In vitro Interconversion of the Soluble and Membrane-Integrated Forms of the *Escherichia coli* Glucose Enzyme II of the Phosphoenolpyruvate-Dependent Sugar-Transporting Phosphotransferase System

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Key Words

Sugar permeases • *Escherichia coli* • Phosphoenolpyruvate-dependent phosphotransferase system • Integral membrane permeases • Solubilization

Abstract

In previous publications, we have shown that integral membrane sugar permeases of the bacterial phosphotransferase system can exist in a 'soluble' (probably micellar) monomeric form (SII) as well as a membrane-integrated dimeric form (MII). We here show that the two forms of the his-tagged glucose permease of *Escherichia coli* can be interconverted in vitro. Conversion of MII to SII is promoted by (1) low protein concentration, (2) detergent, (3) high pH, and (4) phospholipase A₂ treatment. Conversion of SII to MII is promoted by: (1) high protein concentration, (2) adherence to and elution from an Ni²⁺ column, (3) neutral pH, and (4) incorporation into phospholipid liposomes.

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Introduction

In previous publications, we have shown that membrane-integrated permeases (enzymes II) of the bacterial phosphoenolpyruvate-dependent phosphotransferase (PTS) can exist in soluble, cytoplasmic, possibly micellar forms both in vivo and in vitro [Aboulwafa and Saier, 2003, 2004]. The soluble form (SII) appears to be the monomeric protein while the membrane-integrated form (MII) is oligomeric [Aboulwafa and Saier, 2003, 2004; Broos et al., 1998; Erni, 1986; Koning et al., 1999; Leonard and Saier, 1981; Meins et al., 1988; van Montfort et al., 2001, 2002]. These sugar permease enzymes have been shown to catalyze two chemical reactions, both of which can occur in a vectorial fashion:

The second reaction shown is called the transphosphorylation (TP) reaction and is catalyzed by all PTS enzymes II [Saier et al., 1977a–c]. The TP reaction for the glucose enzyme II (II^{Glc}) of *Escherichia coli* has been shown to exhibit differing kinetic properties, depending on whether II^{Glc} is in the SII or MII form [Aboulwafa and Saier, 2004]. Specifically, SII does not exhibit substrate (sugar-P) inhibition when the concentration of the radioactive sugar is maintained constant and that of the sugar-P is increased,

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Fig. 1. Conversion of SII to MII by passage through an Ni^{2+} column followed by concentration. **a** Gel filtration of Ni^{2+} column-purified and concentrated N-his II^{Glc} SII (peak 2). After an HSS was passed through a Biogel filtration column, P2 (SII) was purified on an Ni^{2+} column and concentrated using an Amicon Ultra 30,000 MWCO filter before it was again passed through the gel filtration column. The elution profile reveals conversion of some of SII (P2) to MII (P1) following purification on the Ni^{2+} column and concentration. This experiment was repeated several times, and although conversion to MII (P1) was always observed, variability in the amount of conversion (30–60%) was observed. **b** TP activity of fractions 9 (□), 12 (○) and 16 (△) from the elution profile shown in **a** as a function of glucose-6-phosphate concentration, assayed under standard conditions [Aboulwafa and Saier, 2004]. While fractions 9 and 12 exhibit substrate inhibition (characteristic of MII), fraction 16 does not (characteristic of SII).

but MII does [Aboulwafa and Saier, 2004]. This fact provides a convenient assay for the interconversion of these two forms of the enzyme. We therefore used this assay as well as gel filtration to study the his-tagged form of II^{Glc} , and showed that the two forms can be interconverted in vitro. The conditions promoting the formation of each of these two forms were defined as reported here.

Fig. 2. Effect of liposome association of SII on the catalytic behavior of II^{Glc} in the TP reaction. Purified SII (P2) N-his II^{Glc} was assayed for the TP reaction as a function of glucose-6-phosphate concentration (□). Subsequently, it was incorporated into pre-formed liposomes and assayed again (○).

Results

Conversion of the Soluble Enzyme II (Gel Filtration Peak 2; P2; SII) into the Membrane-Associated Enzyme II (Gel Filtration Peak 1; P1; MII)

E. coli strain BL21GH4 bearing the plasmid encoding the N-terminally his-tagged II^{Glc} (N-his- II^{Glc}) was grown, and the 2-hour high-speed supernatant (HSS) was prepared as described under 'Experimental Procedures'. The 2-hour HSS was passed through a Biogel (Biogel A-1.5 m fine-grade, exclusion limit <10,000–1,500,000, Bio-Rad) filtration column at 4°C yielding two peaks when assayed by the TP assay, peak 1 (P1; MII) and peak 2 (P2; SII), as described previously [Aboulwafa and Saier, 2003]. While peak 1 showed strong substrate inhibition, peak 2 showed none as reported previously. P2 his- II^{Glc} (SII) was purified on a pre-equilibrated (his-tagged protein-binding buffer containing imidazole) Ni^{2+} column. The his-tagged II^{Glc} was washed and eluted as described by Aboulwafa and Saier [2004]. It was then assayed using the TP reaction as a function of glucose-6-phosphate concentration. Mild substrate inhibition was observed [see Aboulwafa and Saier, 2004]. The eluate was concentrated (with an Amicon Ultra 30,000 MWCO filter). The concentrated Ni^{2+} column-purified SII (P2) was again applied to a Biogel filtration column, and two peaks of activity, P1' (MII) and P2' (SII), were eluted in medium 63 (M63) at pH 7.0. Different relative amounts of P1' and P2' activity were obtained in different preparations, but the two peaks were fairly comparable in magnitude, with between 30 and 60% of the activity in P1' (see, for example, fig. 1a).

P1' (fractions 9–12) showed substrate inhibition in the TP reaction, but P2' (fraction 16) did not (fig. 1b). It was therefore concluded that partial conversion of SII (P2; monomeric) into MII (P1; oligomeric) had occurred.

Ni²⁺ column-purified his-II^{Glc} from P2 was passed through the Biogel filtration column, and P2' (SII) was assayed (TP reaction) as a function of glucose-6-phosphate concentration. Virtually no substrate inhibition was observed (fig. 2). This enzyme was incorporated into liposomes as described in the legend to figure 2. The results of the TP assay are shown in figure 2. Although the starting material showed practically no substrate inhibition in the TP reaction, the liposome-associated enzyme exhibited very substantial substrate inhibition. These results suggest that lipid bilayer association tends to convert SII to MII with the expected change in catalytic properties.

Conversion of MII (P1) into Soluble SII (P2) and Back Again

E. coli strain BL21GH4 bearing the plasmid encoding the N-terminally his-tagged II^{Glc} (N-his-II^{Glc}) was grown as described under 'Experimental Procedures', and the pellet fraction was separated by high-speed centrifugation. When the pellet fraction was passed through the Biogel filtration column [Aboulwafa and Saier, 2003], only P1 (not P2) was observed. This pellet fraction was brought to pH 8.9 in M63 buffer, and the enzyme was solubilized with 2% Triton X100 (with stirring at 4°C for 7.5 h) [Aboulwafa and Saier, 2004]. The extract was passed through a 0.45-μm Millipore filter, and 1 or 2.5 ml of the protein solution was loaded onto a Biogel filtration column as described previously [Aboulwafa and Saier, 2003]. The enzyme was eluted with M63 containing 0.1% Triton X100 at pH 8.9. A large peak 1 (P1; MII) was observed as well as a small peak 2 (P2; SII) (fig. 3a). P2 exhibited no substrate inhibition, although P1 did (fig. 3b). These experiments show that detergent treatment at high pH converts some of the membrane-integrated oligomeric MII into the soluble, monomeric SII.

P2 (SII) from the above experiment was collected and concentrated using an Amicon Ultra 30,000 MWCO filter. An aliquot of 2.5 ml of the concentrate produced was loaded onto a Biogel filtration column and eluted under the same conditions described above using the same eluant. Two peaks, a large peak 1 and a smaller peak 2, were produced (fig. 4). Once again, the large peak 1 showed substrate inhibition but the small peak 2 did not (data not shown). His-tagged EII^{Glc} from the solubilized pellet was purified with an Ni⁺ column using standard procedures. Purified his-tagged EII^{Glc} from P2 of the concentrated

Fig. 3. Effect of detergent treatment on the kinetic properties of II^{Glc}. **a** Gel filtration profile of 2% Triton X100-solubilized pellet (incubated for 7.5 h in M63 buffer, pH 8.9 at 4°C). **b** TP assay of the two peaks from the gel filtration column (**a**) as a function of glucose-6-phosphate concentration at a constant (50 μM) concentration of [¹⁴C]methyl α-glucoside. The amounts of protein in the reaction mixtures were 8.5 and 90 μg for P1 and P2, respectively. Peak 1 (P1; MII; ○) shows substrate inhibition, while peak 2 (P2; SII; □) does not.

Fig. 4. Gel filtration of 2.5 ml of concentrated (with an Amicon Ultra 30,000 MWCO filter) P2 (SII) derived from the Triton X100-solubilized pellet of BL21GH4 (gel filtration peak 2). The column was eluted with M63 at pH 8.9 in the presence of 0.1% Triton X100.

Fig. 5. Effects of PLA2 treatment on TP activities of Ni column-purified EII^{Glc}. Peak 2 of solubilized pellet from *E. coli* strain BL21GH4 was passed through the Ni column, concentrated, and passed through the gel filtration column again to yield P1 (MII). PLA2 from porcine pancreas (1,500 units) (Sigma catalogue No. PO861) was incubated for 60 min at pH 8 and 40°C with 3.75 µg of this preparation, with mild shaking. The figure shows the TP reaction as a function of glucose-6-phosphate concentration before (□) and after (○) PLA2 treatment.

Fig. 6. Summary of conditions that promote the reversible inter-conversion of the membrane-integrated form of II^{Glc} (MII) and the soluble form (SII).

detergent-solubilized pellet was twice dialyzed (5 ml against 2 l M63 plus 2 mM DTT, pH 7, over a 24-hour period). This enzyme was assayed for TP activity both before and after dialysis at different concentrations of glucose-6-phosphate and 50 µM [¹⁴C]methyl α-glucoside. Mild substrate inhibition was observed before dialysis, and stronger inhibition was observed after dialysis (data not shown), although the starting material (peak 2 from the solubilized pellet) had shown no substrate inhibition.

An aliquot of the Ni column-purified and dialyzed his-tagged EII^{Glc}, obtained from P2 of the solubilized pellet, was concentrated and loaded onto a Biogel filtration

column and eluted at neutral pH with M63. A peak 1, but no peak 2, was observed (see fig. S1A on our website; <http://www.biology.ucsd.edu/~msaier/supmat/MII-SII>). The TP activities of the different fractions representing peak 1 at different concentrations of glucose-6-phosphate and 50 µM [¹⁴C]methyl-α-glucoside are shown in figure S1B (<http://www.biology.ucsd.edu/~msaier/supmat/MII-SII>). The results show that purification and elution from an Ni⁺ column followed by concentration and dialysis promoted the conversion of SII, obtained from the solubilized pellet, to MII.

The above-described experiments show that both Ni²⁺ column purification and protein concentration/dialysis convert the purified monomeric form of II^{Glc} (SII) to a form exhibiting the properties of the oligomeric form (MII). However, both forms of the enzyme must subsequently be relatively stable, allowing their separation by gel filtration and assay using the TP reaction. Conversion of SII (P2) to MII (P1) therefore occurs slowly and is promoted by both purification on the Ni²⁺ column and concentration of the protein.

Conversion of MII to SII by Treatment with Phospholipase A₂

As shown in figure 5, when the purified P1 (MII), obtained following Ni²⁺ column purification and concentration, was assayed for substrate inhibition in the TP reaction, both before and after phospholipase A₂ (PLA2) treatment, the substrate inhibition observed before PLA2 treatment was largely lost following PLA2 treatment. These observations suggest that purified SII retains phospholipids, that passage through the Ni²⁺ column followed by concentration promotes formation of the oligomeric MII from the monomeric SII obtained initially from the pellet as noted above, and that exposure to PLA2 partially reverses this process, either converting MII to SII, or causing MII to acquire the catalytic properties of SII. Regardless of the agent or procedure used to affect the equilibrium between SII and MII, both forms can subsequently be separated by gel filtration, and P1 (MII) always shows substrate inhibition while P2 (SII) does not (see also fig. S2 on our website; <http://www.biology.ucsd.edu/~msaier/supmat/MII-SII>).

Discussion

The scheme shown in figure 6 summarizes our in vitro findings. Several conditions influence the distribution of II^{Glc} between the MII (oligomeric; probably dimeric)

form and the SII (monomeric) form. These include (1) protein concentration, (2) detergent, (3) pH, (4) passage through an Ni^{2+} column, and (5) the availability of phospholipids in bilayer form. How each of these conditions or agents influences the relative proportions of MII and SII is fully consistent with our bilayer (MII) $\leftrightarrow$ micelle (SII) proposal [Aboulwafa and Saier, 2004]. After PLA treatment, it is possible that the lysophospholipids and/or detergent molecules present participate in micelle formation. These results are in general agreement with our previous results [Aboulwafa and Saier, 2003, 2004] as well as those of Broos et al. [1998].

In summary, we have demonstrated that the 'soluble' and 'membrane-integrated' forms of the enzyme II^{Glc} (SII and MII, respectively) can be interconverted in vitro. The conditions that favor formation of one or the other of these two forms have been defined (fig. 6). Definition of these in vitro conditions are likely to lead to an understanding of the conditions that maintain the distribution of these two forms in vivo [Aboulwafa and Saier, 2003, 2004]. Such an understanding will hopefully lead to the in vivo manipulation and consequent appreciation of the physiological significance, if any, of the cytoplasmic forms of PTS enzymes II.

Experimental Procedures

The methods and the commercially obtained compounds used have been described in our earlier publications [Aboulwafa and Saier, 2003, 2004] or are presented here and in the figure legends. Strain BL21GH4 bears a plasmid encoding the N-terminally his-tagged II^{Glc} (N-his- II^{Glc}) and was made by Dr. Ming Ren Yen. All of the work reported in this paper was performed with this N-his-tagged enzyme.

Bacterial Growth and Crude Enzyme Preparation

Strain BL21GH4 (pET19b- $\text{H}_4\text{-ptsG amp}^r$) was grown in 2 $\times$ TY medium (tryptone, 12 g; yeast extract, 24 g; water, 1 l; 250 rpm). Ampicillin was added to a final concentration of 100 $\mu\text{g}/\text{ml}$, and cells were grown to an OD of 0.8 absorbency units. Then isopropyl- β -D-thiogalactopyranoside was added to the final concentration of 0.2 mM, and incubation was continued for 24 h at 37° with shaking (250 rpm) before harvesting. Cells were washed 3 $\times$ with M63 and resuspended in 35 ml of the same medium. These suspensions were passed through a French press 3 $\times$ at 0°C and 16,000 psi. The cell lysate was centrifuged at 10,000 rpm in a Sorvall® SS-34 rotor at 4°C for 10 min. The supernatants represent the crude extracts which were centrifuged at 40,000 rpm (100,000 g) for 2 h in a Beckman Ti50 rotor to give the pellet and the HSS. The pellet was suspended in 10 ml of M63.

Liposome formation and II^{Glc} incorporation were accomplished as follows: Commercial phosphatidylglycerol (PG, 5 mg)

plus purified *E. coli* phosphatidylethanolamine (PE, 15 mg) both from Sigma in 2 ml of M63/5 mM DTT was vortexed, and an aliquot of 0.5 ml in a 15 $\times$ 1.6 cm (internal diameter) Pyrex tube was flushed with N_2 gas. The tube was sealed, and the suspension was sonicated intermittently for a total of 80 min in an ice bath using a bath sonicator (G11 SP1 special ultrasonic cleaner, Laboratory Supplies, Inc., Hicksville, N.Y., USA). The temperature did not exceed 8°C. Reconstitution was achieved without further sonication using the detergent dilution procedure as described previously [Ambudkar and Maloney, 1986; Leonard and Saier, 1983]. An aliquot (SII; P2) of 0.5 ml (965 μg protein), obtained by gel filtration of dialyzed Ni^{2+} column-purified N-his EII^{Glc} , was added to the liposomes and mixed; 50 μl of 2% Triton X100 was then added to give a final Triton concentration of 0.1%. This mixture was kept on ice for 45 min. It was then diluted 30-fold by pipetting into M63/5 mM DTT, vortexed and centrifuged at 45,000 rpm (Ti50 rotor) for 1 h. The supernatant produced was discarded, and the sedimented proteoliposomes were resuspended in 1 ml of M63/5 mM DTT and immediately used for assay. The TP reaction was assayed as described previously [Aboulwafa and Saier, 2003, 2004] using 50 μM [^{14}C]methyl α -glucoside and variable concentrations of glucose-6-P. The amount of protein in the reaction mixture was 96 μg , both with and without liposomes.

The effect of PLA2 treatment was measured as follows. The pellet fraction or Ni^{2+} column-purified EII^{Glc} was incubated with 1,500 units of PLA2 per ml (PLA2 from porcine pancreas; Sigma catalogue No. PO861) [1 unit of enzyme produces 1 μmol of free fatty acid per min at pH 8 and at 40°C using lecithin phosphatidylcholine (PC) as the substrate. It preferentially hydrolyzes the β -ester bonds of zwitterionic glycerophospholipids (PC and PE), but anionic lipids, PG, phosphatidylinositol (PI) and phosphatidylserine (PS), are also hydrolyzed. The enzyme attacks phospholipids in the membranes of intact cells]. Incubation was for 60 min at 40°C with mild shaking. The preparations before and after PLA2 treatment were either assayed directly (Ni^{2+} column-purified EII^{Glc} ; fig. 5) or the treated preparation (for the pellet fraction) was applied to the gel filtration column, and the two peaks of activity (fig. S2A on our website; <http://www.biology.ucsd.edu/~msaier/supmat/III-SII>) were assayed for the TP reaction as a function of glucose-6-phosphate concentration (fig. S2B on our website; <http://www.biology.ucsd.edu/~msaier/supmat/III-SII>) using standard assay conditions [Aboulwafa and Saier, 2003, 2004]. The assay incubation time was 1 h. The results show that some of the pellet fraction (MII) acquired the properties of the soluble enzyme (SII).

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