The bacterial phosphoenolpyruvate (PEP)-dependent phosphotransferase system (PTS) consists of many integral membrane perases and sugar phosphotransferases, each specific for a different sugar, and a set of cytoplasmic energy-coupling proteins (28, 33, 38, 40–43). Western blot analyses using anti-His tag monoclonal antibodies revealed that although II\textsubscript{Glc} from the two fractions migrated similarly in sodium dodecyl sulfate gels, the two fractions migrated differently on native gels both before and after Triton X-100 treatment. Peak 1 II\textsubscript{Glc} migrated much more slowly than peak 2 II\textsubscript{Glc}. Both preparations exhibited both phosphoenolpyruvate-dependent sugar phosphorylation activity and sugar phosphate-dependent sugar transphosphorylation activity. The kinetics of the transphosphorylation reaction catalyzed by the two II\textsubscript{Glc} fractions were different: peak 1 activity was subject to substrate inhibition, while peak 2 activity was not. Moreover, the pH optima for the phosphoenolpyruvate-dependent activities differed for the two fractions. The results provide direct evidence that the two forms of II\textsubscript{Glc} differ with respect to their physical states and their catalytic activities. These general conclusions appear to be applicable to the His-tagged mannose permease of \textit{E. coli}. Thus, both phosphoenolpyruvate-dependent phosphotransferase system enzymes exist in soluble and membrane-integrated forms that exhibit dissimilar physical and kinetic properties.

Enzyme I, HPr, and IIA are the energy-coupling proteins of the PTS (31). Although these reactions occur in a vectorial fashion in intact cells and bacterial membrane vesicles, they can also be demonstrated in vitro by using broken cell extracts or purified enzymes (28, 33, 38, 40–43).

In a recent communication (2) we presented evidence for two physically distinct forms of the enzymes II, one the membrane-integrated form, extensively characterized previously, and the other a “soluble” form not previously identified. When crude extracts of French-pressed \textit{Escherichia coli} cells or osmotically shocked \textit{E. coli} spheroplasts were centrifuged in an ultracentrifuge at high speed, a small fraction of the Enzyme II activity remained in the high-speed supernatant, and passage of the nonsedimented material through a gel filtration column gave two activity peaks, one in the void volume exhibiting high PEP-dependent and TP activities and a second included peak with high PEP-dependent activities and high (II\textsubscript{Man}), moderate (II\textsubscript{Glc}), or negligible (II\textsubscript{Mtl}) TP activities. The peak 1 enzyme probably consists of tiny membrane vesicles or membrane fragments with integrated enzymes II in a physical state similar to that of pelleted enzymes II.

Long-term exposure of cells to chloramphenicol resulted in selective loss of the soluble fraction with retention of much of the pelleted activity concomitant with extensive protein degradation, suggesting that both forms are present in intact cells. Western blot analyses showed that the soluble II\textsubscript{Glc} exhibited a subunit size of about 45 kDa, and the soluble forms of all three native enzymes II eluted from the gel filtration column with apparent molecular masses of 40 to 50 kDa. We proposed that enzymes II of the PTS exist in two physically distinct forms in the intact \textit{E. coli} cell, one integrated into the membrane and one either soluble or loosely associated with the membrane. We proposed that the membrane-integrated enzymes II are largely dimeric, whereas the soluble enzymes II that were re-
thiogalactopyranoside was added to a final concentration of 100 µM. Incubation was continued until the optical density at 600 nm reached 0.8; then, isopropyl-
chloramphenicol was added to a final concentration of 50 µg/ml, which exhibited low II Glc activity comparable to that produced was recentrifuged at 200,000 g./H11003

This in communication we report further characterization of these two forms of the glucose-specific Enzyme II (IIGlc). Using a His-tagged IIGlc (his-IIGlc) (7, 44) and monoclonal antibody against the His tag, we show that His-IIGlc exists in the same two forms observed previously for the wild-type enzyme. On sodium dodecyl sulfate (SDS) gels, both preparations migrate as a single species, but on native gels the two preparations migrate differently, both before and after detergent treatment, consistent with oligomeric and monomeric forms. We further show that the two forms exhibit different kinetic properties. While the transphosphorylation reaction of the membrane-integrated form exhibits the characteristic substrate inhibition observed previously (41), the soluble form does not. Further, the two forms of the enzyme show different responses to pH. These observations lead to the suggestion that the catalytic properties of a PTS Enzyme II, including the characteristic of substrate inhibition, are strongly influenced by the enzyme oligomeric state in the membrane.

**MATERIALS AND METHODS**

**Growth and assay conditions.** Unless otherwise indicated, strains (Table 1) were grown in 1 liter of Luria-Bertani (LB) broth in 2-liter conical flasks and incubated with shaking (275 rpm) at 37°C for 18 h. Strain ZSC112L(pJFGH11) expresses the ptsG gene constitutively under the control of its own promoter rather than the lac or ara promoter. This plasmid is a low-copy-number plasmid. Consequently, His-tagged IIGlc is not appreciably overproduced.

When working with strain ZSC112L(pJFGH11) (Amp’), a loopful of a fresh culture from a plate with LB plus 100 µg of ampicillin/ml (LB-Amp) was inoculated to 50 ml of LB-Amp broth in a 250-ml Erlenmeyer flask. Cells obtained were inoculated to 1 liter of LB-Amp broth in a 2-liter broth. The flask was incubated at 37°C with shaking at 275 rpm for 18 h before harvesting. The extract, obtained by passage of the cell suspension through a French press at 10,000 lb/in2, was centrifuged at 12,000 g./H9262

When working with strain ZSC112L(pJFGH11), a 1.5 ml sample of the enzyme (peak 1 or peak 2 from the gel filtration column) in either medium 63 (M63) adjusted to pH 8.9 or Tritis-glucose, pH 8.9, containing 1 mM methyl-α-D-glucopyranoside was treated with Triton X-100 (2%) with stirring at 4°C for 1 h in order to solubilize IIGlc from the membrane according to the procedure of Waebet al. (48). The sample was filtered through a 0.45-µm-pore-size Millipore filter. For gel filtration, the solubilized pellet was either applied directly and eluted under alkaline conditions (pH 8.9) in the presence of 0.1% Triton X-100 or it was first diafiltered three times against 2 liters of water for 36 h (total dialysis time) to reduce the detergent and salt concentrations and then loaded onto the column and eluted at neutral pH in the absence of additional detergent. For affinity purification of His-tagged proteins, the pH of the detergent-treated samples (e.g., peaks 1 and 2 after gel filtration of a 2-h HSS at neutral pH and in the absence of detergent) was adjusted to pH 8.0 before loading onto a nickel column (48).

**Purification of His-tagged IIGlc on a Ni 2+ column.** The nickel column was prepared with His-Bind resin from Novagen. A bed volume per column of 1.5 ml was produced from 3 ml of a resin suspension. Column preparation involved washing with sterile deionized water (three times with 1.5 ml, i.e., 4.5 ml), charging the column using charge buffer, 50 mM NaSO4 (five times with 1.5 ml [7.5 ml]), and equilibrating with buffer B (50 mM NaPO4 [pH 8], 300 mM NaCl, 1 mM methyl-α-D-glucopyranoside, 0.1% Triton X-100) (three times with 1.5 ml [4.5 ml]) (7, 48). For a 50-ml sample of the enzyme (peak 1 or peak 2 from the gel filtration column), the following protocol was used. The column was washed with buffer B, pH 8 (20 times with 1.5 ml [30 ml]), then washed with buffer B, pH 6 (10 times with 1.5 ml [15 ml]), and eluted with buffer B, pH 5 (10 times with 1.5 ml [15 ml]). The eluted sample was collected in fractions of about 1 ml and rapidly neutralized with 1 M NaOH before dialyzing against water. Fractions were tested for both activity and protein profile on SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Subsequently, the eluted sample was collected into two fractions, each of about 7.5 ml. These were concentrated using Centriprep columns (cutoff ≈ 30,000 Da) by centrifugation at 1,500 × g for 15 min using a swinging angle rotor. The concentrated fractions were dialyzed against water (three times with 2 liters over a period of 36 h). Removal of detergent tended to cause the monomeric form of IIGlc produced from the solubilized pellet or solubilized peak 1 but not from peak 2 when treated similarly to oligomerize. Dithiothreitol was added to a final concentration of 5 mM after dialysis. Dialyzed fractions were assayed in the presence of phosphatidyl glycerol (1 mg/ml, final concentration) as described by Waebet al. (48).

When working with strain ZSC112L(pJFGH11) except that in addition to ampicillin (100 µg/ml), chloramphenicol was added to a final concentration of 50 µg/ml and incubation was continued until the optical density at 600 nm reached 0.8; then, isopropyl-

β-thiogalactopyranoside was added to a final concentration of 100 µM (10).
RESULTS

Detection of soluble and membrane-integrated His-tagged Enzyme II<sup>Glc</sup> by Western blotting. The His-tagged Enzyme II<sup>Glc</sup> in the 2-h HSS derived from strain ZSC112L(pJFGH11) was passed through the gel filtration column, yielding peaks 1 and 2 (Fig. 1) as reported previously for the wild-type enzyme from <i>E. coli</i> strain 301 (2). It was detected by the Western blotting technique using anti His tag monoclonal antibody. Both pooled fractions (peaks 1 and 2) were treated with detergent (2% Triton X-100) as described in Materials and Methods. Using the Coomassie blue stain, both gel filtration peaks 1 and 2 yielded multiple bands on SDS-PAGE (data not shown). The Western blotting results for peaks 1 and 2 following gel filtration and SDS-PAGE are shown in Fig. 2A. Both fractions yielded a single band corresponding to His-tagged II<sup>Glc</sup>, and the migration rate was the same for II<sup>Glc</sup> from both peaks. However, when II<sup>Glc</sup> was analyzed using native gels, peaks 1 and 2 appeared in different positions, with peak 1 II<sup>Glc</sup> running much more slowly than peak 2 II<sup>Glc</sup> (Fig. 2B).

Chemiluminescent detection. Detection of II<sup>Glc</sup> and II<sup>Man</sup> by using primary mouse anti-His tag antibody and secondary goat anti-mouse immunoglobulin G–HRP involved use of the chemiluminescent detection technique with Immune-Star HRP (catalog no. 170–5040; Bio-Rad) (24) as described by the manufacturer. The membrane was packed between two plastic sheets and developed with autoradiographic film (X-ray film). Exposure times ranged between 1 and 8 min.

Materials. The materials used, their purities, and their sources were as described previously (1, 2) except when noted otherwise. Radioactive sugars were used at a specific activity of 5 μCi/μmol. Phosphatidylglycerol (PG) was obtained from Sigma Chemical Corporation. Acrylamide, bisacrylamide, Tris-glycine buffer, SDS, ammonium persulfate, and Laemmli buffer were from Bio-Rad.

FIG. 1. Profiles of PEP-dependent activity (□) and TP activity (△) as well as protein concentration (△) following gel filtration of a 2-h HSS from an extract of <i>E. coli</i> strain ZSC112L(pJFGH11). Elution was with M63, pH 7.0, containing 2 mM dithiothreitol. ['<sup>3</sup>C]methyl-α-glucoside (20 μM for the PEP reaction; 100 μM for the TP reaction) plus either PEP (10 mM) and a 4-h HSS or glucose-6-P (10 mM) were used for the assay under standard conditions. The fractions pooled for enzyme analysis are shown by the horizontal black bars.
X-100 according to the method of Waeb et al. (48) (see Materials and Methods), and peak 2 II Glc was similarly treated. These preparations were then applied to a Ni²⁺ affinity column as described above, yielding in each case fractions exhibiting both PEP-dependent and TP II Glc activities.

The TP activities of the purified II Glc in peaks 1 and 2 were assayed as a function of glucose-6-P concentration at a constant methyl-α-glucoside concentration (Fig. 3). It can be seen that the two enzyme preparations exhibited strikingly different behaviors. Thus, the peak 2 activity increased with glucose-6-P concentration up to 1,000 mM, while the peak 1 activity peaked at a glucose-6-P concentration of about 10 mM. These results show that these two enzyme preparations, which on the basis of native gel electrophoresis experiments appeared to be in different oligomeric states (Fig. 2), exhibit different kinetic properties.

The radioactive product of the TP reaction obtained with the nickel column-purified II Glc preparations was subjected to treatment with alkaline phosphatase (10 U in 200 μl; solution adjusted to pH 9.8). The solution was incubated at 37°C for 90 min, which resulted in conversion of greater than 95% of the anionic product into a neutral product. These results are consistent with the conclusion that the product of the TP reaction was methyl-α-glucoside-6-phosphate, as expected.

The Ni²⁺ column-purified His-tagged II Glc from peak 2 was assayed for the TP reaction in the presence and absence of the ammonium salt of commercial L-α-phosphatidyl-D,L-glycerol from egg lecithin (catalog no. P5531; Sigma Chemical Corp.). For this purpose, 100 μM [¹⁴C]methyl-α-glucoside and 10 mM glucose-6-P were present as substrates under standard conditions (1). Addition of 20 μl of a 10-mg/ml PG solution in chloroform-methanol (98:2) to a final volume of 200 μl (final PG concentration of 1 mg/ml) stimulated the TP activity optimally. Stimulation was 3.5-fold. Control experiments showed that the organic solvent had no stimulatory effect. We conclude that the purified peak 2 enzyme is lipid deficient.

Characteristics of detergent-solubilized II Glc. Pelleted II Glc (centrifuged at 200,000 × g for 2 h) from a crude extract of strain ZSC112L(pJFGH11) was treated with Triton X-100 (see Materials and Methods). The treated pellet and the original 2-h HSS were passed separately at alkaline pH and in the presence of 0.1% Triton X-100 through the gel filtration column, instead of at neutral pH in the absence of detergent, as performed in the experiment shown in Fig. 1. The elution
profiles are shown in Fig. 4. The solubilized pellet fraction gave three TP activity peaks, while the HSS gave one broad peak. The elution profiles suggest that multiple molecular species of II\textsuperscript{Glc} are present in both preparations. The species from the Triton-solubilized pellet probably included at least two oligomeric forms as well as a monomeric species. The HSS fraction could be monomeric II\textsuperscript{Glc} associated with various amounts of lipid, detergent, and/or other proteins.

The TP kinetics of the different fractions shown in Fig. 4 as a function of glucose-6-P concentration are shown in Fig. 5. The crude solubilized pellet before gel filtration appeared to show a slight activity peak at about 20 mM glucose-6-P, but activity then increased further as the glucose-6-P concentration increased. Peak P1 from the gel filtration column (Fig. 4) showed maximal activity at about 10 mM glucose-6-P with inhibition at higher concentrations (Fig. 5). This behavior re-

FIG. 3. TP activities of Ni\textsuperscript{2+} column-purified preparations of the II\textsuperscript{Glc} from gel filtration peaks 1 (△) and 2 (○). The enzyme preparation applied to the gel filtration column was a 2-h HSS from strain ZSC112L(pJFGH11). Activity was measured as a function of glucose-6-P concentration. The final methyl-α-glucoside concentration was 50 μM. The specific activities, when measured with a final [\textsuperscript{14}C]methyl-α-glucoside concentration of 50 μM and a final glucose-6-P concentration of 5 mM with 1 μg of phosphatidylglycerol/μl present, were 16 and 0.7 pmol of sugar-P formed per mg of protein per min for the peak 1 and peak 2 enzyme preparations, respectively.

FIG. 4. Elution profile of the 2% Triton-solubilized pellet fraction (△) and a 2-h HSS (○), eluted from the gel filtration column with M63, pH 8.9, containing 0.1% Triton X-100 and 2 mM dithiothreitol and monitored using the TP assay. The pellet fraction was solubilized at pH 8.9 with 2% Triton X-100, stirring at 4°C for 2 h, and then filtered through a 0.45-μm Millipore membrane filter. An aliquot of 2.5 ml of the solubilized pellet or 2.5 ml of the 2-h HSS was fractionated through the gel filtration column. The resultant fractions were dialyzed against water for 36 h (three changes; three 2-liter volumes; 12 h each) to reduce the detergent and salt concentrations and then assayed as follows: 100 μl from the dialyzed fractions was mixed with an aliquot of assay mix containing both TP substrates (100 μM [\textsuperscript{14}C]methyl-α-glucoside and 10 mM glucose-6-P). Phosphatidylglycerol was incorporated at 200 μg per reaction mix (1 mg/ml) in the case of the solubilized pellet fractions only. Assay times were 2 and 1.5 h for the solubilized pellet fractions and the HSS fractions, respectively.
sembles that of membrane-integrated II\textsuperscript{Glc} (40, 41). By contrast, the low-molecular-weight enzyme (peak P2 in Fig. 4) showed no substrate inhibition at high glucose-6-P concentrations. These results reveal different kinetic behaviors for the different II\textsuperscript{Glc} preparations, comparable to that with the membrane-integrated and soluble forms of the enzyme obtained from a total crude extract.

Pelleted II\textsuperscript{Glc} from a crude extract of the His-tagged II\textsubscript{CB\textsuperscript{Glc}}-producing strain, ZSC112L(pJFGH11) (Table 1), was solubilized with 2% Triton X-100 at pH 8.9, and 5 ml of the solubilized fraction was three times dialyzed against 2 liters of water to reduce the detergent and salt concentrations. An aliquot (2.5 ml) of this preparation was applied to the gel filtration column and eluted with M63, pH 7. Fractions from this column were assayed for TP activity with and without PG. When present, 4\mu l of a 50-mg/ml PG solution in hexane-ethanol (9:1) (lyophilized PG was from Sigma Corp., catalog no. P8318) was added to the 200-\mu l assay mixture to give a final PG concentration of 1 mg/ml. As shown in Fig. 6, the addition of PG greatly increased the TP activity of the enzyme and shifted the activity peak towards higher-molecular-weight material. This stimulation by PG resembled that noted above for the affinity-purified soluble II\textsuperscript{Glc}. Control experiments showed that the solvent alone was without effect. Thus, both preparations are lipid deficient.

Comparing Fig. 4 with Fig. 6 reveals that the gel filtration profile changed dramatically when the detergent-treated pellet fraction was eluted at alkaline pH in the presence of detergent (Fig. 4) versus when it was dialyzed against water and eluted from the gel filtration column at neutral pH in the absence of added detergent (Fig. 6). The multiple detergent-solubilized enzyme species evidently reassociate into higher-molecular-weight material when substantial amounts of the detergent are removed at neutral pH.

Kinetic properties of soluble versus membrane-integrated enzymes II. As noted above and shown in Fig. 1, passage of the 2-h HSS protein fraction through the gel filtration column gave two activity peaks, an excluded peak (P1) and an included peak (P2). Both II\textsuperscript{Glc} and II\textsuperscript{Man} activities could be identified in both peaks. These enzyme preparations gave linear increases in II\textsuperscript{Glc} and II\textsuperscript{Man} activities when assayed as a function of protein concentration. Figure 7 shows the activities of II\textsuperscript{Glc} and II\textsuperscript{Man} as a function of the glucose-6-P concentration. Regardless of the radioactive substrate used (methyl-\textalpha-glucoside to assay II\textsuperscript{Glc} or 2-deoxyglucose to assay II\textsuperscript{Man}), the membrane-integrated enzymes (peak 1) exhibited substrate inhibition, while the soluble enzymes (peak 2) exhibited an essentially hyperbolic response to substrate concentration. Half-maximal activity was observed at about 10 mM glucose-6-P for both II\textsuperscript{Glc} and II\textsuperscript{Man}. It is therefore apparent that while the two enzyme preparations exhibit strikingly different kinetic characteristics, II\textsuperscript{Glc} and II\textsuperscript{Man} exhibit comparable behaviors. Peak 2 was assayed for sugar-phosphatase activity, but under the conditions of the assay, which included addition of fluoride to inhibit phosphatase activities, no such activity was detected (data not presented).

Distinct pH curves for the soluble and membrane-integrated enzymes II. Figure 8 shows the pH profiles for the PEP-dependent sugar phosphorylation reactions for peaks 1 and 2 when methyl-\textalpha-glucoside (Fig. 8A) or 2-deoxyglucose
(Fig. 8B) was the sugar substrate and PEP (2.5 mM) serving as the phosphoryl donor was used to assay II Glc or II Man, respectively. The results showed that the two peaks of both II Glc and II Man exhibit distinctive responses to pH.

**DISCUSSION**

The PTS consists of integral membrane sugar-phosphorylating permeases (the enzymes II) and the cytoplasmic energy-

![Graph](http://jb.asm.org/)
coupling proteins (31). These PTS proteins are often fused together in various combinations and orders (32, 46). Fusion of PTS protein domains has important consequences with respect to catalytic activity and stability (44). The enzymes II are all believed to exist in the membrane in oligomeric states, and at least some of them are probably dimeric (31).

In a recent publication, we provided evidence for a novel form of the enzymes II (2). This novel form eluted from a gel filtration column as a monomeric protein and appeared to be cytoplasmic or loosely associated with the membrane. The evidence presented suggested that both forms exist in intact wild-type E. coli cells and are not artifacts of enzyme preparation. No evidence for a precursor-product relationship during membrane biogenesis was obtained.

As reported in this communication, we have extended these studies by conducting comparative analyses of different forms of the His-tagged Enzyme II complexes with emphasis on the glucose-specific Enzyme II, II^Glc. The following conclusions resulted from these studies. First, by using the His-tagged II^Glc (7, 48) together with anti-His tag monoclonal antibody, we showed that the two forms of the protein migrated differently in native gels, both before and after detergent treatment, as expected assuming that the membrane-integrated form is oligomeric while the soluble form is monomeric. Both forms of II^Glc migrated at the same rate in denaturing SDS gels. These observations suggest that the gentle Triton X-100 extraction procedure described by Waeber at al (48) and Buhr et al. (7) does not cause extensive subunit dissociation.

Second, the two forms of the enzyme could be affinity purified on a Ni$^{2+}$ column, yielding preparations that approached homogeneity. Both forms of the enzyme exhibited both PEP-dependent and sugar-P-dependent $^{14}$C sugar phosphorylation under our standard assay conditions. However, on native gels, the detergent-solubilized, purified, membrane-integrated form still appeared to be oligomeric, while the purified soluble form seemed to be monomeric.

Third, the two forms of II^Glc exhibited different enzymatic characteristics. They displayed different pH curves for the PEP-dependent reaction and, most strikingly, only the membrane-integrated form exhibited the characteristic substrate...

FIG. 8. PEP-dependent sugar phosphorylation activities of peaks 1 (white bars) and 2 (black bars) at different pH values from 4 to 10 with methyl-α-glucoside (A) or 2-deoxyglucose (B) as the sugar substrate. The assays were conducted as outlined in Materials and Methods, with PEP at 2.5 mM and the $^{14}$C-labeled sugar concentration at 10 μM. The enzyme preparations used were from strain 301, and a 4-h HSS of an extract from strain AD90 was used as a source of the soluble PTS energy-coupling enzymes. The experiments were conducted three times, and the standard deviation values are indicated by the vertical bars.
inhibition when assayed using the TP reaction. This surprising result suggests that the characteristic of substrate inhibition, previously documented for all well-characterized Enzyme II complexes of the PTS, is dependent on enzyme oligomerization or integration into the membrane.

Fourth, in preliminary studies, results with II-Man suggested that the two forms of this nonhomologous enzyme complex exhibit differences similar to those studied in detail with II-Glc. Thus, the two forms of II-Man from strain 301 exhibited clear kinetic differences (Fig. 7 and 8 and our unpublished observations). It therefore seems likely that our observations with II-Glc will prove to be generally relevant to other Enzyme II complexes of the PTS.

Fifth, the soluble II-Glc exhibited low activity that could be stimulated by addition of phosphatidyl glycerol both before and after affinity purification. This result suggests that the soluble II-Glc is lipid deficient. It seems reasonable to propose that oligomerization is dependent on appropriate lipid associations (29).

Finally, several of the experimental results reported as well as our most recent unpublished results support that an equilibrium exists between the monomeric and oligomeric forms of II-Glc in vitro. The soluble monomer is favored by (i) detergent, (ii) low protein concentration, and (iii) high pH, while the membrane-integrated oligomer is favored by phospholipids, high protein concentration, and neutral pH. Since protein conformations and protein-protein interactions are often ligand dependent (3), it might be expected that a ligand-dependent oligomerization process would be dependent, or at least influenced by, protein conformational transitions. However, cause-and-effect relationships are often difficult to establish.

The studies reported here provide new insight into the potential of integral membrane enzymes to exist in alternative conformations. Different conformations of a single polypeptide chain may exhibit drastically different physicochemical and physiological characteristics (15). They may also provide a springboard for the appearance of divergent protein evolutionary pathways (15). To what extent the observations reported here will prove to be applicable to other types of integral membrane proteins has yet to be examined.

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