Purification of the Mannitol-specific Enzyme II of the Escherichia coli Phosphoenolpyruvate:Sugar Phosphotransferase System*

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SUMMARY

The inducible, mannitol-specific Enzyme II of the phosphoenolpyruvate:sugar phosphotransferase system has been purified approximately 230-fold from Escherichia coli membranes. The enzyme, initially solubilized with deoxycholate, was first subjected to hydrophobic chromatography on hexyl agarose and then purified by several ion exchange steps in the presence of the nonionic detergent, Lubrol PX. The purified protein appears homogeneous by several criteria and probably consists of a single kind of polypeptide chain with a molecular weight of 60,000 (±5%). In addition to catalyzing phosphoenolpyruvate-dependent phosphorylation of mannitol in the presence of the soluble enzymes of the phosphotransferase system, the purified Enzyme II also catalyzes mannitol 1-phosphate:mannitol transphosphorylation in the absence of these components. A number of other physical and catalytic properties of the enzyme are described. The availability of a stable, homogeneous Enzyme II should be invaluable for studying the mechanism of sugar translocation and phosphorylation catalyzed by the bacterial phosphotransferase system.

A number of sugars are simultaneously transported and phosphorylated in Escherichia coli and many other prokaryotes via a phosphoenolpyruvate:sugar phosphotransferase system first described by Rosenman and co-workers (1). Extensive biochemical and genetic investigations of the PTS, summarized in several recent reviews (2-4), have shown that at least three proteins are involved in the uptake and phosphorylation steps:

Phosphoenolpyruvate + HPr\[\text{Enzyme I}\] \[\rightarrow\] \[\text{Mg}^{2+}\] Phospho-HPr + pyruvate

Phospho-HPr + sugar \[\text{Enzyme II complex}\] \[\rightarrow\] \[\text{Enzyme III}\] sugar-P + HPr

Enzyme I and HPr are soluble, general components of the system, both of which have been purified from E. coli (5, 6). Enzyme II complexes are tightly associated with the membrane and function as the sugar-specific recognition components of the PTS (7). In addition, at least some substrates of the PTS also require another sugar-specific protein for their transport, Enzyme III, which may be either membrane-associated or soluble, and is itself phosphorylated by phospho-HPr during the reaction (2, 7, 9). Recently, it has also been shown that each of the Enzyme II complexes catalyzes a sugar-phosphate:sugar phosphotransfer reaction which is independent of the soluble components of the PTS (9-11):

\[\text{[\text{C}}^\text{14}]\text{sugar} + \text{sugar-P} \xrightarrow{\text{Enzyme II}} \text{[\text{C}}^\text{14}]\text{sugar-P} + \text{sugar}\]

In whole cells and in membrane vesicles, this reaction can be shown to occur vectorially such that external sugar is taken up and phosphorylated at the expense of internal sugar-P (11, 12). Thus, an Enzyme II complex can apparently catalyze both active group translocation and exchange group translocation (4).

In order to investigate the mechanisms of these transport reactions in detail, it is necessary to have a highly purified, stable Enzyme II. In this communication, we report the purification to apparent homogeneity of the n-mannitol-specific Enzyme II from E. coli. The purified enzyme has a poly peptide chain molecular weight of 60,000 and catalyzes both phosphoenolpyruvate- and mannitol-1-P-dependent phosphorylation of n-mannitol under appropriate conditions. Our preliminary characterization of Enzyme II shows that it should be suitable for detailed catalytic and physicochemical studies.

EXPERIMENTAL PROCEDURES

Materials—Hexyl agarose and &-aminohexyl agarose were from Miles. DEAE-cellulose was a product of Eastman. Lubrol PX (polyoxyethylene derivatives of lauryl alcohol) and sodium deoxycholate were products of Sigma. The latter was recrystallized at least three times from acetone-H$_2$O (5:1, v/v). Mannitol 1-phosphate (Sigma, barium salt) was converted to the sodium salt by passage through a column of Bio-Rad AG 50-X8 (sodium form). n-[^14]C]Mannitol was a product of New England Nuclear Corp. Other compounds were obtained from commercial sources and were of the highest grade available.

Assays—Activities of Enzyme II were assayed as follows. Phosphoenolpyruvate-dependent sugar phosphorylation was determined in a total volume of 100 μl containing 25 mM Tris-HCl, pH 9.1, 5 mM MgCl$_2$, 10 mM KF, 1 mM dithiothreitol, 10 mM phosphoenolpyruvate, 0.1 mM [¹⁴C]mannitol (5 μCi/μmol), and saturating amounts of the soluble enzymes of the PTS (HPr and Enzyme I). The size of the sample to be assayed was chosen such that Enzyme II was present in rate-limiting amounts. Mixtures were incubated for 30 min at 37°C; the reaction was stopped by addition of 5 μl of 1 M nonradioactive mannitol, and 20-μl aliquots were then placed on 2.4-cm discs of DEAE-filter paper (Whatman, DE81) (13, 14). The filters were air-dried, and each was washed with 50 ml of H$_2$O in a vacuum filtration
Homogeneous Enzyme $I_{II}^{"}$

Apparatus. After drying for 10 min on a hotplate, the filters were counted in 5 ml of a toluene-based scintillation fluid. Mannitol-1-P: mannitol transphosphorylation was measured as previously described (10) by the Dowex 1-X2 resin column procedure (5). When purified Enzyme $I_{II}^{"}$ was assayed for either activity, Lubrol PX was present after a dilution of the stock enzyme solution (0.025 to 0.05%, final concentration).

Analytical Procedures—Protein was estimated according to Lowry et al. (15) by the bovine serum albumin as the standard. Solutions containing Lubrol PX gave a precipitate in this procedure. Therefore, samples were centrifuged before reading, and appropriate blanks were run. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate was performed according to Laemmli (16) in 1-mm-thick slabs. Some samples were concentrated by lyophilization after dialysis against 5 mM NH$_4$HCO$_3$ containing 1 mM $\beta$-mercaptoethanol, before being loaded on the gel. Electrophoresis in gels containing Lubrol PX was at 4°C in 6% polyacrylamide slab gels (2.5 x 100 cm) containing 1% Ampholytes (Bio-Rad, pH 3 to 10), 0.5% Lubrol PX, and the protein sample. Gels also contained N,N',N'-tetramethylethylenediamine (0.1%), and flavin mononucleotide (2 $\mu$g/ml) and were photographized. All gels were stained for protein with Coomassie blue. Gel staining was performed after electrophoresis. The gels were sliced, and the stained bands were eluted with 6M GnHCl. The solution was then applied to a DEAE-cellulose column (5). When purified Lubrol PX was at 4°C in 6% polyacrylamide tube gels (2.5 x 100 cm) containing 1% Ampholytes (Bio-Rad, pH 3 to 10), 1 mM dithiothreitol, 0.5% Lubrol PX, and the buffer. The solution was then applied to a DEAE-cellulose column (Fig. 1B) and $\alpha$-aminohexyl agarose (Fig. 1C) resulted in an additional 3- to 4-fold purification. After rechromatography on a small $\alpha$-aminohexyl agarose column, the enzyme had been purified approximately 235-fold relative to crude E. coli membranes. Table I summarizes a typical purification.

The results of polyacrylamide gel electrophoresis in the presence of dodecyl sulfate of samples taken after each purification step are shown in Fig. 2A. After the final step, only a single band, apparent $M_r = 60,000$ (±5%), was visible on the gel. In contrast, if membranes from stationary phase cells were used as starting material, two additional bands ($M_r = 29,000$ and 28,000) co-purified with the larger component (Fig. 2A, Panel 7). This result suggests that proteases present in stationary phase cells may partially cleave the native protein during purification. For this reason, only membranes from midexponential phase cells were used to purify Enzyme $I_{II}^{"}$.

To confirm that the major electrophoretic band in our purified preparation was indeed Enzyme $I_{II}^{"}$, a nondenaturing gel in the presence of Lubrol PX was run. Again, a single band was obtained, and elution of the enzyme from a parallel, unstained gel showed that Enzyme $I_{II}^{"}$ activity co-migrated with this band (Fig. 2B).

Finally, an isoelectric focusing gel in Lubrol PX gave a single major band, although several minor

\[ \text{FIG. 1. Purification of Enzyme $I_{II}^{"}$ by hydrophobic and ion exchange chromatography. Specific conditions for each column are given under "Experimental Procedures." Phosphoenolpyruvate-dependent phosphorylation of mannitol (●●●●) and absorbance at 280 nm (□□□□) are plotted in each case. The bar (-----) indicates fractions pooled for each column. A, hexyl agarose column run in extraction buffer containing 0.5% deoxycholate. Fractions 1 to 4 represent the flow-through and three washes, 50 ml each. Subsequent fractions were 5 ml each. Enzyme $I_{II}^{"}$ activity was eluted with 0.5% Lubrol PX (arrow). B, DEAE-cellulose chromatography in the presence of Lubrol PX (0.5%). Protein was eluted with a gradient from 0 to 0.3 M NaCl (---). C, hexyl agarose column in the presence of 0.5% Lubrol PX. Fractions were diluted 2-fold before being assayed. Activity was again eluted with a salt gradient (---). The flow-through and washes in B and C have been omitted from the profiles since these contained little protein and no Enzyme $I_{II}^{"}$ activity.} \]
bands close to it on the acidic side were evident (Fig. 2C). The major component was slightly acidic with a pI of 6.2. The minor bands possibly represented modified Enzyme II\textsuperscript{m1}'s or related proteins, for example, by oxidation or deamidation during purification or electrofocusing. It seems unlikely that they were contaminants consisting of unrelated proteins since single bands were obtained in the two other gel systems. Judging from these experiments, we estimate that the protein was >95% homogeneous and that it consisted of a single kind of polypeptide chain.\textsuperscript{5} The latter conclusion, however, must await confirmation by other physicochemical methods.

We have also examined several catalytic properties of the purified protein. Phosphoenolpyruvate-dependent sugar phosphorylation was not detected with fructose, mannose, or methyl-\alpha-glucoside as substrates. Sorbitol (D-glucitol) did serve as a substrate with a velocity at 0.1 mM equal to about 3% of that observed with mannitol.\textsuperscript{3,4} Purified Enzyme II\textsuperscript{m1}'s like the activity in crude membranes (10), was very sensitive to sulfhydryl reagents. Phosphoenolpyruvate-dependent activity was rapidly lost during exposure of the enzyme to N-ethylmaleimide. Less than 10% of the original activity remained after a 10-min incubation at 25°C in 20 mM Tris-HCl buffer, pH 8.4, containing 0.1 mM dithiothreitol, 0.5% Lubrol PX, and 0.5 mM N-ethylmaleimide. This result indicated that at least one free sulfhydryl group was necessary for catalytic function.

Biochemical and genetic investigations have provided evidence suggesting that Enzyme II\textsuperscript{m1} complexes of the PTS are responsible for the sugar-P:sugar transphosphorylation reactions detected in intact bacterial cells and isolated membranes (10). We have confirmed this conclusion with purified Enzyme II\textsuperscript{m1} which we have found to catalyze phosphoryl transfer from mannitol-1-P to [\textsuperscript{14}C]mannitol in the absence of the soluble PTS enzymes. The pH-activity profiles of this reaction for both unfractionated membranes and the purified protein gave maxima near pH 7. In contrast, purified Enzyme II\textsuperscript{m1} exhibited two maxima, near pH 7 and pH 9, in the phosphoenolpyruvate-dependent phosphorylation of mannitol (not shown). This was probably due to contributions to the shape of the (subunit $M_1$ = 40,000) only after induction with mannitol and cAMP. However, attempts to determine the quaternary structure of the protein have so far been unsuccessful. For example, gel filtration on Sepharose 6B in the presence of Lubrol PX gave an apparent molecular weight greater than 700,000 suggesting that the enzyme may be included in detergent micelles (unpublished).

We have no evidence for a mannitol-specific Enzyme III that might be required for phosphoenolpyruvate-dependent mannitol phosphorylation. Purified Enzyme II\textsuperscript{m1} and partially purified Enzyme I and HPr (5, 6), both free of glucose-specific Enzyme III activity, are sufficient to catalyze this reaction. Thus, Enzyme II\textsuperscript{m1} may interact directly with phospho-HPr during mannitol phosphorylation.
of the curve by either or both of the soluble PTS enzymes. We have also conducted preliminary initial rate kinetic studies of the transphosphorylation reaction. However, strong substrate inhibition was exhibited by both mannitol and mannitol-1-P which complicated detailed kinetic analyses of the data obtained.

Purification procedures have been reported previously for the membrane-bound Enzyme II specific for glucose and mannose ("Enzyme IIB") from *E. coli* (20) and the lactose-specific Enzyme II from *Staphylococcus aureus* (21). However, evidence for homogeneity other than a single band on a dodecyl sulfate gel was not presented in either case. Furthermore, purification relative to crude membranes was only about 10-fold for the *E. coli* enzyme and 20-fold for the one from *S. aureus*, and both preparations were unstable (20, 21). In contrast, our purified Enzyme II<sup>mut</sup> is stable for several days at 4°C and for at least 2 months at −70°C.

In the present study, we have used hydrophobic chromatography as the most effective step in the purification of Enzyme II<sup>mut</sup>. After subsequent ion exchange chromatographic steps, however, the overall yield was only about 25%, with the greatest loss occurring during DEAE-cellulose chromatography. Nevertheless, we have shown that our preparation of Enzyme II<sup>mut</sup> is apparently homogeneous with a subunit molecular weight of 60,000. Furthermore, we have demonstrated the ability of the purified enzyme to catalyze mannitol-1-P:mannitol transphosphorylation in the absence of the soluble PTS enzymes and the apparent independence of the phosphoenolpyruvate reaction from an Enzyme III requirement. It may be necessary to scale up the purification procedure and to increase the yield in order to obtain enough material for more detailed physical and chemical analyses of the enzyme. However, the method described in this communication yields a sufficient quantity of stable Enzyme II<sup>mut</sup> for a variety of studies which should be important in determining the mechanism of PTS-mediated transport in *E. coli*.

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G R Jacobson, C A Lee and M H Saier, Jr

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