phosphotransferase system exist, in part, as peripheral membrane constituents associated with the integral membrane enzyme II complexes.

Key words: peripheral membrane proteins, sugar transport, energy coupling, bacteria, phosphotransferase system, osmotic shock, membrane vesicles, protein-protein interactions

The bacterial phosphoenolpyruvate-sugar phosphotransferase system (PTS) catalyzes the concomitant transport and phosphorylation of its sugar substrates with phosphoenolpyruvate as the phosphoryl donor [1]. This reaction requires the participation of the soluble energy coupling proteins of the PTS (enzyme I, HPr and in some cases, a sugar-specific enzyme III) as well as a sugar specific integral membrane enzyme II complex. Kaback studied the PTS-catalyzed uptake of [^{14}C]methyl α-glucoside into membrane vesicles isolated from wild type

Abbreviations: PTS, phosphoenolpyruvate-sugar phosphotransferase system; HPr, heat stable phosphoryl carrier protein of the PTS.

Received July 29, 1981; accepted September 13, 1981.

0730-2312/82/1802-0231$02.50

Escherichia coli cells and concluded that phosphoenolpyruvate served as the energy source for group translocation of sugar [2]. Little or no uptake occurred in vesicles derived from a mutant strain that lacked enzyme I of the PTS. Since the vesicles had been subjected to a rigorous osmotic shock procedure, most of the soluble constituents were absent from these preparations. Kaback therefore proposed that a fraction of the cellular enzyme I and HPr was associated with the cytoplasmic membrane and that the association of these soluble proteins with the membrane accounted for their activities in the shocked vesicle preparations in promoting sugar uptake.

In previous communications from this laboratory, it was shown that enzyme I and the membrane-bound enzyme II complexes catalyzed phosphoryl exchange reactions [3,4]. These reactions allowed the development of assays for these proteins that were independent of the other protein constituents of the system. In this paper, we utilize these assay procedures, as well as those for the transport and phosphorylation of sugars with phosphoenolpyruvate as phosphoryl donor, to gain information about the possible interactions that occur between the proteins of the PTS. These studies were prompted by the finding that in an evolutionarily divergent bacterium, Ancalomicrobium adetum, all of the proteins of the PTS were membrane bound [5]. Evidence is presented that a fraction of the soluble enzymes of the E coli phosphotransferase system exists in bacterial vesicles in association with the cytoplasmic membrane. The functional complex of PTS enzymes can be destroyed by detergent treatment, sonication, or passage through a French pressure cell, but repeated osmotic shock does not cause release, eliminating the possibility of intravesicular trapping of the soluble enzymes. The partial reactions catalyzed by enzyme I and enzyme II are not appreciably dependent on these protein associations. We suggest that phosphoenolpyruvate-dependent sugar physphorylation is catalyzed far more efficiently by a membrane-bound multi-PTS enzyme complex than by a mixture of the separated enzymes at corresponding concentrations.

METHODS

Most compounds used in the present study were prepared or purchased as described previously [3,4]. [^{14}C]Phosphoenolypyruvate was purchased from Amersham/Searle and was used at a specific activity of 0.5 mCi/mmole. [^{14}C]Sugars were used at specific activities between 5 and 200 mCi/mmole.

E coli strain ML308 was used throughout these studies. Cells were harvested during exponential growth for preparation of membrane vesicles as described [6]. Vesicles were loaded with phosphate donors by the Ca^{++} EDTA osmotic shock procedure [6]. Because different vesicle preparations showed appreciable variation with respect to uptake activity and sensitivity to various treatments [6], results should be compared only within a series of experiments conducted with a single vesicle preparation.

Phosphoenolpyruvate-dependent sugar phosphorylation and the various partial reactions of the PTS were measured with intact vesicles as the enzyme source before or after the treatments described in the legends to the tables and figures. Activities were linear with time and enzyme concentration under the conditions employed. Unless otherwise stated, the assay mixtures were as follows: 1) enzyme

I catalyzed exchange between [^{14}C]phosphoenolpyruvate and pyruvate: 100 mM potassium phosphate buffer, pH 7.0; 20 mM KF; 10 mM $MgCl_2$; 0.2 mM [^{14}C] phosphoenolpyruvate; 2 mM pyruvate; incubation time: 30 min at 37°C; final volume, 100 μl. [^{14}C]Pyruvate was determined as described [4]. 2) Phosphoenolpyruvate-dependent phosphorylation of [^{14}C]methyl α-glucoside: 100 mM potassium phosphate buffer, pH 7.0; 20 mM KF; 10 Mm $MgCl_2$; 2 mM dithiothreitol; 50 mM phosphoenolpyruvate; 25 μM [^{14}C]methyl α-glucoside (5 mC/mmole); incubation time: 30 min at 37°C; final volume, 200 μl. A preparation of soluble proteins (0.5 mg protein/ml) from a crude extract of wild type E coli cells was used as a source of extravesicular enzyme I and HPr [7]. 3) enzyme II^{glc} catalyzed transphosphorylation: conditions were as described above for the phosphoenolpyruvate-dependent sugar phosphorylation reaction except that the phosphoryl donor was 10 mM glucose-6-phosphate [3]. 4) [^{14}C]methyl α-glucoside uptake was measured as described previously [6] with the incubation temperature at 28°C. Aliquots were removed for filtration and washing 2 and 4 min after addition of the radioactive sugar.

RESULTS

Sonicated whole cell extracts and sonicated membrane vesicles were assayed for the enzymes of the PTS and for glucokinase to ascertain the percentages of these enzymes that were lost during the osmotic shock procedure. Relative activities for enzyme I, HPr, enzyme III^{glc}, enzyme II^{glc}, and glucokinase (expressed in % recovery of vesicles as compared with whole cells), respectively, were as follows: 21%, 12%, 28%, 185%, and 4%. The greater than 100% recovery of enzyme II^{glc} activity presumably resulted from loss of sugar phosphate phosphatase during osmotic shock. The results showed that all of the PTS enzymes were retained by the vesicles to a far greater extent than was the typical soluble enzyme, glucokinase.

Table I summarizes the effects of sonication on the rates of the various reactions catalyzed by the PTS enzymes in membrane vesicles of E coli strain ML308. Sonication did not appreciably reduce the rates of the enzyme I-catalyzed phosphoenolpyruvate-pyruvate phosphoryl exchange reaction, the enzyme II-catalyzed transphosphorylation reaction, or the phosphoenolpyruvate-dependent sugar phosphorylation reaction under conditions where enzyme II^{glc} was present in limiting amounts. By contrast, phosphoenolpyruvate-dependent sugar phosphorylation and uptake by isolated membrane vesicles were effectively reduced to low values. This treatment was shown to fragment the vesicles and release more than 80% of the vesicular enzyme I in a soluble form, as revealed after high-speed centrifugation of the membrane fragments. Similar results were obtained when the vesicles were passed through a French pressure cell (data not shown). None of these reaction rates were appreciably reduced when the vesicles were subjected to repeated osmotic shock, suggesting that the cytoplasmic PTS enzymes were not soluble in the intravesicular compartment, but were membrane associated.

Figure 1 summarizes the effects of two detergents (sodium dodecyl sulfate, Fig. 1A and Triton X-100, Fig. 1B) on four PTS-catalyzed reactions. Low detergent concentrations reduced the extent of sugar uptake under conditions that did not alter the enzyme I- and enzyme II-catalyzed phosphoryl exchange reactions.

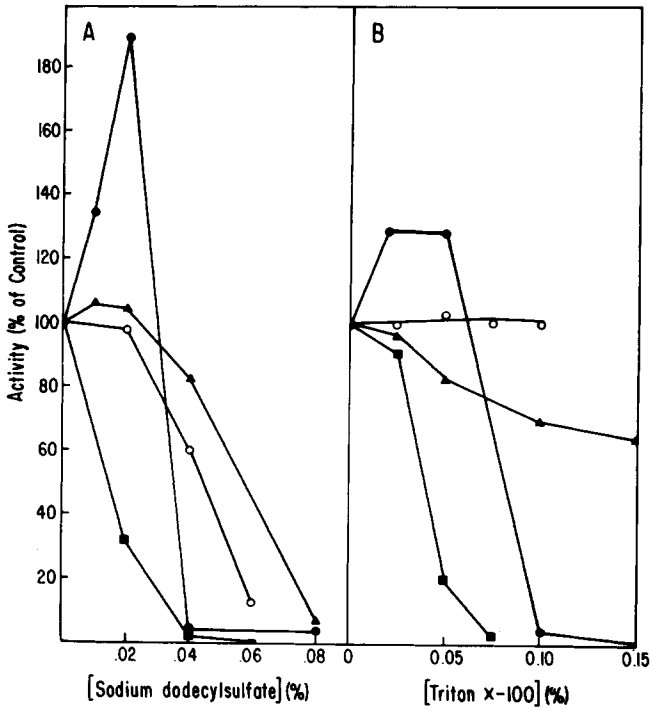

Fig. 1. Effects of detergents on reactions catalyzed by the phosphotransferase system in membrane vesicles of E coli strain ML308. Membrane vesicles (25μl, 10 mg protein/ml) were incubated at 37° for 1 h in the presence of sodium dodecyl sulfate (A) or Triton X-100 (B) at the concentration indicated on the abscissa. Subsequently, the vesicles were diluted 20-fold for enzyme assays as described under Methods. Phosphorylation assays were conducted for 30 min at 37°C. Uptake of [^{14}C]methyl α-glucoside was measured after a 4-min incubation of the vesicles with the radioactive substrate at 28°C. ●, phosphoenolpyruvate-dependent phosphorylation of [^{14}C]methyl α-glucoside; ▲, glucose-6-phosphate-dependent [^{14}C]methyl α-glucoside; transphosphorylation; ○, enzyme I-catalyzed [^{14}C]phosphoenolpyruvate-pyruvate exchange; ■, [^{14}C]methyl α-glucoside uptake.

These same low concentrations stimulated the phosphoenolpyruvate-dependent sugar physphorylation reaction. Both the stimulation of phosphorylation and the inhibition of uptake were presumably due to disruption of the membrane permeability barrier (see below).

High concentrations of sodium dodecyl sulfate (but not of Triton X-100) strongly inhibited the enzyme I- and enzyme II-catalyzed phosphoryl transfer reactions. However, at moderate concentrations of dodecyl sufate (0.04%) or Triton X-100 (0.10%) the phosphoenolpyruvate-dependent sugar phosphorylation reaction was selectively abolished; the two phosphoryl exchange reactions occurred at nearly normal rates at these detergent concentrations. In these experiments, the presence of 0.10% Triton X-100 solubilized approximately 50% of the vesicular enzyme I but none of the enzyme II.

Table II summarizes the effects of $MgCl_2$, phosphoenolpyruvate, and EDTA on the ease with which detergents destroyed the activity of the PTS enzyme complex. It can be seen that Mg^{++} and phosphoenolpyruvate apparently stabilized

TABLE I. Effect of Sonication on Reactions Catalyzed by the Phosphotransferase System in Membrane Vesicle of E coli Strain ML308*

Reaction measured	Remaining activity after sonication (%)
Enzyme I catalyzed exchange (PEP ↔ pyruvate)	88
Enzyme II transphosphorylation (glucose-6-P → [^{14}C]methyl α-glucoside)	77
PEP-dependent phosphorylation (PEP) → [^{14}C]methyl α-glucoside)	21
PEP-dependent phosphorylation + soluble PTS enzymes (PEP) → [^{14}C]methyl α-glucoside)	143
Transport ([^{14}C]methyl α-glucoside uptake)	7

*Vesicles were prepared from E coli strain ML308. Both uptake and phosphorylation of [^{14}C]methyl α-glucoside (5 μM, specific activity, 125 mCi/mmole) were measured as described for uptake measurements under Methods. Vesicles were sonicated with a sonifier Cell Disrupter (Branson Sonic Power Co), operating at the microtip limit, for 1 min at 0°C. When indicated, soluble proteins from a crude extract of wild type E coli cells were added to a final concentration of 0.5 mg protein/ml.

TABLE II. Effects of Phosphoenolpyruvate, $MgCl_2$, and EDTA on the Stability of the Phosphoenolpyruvate-Dependent [^{14}C]Methyl α-Glucoside Phosphorylating Activity in Membrane Vesicles From E coli Strain ML308 Following Detergent Treatment*

	(%) Activity remaining after treatment with	
Addition	0.04% Sodium dodecyl sulfate	0.05% Triton X-100
10 mm $MgCl_2$	66	98
10 mM $MgCl_2$ + 50 mM PEP	127	116
5 mM EDTA	3	39

*Vesicles were preincubated with detergent in the presence of the agents indicated above as described in Figure 1. Subsequently, the preparations were diluted 20-fold for assay of phosphoenolpyruvate-[^{14}C]methyl α-glucoside phosphorylating activity as detailed under Methods. The concentration of detergent that effectively reduced phosphoenolpyruvate-dependent sugar phosphorylating activity depended on the membrane preparation under study and the concentration of membrane protein.

the complex, while EDTA, which chelates divalent cations, had the opposite effect, regardless of the detergent under study. Phosphoenolpyruvate and Mg^{++} did not stabilize the vesicles to disruption by sonication (data not shown).

Figure 2 summarizes results that compare the effects of Triton X-100 on the rates of phosphoenolpyruvate-dependent sugar phosphorylation when the vesicles were preincubated with the detergent at either 37°C or 0°C. Preincubation of the vesicles at 37°C with Triton X-100 at a concentration in excess of 0.04% reduced the rate of sugar phosphorylation much more than when the preincubation was conducted at 0°C. Low detergent concentrations were stimulatory. These results suggest that part (and probably most) of the inhibitory effect of the detergent was due to a time- and temperature-dependent protein-detergent interaction and did not result from inclusion of detergent in the assay solution.

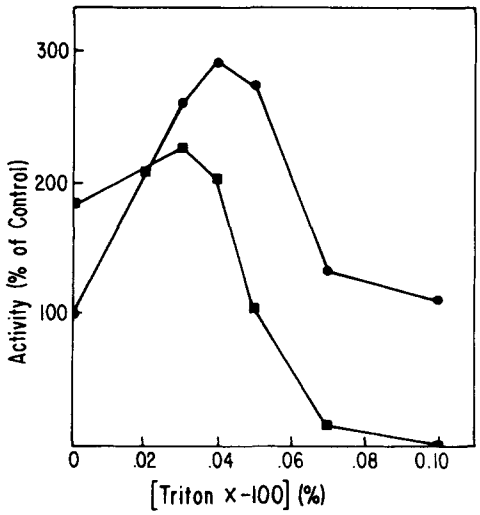

Fig. 2. Effect of preincubation with Triton X-100 on the loss of phosphoenolpyruvate-dependent [^{14}C]methyl α-glucoside phosphorylation activity. The experimental design was as described in the legend to Figure 1. ■, vesicles were preincubated with detergent for 1 h at 37°C before assay; ●, vesicles were preincubated for less than 15 min with detergent at 0°C before assay.

If the stimulatory effect of low detergent concentrations on the rate of phosphoenolpyruvate-dependent sugar phosphorylation was due to an increase in membrane permeability, then toluene, which is known to permeabilize bacterial membranes without destroying PTS function [9], should elicit a similar response. The results depicted in Figure 3 verify this prediction. Toluene greatly stimulated phosphoenolpyruvate-dependent phosphorylation of methyl α-glucoside without appreciably enhancing the rates of enzyme I- or enzyme II-catalyzed phosphoryl exchange. Concentrations of toluene in excess of 0.2% were mildly inhibitory under the conditions employed.

DISCUSSION

In 1968, Kaback published his classic paper on the involvement of the bacterial phosphotransferase system in the transport of sugars into isolated membrane vesicles [2]. He showed that, in the presence of phosphoenolpyruvate, sugars such as methyl α-glucoside were accumulated intravesicularly as the phosphate esters. Since the vesicles had been prepared by an osmotic shock treatment, which resulted in the release of the soluble proteins, and since vesicles derived from an enzyme I-negative mutant could not accumulate the sugars, it was suggested that the soluble protein constituents of the PTS might be associated with the cytoplasmic membrane [2]. Our interest in this possibility was revived when we characterized a phosphotransferase system in the unusual star-shaped bacterium, Ancalomicrobium adetum [5]. While the PTS in this organism appeared to be similar in several respects to that found in E coli, both enzyme I and HPr were localized primarily to the membrane fraction. The solubilization of these proteins

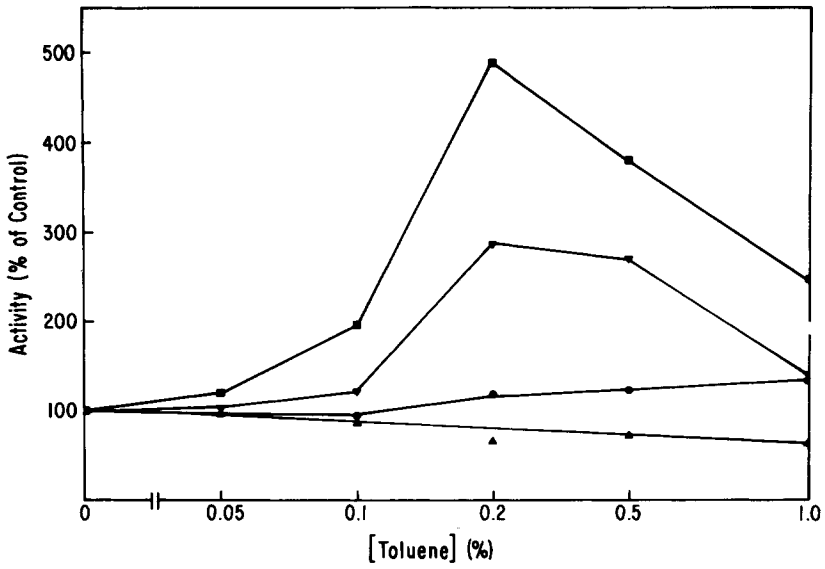

Fig. 3. Effect of toluene on the different reactions catalyzed by the phosphotransferase system in E coli membrane vesicles. Toluene was added to concentrated membrane vesicle suspensions at 0°C to the final concentration indicated on the abscissa. The preparations were mixed vigorously and then diluted 20-fold for assay of enzyme activity at 37°C as described under Methods. Values are plotted relative to the controls, which lacked toluene. ■,Phosphoenolypyruvate-dependent phosphorylation of [^{14}C]methyl α-glucoside by E coli membrane vesicles;▼, phosphoenolpyruvate-dependent phosphorylation of [^{14}C]methyl α-glucoside in the presence of 0.5 mg/ml soluble proteins from wild type E coli as a source of enzyme I and HPr; ●, enzyme I-catalyzed phosphoenolpyruvate-pyruvate exchange; ▲, enzyme II-catalyzed glucose-6-phosphate: [^{14}C]methyl α-glucoside transphosphorylation. The control rates of these four reactions were 2.6, 4.5, 9.1, and 7.3 nmoles of product formed per min per mg protein at 37°C respectively.

from the membrane required extraction with butanol and urea [7]. The fructose-specific phosphotransferase system in Rhodospirillum rubrum was similarly found to be membrane associated, but a peripheral membrane protein constituent of the system could be released in a soluble form by repeated extraction of the membranes with a salt-free solution [10]. These observations suggested that enzyme I and HPr might be peripheral membrane proteins easily released from the membranes of most bacteria by the procedures used to disrupt the cells.

In this communication, evidence is presented that enzyme I and HPr do, in fact, form a functional complex with the membrane in isolated, osmotically shocked vesicle preparations. These associations are apparently stabilized by divalent cations and phosphoenolpyruvate and are destabilized by exposure to divalent cation chelating agents and detergents. The catalytic complex is also destroyed by sonication or by the sheering forces that result when the vesicles are passed through a French pressure cell. Since all of these treatments are relatively mild, it is clear that the forces holding enzyme I and HPr to the membrane are not strong.

The results and conclusions reported in this communication provide an explanation for the high rates of sugar transport catalyzed by the PTS in membrane vesicles in spite of the fact that large proportions of the cellular enzyme I, HPr,

and enzyme III^{glc} are lost from the cytoplasmic fraction during vesicle preparation [2,6]. They also provide confirmatory evidence for the suggestion of Gachelin that an organized complex of PTS enzymes on the cytoplasmic surface of the membrane explains the high rate of PTS-catalyzed sugar phosphorylation in toluenized cells as compared with disrupted cell extracts [9]. That phosphoenolpyruvate must be present intravesicularly to stimulate transport has been established [11], and Owen and Kaback have shown that virtually all of the vesicles in their preparations are sealed and right side out [12]. The results reported in this communication and the previous reports [2,9,11] therefore provide substantial evidence for a membrane-associated complex of PTS proteins that functions in an ordered, 3-dimensional phosphoryl transfer sequence, coupled efficiently to sugar uptake in intact E coli cells.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grant 1 RO1 AI 14176-01 from the National Institute of Allergy and Infectious Diseases.

REFERENCES

1. Postma PW, Roseman S: Biochim Biophys Acta 457:213–257, 1976.
2. Kaback HR: J Biol Chem 243:3711–3724, 1968.
3. Saier MH Jr, Feucht BU, Mora WK: J Biol Chem 252:8899–8907, 1977.
4. Saier MH Jr, Schmidt MR, Lin P.: J Biol Chem 255:8579–8584, 1980.
5. Saier MH Jr, Staley JT: J Bacteriol 131:716–718, 1977.
6. Saier MH Jr, Cox DF, Moczydlowski EG: J Biol Chem 252:8908–8916, 1977.
7. Kundig W, Roseman S: J Biol Chem 246:1407–1418, 1971.
8. Saier MH Jr, Schmidt MR: J Bacteriol 145:391–397, 1981.
9. Gachelin G: Biochem Biophys Res Commun 34:382–386, 1969.
10. Saier MH Jr, Feucht BU, Roseman S: J. Biol Chem 246:7819–7821, 1971.
11. Saier MH Jr, Feucht BU: J Bacteriol 141:611–617, 1980.
12. Owen P, Kaback HR: Proc Natl Acad Sci USA 75:3148–3152, 1978.