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Analysis of Mutations That Uncouple Transport from Phosphorylation in Enzyme II^{Glc} of the *Escherichia coli* Phosphoenolpyruvate-Dependent Phosphotransferase System

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Mutations that uncouple glucose transport from phosphorylation were isolated in plasmid-encoded *Escherichia coli* enzyme II^{Glc} of the phosphoenolpyruvate-dependent phosphotransferase system (PTS). The uncoupled enzymes II^{Glc} were able to transport glucose in the absence of the general phosphoryl-carrying proteins of the PTS, enzyme I and HPr, although with relatively low affinity. K_m values of the uncoupled enzymes II^{Glc} for glucose ranged from 0.5 to 2.5 mM, 2 orders of magnitude higher than the value of normal II^{Glc}. Most of the mutant proteins were still able to phosphorylate glucose and methyl α-glucoside (a nonmetabolizable glucose analog specific for II^{Glc}), indicating that transport and phosphorylation are separable functions of the enzyme. Some of the uncoupled enzymes II^{Glc} transported glucose with a higher rate and lower apparent K_m in a *pts*$^+$ strain than in a Δ*ptsHI* strain lacking the general proteins enzyme I and HPr. Since the properties of these uncoupled enzymes II^{Glc} in the presence of PTS-mediated phosphoryl transfer resembled those of wild-type II^{Glc}, these mutants appeared to be conditionally uncoupled. Sequencing of the mutated *ptsG* genes revealed that all amino acid substitutions occurred in a hydrophilic segment within the hydrophobic N-terminal part of II^{Glc}. These results suggest that this hydrophilic loop is involved in binding and translocation of the sugar substrate.

The bacterial phosphoenolpyruvate (PEP)-dependent phosphotransferase system (PTS) catalyzes uptake and concomitant phosphorylation of carbohydrates (24, 33). Transfer of the phosphoryl group from PEP to the carbohydrate is catalyzed sequentially by the soluble and general proteins enzyme I and HPr and one of a series of substrate-specific and membrane-bound enzymes II (Fig. 1A). Transfer from P-HPr to enzyme II is either direct or via a soluble, carbohydrate-specific enzyme III.

In *Escherichia coli* and *Salmonella typhimurium*, II^{Glc} forms, in conjunction with III^{Glc}, the major glucose-specific permease. The glucose permease catalyzes uptake and phosphorylation of glucose and the nonmetabolizable glucose analog methyl α-glucopyranoside (αMG). Glucose can also be taken up and phosphorylated via the mannose PTS.

II^{Glc} is an integral membrane protein and catalyzes the actual transport and phosphorylation of the sugar substrate. The association of II^{Glc} with III^{Glc} is probably only transient, but it is important for phosphoryl transfer from II^{Glc} to glucose (7, 8). From the sequence of the cloned *E. coli ptsG* gene, the structural gene for II^{Glc}, it was deduced that the protein consists of 477 amino acid residues with a total M_r of 50,645 (9). II^{Glc} is a moderately hydrophobic protein. The N-terminal end of the protein (residues 1 to 385) contains a hydrophobic region that can span the membrane at least six times (9; see also Fig. 2). This region is divided into two parts by an intervening hydrophilic sequence of about 120 residues (residues 180 to 300). The C-terminal end of II^{Glc} consists of about 90 amino acid residues which are also hydrophilic. Sequence comparisons have led to the idea that other enzymes II possess a comparable domain structure (17, 18, 36). Furthermore, it has been suggested that the large hydrophobic domain of the enzymes II is involved in binding of the substrate and its translocation across the membrane (11, 17–19, 22, 42). In the case of II^{Mtl}, the enzyme II responsible for mannitol uptake and phosphorylation, it has been shown that this domain binds mannitol with the same affinity as the complete protein (11, 19). However, within these hydrophobic domains, it is completely unknown which amino acid residues are involved in binding or translocation of the substrate.

All PTS proteins, including enzymes II, are phosphorylated transiently during turnover (24, 33). However, in *ptsHI* deletion mutants, which lack the general phosphoryl-carrying proteins enzyme I and HPr, the phosphoryl transfer pathway is blocked and the enzymes II are not phosphorylated. These mutants are unable to grow on PTS carbohydrates if no other transport systems are available (35). Thus, nonphosphorylated enzymes II are unable to catalyze carbohydrate transport at a rate that is sufficient to support growth. Mutants have been isolated from such *S. typhimurium ptsHI* deletion strains in which transport of glucose via II^{Glc} can occur in the absence of phosphorylation of II^{Glc} (31, 37). The mutated II^{Glc} is able to catalyze facilitated diffusion of glucose in *ptsHI* deletion mutants (38; Fig. 1B). In these uncoupled II^{Glc} mutants it is not known which amino acid residues are changed.

Recently, Lolkema et al. (20, 21) have investigated the mechanism of coupling between mannitol transport and phosphorylation via II^{Mtl}. According to these researchers, phosphorylation of the Cys-384 residue in a hydrophilic domain of the protein accelerates the rate of translocation of mannitol, catalyzed by the hydrophobic N-terminal domain. Thus, phosphorylation of the domain containing Cys-384 modulates the activity of the translocator domain. II^{Glc} is composed of two comparable domains and contains a con-

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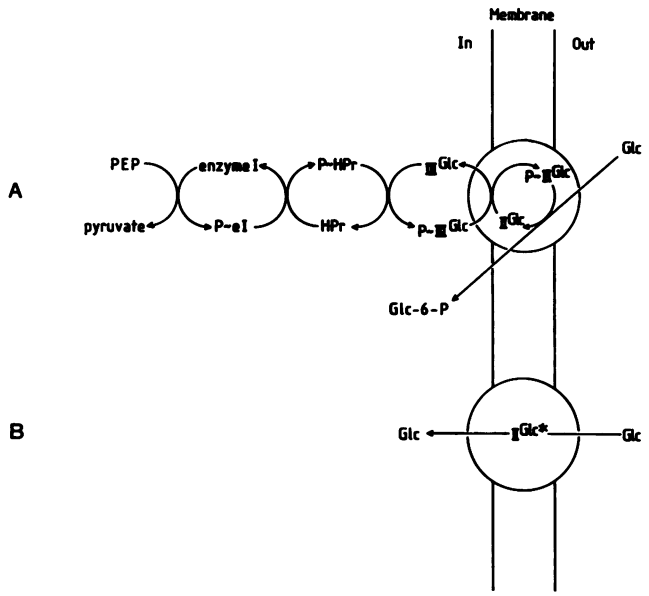

FIG. 1. Glucose transport via the PTS. (A) Transport via III^{Glc}/II^{Glc}. Enzyme I and HPr are the general PTS proteins. P indicates the phosphorylated form of the protein. Glucose is accumulated as glucose 6-phosphate. (B) Uncoupled II^{Glc} (II^{Glc*}) is able to catalyze glucose transport in the absence of PEP-dependent phosphorylation.

served cysteine residue, Cys-421. Possibly, II^{Glc} and II^{Mtl} function by comparable mechanisms.

In this report, we describe the isolation and characterization of mutations in plasmid-encoded *E. coli* II^{Glc} that uncouple transport from phosphorylation. The altered II^{Glc} molecules transported glucose with relatively low affinity, but most mutants were still able to catalyze phosphorylation of the sugar substrate, indicating that these two functions of the protein are separable. Sequencing of the mutated *ptsG* genes revealed that all amino acid substitutions in the uncoupled enzymes II^{Glc} were localized in the hydrophilic segment within the hydrophobic N-terminal part of the protein. These results suggest that this hydrophilic loop (17) is involved in binding and translocation of the sugar substrate.

MATERIALS AND METHODS

Bacterial strains and plasmids. The strains used are listed in Table 1. Plasmid pTSG4 (9) contains the *E. coli* structural gene for enzyme II^{Glc} and was kindly provided by B. Erni (University of Bern, Bern, Switzerland).

Media and growth conditions. Cells were grown at 37°C on a rotary shaker in liquid medium. LB medium (39) was used as the complex medium, and medium A [1 g of $(NH_4)_2SO_4$, 10.5 g of K_2HPO_4, 4.5 g of KH_2PO_4, 1 g of $MgSO_4$ per liter of demineralized water] containing 0.5% glycerol or glucose as the carbon and energy source was used as the defined medium. Amino acids, when required, were present at 20 μg/ml. Plasmid-containing strains were grown in the presence of 25 μg of tetracycline per ml or 50 μg of ampicillin per ml.

Chemicals. [U-^{14}C]methyl α-glucopyranoside (10.8 GBq/mmol) was obtained from the Radiochemical Centre, Amersham, England; [α-^{35}S]dATP (52.7 TBq/mmol) and D-[1-^{3}H] glucose (574 GBq/mmol) were purchased from Dupont, NEN Research Products, Boston, Mass. Isopropyl-β-D-thiogalactopyranoside and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside were obtained from Boehringer GmbH, Mannheim, Germany. All other chemicals were reagent grade.

Genetic methods. Isolation and transformation of plasmid DNA and agarose gel electrophoresis were performed as previously described (39). Restriction enzymes, T4 DNA ligase, and T7 DNA polymerase were obtained from Pharmacia and used as recommended by the supplier.

To sequence the *ptsG* mutations, the complete *ptsG* gene from pTSG4 and its derivatives was cloned into M13mp18 and M13mp19. Plasmid DNA was digested with *Pst*I, *Bam*HI, and *Tth*111I. Plasmid fragments were subsequently separated by using agarose gel electrophoresis. The 3.2-kb *ptsG* fragment was isolated from the gels by using DEAE-

TABLE 1. Strains used in this study

Strain	Genotype	Construction	Reference or source
S. typhimurium			
PP784	*galP283 trpB223 cysA1539*::Tn*10*		34
PP799	*galP283 trpB223*		34
PP1131	*ptsM420 ptsG204 mtl-594 trpB223* (Tn*10* linked to *ptsM*)		32
PP1139	*ptsG415*::Tn*10 trpB223*		31
PP1151	*ptsM420 trpB223* (Tn*10* linked to *ptsM*)	SB3507 × P22(PP1131)	This study
PP1977	*ptsM420 trpB223*	Excision Tn*10* PP1151	This study
PP1980	*ptsM420 ptsG415*::Tn*10 trpB223*	PP1977 × P22(PP1139)	This study
PP2076	Δ(*cysK-ptsHI*)*41 galP283 trpB223*	PP784 × P22(SB2309)	This study
SB2309	Δ(*cysK-ptsHI*)*41 trpB223*		4
SB3507	*trpB223*		31
E. coli			
PPA214	*ptsG2 ptsM1 strA glk-7* (Tn*10* linked to *pts*)	ZSC112 × P1(UE1)	This study
PPA222	*ptsG2 ptsM1 strA* Δ*ptsHI* (Tn*10* linked to *pts*)	ZSC112 × P1(UE1)	This study
PPA223	*ptsG2 ptsM1 strA* Δ*ptsHI glk-7* (Tn*10* linked to *pts*)	ZSC112 × P1(UE1)	This study
UE1	*thi* Δ(*ptsHI-crr*) (Tn*10* linked to *pts*)		W. Boos
WA2127	*ptsLPM recA thr leu met thi*		10
ZSC13	*strA glk-7*		5
ZSC112	*ptsG2 ptsM1 strA glk-7*		5

cellulose membranes as described previously (39) and ligated into M13mp18 and M13mp19 cut with *Bam*HI and *Pst*I. Ligated M13 DNA was transformed to *E. coli* TG1 (39), and a single-stranded DNA template was produced by standard methods (39). DNA sequencing was accomplished via the dideoxy-chain termination method of Sanger et al. (40) by using a commercially available sequencing kit (United States Biochemical, Cleveland, Ohio) in combination with T7 DNA polymerase, with [α-^{35}S]dATP as the incorporated label. The *ptsG* template was primed at approximately 300-bp intervals by using primers provided by B. Erni.

Preparation of P1 transducing lysates and transduction with bacteriophage P1 were performed as described by Arber (2). Preparation of P22 transducing lysates and transduction with phage P22 were performed as described by Ely et al. (6). Excision of transposon Tn*10* from the chromosome was performed as described by Bochner et al. (3).

Oxygen consumption. Oxygen consumption was measured with a Clark-type electrode in medium A and expressed as nanomoles of O_2 consumed per minute per milligram (dry weight) at 25°C.

Preparation of cell extracts and enzyme assays. Cells were ruptured by passage through an Aminco French pressure cell at 1,100 kg/cm^2, and cell extracts were prepared as described earlier (30). The cell extracts were centrifuged at 230,000 × *g* for 1 h at 4°C. The activity of II^{Glc}, measured as PEP-dependent phosphorylation of methyl α-glucoside, was determined in the 230,000 × *g* pellet essentially as described by Kundig and Roseman (15). As a source of the soluble components of the glucose PTS, a high-speed supernatant of strain PP1980 was used.

SDS-PAGE and immunoblotting. Polyacrylamide gel electrophoresis (PAGE) in the presence of sodium dodecyl sulfate (SDS) was performed as described by Laemmli (16). Protein was subsequently electroblotted from the gel onto cellulose nitrate sheets (pore diameter, 0.45 μm; Schleicher & Schuell) as previously described (43), and II^{Glc} was specifically visualized with monoclonal antibody 2D8 (gift from B. Erni; 25) and goat anti-mouse immunoglobulin G conjugated to horseradish peroxidase (Bio-Rad).

Protein. Protein was determined by the method of Peterson (28) with bovine serum albumin as the standard.

Binding of glucose to enzyme II^{Glc}. Binding of radiolabelled glucose to membranes containing II^{Glc} was measured by flow dialysis as described by Lolkema et al. (19) with the following modifications. The volume of the lower compartment of the flow dialysis system was 300 μl, and the upper compartment was loaded with 250 μl of a membrane suspension (1 mg of protein per ml) equilibrated with [1-^{3}H]glucose. The buffer which was used throughout the experiment contained 25 mM potassium phosphate (pH 7.5) and 5 mM dithiothreitol. A flow rate of 1 ml/min and a sampling time of 30 s were used.

RESULTS

Isolation of uncoupled II^{Glc} mutants. To facilitate genetic and recombinant DNA manipulations, plasmid pTSG4 (9), containing the *E. coli* structural gene for enzyme II^{Glc} (*ptsG*), was used to isolate uncoupled II^{Glc} mutants in *S. typhimurium* PP2076. This strain is unable to grow on glucose because it lacks the chromosomal genes that encode the general phosphoryl-carrying proteins of the PTS, enzyme I and HPr. In the absence of PTS-mediated phosphorylation, enzyme II-catalyzed transport is very slow (35). Thus, uncoupled II^{Glc} mutants are defined as containing an altered enzyme II^{Glc} which is able to catalyze glucose transport in the absence of enzyme I and HPr. About 10^8 cells of strain PP2076/pTSG4 were spread on glucose-containing plates. After 3 days of incubation, about 100 to 200 colonies had appeared spontaneously on each plate. To ensure that mutations responsible for growth on glucose were due to alterations in plasmid-encoded II^{Glc} and not caused by chromosomal mutations, plasmid DNA was isolated from a number of colonies and retransformed to strain PP2076. After subsequent testing of the tetracycline-resistant transformants for growth on glucose, seven independent mutants were obtained.

TABLE 2. Nucleotide changes and deduced amino acid substitutions in the isolated plasmid mutants[a]

Plasmid	Mutant allele	Nucleotide change	Amino acid change	No. isolated
pTSG4-1	*ptsG101*	T-617→C	Val-206→Ala	1
pTSG4-2	*ptsG102*	C-607→A	Arg-203→Ser	3
pTSG4-3	*ptsG103*	T-887→A	Ile-296→Asn	2
pTSG4-11	*ptsG104*	A-771→C	Lys-257→Asn	1

[a] The complete *ptsG* gene from the isolated mutants was sequenced as described in Materials and Methods and compared with the wild-type gene. Some mutants were isolated more than once, independently of each other, as indicated in the last column.

Sequencing of *ptsG* mutations. To localize the mutations leading to uncoupling of glucose transport from its phosphorylation via II^{Glc}, the *ptsG* gene from pTSG4 and its derivatives was cloned into M13mp18 and M13mp19. The complete gene was sequenced for each mutant by the strategy described in Materials and Methods. The nucleotide changes and the deduced changes in the amino acid sequence are summarized in Table 2. In the seven independent mutants, four different mutations in *ptsG* were found; two of the mutations were found more than once. The alterations in II^{Glc} were all localized within a stretch of about 100 amino acids (Fig. 2). The segment of II^{Glc} in which the mutations were localized is a relatively hydrophilic region separating two hydrophobic regions that contain the putative membrane-spanning α-helices of II^{Glc} (9).

Glucose transport by uncoupled II^{Glc}. Uncoupled II^{Glc} was able to support growth of PP2076, a strain lacking enzyme I and HPr, on glucose. When we quantitated the maximal growth rate (μ_{max}) on glucose, it appeared that the mutants grew approximately seven times faster than parental strain PP2076/pTSG4, which contains the *ptsG*$^+$ gene, but still only half as fast as wild-type strain PP799, which uses the normal PTS pathway for glucose uptake (Table 3). Apparently,

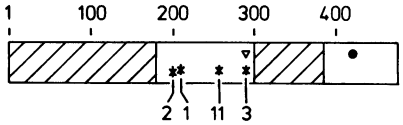

FIG. 2. Locations of *ptsG* mutations. The bar represents the II^{Glc} amino acid sequence. The scale above the bar indicates the number of residues. Open areas represent hydrophilic domains, and hatched areas represent hydrophobic domains, as determined by Erni and Zanolari (9). Mutations are indicated by asterisks, and the numbers correspond to the plasmids in Table 2. The residue that is most likely phosphorylated in II^{Glc}, Cys-421, is indicated by the dot. The triangle represents the conserved GITE motif (18) with unknown function.

TABLE 3. Maximal growth rate on glucose and kinetic parameters of glucose oxidation[a]

Strain	μ_{max} (h^{-1})	K_m (mM)	V_{max} (nmol of O_2/min/mg [dry wt])
PP799	0.66 ± 0.09	<0.03[b]	117 ± 13
PP2076/pTSG4	0.05 ± 0.01	NA[c]	NA
PP2076/pTSG4-1	0.38 ± 0.04	2.6 ± 0.4	47 ± 7
PP2076/pTSG4-2	0.34 ± 0.04	1.0 ± 0.2	52 ± 10
PP2076/pTSG4-3	0.33 ± 0.06	0.6 ± 0.1	41 ± 5
PP2076/pTSG4-11	0.34 ± 0.02	2.3 ± 0.3	56 ± 6

[a] *S. typhimurium* PP799 contains an intact glucose PTS, while PP2076 lacks enzyme I and HPr. PP2076/pTSG4-2, PP2076/pTSG4-4, and PP2076/pTSG4-5 were independently isolated mutants which contained the same mutation in *ptsG*. The results for PP2076/pTSG4-2 were representative for this group and are the only ones given. Similarly, PP2076/pTSG4-3 and PP2076/pTSG4-6 contained the same mutation and only the results for PP2076/pTSG4-3 are given. Cells were grown exponentially in medium A containing 0.5% glucose. Oxidation studies were performed as described in Materials and Methods. Mean values are given together with standard errors.

[b] The K_m of wild-type strain PP799 for glucose oxidation could not be accurately determined but was lower than 30 μM (see text).

[c] NA, not applicable.

TABLE 4. In vitro phosphorylation of αMG by uncoupled II^{Glc}[a]

Strain	Phosphorylation activity (nmol/min/mg of protein)
PP799	50 ± 5
ZSC13	25 ± 7
PP1980	0
PP1980/pTSG4	43 ± 9
PP1980/pTSG4-1	39 ± 9
PP1980/pTSG4-2	29 ± 9
PP1980/pTSG4-3	12 ± 7
PP1980/pTSG4-11	0

[a] Cells of PP1980 and transformants were grown in LB medium. PP799 and ZSC13 were grown in medium A containing 0.2% glucose. Phosphorylation of 0.5 mM [U-^{14}C]αMG (140 cpm/nmol) was measured as described in Materials and Methods. Mean values are given together with standard errors.

uptake of glucose via uncoupled II^{Glc} was not as fast as normal PTS-mediated glucose uptake.

To investigate the characteristics of glucose transport catalyzed by uncoupled II^{Glc}, we determined the K_m and V_{max} values of glucose oxidation by the mutants (Table 3). Strain PP2076/pTSG4 oxidized glucose only slowly at very high glucose concentrations. For instance, at 80 mM glucose, a rate of 2 nmol of O_2 consumed per min per mg of dry weight was reached. Therefore, no K_m and V_{max} values were determined for strain PP2076/pTSG4. The strains containing the plasmids encoding uncoupled II^{Glc} oxidized glucose but with a maximal rate and affinity which were lower than those of wild-type strain PP799 (Table 3). The maximal rates of glucose oxidation exhibited by the mutants were approximately half that of the wild-type strain and corresponded to the equally decreased maximal growth rates (Table 3). In strain PP799, the apparent K_m for glucose, as measured by glucose oxidation, was lower than 30 μM but could not be accurately determined by this method because at very low glucose concentrations, all glucose was consumed before the maximal rate of oxidation was reached. The apparent K_m values of the uncoupled mutants were in the range of 0.6 to 2.6 mM, 2 orders of magnitude higher than that of the wild-type strain. Wild-type II^{Glc} phosphorylates glucose during transport, while glucose, transported by uncoupled II^{Glc} in PP2076, is phosphorylated intracellularly by glucokinase. Both pathways yield glucose 6-phosphate. Since the metabolism of glucose 6-phosphate is the same in PP799 and PP2076 and because the affinity of glucokinase for glucose is high (K_m, approximately 70 μM; 37), the relatively high K_m values of uncoupled II^{Glc} mutants for glucose, as measured by glucose oxidation, suggested that uncoupled II^{Glc} had a low affinity for glucose compared with wild-type II^{Glc} (according to Adler and Epstein [1], the K_m of wild-type II^{Glc} for glucose, measured by glucose transport, is 20 μM in *E. coli*).

PEP-dependent phosphorylation catalyzed by uncoupled II^{Glc}. To determine whether uncoupled II^{Glc} could still catalyze PEP-dependent phosphorylation of its substrates, we measured in vitro phosphorylation of 0.5 mM αMG catalyzed by uncoupled II^{Glc} and compared it with the activity of wild-type II^{Glc}. The specific activity of uncoupled II^{Glc} from PP1980/pTSG4-1 was close to that of wild-type II^{Glc}, while PP1980/pTSG4-2 and PP1980/pTSG4-3 had lower activities (Table 4). The rate of αMG phosphorylation by II^{Glc} from PP1980/pTSG4-11 was very low. We also measured αMG phosphorylation at high concentrations (10 mM), which did not result in higher rates of phosphorylation for either uncoupled or wild-type II^{Glc} (data not shown). Thus, although uncoupled II^{Glc} exhibited a lowered affinity for glucose than did wild-type II^{Glc} in an oxidation assay (K_m values decreased 20- to 100-fold), the apparent K_m for αMG as measured by phosphorylation was affected much less (at most, 10-fold).

We investigated whether the expression or stability of uncoupled II^{Glc} differed from that of wild-type II^{Glc}. The amounts of II^{Glc} expressed by pTSG4, its derivatives, and *E. coli* ZSC13 containing a wild-type chromosomal copy of *ptsG*$^+$ were determined semiquantitatively on a Western immunoblot (Fig. 3). pTSG4-1 and pTSG4-2 expressed amounts of II^{Glc} comparable to that of pTSG4, while pTSG4-3 expressed somewhat less II^{Glc}. Because the decrease in activity of II^{Glc} from PP1980/pTSG4-3 was more pronounced than the decrease in the amount of protein synthesized, we conclude that the phosphorylation activity of II^{Glc} from mutant pTSG4-3 was lower than that of wild type II^{Glc}. We were not able to detect II^{Glc} on blots containing membrane proteins from strain PP1980/pTSG4-11. It is possible that II^{Glc} expressed from pTSG4-11 was less stable than wild-type II^{Glc}.

Is uncoupled II^{Glc} able to phosphorylate glucose in vivo? *E. coli* PPA214 does not contain any functional II^{Glc}, II^{Man}, or glucokinase and, as a consequence, is unable to take up or metabolize glucose. Introduction of both pTSG4 and its mutant derivatives, expressing uncoupled II^{Glc}, into PPA214, restored growth of this strain on glucose, suggesting that uncoupled II^{Glc} was able to phosphorylate glucose. When the genes for enzyme I and HPr were deleted from PPA214, yielding strain PPA223 and blocking PEP-dependent phosphorylation, pTSG4 and its derivatives were no longer able to restore growth on glucose, confirming that phosphorylation of glucose in PPA214 was mediated by the PTS.

PEP-dependent phosphorylation could have an effect on the kinetic characteristics of glucose transport via uncoupled II^{Glc}. pTSG4 derivatives expressing uncoupled II^{Glc} were introduced into both PPA214 (*pts*$^+$ *glk*) and isogenic Δ*ptsHI glk*$^+$ strain PPA222, and the K_m and V_{max} values of glucose oxidation were determined. In both strains, transport is catalyzed by uncoupled II^{Glc} but phosphorylation is cata-

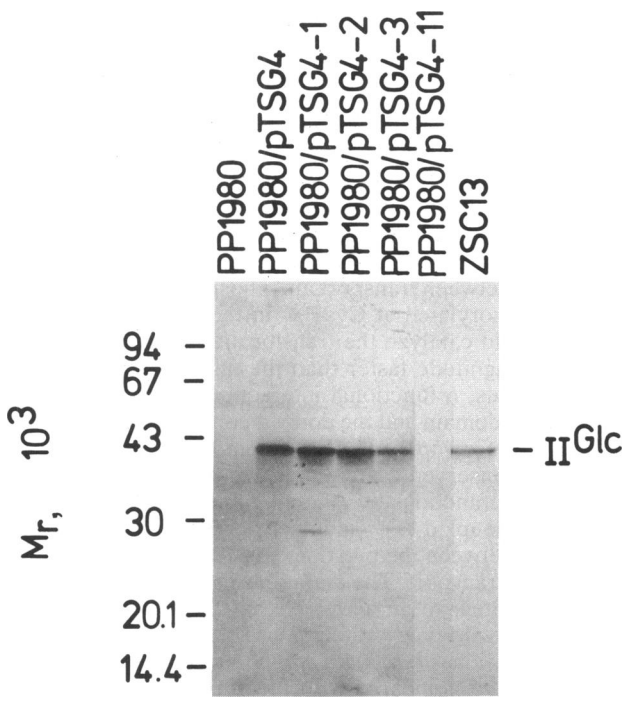

FIG. 3. Expression of II^{Glc}. *S. typhimurium* PP1980 (*ptsG*::Tn*10*) does not contain II^{Glc} and was transformed with the four different pTSG4 derivatives (indicated by the numbers 1, 2, 3, and 11) that express uncoupled II^{Glc}. PP1980 and transformants were grown in LB medium, and ZSC13 was grown in medium A supplemented with 0.2% glucose. After SDS-PAGE and electroblotting of membrane proteins (50 μg of protein in each case), II^{Glc} was visualized with a monoclonal antibody against II^{Glc} as described in Materials and Methods.

lyzed by II^{Glc} in PPA214 and by glucokinase in PPA222. The K_m values as determined for the different mutant proteins in *E. coli* PPA222 (Table 5) were comparable to the values in *S. typhimurium* PP2076 (Table 3). However, for PPA214/pTSG4-1 and PPA214/pTSG4-2, the apparent K_m for glucose, as measured by glucose oxidation, was higher than in PPA222/pTSG4-1 and PPA222/pTSG4-2. The presence of enzyme I, HPr, and III^{Glc} in PPA214 resulted in a 60-fold-

TABLE 5. Effect of PEP-dependent phosphorylation on the kinetic parameters of glucose oxidation[a]

Strain	K_m (mM)	V_{max} (nmol of O_2/min/mg [dry wt])
PPA222/pTSG4-1	2.3	23
PPA214/pTSG4-1	0.04	40
PPA222/pTSG4-2	0.7	24
PPA214/pTSG4-2	0.16	36
PPA222/pTSG4-3	0.4	22
PPA214/pTSG4-3	0.5	24
PPA222/pTSG4-11	2.1	18
PPA214/pTSG4-11	2.0	25

[a] *E. coli* PPA222 (*ptsG ptsM ΔptsHI*) and PPA214 (*ptsG ptsM glk*) containing pTSG4 derivatives expressing uncoupled II^{Glc} were grown exponentially in medium A containing 0.5% glucose. Oxidation studies were performed as described in Materials and Methods.

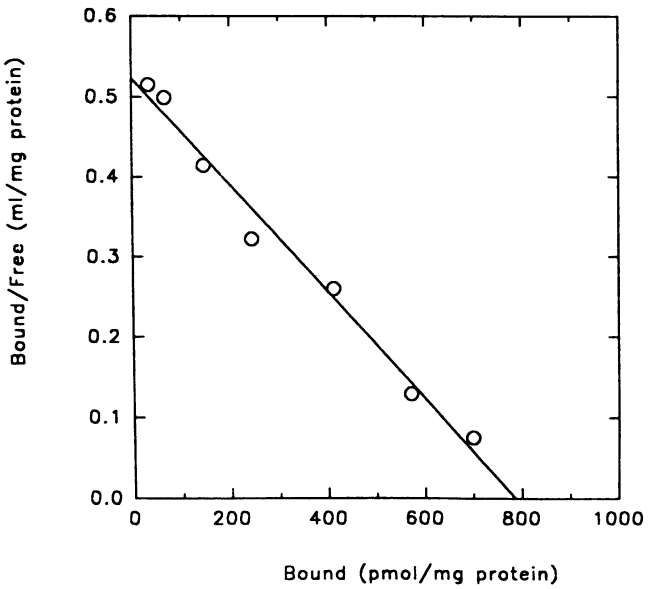

FIG. 4. Scatchard analysis of glucose binding to II^{Glc}. Membrane vesicles were isolated from *E. coli* WA2127/pTSG4 grown in LB medium. Membrane vesicles at a total protein concentration of 1 mg/ml were incubated with 0.1 to 10 μM [1-^{3}H]glucose. Free [1-^{3}H]glucose was measured before and after addition of a 1,000-fold excess of unlabeled glucose by flow dialysis as described in Materials and Methods. Each datum point is the mean of at least three determinations.

decreased K_m for mutant pTSG4-1. The effect for mutant pTSG4-2 was less pronounced, i.e., a fivefold decrease in the K_m value. Simultaneously with the decreased K_m, the maximal rate of glucose oxidation for mutants pTSG4-1 and pTSG4-2 was increased in PPA214 compared with PPA222 (Table 5). No change in the K_m for glucose was observed for II^{Glc} expressed from pTSG4-3 and pTSG4-11.

Binding of glucose to II^{Glc}. During glucose oxidation in PP2076, uncoupled II^{Glc} mutants exhibited high K_m values in comparison with strains containing wild-type II^{Glc}. We performed binding assays to investigate whether uncoupled II^{Glc} had an altered binding affinity for glucose. Binding of glucose to II^{Glc} was measured by flow dialysis. Because binding experiments required large amounts of II^{Glc}, we used *E. coli* WA2127 containing pTSG4 or one of its derivatives expressing uncoupled II^{Glc} in these assays. When grown in LB medium, WA2127 contained very little II^{Glc}, whereas in the case of WA2127/pTSG4, growth in LB medium resulted in overproduction of II^{Glc}. From the Scatchard plot shown in Fig. 4, for wild type II^{Glc} a K_d of 1.5 μM for glucose was calculated. In contrast, membranes of WA2127 or membranes of WA2127/pTSG4-1, WA2127/pTSG4-2, WA2127/pTSG4-3, and WA2127/pTSG4-11 containing uncoupled II^{Glc} bound no or very little glucose even at glucose concentrations as high as 10 μM (data not shown). Because of technical limitations of the flow dialysis assay, no K_d was determined for uncoupled II^{Glc}. However, the results suggested that uncoupled II^{Glc} had an elevated K_d for glucose.

DISCUSSION

The PTS catalyzes the transport and concomitant phosphorylation of its substrates. Transport and phosphorylation

are tightly coupled. In the absence of PTS-mediated phosphorylation, the carbohydrate-specific enzymes II are virtually unable to catalyze the transport of their substrates (rates are less than 1% of that in the presence of enzyme I and HPr). In this report, we describe the isolation and characterization of a class of mutations in enzyme II^{Glc} of the PTS in *E. coli* which resulted in uncoupling of carbohydrate transport from phosphorylation. Thus, contrary to the wild-type protein, uncoupled II^{Glc} can catalyze glucose transport in the absence of the general PTS proteins enzyme I and HPr. In these uncoupled II^{Glc} mutants, glucose is subsequently phosphorylated by glucokinase. Previously, we have isolated comparable mutations in *S. typhimurium* (31, 37) and subsequently shown that in these mutants the mechanism of glucose transport via uncoupled II^{Glc} is facilitated diffusion (38).

In the mutants described in this report, the apparent K_m of uncoupled II^{Glc} for glucose was increased 2 orders of magnitude compared with that of wild-type II^{Glc}. In addition, uncoupled II^{Glc} bound little or no glucose at glucose concentrations of up to 10 μM, while normal II^{Glc} exhibited a K_d of 1.5 μM. Previously isolated uncoupled II^{Glc} mutants (31, 37) also exhibited high K_m values. It should be pointed out that some enzymes II which catalyze the entry of the non-PTS carbohydrates galactose and trehalose exhibit high apparent K_m values for these carbohydrates. In *S. typhimurium*, facilitated diffusion of galactose (29) and trehalose (32) is catalyzed by enzyme II^{Man}, while in *E. coli*, facilitated diffusion of galactose proceeds via II^{Glc} (14). A high K_m could be a functional characteristic of enzymes II which catalyze transport via a mechanism of facilitated diffusion. Facilitators do not accumulate their substrates but only equilibrate them across the cytoplasmic membrane in an unmodified form. For instance, in the case of glucose transported by uncoupled II^{Glc}, a low K_m for glucose would result in a continuously loaded facilitator, even if the intracellular glucose concentration is a few micromolar. This low intracellular glucose concentration might not be high enough for growth. A high K_m circumvents this problem and leads to viable mutants.

Although the uncoupled enzymes II^{Glc} had altered transport characteristics, most of the mutant proteins were able to catalyze phosphorylation of glucose and αMG. This indicates that transport and phosphorylation by the PTS permeases are separable functions. One of the mutant enzymes II^{Glc} did not phosphorylate αMG, however. Although no protein was detected in this mutant on a Western blot, it is possible that this mutant resembled previously isolated uncoupled II^{Glc} mutants that were also not able to phosphorylate αMG (31). Apparently, different types of uncoupled II^{Glc} mutants, in which the phosphorylation activity varies from 0 to 100% (i.e., comparable to wild-type II^{Glc}), can be isolated.

Uncoupled II^{Glc} is able to catalyze glucose transport in the absence of enzyme I and HPr. Interestingly, when two of the mutant proteins (those encoded by *ptsG101* and *ptsG102*) were expressed in a pts^+ strain, the K_m for glucose, as measured by glucose oxidation, was lower than in a Δ*ptsHI* strain. Thus, phosphorylation via enzyme I, HPr, and III^{Glc} improved the affinity for glucose. Possibly, phosphorylation of II^{Glc} induced a conformational change that resulted in a higher affinity. It appears that these mutants are conditionally uncoupled.

Recently, Lolkema et al. (20, 21) have investigated the mechanism of coupling between transport and phosphorylation in enzyme II^{Mtl}. II^{Mtl} is the best-characterized PTS permease. It consists of three domains (11, 19, 44–46). (i) The most C-terminal domain (residues 475 to 637) is comparable to the autonomous subunit III^{Glc} of the glucose permease. In this domain of II^{Mtl}, His-554 is phosphorylated by P-HPr (27). (ii) A second hydrophilic domain (residues 357 to 474) contains the Cys-384 residue, which is phosphorylated by His-554 (27). (iii) A hydrophobic transmembrane domain (residues 1 to 356) binds mannitol (11, 19) and catalyzes its translocation across the membrane. The model of Lolkema et al. (20, 21) relates this domain structure to the mechanism of coupling between transport and phosphorylation. Enzyme II^{Mtl}, phosphorylated at Cys-384 in the second hydrophilic domain, could catalyze the translocation of mannitol 2 to 3 orders of magnitude faster than the unphosphorylated protein (21). Thus, a functional interaction exists between the translocator domain and the domain containing Cys-384 (20). Since II^{Glc} is composed of two comparable domains and contains a conserved cysteine residue (Cys-421), it is possible that II^{Glc} functions by a comparable mechanism.

In the uncoupled II^{Glc} mutants described in this report, interaction between the two domains is apparently no longer required for transport. The mutations in uncoupled II^{Glc} may bring the protein to an activated state, as phosphorylation does for the wild-type protein. Possibly, the energy barrier for translocation of a loaded binding site is lowered in uncoupled II^{Glc} compared with the unphosphorylated wild-type protein. Alternatively, a hypothetical pore in II^{Glc} may always be open, regardless of the phosphorylation state of the protein, as proposed originally (31).

Apart from phosphorylation, alkylation of Cys-384 in II^{Mtl} by *N*-ethylmaleimide or substitution of Cys-384 by a serine residue resulted in a 5- to 10-fold-increased mannitol translocation rate (20). Since these two modifications of Cys-384 abolish the phosphorylating activity of II^{Mtl}, transport is uncoupled from phosphorylation and the protein is converted into a facilitator. A similar effect has been reported for the homologous Cys residue in enzyme II^{Bgl}. Substitution of Cys-24 in II^{Bgl} by a serine residue abolishes the phosphorylation activity of II^{Bgl} but allows facilitated diffusion of glucose, as measured by growth (41). According to Nuoffer et al. (26), II^{Glc} behaves differently. II^{Glc}, in which Cys-421 is replaced by Ser, is not able to phosphorylate its substrates like the comparable II^{Mtl} and II^{Bgl} mutant proteins. However, a Cys-421-to-Ser mutant of II^{Glc} did not facilitate glucose entry into the cell. Thus, a mutation in the Cys-421 residue of II^{Glc} did not uncouple transport from phosphorylation. In this report, we describe other substitutions in II^{Glc} that do result in uncoupling of transport and phosphorylation. Clearly, other residues apart from the essential Cys are involved in the process of coupling between transport and phosphorylation.

In one of the mutant enzymes II^{Glc} (encoded by *ptsG101*), Val-206 was substituted by Ala. It is striking that this conservative change caused such drastic effects on the properties of II^{Glc}. However, similar examples have been reported. In the high-affinity glucose transporter of *Saccharomyces cerevisiae*, substitution of Val-402 by an Ile residue inactivated the carrier completely (23). Substitution of Ala-177 by Val in the lactose permease of *E. coli* led to uncoupling of lactose and proton transport and to an altered substrate specificity of the carrier (12, 13). Apparently, conservative amino acid substitutions in proteins can lead to substantial changes in their properties.

PTS permeases are composed of several domains (17, 18, 36). The structure of the glucose permease resembles that of II^{Mtl}, II^{Bgl}, II^{Nag}, and II^{Scr}/III^{Glc}, which are specific for

mannitol, β-glucosides, *N*-acetylglucosamine, and sucrose, respectively. In addition to two hydrophilic domains, these permeases contain a hydrophobic domain of about 350 amino acids. It has been suggested that the hydrophobic domain contains the substrate-binding site and the residues involved in translocation of the carbohydrate (11, 17–19, 22, 42). In II^{Glc}, this domain is located at the N terminus. Within this hydrophobic domain, an intervening hydrophilic segment is present (Fig. 2). Interestingly, all mutations in II^{Glc} that resulted in uncoupled glucose transport were located in this hydrophilic loop. Since uncoupled II^{Glc} translocated, and probably also bound, glucose with low affinity, these results suggest that the hydrophilic loop is involved in binding and translocation of the substrate. Recently, Manayan et al. (22) described a mutation in II^{Mtl} that led to a transport-negative phenotype, while the altered enzyme II^{Mtl} could still phosphorylate mannitol. This mutation was sequenced and appeared to be located in the hydrophilic loop within the hydrophobic domain of II^{Mtl}. These comparable results for II^{Glc} and II^{Mtl} suggest that in general, the hydrophilic loop of the PTS permeases is involved in substrate translocation.

An interesting observation by Lengeler et al. (18) is that PTS permeases contain a highly conserved GITE motif, of which the function is unknown. In II^{Glc}, residues 295 to 298 form the GITE motif, and interestingly, in one of the uncoupled mutants (*ptsG103*) Ile-296 (I) was substituted by Asn. Possibly, the GITE motif is important for the coupling between transport and phosphorylation. It would therefore be interesting to introduce mutations in the GITE motif in other enzymes II and investigate the effects on phosphorylation activity and coupling.

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