Glucose Permease of *Escherichia coli*

PURIFICATION OF THE II\(^{Glc}\) SUBUNIT AND FUNCTIONAL CHARACTERIZATION OF ITS OLIOMERIC FORMS*

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The membrane subunit (II\(^{Glc}\)) of the glucose permease has been purified from overproducing *Escherichia coli*. About 2 mg of pure protein was obtained from 10 g (wet weight) of cells. II\(^{Glc}\) of *E. coli* and *Salmonella typhimurium* are functionally indistinguishable. A small difference was revealed, however, by a monoclonal antibody which neutralizes glucose phosphorylation activity of II\(^{Glc}\) from *S. typhimurium*, but does not cross-react with II\(^{Glc}\) of *E. coli*. A dimeric form of purified II\(^{Glc}\) can be detected by chemical cross-linking and by zonal sedimentation at 4 °C. Upon mild oxidation a disulfide bond is formed between the subunits of the dimer. Oxidized II\(^{Glc}\) is more stable than the reduced form but is inactive because it cannot be phosphorylated by the cytoplasmic subunit (III\(^{Glc}\)) of the glucose permease. Cys-421 could be identified as the oxidation-sensitive residue, using a novel assay to detect III\(^{Glc}\)-dependent phosphorylation of nitrocellulose-bound II\(^{Glc}\) that has been purified by gel electrophoresis. No dimeric form of phosphorylated II\(^{Glc}\) could be detected. Because phosphorylated II\(^{Glc}\) is a catalytic intermediate it is concluded that catalytically active II\(^{Glc}\) is a monomer and that the dimeric form is an artifact observed only with purified resting II\(^{Glc}\). That II\(^{Glc}\) is active as a monomer is further supported by the observation that monomeric II\(^{Glc}\) catalyzes phosphoryl exchange between glucose and glucose 6-phosphate at equilibrium and that an excess of inactive II\(^{Glc}\) with a serine replacing Cys-421 does not interfere with the activity of wild-type II\(^{Glc}\) as would be expected if interaction between the subunits in a dimer were essential for activity.

The uptake of a number of hexoses and hexitols in bacteria is mediated by the permeases of the bacterial phosphotransferase system (for review see Postma and Lengeler, 1985; Postma, 1987). They transport sugars by a mechanism involving the concomitant phosphorylation of the substrate (Fig. 1) and they act as chemoreceptors monitoring the environment for changes in sugar concentration (Adler and Epstein, 1974; Niwano and Taylor, 1982). The phosphoryl group is transferred from phosphoenolpyruvate to the various permeases by two unique cytoplasmic phosphoryl carrier proteins, enzyme I and HPr. Eight PTS permeases are known in *Escherichia coli* which differ in sugar specificity, subunit composition (enzymes II and III; Saier et al., 1988), and in the way their expression is controlled.

The glucose permease consists of two subunits, cytoplasmic III\(^{Glc}\) (Nelson et al., 1984; Meadow and Rosemanuel, 1982; Saffant et al., 1987) and transmembrane II\(^{Glc}\). The gene *ptsG* for II\(^{Glc}\) of *E. coli* has been cloned and sequenced (Erni and Zanolari, 1986; Bouma et al., 1987) and II\(^{Glc}\) of *Salmonella typhimurium* has been purified (Erni et al., 1982). A phosphorylated, catalytic intermediate of II\(^{Glc}\) (P-II\(^{Glc}\)) exists (Begley et al., 1982) and can be isolated in active form (Erni, 1986). The phosphoryl group is transferred from phosphorylated III\(^{Glc}\) (P-III\(^{Glc}\)) to II\(^{Glc}\) in a reversible reaction, and from P-II\(^{Glc}\) to glucose in a reaction which is strongly stimulated by the presence of III\(^{Glc}\), indicating allosteric enhancement between the two subunits in the complex (Erni, 1986).

A procedure, modified for the purification of II\(^{Glc}\) from overproducing *E. coli* (Erni and Zanolari, 1986), along with differences between II\(^{Glc}\) of *E. coli* and *S. typhimurium*, will be described in the first part of this report.

In the second part, different oligomeric forms of II\(^{Glc}\) will be characterized and the question whether II\(^{Glc}\) is active as monomer or as dimer will be discussed. This question has been discussed previously for both the glucose permease and the mannitol permease of the PTS. Leonard and Saier (1983), Roossien et al. (1984), Stephan and Jacobson (1986), and Robillard and Blauw (1987) postulated subunit interaction on the basis of the sigmoidal relation between activity and concentration of the mannitol permease. Under certain experimental conditions, they could also extract a dimeric form of the mannitol permease (Roossien and Robillard, 1984b; Stephan and Jacobson, 1986). Robillard and Konings (1982) and Roossien and Robillard (1984a) discussed the possibility that the glucose permease and the mannitol permease are composed of two subunits linked by a disulfide bridge and that a dithio-disulfide interchange is part of the reaction mechanism of the permeases.

Disulfide bridges are also thought to have a function in other oligomeric proteins. Structural roles have been attributed to disulfide bridges between the δ subunits of the acetyl-

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1 The abbreviations used are: HPr, histidine-containing phosphoryl carrier protein of the PTS; PTS, phosphoenolpyruvate-sugar phosphotransferase system; II\(^{Glc}\), transmembrane subunit of the glucose permease; III\(^{Glc}\), cytoplasmic subunit of the glucose permease, glucose-specific enzyme III of the PTS; P-I\(^{II}\) and P-I\(^{III}\), phosphorylated intermediates of II\(^{Glc}\) and III\(^{Glc}\); II\(^{Glc}\)(421), II\(^{Glc}\) mutant with serine instead of cysteinyl residue 421; enzyme L, enzyme I of the PTS; PEP, phosphoenolpyruvate; aMG, methyl-a-D-glucopyranoside; octyl-POE, polydisperse octyl-oligooxyethylene; NP-40, Nonidet P-40; DTT, dithiothreitol.
Glucose Permease of *E. coli*

**Enzymology of the bacterial phosphotransferase system.** Enzyme I and HPr are the cytoplasmic phosphoryl carrier proteins. III*Gc* is the cytoplasmic subunit, II*Gc* the transmembrane subunit of the glucose permease. II*Gc* transports glucose across the cytoplasmic membrane (dotted areas) and concomitantly phosphorylates the transported sugar. Each subunit of the glucose permease contains one phosphorylation site. In some other permeases of the bacterial phosphotransferase system the two phosphorylation sites are localized on a single protein subunit. The solid arrows show the sequential transfer of a phosphoryl group from phosphoenolpyruvate (PEP) to glucose as it occurs in the P-enolpyruvate:glucose phosphotransferase assay. The broken arrows show II*Gc* catalyzed phosphoryl exchange between glucose 6-phosphate and radiolabeled glucose or α-MG (Glc*, transphosphorylation reaction). Pro, pyruvate.

Choline receptor (Zingsheim et al., 1982) and between the subunits of the human transferrin receptor (Jing and Trowbridge, 1987), and a possible role in signaling has been attributed to disulfide/sulfhydryl exchanges between subunits of the insulin receptor (Maturo et al., 1983).

**EXPERIMENTAL PROCEDURES**

**RESULTS**

*Purification of II* *Gc*—The procedure used to purify II*Gc* from *S. typhimurium* was followed (Erni et al., 1982). It consists of membrane solubilization at alkaline pH, isoelectric focusing in a sucrose density gradient, and chromatofocusing. Zonal sedimentation on a glycerol gradient is no longer necessary for preparations from overproducing *E. coli* strains. Because of the increased II*Gc* concentration in the membrane extracts, the amount of protein applied to the isoelectric focusing columns had to be reduced by 40% and the detergent concentration increased from 1 to 2% final concentration. The amount of protein per batch is limited by the capacity of the isoelectrofocusing column, and increasing the column volume is limited by the concomitant increase of the focusing time. II*Gc* of *E. coli* is unstable at the alkaline pH where it focuses, and it slowly loses activity during the time required for complete focusing of larger volumes. To prevent loss of activity, the pH of II*Gc*-containing fractions was lowered to 7.0 immediately after collection. For chromatofocusing, II*Gc* was equilibrated with the alkaline starting buffer by gel filtration rather than by dialysis, and the preparation was then chromatofocused without delay. After isoelectric focusing, II*Gc* was approximately 80% pure, and after chromatofocusing it appears homogeneous on polyacrylamide gels stained with alcaline silver nitrate (see Fig. 3, below) or Coomassie Blue (not shown). The total recovered activity is between 60 and 100% of the activity originally present in the membrane fraction (Table II, Miniprint). The specific activity of purified II*Gc* varies depending on the assay conditions. It is five times as high if partially purified enzyme I, HPr, and III*Gc* are used instead of the crude cytoplasmic extract which is routinely used in assays to monitor the progress of II*Gc* purification.

**Differences between II*Gc* of *E. coli* and *S. typhimurium*—II*Gc* of *E. coli* and *S. typhimurium* have the same electrophoretic mobility on polyacrylamide gels (Fig. 2a), are equally active in combination with III*Gc* originating from either *E. coli* or *S. typhimurium*, and have amino acid compositions indistinguishable by routine amino acid analysis (Erni and Zanolari, 1986). They focus, however, at different pH values during isoelectric focusing and chromatofocusing. A difference between the two proteins could be detected with monoclonal antibodies and on tryptic fingerprints. Of six monoclonal antibodies raised against II*Gc* of *S. typhimurium*, four cross-reacted with II*Gc* of *E. coli* on immunoblots but two did not (Fig. 2a). A small difference between II*Gc* of *E. coli* and *S. typhimurium* could also be detected on tryptic fingerprints of the purified, radioiodinated proteins (Fig. 2b).

The Dimeric Forms of II*Gc*—When purified II*Gc* was chromatofocused in the absence of DTT or dialyzed against a buffer from which DTT had been omitted and then analyzed by glycerol gradient centrifugation, two forms with different sedimentation velocity could be distinguished and identified as dimer and monomer from their electrophoretic mobilities on polyacrylamide gels (Fig. 3). Because the dimeric form appeared only when DTT had been omitted from the sample buffer, the two subunits must be cross-linked by one or several disulfide bridges. If II*Gc* was kept reduced with DTT, its sedimentation velocity varied with temperature. At 4°C, reduced II*Gc* co-sedimented with the oxidized dimeric form (Fig. 3c), while at 15°C it sedimented as a monomer (Fig. 3d). Purified II*Gc* therefore exists in at least three different forms: as a disulfide-cross-linked dimer, as a noncovalently bonded dimer, and as a monomer. All three forms have P-enolpyruvate:glucose phosphotransferase activity if assayed in the presence of DTT (Figs. 1 and 3).

Disulfide-Bridged Dimeric II*Gc* Is Inactive—To assay the activity of disulfide-bonded dimeric II*Gc* P-enolpyruvate as the phosphoryl donor was replaced by P-III*Gc* which unlike enzyme I does not contain cysteines (Nelson et al., 1984; Saffent et al., 1987) and is insensitive to oxidizing conditions.

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1 Portions of this paper (including "Experimental Procedures," part of "Results," Figs. 8-12, and Table II) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.

2 B. Erni, unpublished data.

3 B. Erni and B. Zanolari, unpublished results.
Fig. 3. Characterization of the monomeric (M) and dimeric (D) forms of II\textsuperscript{Glc}. Oxidized II\textsuperscript{Glc} (a and b) and reduced II\textsuperscript{Glc} (c and d) were analyzed by zonal sedimentation on glycerol gradients at 4 and 15 °C. Of each II\textsuperscript{Glc}-containing fraction, aliquots were analyzed by gel electrophoresis in the absence (a and b) or presence of DTT (c and d) and assayed for P-enolpyruvate:glucose phosphotransferase activity in the presence of DTT (open bars). The oxidized II\textsuperscript{Glc} was obtained by chromatofocusing in the absence of DTT. The reduced II\textsuperscript{Glc} was obtained from oxidized II\textsuperscript{Glc} by incubation in 5 mM DTT for 45 min prior to centrifugation. Sedimentation was from right to left.

Fig. 4. Sugar phosphorylation activity of oxidized and reduced II\textsuperscript{Glc}. a, activity of reduced II\textsuperscript{Glc} (fraction 8 of a glycerol gradient, Fig. 3d) measured in the absence (O) and in the presence (●) of 2.5 mM DTT. b, activity of oxidized II\textsuperscript{Glc} (fraction 4 of a glycerol gradient, Fig. 3b) measured in the absence (O) and presence (●) of DTT. P-III\textsuperscript{Glc} was the phosphoryl donor, [\textsuperscript{32P}]P-PIP-II\textsuperscript{Glc} the phosphoryl acceptor.

As shown in Fig. 4, disulfide-bonded II\textsuperscript{Glc} from fraction 4 of a glycerol gradient (Fig. 3b) was inactive unless DTT was added to the incubation mixture. Monomeric II\textsuperscript{Glc} taken from fraction 8 of a glycerol gradient (Fig. 3d) had reduced activity if assayed without added DTT (30 μM was introduced with the II\textsuperscript{Glc} aliquot), indicating that II\textsuperscript{Glc} is oxidation sensitive and that oxidation sensitivity and disulfide bridge formation might be related to the same cysteines. II\textsuperscript{Glc} which sedimented as oxidized dimer had a higher specific activity than reduced II\textsuperscript{Glc}. It appears that during sedimentation of oxidized II\textsuperscript{Glc} at 15 °C, denatured II\textsuperscript{Glc} which can no longer dimerize is left behind and thus separated from active II\textsuperscript{Glc} (Fig. 3, a and b).

Cys-421 Forms the Disulfide Bridge between II\textsuperscript{Glc} Monomers—Two catalytic steps can be distinguished experimentally in II\textsuperscript{Glc}-dependent phosphorylation of glucose (Fig. 1). They are, first, P-III\textsuperscript{Glc}-dependent phosphorylation of II\textsuperscript{Glc} and, second, phosphoryl transfer from P-II\textsuperscript{Glc} to glucose, which is believed to occur coupled with vectorial glucose transport. Formation of P-II\textsuperscript{Glc} can be easily assayed if II\textsuperscript{Glc} is immobilized on nitrocellulose paper and then incubated with a soluble phosphoryl donor, either with [\textsuperscript{32P}]P-enolpyruvate in the presence of catalytic amounts of the phosphoryl carrier proteins or with [\textsuperscript{32P}]P-III\textsuperscript{Glc}. After washing of the nitrocellulose discs with buffer, the \textsuperscript{32P} transferred to II\textsuperscript{Glc} can be determined by liquid scintillation counting. Disulfide-cross-linked dimeric II\textsuperscript{Glc} and reduced monomeric II\textsuperscript{Glc} were separated by polyacrylamide gel electrophoresis, electroblotted to nitrocellulose, and then assayed for [\textsuperscript{32P}]P-III\textsuperscript{Glc}-dependent phosphorylation both in the presence and in the absence of DTT. As shown in Table I, II\textsuperscript{Glc} purified as oxidized dimer and II\textsuperscript{Glc} purified as reduced monomer were both phosphorylated under reducing conditions but not (or only very little) under oxidizing conditions. Oxidation of II\textsuperscript{Glc} prevented phosphorylation of II\textsuperscript{Glc} by P-III\textsuperscript{Glc}. To identify the oxidation-sensitive cysteine(s), phosphorylation of wild-type II\textsuperscript{Glc} was compared with the phosphorylation of four mutant II\textsuperscript{Glc} which had one or two of the three cysteines replaced by serines (Nuoffer et al., 1988). The five different variants of II\textsuperscript{Glc} were purified from crude membranes by polyacrylamide gel electrophoresis, electroblotted to nitrocellulose, and assayed under reducing and oxidizing conditions. Wild-type II\textsuperscript{Glc} and, similarly, the mutants with serines in place of Cys-204 and Cys-326 were phosphorylated under reducing conditions, but not under oxidizing conditions (Fig. 5). The mutant with serine in place of Cys-421, in contrast, is inactive (Nuoffer et al., 1988) and could not be phosphorylated at all. Because Cys-421 is present in all oxidation-sensitive forms of II\textsuperscript{Glc}, while Cys-204 and/or Cys-326 can be replaced by serine, Cys-421 must be the oxidation-sensitive residue and also involved in formation of a disulfide bridge. Iodoacetamide did not react with oxidized, dimeric II\textsuperscript{Glc} (Fig. 8, Miniprint), as expected if the catalytically essential Cys-421 is oxidized to a disulfide

### Table I

<table>
<thead>
<tr>
<th>Presence of 2.5 mM DTT</th>
<th>Dimeric II\textsuperscript{Glc}</th>
<th>Monomeric II\textsuperscript{Glc}</th>
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<td>Absence of DTT</td>
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<td>0.09</td>
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<td>0.0035</td>
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Phosphorylation of immobilized II\textsuperscript{Glc} by soluble [\textsuperscript{32P}]P-III\textsuperscript{Glc}

Oxidized dimeric and reduced monomeric II\textsuperscript{Glc} were separated by gel electrophoresis and blotted to nitrocellulose. The two forms of immobilized II\textsuperscript{Glc} were incubated with soluble [\textsuperscript{32P}]P-III\textsuperscript{Glc} in the presence and in the absence of 2.5 mM DTT. Given are the molar ratios of \textsuperscript{32P} bound per II\textsuperscript{Glc} monomer, calculated from the specific activity of \textsuperscript{32P} and the amount of purified II\textsuperscript{Glc} (20 pmol) applied to the polyacrylamide gel. It was assumed that II\textsuperscript{Glc} was quantitatively transferred from the gel to nitrocellulose.
and thus protected against alkylation.

**II^{Gle} Is Active as a Monomer**—The preceding experiments indicated that disulfide-linked dimeric II^{Gle} is inactive, but not whether the reduced II^{Gle} is active in the monomeric or in the dimeric form. It can be expected that, if II^{Gle} were active as a dimer, any catalytic intermediate of II^{Gle} should be dimeric, too. We therefore looked for the dimeric form of phosphorylated II^{Gle} by oxidizing P-II^{Gle} and by cross-linking it with glutaraldehyde. All phosphorylated II^{Gle} was monomeric, and no phosphorylated dimers could be detected (Fig. 6, lanes 4 and 8), although substantial amounts of nonphosphorylated II^{Gle} were trapped as dimers (Fig. 6, lanes 2 and 6). The increased electrophoretic mobility of some of the cross-linked samples (Fig. 6, lanes 2 and 4) indicates that glutaraldehyde did react with P-II^{Gle} and presumably prevented the complete unfolding of the polypeptide chain. The failure to detect the phosphorylated dimer can therefore not be due to lack of reactivity of the phosphorylated form of II^{Gle}.

On the other hand, cross-linking via a disulfide might depend on the distance between the reactive cysteines, and in this case failure to detect cross-linking could have steric reasons, e.g. be due to a different conformation or a decreased flexibility of the phosphorylated subunits.

The following two observations are compatible with II^{Gle} being active as a monomer. 1) Monomeric II^{Gle} catalyzed the phosphoryl exchange between glucose 6-phosphate and [14C]αMG (transphosphorylation reaction, Fig. 1; Saier et al., 1977). If glucose 6-phosphate and [14C]αMG were present during centrifugation at 15 °C, [14C]αMG 6-phosphate was found in the fractions of the gradient through which monomeric II^{Gle} had swept (Fig. 7c). Although indicating that II^{Gle} is active as a monomer, this observation is not conclusive. In a control experiment where 0.0025% glutaraldehyde was present in the gradient, only 60% of II^{Gle} was monomeric, while 30% was covalently cross-linked at the end of the centrifugation experiment (Fig. 7b). It must therefore be assumed that dimeric II^{Gle} can form even at 15 °C, although the amount present at any time of the experiment must be exceedingly small. 2) II^{Gle} with a serine in place of Cys-421 did not inhibit wild-type II^{Gle}, even if the former was expressed in excess (Fig. 9, Miniprint). This means that II^{Gle} either does not form (hetero)dimers in vivo, or that, if formed, dimerization does not affect the P-enolpyruvate:glucose phosphotransferase activity of II^{Gle}. This single and conservative amino acid substitution should not impair the ability of II^{Gle}(C421S) to associate with wild-type II^{Gle}, particularly, as it has been demonstrated by Nuoffer et al. (1988), that II^{Gle}(C421S) is normally expressed, inserted into the membrane, and also has a distinct effect in the metabolism of maltose.

**FIG. 6. Phosphorylated II^{Gle} is a monomer.** a, [32P]P-II^{Gle} was incubated for 40 min at 4 °C without (-) and with (+) 0.0025% glutaraldehyde. The products were separated by gel electrophoresis and the gels autoradiographed (lanes 3 and 4) and silver stained (lanes 1 and 2). b, [32P]P-II^{Gle} was oxidized by 3-fold dilution into electrophoresis sample buffer without DTT (-) or kept reduced with 2 mM DTT present in the buffer (+). The products were electrophoresed: autoradiography (lanes 7 and 8), silver-stained gel (lanes 5 and 6). Note that II^{Gle} (*) is not stained by alkaline silver nitrate (lanes 1, 2, 5, and 6) but visible on the autoradiographs (lanes 3, 4, 7, and 8). D, dimeric II^{Gle}; M, monomeric II^{Gle}. [32P]P-II^{Gle} was freshly prepared before cross-linking as follows: 0.6-1.0 μg of purified II^{Gle} was incubated for 5 min at 37 °C in 10 μl containing 50 mM NaPi, pH 7.5, 2.5 mM DTT, 1% polysine, 0.05% octyl-oligoxyethylene, 5 mM MgCl₂, 2.5 mM NaN₃, 2 μM [32P]P-enolpyruvate, and enzyme I, HPr, and II^{Gle} at 1/100 the concentration present in the P-enolpyruvate:glucose phosphotransferase assay.

**FIG. 7. Characterization of monomeric II^{Gle}.** Reduced II^{Gle} was sedimented at 15 °C through a glycerol gradient containing 1 mM DTT (a) and 1 mM DTT plus 0.0025% glutaraldehyde (b). Aliquots of each fraction were analyzed by gel electrophoresis in the presence of DTT and assayed for protein concentration by the method of Bradford (1976). A small amount of larger oligomers is also formed (b, fractions 2-6). Note that glutaraldehyde-cross-linked dimeric II^{Gle} (in fractions 7-9 containing 50% of total II^{Gle} applied to the gradient) and monomeric II^{Gle} (in fractions 10-12 containing 60% of II^{Gle}) have increased electrophoretic mobilities with respect to free monomeric II^{Gle} (b, left lane) and disulfide-cross-linked dimeric II^{Gle} (a, left lane). c, transphosphorylation activity of II^{Gle} during zonal sedimentation through the glycerol gradient shown in a. The amount of [14C]αMG-6-P formed in the region through which II^{Gle} has sedimented is shown (o). P-enolpyruvate:glucose phosphotransferase activity (open bars) was assayed at the end of the experiment. Due to diffusion, a concentration gradient of [14C]αMG (○) at the boundary between the upper and the lower part of the gradient. Sedimentation in all panels is from right to left.

**DISCUSSION**

With an overproducing *E. coli* strain and an improved purification procedure, up to 2 mg of II^{Gle} can be obtained from 10 g (wet weight) of cells, and the recovered P-enolpyruvate:glucose phosphotransferase activity approaches 100% of the activity present in the crude membrane fraction. As far as we can conclude from our studies which started with II^{Gle} of *S. typhimurium* and now continue with II^{Gle} of *E. coli*, the
two proteins are functionally indistinguishable. The small chemical difference, which was discovered using a monoclonal antibody, could be due to the replacement at the surface of the protein of, e.g. a negatively charged by a positively charged amino acid (Geyser et al., 1987). Small differences in the amino acid sequences of the PTS proteins III\textsuperscript{Glc} and enzyme I, and HP\textsubscript{r} of \textit{E. coli} and \textit{S. typhimurium} have recently been reported by Seffen et al. (1987).

None of the monoclonal antibodies inhibits phosphorylation of II\textsuperscript{Glc} by P-III\textsuperscript{Glc}. Rather, they appear to lock P-II\textsuperscript{Glc} in a conformation where the bound phosphor group is stabilized (Fig. 11, Miniprint) and can no longer be transferred to glucose (Fig. 10, Miniprint). Polyclonal anti-II\textsuperscript{Glc} sera from rats have the same properties (results not shown). The antiserum and the monoclonal antibodies do not cross-react on nitrocellulose blots either with the \textit{N}-acyethylglucosamine-specific permease, which has a very similar amino acid sequence, or with the mannose permease which, like II\textsuperscript{Glc}, has glucose as a substrate.

Purified II\textsuperscript{Glc} forms dimers, and a disulfide bridge can be established between functionally active subunits. However, the disulfide-linked dimeric form of II\textsuperscript{Glc} itself cannot be phosphorylated by P-III\textsuperscript{Glc} and is therefore completely inactive. In practice, this property may be exploited to remove the denatured protein (which does not dimerize) from active II\textsuperscript{Glc}, and it can also be used to stabilize II\textsuperscript{Glc} during storage. Over periods of up to three weeks, oxidized II\textsuperscript{Glc} retained more than 70\% of the original activity, while reduced II\textsuperscript{Glc} retained less than 10\% on standing at 20 \degree C. Because Cys-421 is the only catalytically essential cysteine (Nuoffer et al., 1988) and also the only sulfhydryl involved in disulfide bridge formation, its reversible oxidation might be used to selectively protect it.

To understand the molecular mechanism of glucose transport and phosphorylation, it is important to know whether II\textsuperscript{Glc} is active as a monomer or as a noncovalently bound dimer. The dimeric form could be detected only in highly purified preparations of II\textsuperscript{Glc}, but not in crude membrane preparations or membrane extracts containing sodium dodecyl sulfate or the non-ionic detergent polydisperse octyloligooxyethylene. No dimeric form of phosphorylated II\textsuperscript{Glc} could be detected only in highly purified preparations of II\textsuperscript{Glc}, containing sodium dodecyl sulfate or the non-ionic detergent polydisperse octyloligooxyethylene. No dimeric form of phosphorylated II\textsuperscript{Glc} could be trapped by cross-linking, strongly suggesting that the catalytically active intermediate of II\textsuperscript{Glc} is actually a monomer. Also, all attempts failed to find a sigmoidal dependence between activity and concentration of II\textsuperscript{Glc}, a relation which could indicate cooperativity between the subunits in an oligomer. These results suggest that the monomeric form of II\textsuperscript{Glc} is fully active and that dimerization may well be an artefact of purification.

The lack of detectable cooperativity and the conclusions drawn from the above results contrast with results obtained with the mannitol permease, another enzyme II of the bacterial phosphotransferase system (see the Introduction). Although the two permeases have little amino acid sequence similarity (Saier et al., 1988), their mode of action is believed to be analogous. The discrepancy could be due either to functional differences between the two permeases or to in vitro artefacts. The sigmoidal activity/concentration dependence observed with the mannitol permease occurred only at very low protein concentrations. The possibility that this nonlinear relationship was caused by adsorption to the test tube of the initially added protein cannot therefore be excluded. Nonlinearity was observed only in the transphosphorylation reaction (Leonard and Saier, 1983; Roosen et al., 1984; Stephan and Jacobson, 1986) where purified mannitol permease was the only protein present, but not in the P-enolpyruvate-dependent phosphorylation of mannitol, when the soluble phosphoryl carrier proteins were present in excess over the permease. Note, however, one exception recently described by Robillard and Blaauw (1987). Also, addition of bovine serum albumin or increasing the phosphate buffer concentration abolished the sigmoidal activity/concentration profile of the transphosphorylation reaction, as expected if bovine serum albumin acted as a competitor for nonspecific protein binding, and the increased ionic strength of the buffer weakened protein-test tube interactions. On the other hand, there may be functional properties which could explain the difference of oligomeric structure. The glucose permease consists of two different polypeptide subunits (III\textsuperscript{Glc} and III\textsuperscript{II}) which are each phosphorylated once. The mannitol permease consists of one large polypeptide which is thought to contain two phosphorylation sites (Saier et al., 1985, 1988). Two phosphorylation sites on a single polypeptide chain have been identified in the III\textsuperscript{Mann} subunit of the mannose permease, yet another permease of the PTS (Erni et al., 1987). In addition, phosphoryl transfer can occur between two different III\textsuperscript{Mann} subunits. If the phosphoryl transfer corresponded to that between III\textsuperscript{Glc} and III\textsuperscript{II} occurred between sites located on different mannositol permease (or III\textsuperscript{Mann}) molecules, then the mannitol permease (and III\textsuperscript{Mann}) obligatorily would have to be a dimer during catalysis of P-enolpyruvate-dependent phosphotransferase activity. In contrast, noncovalent interaction between II\textsuperscript{Glc} and III\textsuperscript{II} during phosphoryl transfer does not require dimerization of the identical subunits. Although this model of phospho transfer between rather than within the permease polypeptides remains to be tested experimentally in vivo, it suggests how divergent results from different PTS permeases may reflect differences in their respective modes of action.

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by Heinrich Mecke, Neta Safran, and Daud Erez

EXPERIMENTAL PROCEDURES AND RESULTS

Bacterial strains and growth conditions. - E. coli strain A177(pGEM7) was used to transform recipients. Glucose and fructose were used as carbon sources. A177 was transformed with plasmids carrying a mutated gpm gene (Safran et al., 1984). E. coli strain 12991, 5139, and 1101 (Hecht, 1980) were used for the construction of deletion mutants. These bacteria were kept frozen at -70°C. For the purification of wild-type GPM, E. coli strain J53(pGEM7) was streaked out on McIlvaine plates, supplemented with 0.2% glucose and 100 mg/ml ampicillin. A single colony was picked and grown overnight in 5 ml LB broth. 0.5 ml of cell homogenate, 0.5 ml of cell extract, and 1 ml of cell extract were supplemented with 100 mg/ml ampicillin in a test tube on a rotary shaker at 37°C. The culture was diluted 1:10 with fresh medium and allowed to grow for another hour. One ml of this culture was incubated with 1.5 ml of 100 mM D-glucose containing 100 mM ampicillin in a 5 ml Eppendorf flask. This culture was grown to late log phase (OD 600 = 3.5). The cells were harvested by washing with 0.9% NaCl, processed, and frozen at -70°C. About 8 g of packed cells were obtained per liter of medium.

Purification of GPM - All manipulations were performed at 4°C. Following growth, membrane extracts were prepared as described (Roseman et al., 1987) except that only 0.5 g of packed cells were used for the preparation of 10 ml of supernatant fraction. Concentration of the membrane extract was carried out by ultracentrifugation at 200,000 g for 1 hr at 4°C. The 1552 KDa monomer was obtained by gel filtration and dialysis against 100 mM NaCl, 50 mM Tris, pH 7.5. The active fractions were pooled and the pH was adjusted to 7.0 by the addition of 1 N NaOH. The precipitate was removed by centrifugation at 20,000 g for 30 min. The supernatant was dialyzed overnight against 100 mM NaCl, 50 mM Tris, pH 7.5. The active fractions were pooled and dialyzed against 100 mM NaCl, 50 mM Tris, pH 7.5. The precipitate was removed by centrifugation.

TABLE 1

Activity of 1552 KDa during purification

<table>
<thead>
<tr>
<th>Protein Activity</th>
<th>Specific Activity</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>Membranes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Activity</td>
<td>570</td>
<td>100</td>
</tr>
<tr>
<td>Specific activity</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chromatofocusing</td>
<td>4.4</td>
<td>330</td>
</tr>
</tbody>
</table>

Preparation of 1552 KDa GPM was modified by the method of Schulte et al. (1988). From DEAE chromatography, was precipitated with 500 mg/4 ml of the precipitate, the precipitate was dissolved in 50 mM NaCl, pH 7.5 (10 mg/ml containing 10 mg/ml polyethylene glycol 4000). 1 ml of the dialyzate (0.5 - 1.0 mg of protein) was dialyzed overnight against 100 mM NaCl, 50 mM Tris, pH 7.5. The active fractions were obtained by gel filtration (Pharmacia) at a flow rate of 2 ml/min. The active fractions were concentrated to the desired volume and dialyzed against 100 mM NaCl, 50 mM Tris, pH 7.5. The active fractions were concentrated by gel filtration and dialyzed against 100 mM NaCl, 50 mM Tris, pH 7.5. The protein concentration was determined by measuring the absorbance at 280 nm.

Glucose Permease of E. coli

12991


Glucose Permease of E. coli

**PF-P**

Glucose phosphotransferase assay - Sugar phosphorylation activity was assayed by the ion exchange method of Huntig and Morgan (1971) modified as described by Anderson et al. (1973). The reaction mixture contained, per ml: 100 µg glucosephosphorylase from Paenibacillus macerans (5 mg), 10 µM ATP, pH 7.5, 3.75 µl 10X reaction buffer (pH 8.0) containing 0.5 mM MgCl₂ and 10 µM EDTA, 1 µM [3H]ADP, and 10 µM [32P]ATP. The reaction was initiated by the addition of soluble phosphohexose isomerase, glucose 1-phosphate dehydrogenase, and glucose 6-phosphate dehydrogenase, with the concentrations of each enzyme being 10 units of isomerase, 1 unit of glucose dehydrogenase, and 0.6 units of glucose 6-phosphate dehydrogenase, as purified through the first octyl-sulfate step. After incubation at pH 7.8, the reaction was terminated by the addition of either 10 µl of phosphatase or 10 µl of ATPase. The reaction was stopped by the addition of 10 µl of 80% trichloroacetic acid (TCA) and the formation of phosphorus was determined by the addition of 50 µl of 1 N HCl and 10 µl of 0.5 N NaOH. The mixture was then centrifuged for 10 min at 15000 rpm and the supernatant was analyzed for phosphorus content. The assay was linear for at least 10 min. The results were expressed as units of activity per milliliter of the reaction mixture. The assay was performed at 30°C.

**PF-P**

Assay for P-IIIc**

Dependent sugar phosphorylation activity was assayed by the ion exchange method of Huntig and Morgan (1971) modified as described by Anderson et al. (1973). The reaction mixture contained, per ml: 100 µg glucosephosphorylase from Paenibacillus macerans (5 mg), 10 µM ATP, pH 7.5, 3.75 µl 10X reaction buffer (pH 8.0) containing 0.5 mM MgCl₂ and 10 µM EDTA, 1 µM [3H]ADP, and 10 µM [32P]ATP. The reaction was initiated by the addition of soluble phosphohexose isomerase, glucose 1-phosphate dehydrogenase, and glucose 6-phosphate dehydrogenase, with the concentrations of each enzyme being 10 units of isomerase, 1 unit of glucose dehydrogenase, and 0.6 units of glucose 6-phosphate dehydrogenase, as purified through the first octyl-sulfate step. After incubation at pH 7.8, the reaction was terminated by the addition of either 10 µl of phosphatase or 10 µl of ATPase. The reaction was stopped by the addition of 10 µl of 80% trichloroacetic acid (TCA) and the formation of phosphorus was determined by the addition of 50 µl of 1 N HCl and 10 µl of 0.5 N NaOH. The mixture was then centrifuged for 10 min at 15000 rpm and the supernatant was analyzed for phosphorus content. The assay was linear for at least 10 min. The results were expressed as units of activity per milliliter of the reaction mixture. The assay was performed at 30°C.

**PF-P**

Glucose phosphotransferase assay - Sugar phosphorylation activity was assayed by the ion exchange method of Huntig and Morgan (1971) modified as described by Anderson et al. (1973). The reaction mixture contained, per ml: 100 µg glucosephosphorylase from Paenibacillus macerans (5 mg), 10 µM ATP, pH 7.5, 3.75 µl 10X reaction buffer (pH 8.0) containing 0.5 mM MgCl₂ and 10 µM EDTA, 1 µM [3H]ADP, and 10 µM [32P]ATP. The reaction was initiated by the addition of soluble phosphohexose isomerase, glucose 1-phosphate dehydrogenase, and glucose 6-phosphate dehydrogenase, with the concentrations of each enzyme being 10 units of isomerase, 1 unit of glucose dehydrogenase, and 0.6 units of glucose 6-phosphate dehydrogenase, as purified through the first octyl-sulfate step. After incubation at pH 7.8, the reaction was terminated by the addition of either 10 µl of phosphatase or 10 µl of ATPase. The reaction was stopped by the addition of 10 µl of 80% trichloroacetic acid (TCA) and the formation of phosphorus was determined by the addition of 50 µl of 1 N HCl and 10 µl of 0.5 N NaOH. The mixture was then centrifuged for 10 min at 15000 rpm and the supernatant was analyzed for phosphorus content. The assay was linear for at least 10 min. The results were expressed as units of activity per milliliter of the reaction mixture. The assay was performed at 30°C.
**FIG. 13.** Neutralization of PEPl glucone phosphatase activity of $i_{\text{Glc}}^{\text{Glc}}$ by monoclonal antibodies A-F. $i_{\text{Glc}}^{\text{Glc}}$ preparations: medium - and purified $i_{\text{GPI}}^{\text{Glc}}$ of E. coli strain 12983. $i_{\text{Glc}}^{\text{Glc}}$ 12983 medium proteins, 0.1 mg purified protein, were incubated with the indicated amounts of IgG in 50 μl of assay buffer without enzymes. 100 μl of 100 μl of assay buffer containing all components to complete the assay mixture were added and incubated at 37°C for 30 min.

**FIG. 14.** Stabilization of phosphoprotein $i_{\text{Glc}}^{\text{Glc}}$ by antibodies A-F. Purified $i_{\text{Glc}}^{\text{Glc}}$ (10 μg) in 30 μl assay buffer without enzymes, 1 μg, $i_{\text{Glc}}^{\text{Glc}}$, 10 μg IgG and FEP was incubated with 20 μg of IgG A-F, cat anti-mouse IgG (IgG) and without IgG (C). After 30 min at 4°C, 10 μl of assay buffer containing enzyme 1, 0.1 μg, $i_{\text{Glc}}^{\text{Glc}}$ and $i_{\text{Glc}}^{\text{Glc}}$ were added and incubation continued at 4°C. 30 μl aliquots were withdrawn after 2 min (left lanes), 5 min (middle lanes), and 10 min (right lanes), chilled on ice, and diluted with 5 ml cold concentrated gel electrophoresis sample buffer. Proteins were separated by gel electrophoresis, electroblotted to nitrocellulose, and the phosphorylated proteins were visualized by autoradiography. The concentration of soluble phosphorylated protein was five times higher than in the PEPl-glucose phosphotransferase assay.