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Glucose-Specific Permease of the Bacterial Phosphotransferase System: Phosphorylation and Oligomeric Structure of the Glucose-Specific II^{Glc}–III^{Glc} Complex of *Salmonella typhimurium*†

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ABSTRACT: The glucose-specific membrane permease (II^{Glc}) of the bacterial phosphoenolpyruvate-dependent phosphotransferase system (PTS) mediates active transport and concomitant phosphorylation of glucose. The purified permease has been phosphorylated in vitro and has been isolated (P-II^{Glc}). A phosphate to protein stoichiometry of between 0.6 and 0.8 has been measured. Phosphoryl transfer from P-II^{Glc} to glucose has been demonstrated. This process is, however, slow and accompanied by hydrolysis of the phosphoprotein unless III^{Glc}, the cytoplasmic phosphoryl carrier protein specific to the glucose permease (II^{Glc}) of the PTS, is added. Addition of unphosphorylated III^{Glc} resulted in rapid formation of glucose 6-phosphate with almost no hydrolysis of P-II^{Glc} accompanying the process. A complex of II^{Glc} and III^{Glc} could be precipitated from bacterial cell lysates with monoclonal anti-II^{Glc} immunoglobulin. The molar ratio of II^{Glc}:III^{Glc} in the immunoprecipitate was approximately 1:2. Analytical equilibrium centrifugation as well as chemical cross-linking showed that purified II^{Glc} itself is a dimer (106 kDa), consisting of two identical subunits. These results suggest that the functional glucose-specific permease complex comprises a membrane-spanning homodimer of II^{Glc} to which four molecules of III^{Glc} are bound on the cytoplasmic face.

The bacterial phosphotransferase system (PTS)[1] comprises a number of proteins with interdependent metabolic and regulatory functions. One primary function is the active uptake of sugars and hexitols. Depending on the availability of these substrates, components of the PTS regulate the uptake of nutrients through other transport systems [by inducer exclusion and/or by regulation of gene expression; for reviews, see Postma & Roseman (1976) and Saier (1977, 1985)]. A functional PTS is further required for PTS substrate-directed chemotaxis (Adler & Epstein, 1974; Niwano & Taylor, 1982). Genetic and biochemical analysis revealed that the PTS consists of two cytoplasmic proteins and several membrane permeases with different sugar specificities. The permease proteins (enzymes II) span the cytoplasmic membrane, mediating sugar transport concomitant with sugar phosphorylation. As depicted schematically in Figure 1, the cytoplasmic proteins, enzyme I and HPr, are the phosphoryl carriers that transfer phosphoryl groups sequentially from phosphoenolpyruvate to the sugars in an enzyme II dependent reaction. Uptake of glucose in particular requires a third cytoplasmic protein, designated III^{Glc}. It functions as the phosphoryl carrier

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[1] Abbreviations: PTS, phosphoenolpyruvate sugar–phosphotransferase system; enzyme I, enzyme I of the PTS; HPr, histidine-containing phosphocarrier protein of the PTS; P-II^{Glc}, phosphorylated form of the glucose-specific enzyme II of the PTS; P-III^{Glc}, phosphorylated form of the glucose-specific enzyme III of the PTS; αMG, methyl α-glucopyranoside; octyl-POE, polydisperse octyloligo(oxyethylene); Glc-6-P, glucose 6-phosphate.

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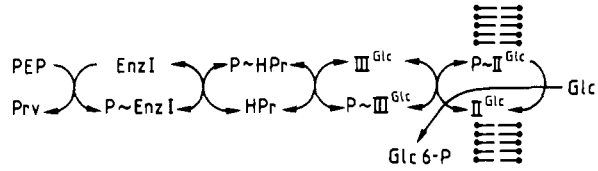

FIGURE 1: Glucose-specific phosphoenolpyruvate-dependent phosphotransferase system. Enzymes I and HPr are the general phosphoryl carrier proteins. Enzyme I transfers a phosphoryl group from phosphoenolpyruvate (PEP; Prv, pyruvate) to HPr. From P-HPr, the phosphoryl group is transferred to III^{Glc} (and enzymes II and III of other sugar specificities not shown in this scheme) and then to II^{Glc}. II^{Glc} mediated sugar transport across the cytoplasmic membrane, concomitant with sugar phosphorylation.

between HPr and II^{Glc}. It is also considered the effector of most if not all of the PTS-dependent regulatory events mentioned above. Thus, it has been demonstrated that III^{Glc} regulates the activities of adenylcyclase (Peterkofsky & Gazdar, 1975; Feucht & Saier, 1980, Scholte et al., 1982), of lactose permease (Osumi & Saier, 1982; Nelson et al., 1983), and of the glycerol- and maltose-uptake systems (Nelson & Postma, 1984; Postma et al., 1984). It has been suggested that the components of the PTS can exist as membrane-associated multiprotein complexes rather than as individual components (Scholte et al., 1982; Brouwer et al., 1982; Saier et al., 1982; Misset et al., 1983).

The soluble phosphoryl carrier proteins enzyme I (Robillard et al., 1979; Weigel et al., 1982a), HPr (Dooijewaard et al., 1979; Beneski et al., 1982), and III^{Glc} (Scholte et al., 1981; Meadow & Roseman, 1982) and the membrane protein II^{Glc} (Erni et al., 1982) have been purified to homogeneity. While the phosphorylated forms of the cytoplasmic phosphoryl carrier proteins have been isolated and characterized early (Anderson et al., 1971; Kundig & Roseman, 1971; Hays et al., 1973; Stein et al., 1974; Meadow & Roseman, 1982; Weigel et al., 1982b; Waygood & Mattoo, 1983), it has long been controversial whether enzymes II are transiently phosphorylated or whether they catalyze the transfer of the phosphoryl group from P-III^{sugar} (or the P-HPr) to the sugar directly (Simoni et al., 1973; Perret & Gay, 1979; Hüdig & Hengstenberg, 1980; Rephaeli & Saier, 1980). First evidence for the existence of a phosphorylated II^{Glc} came from the elegant experiment of Begley et al. (1982), who observed inversion of the chiral phosphoryl group during PTS-mediated transfer from phosphoenolpyruvate to glucose. Such a result would be expected if an even number of phosphoryl carriers participated in the reaction. Since three carriers, enzyme I, HPr, and III^{Glc}, were known to be phosphorylated, they inferred that II^{Glc} was most likely the fourth phosphorylated intermediate. More recently, direct evidence for ^{32}P-labeled II^{Glc} has been obtained by gel autoradiography (Zanolari & Erni, 1984; Peri et al., 1984).

In the first part of this paper we describe the conditions under which P-II^{Glc} can be isolated and show that the phosphoryl group of P-II^{Glc} is transferred to glucose in the absence of III^{Glc}. The relatively low rate and efficiency of this phosphoryl-transfer reaction is increased drastically by the presence of III^{Glc}. In the second part, we present structural evidence for a II^{Glc}–III^{Glc} complex, and we propose a model accounting for its stoichiometry.

Experimental Procedures

Purification of PTS Proteins. II^{Glc} was purified from *Salmonella typhimurium* PP1133 through the glycerol gradient step (Erni et al., 1982) and freed of αMG by dialysis against 10 mM Tris-HCl, pH 7.5, 1% octyl-POE (obtained from J. P. Rosenbusch; Rosenbusch et al., 1982), 1 mM dithiothreitol, and 0.1 mM EDTA. Of the cytoplasmic PTS proteins, HPr was purified through the DEAE-cellulose step (Beneski et al., 1982) and III^{Glc} through the gel filtration step (Scholte et al., 1981). A crude enzyme I containing preparation was obtained by fractionation of a cytoplasmic extract on DEAE-cellulose (Robillard et al., 1979).

Preparation of [^{32}P]Phosphoenolpyruvate. [^{32}P]-Phosphoenolpyruvate was prepared exactly as described by Roossien et al. (1983). The specific activity and the concentration of the [^{32}P]phosphoenolpyruvate was determined by titration with ADP in the presence of pyruvate kinase (Cheung & Marcus, 1975).

Protein Phosphorylation. The incubation mixtures contained 50 mM sodium phosphate, pH 7.5, 2.5 mM dithiothreitol, 2.5 mM NaF, 5 mM $MgCl_2$, 0.5 μM [^{32}P]-phosphoenolpyruvate (50–300 cpm/pmol), and, where indicated, either 0.1 mg/mL L-α-phosphatidylglycerol from egg yolk (Sigma) or 4.5% octyl-POE (Rosenbusch et al., 1982). II^{Glc}, III^{Glc}, enzyme I, and HPr were added as specified for each experiment. Incubation was at 37 °C for the time indicated in each experiment. The reaction was started by the addition of [^{32}P]phosphoenolpyruvate and was stopped by chilling the mixture on ice, immediately followed by addition of 5-fold concentrated gel electrophoresis sample buffer. Samples were not boiled prior to polyacrylamide gel electrophoresis (Anderson et al., 1973). Electrophoresis was performed at 4 °C. The gel was soaked for 10 min in 2% gycerol, dried without fixation on blotter paper, and autoradiographed on Fuji RX films with Kyokko HS intensifying screens. In some experiments, the proteins were electrophoretically transferred from the polyacrylamide gel to nitrocellulose paper (Towbin et al., 1979) and protein blots autoradiographed.

In Vivo Protein Labeling, Antisera, and Immunoprecipitation. Cells were grown on sulfur-limiting minimal medium in the presence of $^{35}SO_4^{2-}$ (Crawford & Gesteland, 1973). They were harvested by centrifugation and washed with 0.9% sodium chloride solution; 2×10^9 cpm of ^{35}S-labeled cells was mixed with 1.25 mg (wet weight) unlabeled cells in 60 μL of 10 mM Tris–glycine, pH 9.3. A volume of 10 μL of 10% sodium dodecyl sulfate was added to the suspension, which was then freeze-thawed 3 times and sonicated in a bath-type sonicator for 3 min. The suspension was diluted with 550 μL of Tris-buffered saline (TBS) containing 0.5% Nonidet NP-40, and insoluble material was removed by centrifugation for 5 min in an Eppendorf table-top centrifuge. The supernatant was withdrawn and divided into two equal portions. To one portion 30 μg of purified monoclonal IgG against II^{Glc} [2] was added; to the other, 90 μL of a polyclonal rabbit serum against III^{Glc} (obtained from Dr. P. Postma; Scholte et al., 1981) was added. After 6 h at 4 °C a second antibody, 30 μL of rabbit anti-mouse IgG (DAKO), and 30 μL swine anti-rabbit IgG (DAKO), respectively, were added. After 12 h at 4 °C, the immunoprecipitates were collected by centrifugation and washed in TBS 3 times with 2.5 M KCl and 0.5% Nonidet NP-40, once with 0.5% deoxycholate, and once with 0.1% sodium dodecyl sulfate (Hughes & August, 1981). The washed pellets were resuspended in 50 μL of sample buffer containing sodium dodecyl sulfate and heated to 100 °C, and 10-μL aliquots were analyzed by polyacrylamide gel electrophoresis. The dried gels were autoradiographed. Densitometric scans of the films were used for quantification. The relative amounts of III^{Glc} and II^{Glc} in the gel were determined

[2] Monoclonal antibodies against II^{Glc} were prepared in mice by standard procedures (Zanolari & Erni, 1984). A detailed report on their characterization will be submitted separately.

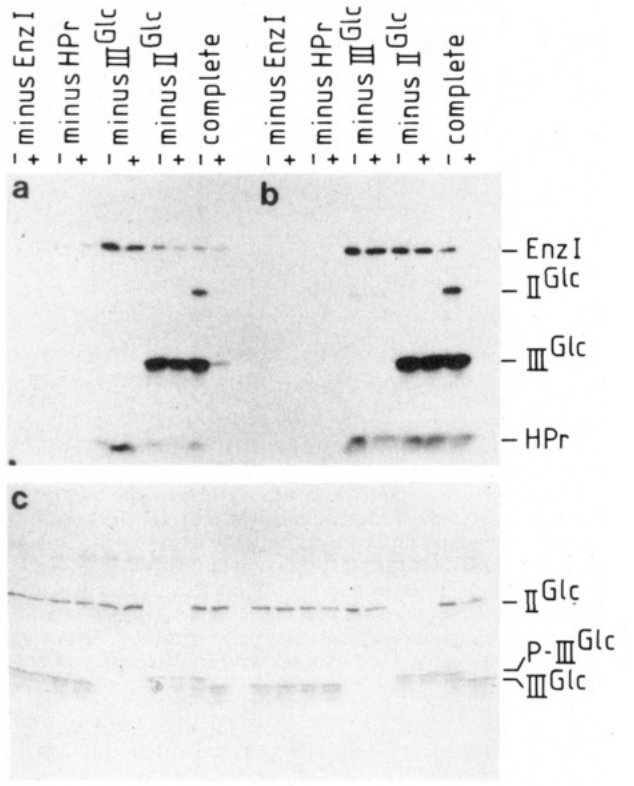

FIGURE 2: Identification of phosphorylated II^{Glc}. (a and b) Phosphorylated proteins of the glucose-specific phosphotransferase system. The results obtained with the complete system are compared with the effect of single omission of each protein component on the phosphorylation of all the other proteins in the presence (+) and in the absence (−) of αMG as a substrate of II^{Glc}. Incubation was for 1 (a) and 10 min (b). (c) Immunochemical identification of II^{Glc} and III^{Glc} with anti-II^{Glc} and anti-III^{Glc} antibodies. The complete incubation mixture contained per 0.05 mL 0.8 μg of enzyme I fraction, 0.2 μg of HPr, 0.2 μg of III^{Glc}, 0.7 μg of II^{Glc}, 50 μg of phosphatidylglycerol, residual octyl-POE (0.05%), and where indicated (+) 0.4 mM αMG. Aliquots (20 μL) were withdrawn from the incubation mixtures after 1-min (a) and 10-min (b) incubation at 37 °C. Proteins were separated by gel electrophoresis and transferred electrophoretically to nitrocellulose. ^{32}P-Labeled proteins were first detected by autoradiography of the protein blot (a and b), and II^{Glc} and III^{Glc} were subsequently identified with anti-II^{Glc} and anti-III^{Glc} antibodies (c). Bound antibodies were visualized by the reaction of lactoperoxidase coupled to antiimmunoglobulin antibodies (Towbin et al., 1979). Phosphorylated enzyme I and P-HPr were identified on the basis of their electrophoretic mobility (Beneski et al., 1982; Kukuruzinska et al., 1982).

from the ratio of ^{35}S in the corresponding peaks and from the molecular weights and the known amounts of sulfur-containing amino acids in each protein (Meadow & Roseman, 1982; Erni et al., 1982).

Equilibrium Centrifugation. The molecular weight of detergent-solubilized II^{Glc} was determined by equilibrium centrifugation according to Ludwig et al. (1982). The rotor speed was 14000 rpm, the temperature was 7 °C, and the partial specific volume of II^{Glc} is 0.73 $cm^3\ g^{-1}$ as calculated (Schachmann, 1957) from its amino acid composition (Erni et al., 1982). Protein concentration was approximately 0.15 mg/mL. The buffer was 20 mM NaP_i, pH 7.8, 0.1 M NaCl, 0.2 mM DTT, and 1% octylpentakis(oxyethylene) (BACHEM, Switzerland). Values for density of buffer and partial specific volume of the detergent micelle were taken from Ludwig et al. (1982).

RESULTS

Phosphorylation of II^{Glc}. Enzyme I, HPr, III^{Glc}, and pure II^{Glc} were incubated in the presence of [^{32}P]phosphoenol-

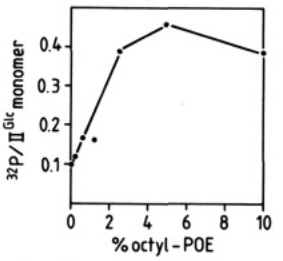

FIGURE 3: Stabilization of P-II^{Glc} by detergent. II^{Glc} was phosphorylated in the presence of different concentrations of octyl-POE. P-II^{Glc} was separated by polyacrylamide gel electrophoresis. P-II^{Glc}-containing bands were cut out from the gel, and the P:II^{Glc} ratio was calculated from the amount of radioactivity in the gel slice and the known amount of II^{Glc} applied to the gel. The incubation mixtures contained the same components as indicated in Figure 2 except for octyl-POE. Incubation was for 5 min at 37 °C. Aliquots (20 μL) were analyzed by gel electrophoresis, and [^{32}P]P-II^{Glc} was identified by autoradiography of the dried gel.

pyruvate and phosphatidylglycerol and at a octyl-POE concentration of less than 0.02%, conditions that are optimal for phosphorylation of methyl α-glucoside (Erni et al., 1982). After gel electrophoretic separation and transfer of the proteins to nitrocellulose, autoradiography of the replica blot showed four phosphorylated bands (Figure 2a,b). Of these, two could be immunochemically identified as P-II^{Glc} and P-III^{Glc} with a monoclonal antibody against II^{Glc} and an antiserum against III^{Glc} (Figure 2c). Single omission of one of the three phosphoryl carrier proteins abolished phosphorylation of II^{Glc}. In the complete system, the presence of the transport substrate methyl α-glucoside in molar excess over [^{32}P]phosphoenolpyruvate resulted in a time-dependent dephosphorylation of all four PTS proteins, including II^{Glc}. A decrease of phosphorylation is already visible after 1 min of incubation (Figure 2a), and dephosphorylation is complete after less than 10 min (Figure 2b). These observations suggest that P-II^{Glc} itself is a component of this phosphoryl-transfer chain rather than an enzyme catalyzing the direct transfer of a phosphoryl group from P-III^{Glc} to glucose. The slow rate of dephosphorylation is due to the relatively high concentration of octyl-POE, which has been shown to reduce sugar phosphorylation activity (Erni et al., 1982). Comparison of immunochemically detected II^{Glc} (Figure 2c) and radiolabeled II^{Glc} (Figure 2a,b) indicates that P-II^{Glc} and nonphosphorylated II^{Glc} have the same electrophoretic mobility, while the mobilities of III^{Glc} and P-III^{Glc} differ significantly (Figure 2c). The multiple protein bands of both species, visualized with the anti-III^{Glc} serum in Figure 2c, are most likely different forms of III^{Glc}, as they have been described by Meadow & Roseman (1982). In Figure 2a,b it is also seen that phosphorylation of enzyme I is strongly enhanced in the presence of HPr, indicating cooperative interaction between enzyme I and HPr and thus supporting the notion of functional association between these proteins reported by Saier et al. (1982).

A comparison of the molar amounts of II^{Glc} loaded onto the gel and of ^{32}P present in the II^{Glc} band after electrophoresis indicated that only about 5–15% of the II^{Glc} in the gel was phosphorylated. This low yield could be due either to the presence in the preparation of inactive II^{Glc} that is no longer susceptible to phosphorylation or to rapid hydrolysis of the phosphoprotein bond in II^{Glc}. The latter possibility is more likely in view of the observation that monoclonal anti-II^{Glc} antibodies that completely inhibited II^{Glc}-dependent sugar phosphorylation increased the extent of II^{Glc} phosphorylation (Zanolari & Erni, 1984). It was then realized that detergents known to inhibit the activity of II^{Glc} also increased the extent of II^{Glc} phosphorylation. Figure 3 shows that the addition of

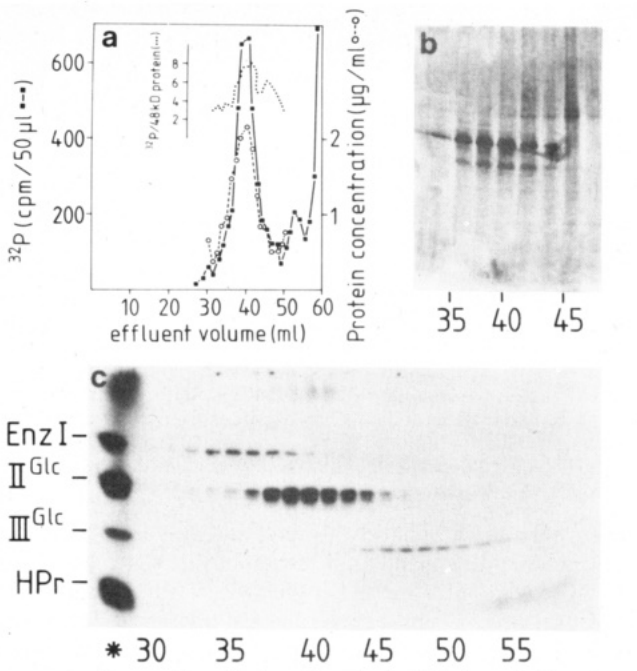

FIGURE 4: Separation of phosphorylated II^{Glc} by gel filtration chromatography. (a) Elution profile of the gel filtration column. Protein concentrations were determined in 0.4-mL aliquots of the fractions indicated, according to Schaffner & Weissmann (1973). Bovine serum albumin was used as standard. Radioactivity was determined by liquid scintillation counting in 50-μL aliquots. The P:II^{Glc} ratio was calculated by assuming that II^{Glc} was the only protein present in the relevant fractions and that the ^{32}P in these fractions was bound to II^{Glc}. Aliquots (25 μL) of the column fractions indicated were analyzed by gel electrophoresis. One gel (b) was stained with alkaline silver nitrate (Wray et al., 1981); a second gel (c) was dried and autoradiographed. In the lane designated (*) 10 μL of the unfractionated incubation mixture is analyzed. The incubation mixture (1 mL) contained 6 μg of enzyme I, 3 μg of HPr, 3 μg of III^{Glc}, 15 μg of II^{Glc}, 4.5% octyl-POE, and 2.8 μM [^{32}P]phosphoenolpyruvate (350 cpm/pmol). Incubation at 37 °C was stopped after 2.5 min by chilling in an ice–salt bath followed by addition of sodium dodecyl sulfate to 1% final concentration. The mixture was loaded to the gel filtration column (AcA34, LKB, 1 cm × 68 cm) equilibrated with 50 mM Tris-HCl, pH 7.5, 0.1% sodium dodecyl sulfate, 0.1 mM EDTA, and 0.1 mM DTT at 4 °C. The column was eluted at a rate of 0.75 mL/10 min. Note the trailing of the radioactivity peak behind the protein peak, which could indicate that some hydrolysis of P-II^{Glc} occurred even under these experimental conditions. The numbers in panels b and c refer to the fraction numbers (a).

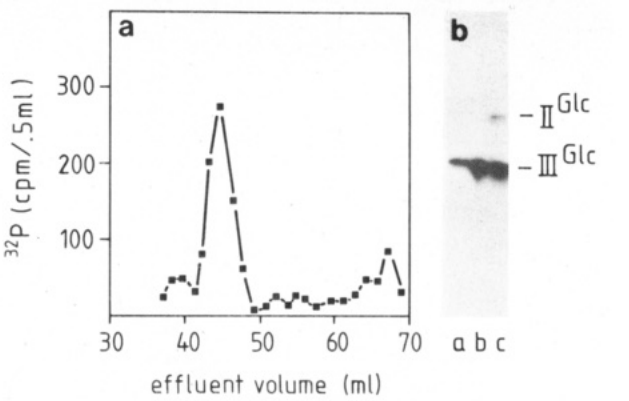

FIGURE 5: Phosphoryl transfer from P-II^{Glc} to III^{Glc}. [^{32}P]P-II^{Glc} (prepared as described in Figure 4) and III^{Glc} were incubated, and the reaction mixture was analyzed by gel filtration (a). The peak fraction (44) was concentrated by vacuum dialysis, diluted with gel electrophoresis sample buffer, and analyzed by gel electrophoresis (b, lane b). It is compared with authentic P-III^{Glc} (lane a; Meadow & Roseman, 1982) and the incubation mixture prior to gel filtration (lane c). The incubation mixture (1 mL) contained 0.6 μg of [^{32}P]P-II^{Glc}, 30 μg of partially puified III^{Glc}, 2 mg of phosphatidylglycerol, and salts as indicated under Experimental Procedures. Incubation at 37 °C was for 10 min. Gel filtration was performed as described in Figure 4.

Table I: Rate and Extent of Phosphoryl Transfer from P-II^{Glc} to Glucose: Stimulatory Effect of Nonphosphorylated III^{Glc} [a]

	^{32}P from P-II^{Glc} found as			
	15-min incubation		12-h incubation	
	Glc-6-P	P_i	Glc-6-P	P_i
minus III^{Glc}	8	4	47	30
plus III^{Glc}	47	7	77	9

[a] Values are given as percent of the total amount of [^{32}P]P-II^{Glc} added to the incubation mixture either in the presence or in the absence of III^{Glc}. The incubation mixture (5 mL) contained [^{32}P]P-II^{Glc}, (12 pmol, 2400 cpm), III^{Glc} (10 μg) where indicated, 1 mg/mL asolectin, and salts as specified for protein phosphorylation under Experimental Procedures. The reaction mixture was incubated at 22 °C for the time indicated and then extracted with chloroform–methanol (Bligh & Dyer, 1959). The aqueous phase was concentrated by rotary evaporation and loaded on a 10-mL AG 1×8 Dowex column (formate form). The column was eluted with an exponential gradient of 1.5–3 M formic acid at a flow rate of 0.44 mL/min. The amount of Glc-6-P and P_i was calculated from the peak areas in the ion-exchange chromatogram. Note that phosphoryl transfer after 15 min was incomplete and that unreacted P-II^{Glc} was removed in the $CHCl_3$–MeOH layer during processing of the incubation mixture.

octyl-POE to the incubation mixture resulted in a 5-fold increased phosphorylation of II^{Glc}. Phosphorylation of II^{Glc} in the presence of 4.5% octyl-POE afforded a preparation of nearly stable P-II^{Glc}, which could be fractionated by gel filtration (Figure 4a). This figure also shows that fractions of P-II^{Glc} could be obtained that were more than 90% pure (panel b), free of detectable amounts of P-III^{Glc}, and only slightly contaminated with phosphorylated enzyme I (panel c, fractions 36–40). Determinations of protein and radioactivity yielded a molar phosphate:II^{Glc} ratio of 0.6–0.8. P-II^{Glc} elutes slower than enzyme I (68 kDa) but faster than III^{Glc} (20 kDa) during gel filtration in the presence of sodium dodecyl sulfate (Figure 4a, analyzed in Figure 4c). This indicates that P-II^{Glc} is dissociated into its subunits of approximately 50 kDa (see below), in contrast to gel filtration in the presence of octyl-POE where II^{Glc} eluted as a complex of approximately 150-kDa apparent molecular mass (Erni et al. 1982).

Phosphoryl-Transfer Reactions. Genetic and biochemical evidence predict that II^{Glc} interacts with at least two components, its sugar substrate and the phosphoryl carrier protein III^{Glc}. When purified P-II^{Glc} was incubated with III^{Glc}, phosphoryl transfer from P-II^{Glc} to III^{Glc} occurred rapidly, indeed. Phosphorylated III^{Glc} could be separated from the almost completely dephosphorylated II^{Glc} by gel filtration (Figure 5a) and identified by gel electrophoresis. Phosphoryl transfer between P-II^{Glc} and III^{Glc} occurred to the same extent and at a comparable rate when phosphatidylglycerol in the incubation mixture was replaced by detergents (results not shown). In contrast, phosphoryl transfer between P-II^{Glc} and glucose occurred only after the P-II^{Glc} preparation had been diluted into a buffered phospholipid-containing solution, which lowered the detergent below the critical micellar concentration. After incubation in the presence of glucose, two peaks of radioactivity with retention times corresponding to those of Glc-6-P and of P_i could be separated by ion-exchange chromatography, and the identity of Glc 6-P was confirmed by thin-layer chromatography (Thompson, 1978) of an aliquot in parallel with an authentic sample of [^{14}C]Glc-6-P (results not shown). As evident from Table I, phosphorylation of glucose by purified P-II^{Glc} was however accompanied by the

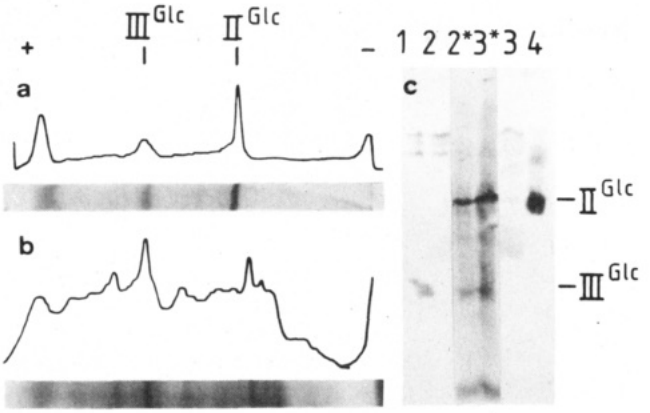

FIGURE 6: Immunoprecipitation of ^{35}S-labeled II^{Glc}–III^{Glc} complex. ^{35}S-Labeled cells were lysed and immunoprecipitated with either anti-II^{Glc} monoclonal immunoglobulin (a) or with anti-III^{Glc} polyclonal antiserum (b) as described under Experimental Procedures. Immunoprecipitated proteins were fractionated by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. Shown are the autoradiograms and their densitometric scans. (c) Two immunoprecipitates obtained with anti-II^{Glc} antibodies were separated by gel eletrophoresis in parallel with authentic II^{Glc} and III^{Glc} and then electrophoretically transferred to a nitrocellulose sheet. Immunoprecipitated proteins were first detected by autoradiography of the protein blot (shown in lanes 2* and 3*), and II^{Glc} and III^{Glc} were then immunochemically labeled with their respective antibodies and visualized with lactoperoxidase coupled to anti-immunoglobulin antibodies (shown in lanes 1–4). (Lane 1) Immunoblot of authentic III^{Glc}; (lanes 2 and 3) immunoblots of immunoprecipitated proteins; (lanes 2* and 3*) autoradiograms of the same immunoprecipitated proteins shown in lanes 2 and 3; (lane 4) immunoblot of authentic II^{Glc}. Note that II^{Glc} could be identified by both autoradiography (lanes 2* and 3*) and immunolabeling (lanes 2 and 3), while III^{Glc} could be identified by autoradiography but, for lack of sensitivity, not by immunolabeling.

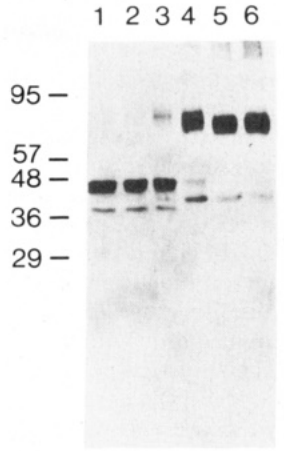

FIGURE 7: Oligomeric structure of II^{Glc}. Gel electrophoretic analysis of the II^{Glc} monomer and of the II^{Glc} dimer stabilized by cross-linking with glutaraldehyde. II^{Glc} that had been used for equilibrium centrifugation (see Experimental Procedures) was diluted in centrifugation buffer to a final concentration of 0.012 mg/mL. Aliquote (30 μL) were incubated with glutaraldehyde at concentrations of 0.0003, 0.0013, 0.005, 0.02, and 0.08% (lanes 2–6). No glutaraldehyde was added to the aliquot analyzed in lane 1. After 40-min incubation at room temperature, excess glutaraldehyde was quenched with ethanolamine (0.1 M final concentration). The samples were analyzed by gel electrophoresis (17.5% polyacrylamide) in the presence of sodium dodecyl sulfate and proteins visualized by alkaline silver staining (Wray et al., 1981). The minor 38-kDa band is probably a proteolytic fragment of II^{Glc} (Erni et al., 1982). The molecular weight standards were phosphorylase (M_r 95 000), catalase (M_r 57 000), fumarase (M_r 48 000), glyceraldehyde-phosphate dehydrogenase (M_r 36 000), and basic carbonic anhydrase (M_r 29 000).

formation of a large amount of inorganic phosphate. Thus, phosphoprotein hydrolysis was competing with sugar phosphorylation under these experimental conditions. The precision as well as the rate of phosphoryl transfer was markedly increased when III^{Glc} was added to the incubation mixture (Table I). In the presence of unphosphorylated III^{Glc}, almost no free P_i was formed, and phosphoryl transfer was complete in less than 15 min. It therefore appears that III^{Glc} not only functions as a phosphoryl carrier between HPr and II^{Glc} but is directly affecting the catalytic activity of II^{Glc}, indicating that a complex between II^{Glc} and III^{Glc} may be the functional unit of glucose phosphorylation and transport.

Structure of the II^{Glc}–III^{Glc} Complex. The notion of functional association between II^{Glc} and III^{Glc} gained support when binding between II^{Glc} and III^{Glc} was demonstrated directly by immunoprecipitation of a II^{Glc}–III^{Glc} complex. With a monoclonal anti-II^{Glc} antibody (purified IgG), two proteins were precipitated from cell lysates of bacteria labeled in vivo with ^{35}S (Figure 6a). Of the two proteins, the major band comigrates with purified II^{Glc} (Figure 6c, lanes 3* and 4) while the minor band comigrates with III^{Glc} (Figure 6c, lanes 1 and 2*).

As a control an immunoprecipitation was also performed with a polyclonal anti-III^{Glc} antiserum under the same experimental conditions. The precipitate thus obtained is of low quality (Figure 6b), most likely because a crude antiserum was used rather than purified monoclonal immunoglobulins. Nevertheless, III^{Glc} as the major band in Figure 6b comigrates with the minor band in Figure 6a, and a minor yet prominent band in Figure 6b comigrated with II^{Glc} in Figure 6a. Other bands in the precipitate could be contaminants or are proteins functionally interacting and therefore coprecipitating with III^{Glc} [for interaction of III^{Glc} with other proteins, see Osumi & Saier (1982), Nelson et al. (1983), Nelson & Postma (1984), and Postma et al. (1984)]. From the densitometric scans of autoradiograms as shown in Figure 6a, the ratio of ^{35}S in the two peaks was determined, yielding a ratio of III^{Glc}:II^{Glc} of approximately 2. This value did not change whether the immunoprecipitate was washed 10 times instead of 5 times or whether the pH of the wash buffer was 9.3 instead of 7.5. An averaged value of 1.8 ± 0.3 was calculated from the results of these various immunoprecipitation experiments.

Sedimentation equilibrium centrifugation and chemical cross-linking were used to investigate the oligomeric structure of purified II^{Glc} itself. II^{Glc} analyzed by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate exhibits a mobility corresponding to an apparent molecular mass of 45–48 kDa (Figure 7). During gel filtration in the presence of 1% octyl-POE, the pure II^{Glc}–detergent complex exhibited a Stokes radius of 4.7 nm (Erni et al., 1982), indicating an oligomeric structure of II^{Glc}. However, an exact subunit stoichiometry could not be derived because the Stokes radius is a function of size and shape of the protein–detergent mixed micelle rather than of the molecular weight of the protein oligomer. To obtain an accurate estimate of the molecular weight, homogeneous II^{Glc} was analyzed by sedimentation equilibrium centrifugation. A molecular mass of 106 ± 8 kDa was found for detergent-solubilized II^{Glc}, thus indicating that detergent-solubilized II^{Glc} itself was most likely a dimer of two identical 53-kDa subunits. The discrepancy between the subunit molecular weight calculated from equilibrium centrifugation and the apparent molecular mass of 45 kDa found by polyacrylamide gel electrophoresis is not surprising. On several occasions, it has been found that the apparent molecular weights of membrane proteins determined by gel electrophoresis in the presence of sodium dodecyl sulfate deviate from their true molecular weight (Ludwig et al., 1982).

To confirm the dimeric structure of II^{Glc}, aliquots of the preparation used for equilibrium sedimentation were cross-linked with increasing concentration of glutaraldehyde and then analyzed by polyacrylamide gel electrophoresis (Figure 7). Only two bands were visible, one of an apparent molecular mass of 45 kDa (corresponding to the II^{Glc} monomer) while the other exhibited an electrophoretic mobility corresponding to a molecular mass of 70 kDa. The first band was dominant at low concentrations of glutaraldehyde. An increase of the glutaraldehyde concentration from 0.001 to 0.005% caused almost complete disappearance of the 45-kDa band with concomitant appearance of a 70-kDa band. From the observation that no band of intermediate mobility was formed and that no band of higher molecular weight appeared, even if the glutaraldehyde concentration was further increased, it is inferred that II^{Glc} consists of two identical, noncovalently bound subunits. That the mass of the cross-linked dimer appears to be 70 kDa rather than twice that of the monomer is likely to reflect incomplete unfolding of the cross-linked dimer. A low protein concentration (0.012 mg/mL) was chosen in the cross-linking experiments to minimize the effect of interdimer cross-linking. The latter occurred only at a very high concentration (1.5%) of glutaraldehyde, resulting in a smear of protein that only partly entered the separating gel (not shown).

Discussion

The glucose-specific permease (II^{Glc}) of the PTS is of interest as a multifunctional membrane protein with a role in active glucose transport, in phosphorylation of glucose, and in chemotactic signaling. It has long been controversial whether II^{Glc} catalyzes the direct transfer of a phosphoryl group from P-III^{Glc} to glucose or whether phosphoryl transfer proceeds via a phosphorylated II^{Glc} intermediate. Strong though indirect evidence for II^{Glc} being phosphorylated came from studies on the stereochemistry of the phosphoryl-transfer reaction (Begley et al., 1982). Subsequently, it was shown that purified II^{Glc} could be phosphorylated in vitro and detected as a ^{32}P-labeled band on polyacrylamide gels (Zanolari & Erni, 1984). Peri et al. (1984) identified a 48-kDa phosphoprotein in crude membrane preparations as P-II^{Glc}, on the basis of the observation that phosphorylation dependend on added III^{Glc}. The occurrence of a phosphorylated mannitol-specific enzyme II has been postulated on the basis of pyruvate burst, pH drop, and ^{32}P-labeling experiments (Misset et al., 1983; Roossien et al., 1984; Roossien & Robillard, 1984b).

We have now purified ^{32}P-labeled II^{Glc}, determined a P:II^{Glc} ratio of between 0.6 and 0.8, and demonstrated in vitro phosphoryl transfer from P-II^{Glc} to glucose. P-II^{Glc} is more sensitive to hydrolysis than are the phosphorylated forms of the cytoplasmic PTS proteins. It can be stabilized by the addition of detergents, which block II^{Glc}-catalyzed sugar phosphorylation completely yet do not prevent the (reversible) transfer of a phosphoryl moiety from P-III^{Glc} to II^{Glc}. Thus, it appears that detergents do not irreversibly inactivate the catalytic site but rather lock the protein in a conformation that either renders the active site inaccessible to glucose or stabilizes the phosphoprotein bond by specific noncovalent interactions, e.g., between the phosphorylated side chain (most likely of histidine; Saier et al., 1985) and other parts of the protein.

When detergent-stabilized P-II^{Glc} was diluted in the presence of glucose and phospholipids, transfer of ^{32}P from P-II^{Glc} to the sugar substrate could be observed. However, phosphoryl transfer to glucose was slow and accompanied by nonspecific phosphoprotein hydrolysis. The presence of III^{Glc} in the incubation mixture increased the rate of phosphorylation of glucose by P-II^{Glc} and suppressed hydrolysis of P-II^{Glc} almost completely.

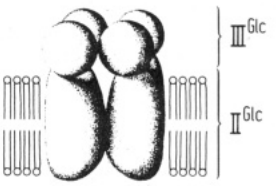

FIGURE 8: Proposed model of the glucose-specific permease (II^{Glc}–III^{Glc}) complex.

Although this result could still be interpreted as meaning that P-III^{Glc} is the ultimate phosphoryl donor to glucose, with II^{Glc} serving only as a transiently phosphorylated cofactor, such a mechanism is unlikely for two reasons: (i) phosphorylation of glucose by III^{Glc} in the absence of II^{Glc} could not be detected (e.g., Figure 2a and unpublished observations), and (ii) such a mechanism would be incompatible with the stereochemical course of the phosphoryl-transfer reaction as described by Begley et al. (1982). We therefore conclude that II^{Glc} and III^{Glc} form a functional complex and that III^{Glc} increases the phosphoryl-transfer activity of P-II^{Glc}.

The reverse transfer of a phosphoryl group from P-II^{Glc} to III^{Glc} is much faster than phosphoryl transfer to glucose and is strikingly insensitive to the presence of detergents. The following experiment (results not shown) might illustrate this: P-II^{Glc} was purified by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate and then electroblotted to nitrocellulose. When the nitrocellulose sheet containing P-II^{Glc} was submerged in a buffer containing III^{Glc}, rapid and strictly III^{Glc}-dependent dephosphorylation of II^{Glc} could be observed. While formation of Glc-6-P is favored over formation of P-III^{Glc} at equilibrium, the results indicate that the latter reaction is kinetically favored in vitro. This kinetic bias for III^{Glc} phosphorylation could be the consequence of the experimental conditions, which were chosen to stabilize P-II^{Glc}. For example, the use of a high concentration of octyl-POE during incubation and the presence of sodium dodecyl sulfate during gel filtration causes dissociation of II^{Glc} into its monomers and might have led to a loss of tightly bound phospholipids. Dissociation of the II^{Glc} dimer and loss of endogeneous phospholipids might not be fully reversible under the conditions used to reconstitute the sugar phosphorylation activity of P-II^{Glc}. It is therefore conceivable that such in vitro alterations differentially affect the processes of (i) reversible phosphoryl transfer between II^{Glc} and III^{Glc} within a stable protein–protein complex and (ii) sugar phosphorylation, which is coupled to sugar translocation.

A functional association of II^{Glc} and III^{Glc} is reflected by structural evidence from immunoprecipitation, chemical cross-linking, and ultracentrifugation studies. Immunoprecipitation with a monoclonal anti-II^{Glc} antibody afforded a complex composed of II^{Glc} and III^{Glc} in an molar ratio of approximately 1:2. Chemical cross-linking and immunoprecipitation indicated that II^{Glc} itself is a dimer composed of two identical 53-kDa subunits. Taken together, these results suggest a II^{Glc}–III^{Glc} complex composed of two II^{Glc} and four III^{Glc} subunits as depicted in Figure 8. A model of III^{Glc} anchored to the membrane through contact with II^{Glc} could explain earlier observations of a membrane-bound form of III^{Glc}, which was found in addition to the cytoplasmic form (Scholte et al., 1982; Saier et al., 1982). Deutscher et al. (1982) demonstrated that a functional analogue of III^{Glc}, the lactose-specific enzyme III of *Staphylococcus aureus*, exists as a trimer. Erni & Zanolari (1985) have purified from *Escherichia coli* the mannose-specific enzyme II^{Man}–III^{Man}

complex, which contains a dimeric form of III^{Man}. Dimeric forms of enzyme II permeases have also been observed. Leonard & Saier (1983) observed a sigmoidal relation between transphosphorylation activity and concentration of the mannitol-specific enzyme II, the analysis of which indicated that the conformation of II^{Mtl} most active is a dimer. Roossien & Robillard (1984b) extracted the dimeric form of II^{Mtl} from bacterial membranes. This permease of the PTS is believed to be functionally analogous to II^{Glc} (Saier et al., 1985). We have recently observed the dimeric form of II^{Glc} when purified protein was air-oxidized and then analyzed on polyacrylamide gels in the absence of disulfide-reducing reagents (unpublished observation). It remains to be shown whether disulfide-mediated cross-linking is an in vitro artifact or whether intersubunit disulfide bridges are functional in sugar transport and phosphorylation by II^{Glc} as it has been suggested by Roossien & Robillard (1984a) for the mannitol-specific enzyme II.

The methods used to stabilize P-II^{Glc} should lend themselves to the better characterization of II^{Glc} as well as other membrane permeases of the PTS. The next steps, isolation of phosphorylated peptides of II^{Glc} and identification of the phosphorylated amino acid residue and of its position in the amino acid sequence of II^{Glc}, are now in progress. Such identification could be the first step in delineating the translocation pathway of glucose across the membrane permease and in elucidating its mechanism.

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Registry No. PTS, 56941-29-8; II^{Glc}, 37278-09-4; glucose, 50-99-7.

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Basement Membrane Complexes with Biological Activity

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ABSTRACT: We have studied the reconstitution of basement membrane molecules from extracts prepared from the basement membrane of the EHS tumor. Under physiological conditions and in the presence of added type IV collagen and heparan sulfate proteglycan, gellike structures form whose ultrastructure appears as interconnected thin sheets resembling the lamina dense zone of basement membrane. The major components of the reconstituted structures include laminin, type IV collagen, heparan sulfate proteoglycan, entactin, and nidogen. These components polymerize in constant proportions on reconstitution, suggesting that they interact in defined proportions. Molecular sieve studies on the soluble extract demonstrate that laminin, entactin, and nidogen are associated in large but dissociable complexes which may be a necessary intermediate in the deposition of basement membrane. The reconstituted matrix was biologically active and stimulated the growth and differentiation of certain cells.

Connective tissues contain different species of collagens, glycoproteins, and proteoglycans (Eyre, 1980; Bornstein & Sage, 1980; Kleinman et al., 1981; Hay, 1982). These macromolecules form the matrix structures that contribute to the physical characteristics of tissues as well as provide unique substrates for the resident cells. For example, the matrix produced by cultered fibroblasts consists of a dissociable complex of type I collagen, fibronectin, and heparan-containing and chondroitin sulfate containing proteoglycans (Hedman et al., 1983). Similarly, Schubert & LaCorbiere (1980) demonstrated the presence of complexes containing collagen, proteglycan, and glycoprotein in the media of cultured myoblasts and neural retinal cells. These complexes which they termed adherons also supported the attachment of cells (Schubert & LaCorbiere, 1982; Schubert et al., 1983).

In the present paper, we examine the macromolecular complexes involved in the formation of basement membranes. Basement membranes are thin but continuous sheets that separate epithelium from stroma and surround nerves, muscle fibers, smooth muscle cells, and fat cells (Kefalides, 1973; Vracko, 1974; Timpl & Martin, 1982; Laurie et al., 1983). Basement membranes contain type IV collagen (Kefalides, 1973; Orkin et al., 1976), the glycoproteins laminin (Timpl et al., 1979; Chung et al., 1979), entactin (Carlin et al., 1981), and nidogen (Timpl et al., 1983), and heparan sulfate proteoglycans (Kanwar & Farquhar, 1979; Hassell et al., 1980, 1985). In various studies, these materials show a codistribution (Leivo et al., 1982; Hayman et al., 1982; Laurie et al., 1982, 1984b) both within the lamina densa and within its extensions across the lamina lucida. Using electron microscopy, the components appear as a network of 5-nm-wide cords (Laurie et al., 1984), and by using electron microscopy, their codistribution suggests that the formation of basement membrane occurs through their interactions. Type IV collagen molecules form intermolecular disulfide bonds and associate in a continuous network (Timpl et al., 1981; Veis & Schwartz, 1981; Fessler & Fessler, 1982; Bächinger et al., 1982; Yurchenko & Furthmayr, 1984) which can be visualized in basement membranes digested with plasmin (Inoue et al., 1983).

Various components of the basement membrane are known to interact with each other. In vitro studies with purified components show that laminin binds through its short chains to native but not to denatured type IV collagen and through a domain in its long chain to the heparan sulfate proteoglycan (Terranova et al., 1980; Woodley et al., 1983; Rao et al., 1983). Alone each of these basement membrane components is soluble. When these macromolecules, however, are mixed together in vitro, they form a floccular precipitate containing laminin, type IV collagen, and heparan sulfate proteoglycan in a 1:1:0.1 molar ratio (Kleinman et al., 1983). However, this precipitate lacks the resiliency and consistency expected of basement membrane structures.

In this study, we have incubated extracts of the EHS tumor containing a mixture of proteins under physiological conditions and analyzed the components that interact and gel. These studies show that under physiological conditions certain components of the basement membrane including type IV collagen, laminin, heparan sulfate proteoglycan, nidogen, and entactin