The Glucose Transporter of the *Escherichia coli* Phosphotransferase System

**MUTANT ANALYSIS OF THE INVARIANT ARGININES, HISTIDINES, AND DOMAIN LINKER**

(Received for publication, December 16, 1997)

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The glucose transporter of the bacterial phosphotransferase system (PTS) consists of a hydrophilic (IIA-Glc) and a transmembrane subunit (IICB-Glc). IICB-Glc has two domains (C and B), which are linked by a highly invariant sequence. Transport of glucose by IIC and phosphorylation by IIB are tightly coupled processes. Three motifs that are strongly conserved in 12 homologous PTS transporters, namely two invariant arginines (Arg-424 and Arg-426) adjacent to the phosphorylation site (Cys-421), the invariant interdomain sequence KT-PGRED, and two conserved histidines (His-211 and His-212) in the IIC domain were mutated and the mutant proteins characterized in vivo and in vitro for transport and phosphorylation activity. Replacement of the strongly β-turn favoring residues Thr and Gly of the linker by α-helix favoring Ala results in strong reduction of activity, whereas the substitutions of the other residues have only minor effects. The R424K and R426K mutants can be phosphorylated by IIA-Glc but can no longer donate the phosphoryl group to glucose. The H211Q and H212Q mutants continue to phosphorylate glucose at a reduced rate but H212Q can no longer transport glucose. Mixtures of purified R424K/H212Q and R426K/H212Q have 10% of wild-type phosphorylation activity and when coexpressed in *Escherichia coli* support glucose transport.

*Escherichia coli* has two transporters for glucose (1, 2). They act by a mechanism that couples translocation with phosphorylation of the substrate. Both transporters are components of the bacterial phosphoenolpyruvate-sugar phosphotransferase system (PTS). The IIA-Glc-IIICB-Glc complex is specific for glucose, the IIAB-Man-IIIC-Man-IIID-Man complex has a broad substrate specificity for Glc, Man, GlcNAc, and other derivatives of Glc altered at the C2 carbon. The PTS comprises two cytosolic proteins, enzyme I and HPr, which sequentially transfer phosphoryl groups from PEP to the different carbohydrate-specific transporters (enzymes II). Number and substrate specificity of the transporters varies for different bacterial species (for review see Refs. 3–5). They have been grouped into four families (6) based on amino acid sequence comparisons. IIICB-Glc belongs to the glucose family presently comprising 25 members (Protein Domain Data base; http://protein.toulouse.inra.fr/promod/ promod.html), the IIAB-Man-IIIC-Man-IIID-Man belongs to the mannose family comprising 6 members (7–12).

All PTS transporters consist of three functional units (IIA, IIB, IIC), which occur either as protein subunits or domains of a multidomain polypeptide (13). The glucose transporter consists of two subunits IIA-Glc and IIICB-Glc. IIA-Glc is a 18-kDa hydrophilic protein that is phosphorylated at His-90 (14). The 51-kDa IICB-Glc subunit consists of two domains (15). The hydrophobic domain (IIC, residues 1–380) spans the membrane eight times and contains the glucose binding site (16). The hydrophilic domain (IIB, residues 380–477) is phosphorylated at Cys-421 (17). Phosphoryl groups are transferred from HPr via His-90 of IIA-Glc to Cys-421 and hence to glucose (18). The IIC and IIB domain are connected by an heptapeptide sequence that is highly conserved in those two- and three-domain transporters of the glucose family that have the domain order CB(A). A chimeric protein consisting of the IIC-Glc domain of the glucose transporter and the IIBGlcNAc domain of the GlcNAc transporter is active and glucose-specific (19). The IIC and IIB domains of IICB-Glc can be expressed as separate polypeptides. The purified subclonal domains retain 2% of wild-type phosphotransferase activity when they are combined in vitro (20).

The three-dimensional structure of the IIB-Glc domain, its interaction with the IIA-Glc subunit and the structural consequences of its phosphorylation at Cys-421 have been analyzed by heteronuclear NMR spectroscopy (21, 22). A model of the transmembrane topology of the IIC domain has been derived from protein fusion studies and further confirmed by linker insertion mutagenesis (16). Point mutants of IICB-Glc have been selected that facilitate Glc transport uncoupled of phosphorylation (23) and that retain glucose phosphorylation activity but have a strongly reduced translocation activity (24). All of these mutations are located in the IIC domain, and none was found so far in the IIB domain.

When the amino acid sequences of the transporters belonging to the glucose family are compared, several regions of strong amino acid similarity can be discerned (Fig. 1). One includes the active site Cys-421 which is phosphorylated by IIA-Glc and donates the phosphoryl group to the transported glucose. Besides Cys-421, Arg-424, and Arg-426 are also invariant. They are the only invariant arginines in the transporters of the glucose family. Arginines are frequently found in phosphate-binding and phosphate-catalytic sites where they can stabilize phosphate through hydrogen bonding and electro-

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* This work was supported by Grant 31-45838.95 from the Swiss National Science Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: PTS, phosphoenolpyruvate-dependent carbohydrate/phosphotransferase system (EC 2.7.1.69); IIA-Glc, hydrophilic subunit of the glucose transporter; IICB-Glc, transmembrane subunit of the glucose transporter; IIAB-Man, IIIC-Man, IIID-Man, subunits of the mannose transporter of the PTS; IICBA-Mtl, mannitol transporter of *E. coli*; HPr, histidine-containing phosphocarrier protein of the PTS; PEP, phosphoenolpyruvate; αMG, α-methyl-D-glucopyranoside.

2 R. Beutler and B. Erni, unpublished results.
prolines, glycines or glutamines (26–28). Their amino acid sequence is usually not conserved. Here we address the question of how important the residues of this heptapeptide are for the function of IICB\textsubscript{Glc}. A third conserved region surrounds the KTPGRED motif, the putative linker between the IIC and IIB domains. Surprisingly, the two invariant arginines in the IIB domain are not conserved in any of the enzymes belonging to the glucose family of the PTS (Fig. 1). Six of the seven residues were replaced one at the time by alanine. Pro-384 was replaced with glycine because several attempts to replace it with Ala by gapped duplex and polymerase chain reaction-based mutagenesis methods failed. The reason for this failure is not known. Mutant proteins were expressed in strain ZSC112\textsubscript{A2}, which carries a chromosomal deletion of \textit{ptsG}. Six transformants produced red colonies on McConkey glucose indicator plates, and only the G385A mutant produced yellow colonies with a small red center. All the IICB\textsubscript{Glc} mutants could be overexpressed in the same amount as wild-type protein and purified by Ni\textsuperscript{2+} chelate affinity chromatography (results not shown). The purified G385A protein displayed only 0.8% of wild-type activity, T383A had 10% of wild-type phosphotransfer activity (Fig. 2). The purified G385A protein was phosphorylated in amounts sufficient to support fermentation of glucose (results not shown). The H212Q mutant produced yellow colonies with a red center. Uptake of \[^{14}C\]MG by bacteria was assayed as described (24). Cells were grown to \(A_{600} = 0.5\) in 250 ml of M9 minimal medium supplemented with 1% glycerol and 20 \(\mu\)M isopropryl-\(\beta\)-D-galactopyranoside, collected by centrifugation, and resuspended in M9 medium without supplemental glucose. 0.3 ml of concentrated cell suspension were diluted with 0.77 ml of M9 medium and aerated for 10 min at 22°C. Uptake was started by the addition of 12 \(\mu\)M \[^{14}C\]MG (6000 cpm/nmol). Aliquots of 100 \(\mu\)l were withdrawn, diluted into 5 ml of ice-cold M9 containing 0.4 M NaCl, filtered through GF (Whatman) glass fiber filters under suction, and washed with 20 ml of ice-cold 0.15 M NaCl. The filters were dried and counted. The dry weight was determined from 0.2 ml of the concentrated cell suspension. In vitro phosphorylation of \[^{14}C\]Glc was assayed by ion-exchange chromatography as described (1). 100 \(\mu\)l of incubation mixture contained 4 \(\mu\)l of cell extract as source of enzyme I, HPr, and IIA\textsubscript{Glc}. 1 \(\mu\)mol of PEP, 1 \(\mu\)mol \[^{14}C\]Glc (1000 dpm/nmol), 0.1 mg of \textit{E. coli} phospholipids, and 5 \(\mu\)l of purified IICB\textsubscript{Glc} and where indicated 3 \(\mu\)M Glc. \[^{32}P\]PEP was prepared as described (37).

RESULTS

Alanine-scanning Mutagenesis of the Interdomain Linker—The amino acid sequence KTPGRED between the IIC and IIB domains is the most highly conserved region in the transporters belonging to the glucose family of the PTS (Fig. 1). Six of these seven residues were replaced one at the time by alanine. Pro-384 was replaced with glycine because several attempts to replace it with Ala by gapped duplex and polymerase chain reaction-based mutagenesis methods failed. The reason for this failure is not known. Mutant proteins were expressed in strain ZSC112\textsubscript{A2}, which carries a chromosomal deletion of \textit{ptsG}. Six transformants produced red colonies on McConkey glucose indicator plates, and only the G385A mutant produced yellow colonies with a small red center. All the IICB\textsubscript{Glc} mutants could be overexpressed in the same amount as wild-type protein and purified by Ni\textsuperscript{2+} chelate affinity chromatography (results not shown). The purified G385A protein displayed only 0.8% of wild-type activity, T383A had 10% of wild-type phosphotransferase activity, whereas the other mutants had between 40 and 120% of wild-type activity (Fig. 2). The very low activity of the G385A mutation was not due to protein degradation, since the protein could be purified in the same amount as the other IICB\textsubscript{Glc} mutants.

Function of the Invariant Arginines and Histidines—The IICB\textsubscript{Glc} mutants H211Q, H212Q, R424K, and R426K were expressed in strain ZSC112\textsubscript{A2}. The H212Q, R424K, and R426K mutants produced yellow colonies on McConkey glucose indicator plates indicating that they were unable to transport and phosphorylate glucose in amounts sufficient to support fermentation of glucose (results not shown). The H211Q mutant produced yellow colonies with a red center. Uptake of \[^{14}C\]MG by whole cells expressing H211Q was reduced to less than 10% of the wild-type control, whereas the H212Q, R424K, and R426K mutants had no transport activity (Fig. 3).

The four mutant proteins could be overexpressed in the usual amounts and purified by Ni\textsuperscript{2+} chelate affinity chromatography. H211Q, H212Q, R424K, and R426K could be phosphorylated in the presence of \[^{32}P\]PEP, enzyme I, HPr, and IIA\textsubscript{Glc}, indicating that none of these residues is required for phosphoryltransfer from IIA\textsubscript{Glc} to Cys-421 on the IIB domain (Fig. 4). Upon addition of glucose to the incubation mixture the H211Q and H212Q mutants are dephosphorylated like the wild-type protein indicating that phosphoryl transfer between Cys-421 of IIB and glucose is still possible. In contrast, the...
Figure 2. Phosphotransferase activities of IICBGlc linker mutants. A, the activity of purified IICBGlc was titrated in the presence of a cytosplasmic extract containing saturating concentrations of enzyme I, HPr, and IIA^Glc. B, activity of the alanine-scanning mutants in percent of wild-type IICBGlc. Wild-type (●); K382A ( ◦ ); T383A (△); P384G ( ▼ ); G385A (○); R386A (△); E387A (○); D388A (□).

Figure 3. Uptake of αMG by intact cells expressing His and Arg mutants of IICBGlc. Cells expressed either one mutant protein alone or two mutant proteins (H211Q plus C421S). Wild-type (●); R211Q (□); H211Q (△); C421S (▼); R424K (○); R426K (□); H212Q plus C421S (○). The uptake reaction was started by the addition of [14C]αMG to a cell suspension at room temperature. 100-μl aliquots (1.2 μg to 1.4 μg dry weight) were withdrawn at the indicated time points, filtered through glass fiber filters, and counted.

Figure 4. Phosphorylation of mutant IICBGlc. Purified IICBGlc was incubated with [14C]P-PGP in the presence of enzyme I, HPr, and IIA^Glc for 10 min at 37 °C. The samples were then split into two aliquots, to which either buffer (−) or Glc (+, 1 mM final concentration) was added. The proteins were separated by gel electrophoresis and analyzed by phosphorimaging. The first lane contains all components with the exception of IICBGlc. Wt., wild-type; Enzl, enzyme I.

R424K and R426K mutants remain phosphorylated in the presence of glucose indicating that both residues are required for the phosphoryl transfer from Cys-421 to glucose. The C421S mutant, which lacks the active site cysteine, is not phosphorylated (34). The glucose phosphotransferase activities of the purified proteins were determined in the standard sugar phosphotransferase assay (Fig. 5A). The H211Q mutant had almost the same specific activity as the wild-type control, the H212Q mutant retained 15% phosphotransferase activity, whereas the R424K and R426K mutants were inactive. Note, that the vectorial transport (uptake) and the nonvectorial phosphorylation activities are differentially affected by the His substitutions in the C domain, whereas both activities are completely inhibited by the Arg substitutions in the B domain.

Since IICBGlc is a homodimeric protein (18), interallelic complementation between different mutant subunits is possible. Cells expressing the H211Q, H212Q, R424K, and R426K mutant were transformed with a second plasmid encoding the inactive C421S mutant of IICBGlc and analyzed for glucose fermentation on McConkey plates. R424K/C421S and R426K/C421S formed yellow colonies. However, the H212Q/C421S double transformants formed red colonies (results not shown) and showed transport activity in the whole cell uptake assay (Fig. 3). Interallelic complementation could also be observed with purified proteins in vitro. Mutant proteins were mixed in different ratios, whereas the total IICBGlc concentration was kept constant. The H212Q/R424K and H212Q/R426K combinations showed complementation (Fig. 5B). The heterodimers had approximately 10% of wild-type PTS activity when the mutant ratio was 1:1 (binomial distribution of heterodimers and homodimers). The mixture of purified R424K and R426K remained inactive. Mixtures of H211Q with inactive H212Q or R424K showed no complementation. The activity, which is 15% of wild-type activity for pure H211Q, decreased in proportion to the decreasing concentration of H211Q. H211Q/C421S and H211Q/R426K mixtures showed only weak complementation of phosphotransferase activity (Fig. 5C).

Discussion

Point mutations in three strongly conserved regions of the IICBGlc subunit of the glucose transporter were characterized with respect to their effect on protein phosphorylation, nonvectorial glucose phosphorylation, and transport activity. The first region of interest is the KTPGRED linker between the IIC and the IIB domains, which is predicted to assume a loop structure (38, 39). The T383A and G385A substitutions reduced the activity to less than 10% of the control. Thr and Gly favor β-turn structures, whereas Ala favors α-helices. Substitution of these β-turn residues at the center of the hinge by Ala could lead to a stiffening of the linker with concomitant reduced domain mobility. The G385A mutation is less active than the two domains expressed as completely separated subunits (20) or the transposition of the B domain from the carboxyl-terminal to the amino-terminal end of the IIC domain. IIBC^Glc with circularly permuted domains has 40–70% activity provided the two domains are essential for vectorial transport of glucose. Phosphoryl transfer from Cys-421 to glucose is completely blocked in the arginine mutants and slow in the H212Q mutant. A mutant with a similar phenotype as R424K and R426K was also found in the IIB domain of the IIA^Man subunit of the structurally

3 R. Gutknecht and B. Erni, unpublished results.
unrelated mannose transporter (41). IIAB\textsuperscript{Man} R172Q can be phosphorylated at His-175 but cannot donate the phosphoryl group to glucose. It is not clear what function Arg-424 and Arg-426 serve. In view of their proximity to the active site cysteine (Fig. 6) they could stabilize the phosphate in the bonded ground state or in the transition state during transfer from Cys-421 to Glc. Phosphotyrosine phosphatases, the second group of proteins that form phosphocysteine intermediates, provide strong evidence for such an interaction (42). Their active sites contain a cysteine and an arginine, separated by five residues. X-ray structures of these proteins indicate that the invariant arginine stabilizes the transition state with three hydrogen bonds between the guanidino group and two phosphate oxygens (43). However, since neither the IIB domains of the mannitol family of PTS transporters. Although complementation between IIC and IIB as well as between IIB and IIA domains therefore appears to be a general property of the dimeric PTS transporters. Although complementation between sites on the same domain has both also been observed (36).

Acknowledgment—We thank Ruedi Beutler for helping with the preparation of the similarity plot.

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