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Topography of the Surface of the *Escherichia coli* Phosphotransferase System Protein Enzyme IIA^{glc} that Interacts with Lactose Permease

Melissa Sondej,[‡] Yeong-Jae Seok,[§] Paul Badawi,[‡] Byoung-Mo Koo,[§] Tae-Wook Nam,[§] and Alan Peterkofsky*[,‡,||]

Laboratory of Biochemical Genetics, National Heart, Lung and Blood Institute, National Institutes of Health, Bethesda, Maryland 20892 and Department of Microbiology, College of Natural Sciences, Seoul National University, Seoul 151-742, Korea

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ABSTRACT: The unphosphorylated form of enzyme IIA^{glc} of the *Escherichia coli* phosphoenolpyruvate: sugar phosphotransferase system inhibits transport catalyzed by lactose permease. We (Seok et al. (1997) *Proc. Natl. Acad. Sci. U.S.A. 94*, 13515−13519) previously characterized the area on the cytoplasmic face of lactose permease that interacts with enzyme IIA^{glc}, using radioactive enzyme IIA^{glc}. Subsequent studies (Sondej et al. (1999) *Proc. Natl. Acad. Sci. U.S.A. 96*, 3525−3530) suggested consensus binding sequences on proteins that interact with enzyme IIA^{glc}. The present study characterizes a region on the surface of enzyme IIA^{glc} that interfaces with lactose permease. Acetylation of lysine residues by sulfosuccinimidyl acetate treatment of enzyme IIA^{glc}, but not lactose permease, reduced the degree of interaction between the two proteins. To localize the lysine residue(s) on enzyme IIA^{glc} that is(are) involved in the regulatory interaction, selected lysine residues were mutagenized. Conversion of nine separate lysines to glutamic acid resulted in proteins that were still capable of phosphoryl acceptance from HPr. Except for Lys69, all the modified proteins were as effective as the wild-type enzyme IIA^{glc} in a test for binding to lactose permease. The Lys69 mutant was also defective in phosphoryl transfer to glucose permease. To derive further information concerning the contact surface, additional selected residues in the vicinity of Lys69 were mutagenized and tested for binding to lactose permease. On the basis of these studies, a model for the region of the surface of enzyme IIA^{glc} that interacts with lactose permease is proposed.

In *Escherichia coli*, the bacterial phosphoenolpyruvate: sugar phosphotransferase system (PTS)[1] effects the coupled transport and phosphorylation of numerous sugars. The pathway involves the PEP-dependent phosphorylation of EI; there is then a sequential phosphoryl transfer to HPr, then to sugar-specific enzymes II. The PTS also plays an important role in several regulatory mechanisms (*1*). Catabolite repression allows for the preferential utilization of sugars transported by the PTS. Consequently, when *E. coli* are cultured in a medium containing both glucose and lactose, the glucose is consumed first. The currently accepted mechanism for this effect is that, when PTS sugars are transported, the steady-state condition of IIA^{glc} is mainly in the dephospho-form. It is believed that dephospho-IIA^{glc} binds to and inhibits lac permease. This results in exclusion of lactose, the precursor of the inducer for the lactose operon. Upon depletion of the pool of glucose, IIA^{glc} reverts to the phospho-form and the inhibition of lac permease is relieved. Consequently, lactose can be taken up and the operon for lactose utilization is induced.

Another aspect to the preferential utilization of PTS sugars deals with the PTS-dependent regulation of adenylyl cyclase (*2*). Many of the inducible sugar transport pathways, such as that for lactose, are positively regulated by the cAMP−CRP system. The phosphorylated form of IIA^{glc} is believed to be a positive activator of adenylyl cyclase. Consequently, when PTS sugars are transported, there is concomitant dephosphorylation of IIA^{glc}, the activation of adenylyl cyclase is diminished and cAMP levels fall.

A number of studies have been carried out in order to elucidate the structural features of lac permease necessary for the interaction with IIA^{glc} (*3*−*5*). We recently described an assay, using radioactively labeled IIA^{glc}, for quantitation of the lac permease−IIA^{glc} interaction and confirmed the necessity for a substrate-induced conformational change (*6*). A collection of insertions in both cytoplasmic and periplasmic loops of this transporter were evaluated for binding to IIA^{glc}. That study established the importance of both the central cytoplasmic loop (VI/VII) as well as the adjoining loop (IV/V). A model for the surface of lac permease that contacts IIA^{glc} was proposed from those data.

[||] This paper is dedicated to the memory of a dear friend and colleague, Dr. Jonathan Reizer, who died on December 31, 1999. He was a pioneer in the PTS field and his name has become synonymous with that research area.

[‡] National Institutes of Health.

[§] Seoul National University.

* Address correspondence to this author at the National Institutes of Health, Building 36, Room 4C-11, Bethesda, MD 20892. Telephone: 301-496-2408. Fax: 301-480-0182. E-mail: alan@codon.nih.gov.

[1] Abbreviations: PTS, phosphoenolpyruvate:sugar phosphotransferase system; PEP, phosphoenolpyruvate; HPr, histidine-containing phosphocarrier protein of the PTS; EI, enzyme I of the PTS; IIA^{glc}, enzyme IIA specific for glucose of the PTS; SSA, sulfosuccinimidyl acetate; TDG, β-D-galactopyranosyl 1-thio-β-D-galactopyranoside; mel, melibiose; lac permease, *Escherichia coli* lactose permease; EtN, ethanolamine, CRP, catabolite receptor protein.

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Table 1: Primers for Mutagenesis of IIA^{glc} [a]

mutagenesis	primers	new restriction site created
vector primers	1162-AACCACACCTATGGTGTATGCA-1183 1355-GAAGACAGTCATAAGTGCGGCGA-1333	
K53E	2428-GATGGTATTGCTATC*GAA*CCA**ACCGGT**AACAAA-2460 2460-TTTGTT**ACCGGT**TGG*TTC*GATAGCAATACCATC-2428	*Pin*AI
K58E	2443-AAACCA**ACCGGT**AAC*GAA*ATGGTCGCGCCAGTA-2475 2475-TACTGGCGCGACCAT*TTC*GTT**ACCGGT**TGGTTT-2443	*Pin*AI
K69E	2476-GACGGCA**CAATTG**GT*GAA*ATCTTTGAAACC-2505 2505-GGTTTCAAAGAT*TTC*AC**CAATTG**TGCCGTC-2476	*Mun*I
K69L	2476-GACGGCA**CAATTG**GT*CTG*ATCTTTGAAACC-2505 2505-GGTTTCAAAGAT*CAG*AC**CAATTG**TGCCGTC-2476	*Mun*I
K99E	2560-GGTATCGACACCGTT**GAGCTC***GAA*GGCGAAGGC-2592 2601-ACGCTTGAAGCCTTCGC*TTC***GAGCTC**AACGGT-2569	*Sac*I
K104E	2575-GAACTGAAAGGCGAAGGC**TTCGAA**CGTATTGCT-2607 2616-ACCTTCTTCAGCAATACG***TTCGAA***GCCTTCGCC-2584	*Sfu*I
K114E	2611-GAAGGTCA**ACGCGT**G*GAA*GTTGGCGATACTGTC-2643 2643-GACAGTAT*TTC*GCCAAC*TTC*C**ACGCGT**TGACCTTC-2611	*Mlu*I
K130E	2653-GATCTGCCGCTG**CTCGAG**GAG*GAA*GCCAAGTCT-2685 2694-AGTCAGGGTAGACTTGG*TTC*CTC**CTCGAG**CAG-2662	*Xho*I
K132E	2656-CTGCCGCTG**CTCGAG**GAGAAAGCC*GAA*TCTACC-2688 2694-AGTCAGGGTAG*ATTC*GGCTTTCTC**CTCGAG**CAG-2662	*Xho*I
K168E	2770-CCGGTTATCCGCATCAAG*GAG*TAAT**GCTAGC**−2800 2800-**GCTAGC**ATTA*CTC*CTTGATGCGGATAACCGG-2770	*Nhe*I
F71S	2471-CAGTAGAC**GGTACC**ATTGGTAAAATC*TCT*GAAACC-2505 2505-GGTTTC*AGA*GATTTTACCAAT**GGTACC**GTCTACTG-2471	*Kpn*I
F71K	2471-CAGTAGAC**GGTACC**ATTGGTAAAATC*AAA*GAAACC-2505 2505-GGTTTC*TTT*GATTTTACCAAT**GGTACC**GTCTACTG-2471	*Kpn*I
E86K	2531-ATAGCGGCGTT*AAA*CTGTTC**GTGCAC**TTCG-2560 2560-CGAA**GTGCAC**GAACAG*TTT*AACGCCGCTAT-2531	*Apa*LI
E86L	2531-ATAGCGGCGTT*TTA*CTGTTC**GTGCAC**TTCG-2560 2560-CGAA**GTGCAC**GAACAG*TAA*AACGCCGCTAT-2531	*Apa*LI
D94G	2545-CTGTTC**GTGCAC**TTCGGTATC*GGC*ACCGTTGAACTG-2580 2580-CAGTTCAACGGT*GCC*GATACCGAA**GTGCAC**GAACAG-2545	*Apa*LI

[a] The vector primers correspond to sequences in pREI (*14*). The sequences from bases 1162−1183 and 1355−1333 are upstream and downstream, respectively, of the multiple cloning region (bases 1229−1269). The sequence numbers of the mutagenic primers correspond to the numbering in the ecoptshi.genbank sequence. The bases in bold correspond to the designated new restriction site created. Underlined bases correspond to bases changed, without changing the translation, to create the new restriction site. The designated codon which was changed is shown italicized.

More recently, cysteine scanning mutagenesis of lac permease was used to deduce residues in loops IV/V and VI/VII necessary for interaction with IIA^{glc} (*7*). The requirement for two interaction regions is consistent with a model for a substrate-induced conformational change that creates an orientation of lac permease that effectively binds IIA^{glc}.

Aside from the elucidation of the sequence of genes encoding some spontaneous mutants of IIA^{glc} resistant to catabolite repression (*8*, *9*), there is little information concerning the structural features of IIA^{glc} necessary for binding to lac permease. The work presented here highlights a region of IIA^{glc} important in this process.

EXPERIMENTAL PROCEDURES

Materials. EI and HPr were purified as previously described (*10*). Oligonucleotides were synthesized using a Model 394 DNA/RNA synthesizer (Applied Biosystems). Restriction endonucleases and T4 DNA ligase were purchased from New England Biolabs. Tryptophan was purchased from Bethesda Research Laboratories. SSA was obtained from Pierce. [D-Glucose-1-^{14}C]lactose (55 mCi/mmol) was from American Radiolabeled Chemicals Inc. (St. Louis, MO).

Preparation of Membranes Enriched in lac Permease. Membrane vesicles were prepared from *E. coli* K12 strain T184 transformed with pT7-5/*lacY* for overproduction of wild-type lac permease (*11*, *12*). The transformed strain was grown, and lac permease was induced as described (*6*). The harvested cells were disrupted in a French pressure cell, and membrane vesicles were purified by centrifugation as described (*6*). The resulting vesicle preparations, approximately 20 mg protein/mL, were stored frozen at −80 °C until use. Protein concentration was estimated using bicinchoninic acid protein assay reagents (Pierce).

Mutagenesis of Specific Residues in IIA^{glc}. pPR3 is an expression vector for *E. coli* IIA^{glc} (10). A two-step PCR mutagenesis method, previously described (*13*), used pPR3 as the initial template for two PCR reactions. One contained a primer upstream of the *Nde*I site of the vector pRE1 (*14*) (1162−1183, see Table 1) and the reverse mutagenic primer

for generating the mutations specified in Table 1 (the reverse mutagenic primer is the second of the two primers listed for each mutation). The other PCR reaction contained a reverse primer downstream of the multiple cloning site of the pRE1 vector (1355−1333, see Table 1) and the forward mutagenic primer for generating the mutations specified in Table 1 (the forward mutagenic primer is the first of the two primers listed for each mutation). For each mutation constructed, the products of the two PCR reactions were annealed and used as the template for a third PCR reaction using the two vector primers. The PCR product (∼950 bp) was purified and digested with *Nde*I and *Sal*I and cloned into the same sites of pRE1 (*14*), a vector designed for the high level expression of proteins. All the constructs were verified by DNA sequencing by the dideoxy method of Sanger (*15*) using an Applied Biosystems automated sequencer.

Expression and Purification of Wild-type and Mutated Forms of IIA^{glc}. Strain GI698 Δpts (*16*) is an *E. coli* strain deleted for the *pts* operon and contains the gene encoding the λ repressor under control of the *trp* promoter; this strain, transformed with the various derivatives of pPR3 expressing wild-type or mutated IIA^{glc}s, was grown in defined medium and IIA^{glc} expression was induced with tryptophan (*17*). Cells were harvested by centrifugation, resuspended in 10 mM Tris, pH 7.5, and then ruptured by two passages through a French pressure cell at 10 000 psi. After centrifugation, the supernatant solutions were chromatographed on a MonoQ HR 10/10 column (Pharmacia) using 10 column volumes of 10 mM Tris, pH 7.5, with a linear salt gradient to 0.3 M NaCl. IIA^{glc}-containing fractions were identified by SDS-PAGE, and they were pooled, concentrated, and further purified on a Superose 12 gel filtration column (1.6 × 50 cm)(Pharmacia) in 10 mM Tris, pH 7.5, containing 100 mM NaCl. The final products were ∼95% pure, as judged by SDS-PAGE. Since the protein expression was carried out in a Δpts strain, all the preparations of IIA^{glc} were completely in the dephospho-form.

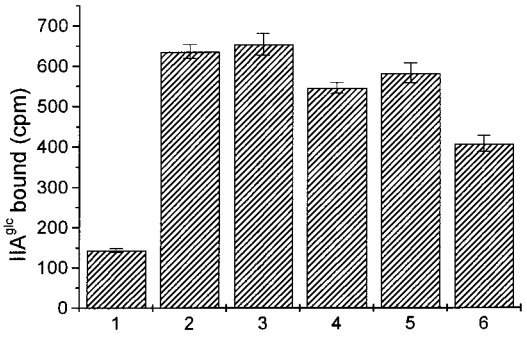
Phosphoryl Acceptance Activity for IIA^{glc} Proteins. Incubation mixtures, in a total volume of 10 μL, contained: 20 mM $NaPO_4$ buffer, pH 7.0; 2 mM $MgCl_2$; 1 mM EDTA; 100 mM KCl; 1 mM DTT; 1 mM PEP; 0.1 μg HPr; 1.2 μg of the indicated IIA^{glc} protein. Paired incubation mixtures were deficient in or supplemented with 0.5 μg of EI. Incubations were initiated by the addition of the IIA^{glc} proteins and carried out at room temperature for 5 min, then stopped by addition of an equal volume of double strength gel loading buffer. The samples were then deposited onto Tris-Glycine SDS 4−20% gradient gels (Novex) and electrophoresis was carried out at 120 V for about 105 min. The gels were then stained with Coommassie Blue. Conversion of the dephospho-form of IIA^{glc} to the phospho-form is associated with an EI-dependent shift of the protein's position on the stained gel (*18*, *19*).

Sugar Phosphorylation Assay using IIA^{glc} Proteins. Details of the method are described in the legend to Figure 3.

Binding of IIA^{glc} to lac Permease. The binding of radioactive IIA^{glc} to membranes enriched in lac permease has been described (*6*). Briefly, the procedure involves incubation of membranes with [^{3}H]-labeled IIA^{glc}, pH 6.3 buffer, Mg, DTT, and TDG or melibiose, and then separating the bound from free IIA^{glc} in a Beckman Airfuge. The pelleted membranes containing IIA^{glc} bound to lac permease are then solubilized and counted in a Beckman liquid scintillation counter (Model LS6500). Data are presented as cpm; each experimental sample was checked for efficiency of counting which, in all cases, was 50 ± 1%. All of the binding experiments, except those of Figure 5, used 2.5 μg of [^{3}H]-IIA^{glc}, while that in Figure 5 used 1.25 μg of [^{3}H]-IIA^{glc}. The blank binding (that in the absence of added sugar) is dependent on the amount of added [^{3}H]-IIA^{glc}. For 1.25 μg of added [^{3}H]-IIA^{glc}, that value is 93 cpm (see Figure 5). For 2.5 μg of added [^{3}H]-IIA^{glc}, (Figures 1, 4, and 6), the mean value is 259 cpm with a standard error of 45 cpm. The sugar-dependent binding reported in Figures 1, 4, and 6 was consistent from experiment to experiment (mean binding = 439 cpm; standard error = 21 cpm). The data in Figure 1 are the mean of two experiments and that in Figures 4, 5, and 6 are representative of experiments carried out at least two times.

Regulation of lac Permease in Intact Cells. E. coli TP2860 (*20*) was cotransformed with pRK248 (*21*), which encodes a temperature-sensitive form of the λ repressor, along with various derivatives of the expression vector pRE1 (*14*), which controls protein expression under the λ P_L promoter. The pRE1 derivatives used in this study were the original pRE1 (control) and constructs encoding both wild-type and mutant forms of IIA^{glc} (K53E, K69E, F71K, D94G, E86K, and K114E). Transformants were prepared by electroporation of both plasmids together, followed by screening for both tetracycline (for pRK248) and ampicillin (for pRE1 derivatives) resistance. The various transformants were grown overnight at 30 °C in M63 medium (*22*) supplemented with thiamine (3 μg/mL), arginine (20 μg/mL), isoleucine (20 μg/mL), shikimic acid (20 μg/mL), tetracycline (10 μg/mL), ampicillin (50 μg/mL), and 0.4% lactose (to induce the lac operon). The cells were harvested, resuspended in fresh medium to a density at A_{600} of 0.75, and, depending on the transformant, incubated at 38−42 °C to induce approximately equal levels of expression of IIA^{glc}. At different time intervals (30, 60, and 90 min), an aliquot (200 μL) was removed for running on an SDS-PAGE gel. Additionally, another aliquot (1 mL) was removed to analyze for lactose transport. The cells were centrifuged, washed, resuspended in 500 μL of M63 medium minus the amino acid and lactose supplements, and then allowed to equilibrate with shaking at room temperature. The transport assays were initiated by the addition of 2.5 μmoles of radioactive lactose (0.08 μCi/μmole). Four aliquots (100 μL) were removed at 0.5 min intervals and the cells were diluted into 10 mL of M63 and quickly collected by vacuum filtration onto GF/C filters. The filters were suspended in 10 mL of scintillation fluid and counted in a Beckman scintillation counter at 96% efficiency. The uptake was corrected for the background (about 1200 cpm) observed in incubation mixtures devoid of cells. Under these conditions, the corrected uptake rate was linear with time. The expression of IIA^{glc} was evaluated by SDS-PAGE (4−20% Tris-Glycine, Novex). The gels were stained with Coommassie Blue and dried. The band corresponding to IIA^{glc} was easily visible in a region of the gel, corresponding to ∼20 kDa where there were few other proteins.

Computer Analyses. Analyses of protein sequences were performed using the GCG programs, version 7.2 (*23*).



lacY	lacY	SSA	lacY	lacY	IIA^{glc}
SSA	DTT	EtN	SSA	Mel	SSA
EtN	Mg	lacY	EtN	SSA	EtN
DTT	IIA^{glc}	DTT	DTT	EtN	lacY
Mg	Mel	Mg	Mg	DTT	DTT
IIA^{glc}		IIA^{glc}	IIA^{glc}	Mg	Mg
		Mel	Mel	IIA^{glc}	Mel

FIGURE 1: Effect of SSA on binding of IIA^{glc} to lac permease. Membrane vesicles were prepared from strain T184/pT7-5/cassette *lacY* harvested 4 h after induction with 1 mM isopropyl 1-thio-β-D-galactopyranoside as described in Experimental Procedures. Incubation mixtures contained, in a total volume of 100 μL: membrane vesicles (0.6 mg of protein), 2.5 μg of [^{3}H]IIA^{glc} (1080 cpm/μg of protein), 50 mM Na-phosphate buffer, pH 7.5, 2 mM DTT, 2 mM $MgCl_2$. Where indicated, reaction mixtures were supplemented with 4 mM melibiose, 1 mM SSA, or 5 mM ethanolamine. The chart below the figure details the order of addition of the reaction components. The reaction mixture was incubated at room temperature for 10 min after the addition of SSA, then for another 10 min after the ethanolamine addition to inactivate the residual SSA. After all reaction components were added, the mixture was incubated for 5 min at room temperature. The membranes were then pelleted in an Airfuge and the bound IIA^{glc} was determined as described in Experimental Procedures. Data are presented as the mean of duplicate experiments. The error bars correspond to the standard error of the mean.

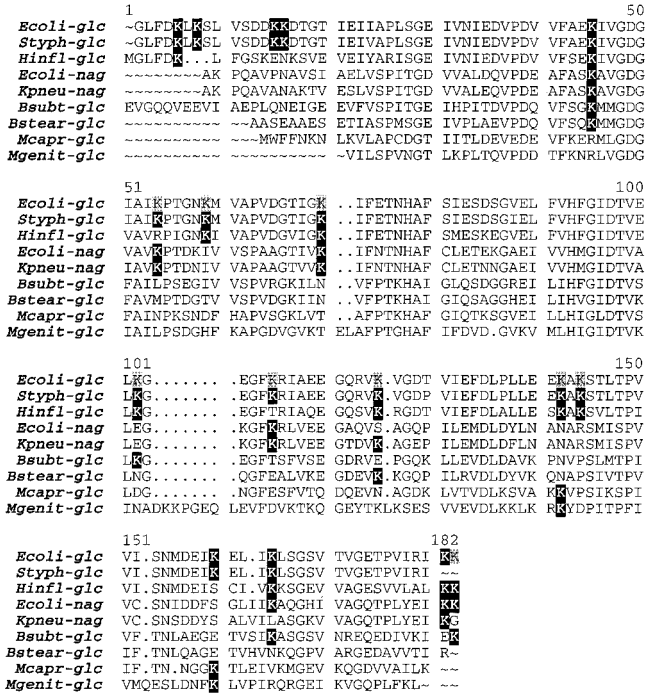


FIGURE 2: Sequence alignment of IIA proteins. Numbering above the aligned sequences corresponds only to the residue in the alignment rather than to a residue number in any of the aligned proteins. The seventeen Lys residues of *E. coli* IIA^{glc} are reverse shaded. Those shaded in gray correspond to the residues mutated in this study. Where there is a corresponding Lys residue in other proteins, it is also shown in reverse shading. Abbreviations used and accession numbers for the various IIAs are: *Escherichia coli* IIA^{glc} (*Ecoli-glc*) P08837; *Salmonella typhimurium* IIA^{glc} (*Styph-glc*) P02908; *Haemophilus influenzae* IIA^{glc} (*Hinfl-glc*) L42023; *Escherichia coli* IIA^{nag} (*Ecoli-nag*) P09323; *Klebsiella pneumoniae* IIA^{glc} (Kpneu-nag) S18607; *Bacillus subtilis* IIA^{glc} (*Bsubt-glc*) P20166; *Bacillus stearothermophilus* IIA^{glc} (*Bstear-glc*) U12340; *Mycoplasma capricolum* IIA^{glc} (Mcapr-*glc*) U15110; *Mycoplasma genitalium* IIA^{glc} (*Mgenit-glc*) L43967.

RESULTS

Acetylation of IIA^{glc}. The acetylating agent sulfosuccinimidyl acetate is specific for ϵ-amino groups of lysine residues and the amino-terminus in proteins (*24*). Experiments were carried out to determine whether SSA modification of lactose permease or IIA^{glc} resulted in disruption of the lac permease−IIA^{glc} interaction (Figure 1). The previously described sensitive radiochemical method for quantitating the binding of IIA^{glc} to lac permease (*6*) was used for the analyses. The results showed that modification of the transporter (incubation # 4) had little effect on the binding reaction. However, acetylation of lysine residues in IIA^{glc} led to a significant partial reduction (corresponding to about 45% of the sugar-dependent binding) in its capability to interact with the permease (incubation # 6). Appropriate controls showed that the presence of SSA inactivated with ethanolamine either before or after the addition of lac permease had no effect on the binding reaction (incubations 3 and 4). Pretreatment of the permease with melibiose before modification had no effect on the binding of IIA^{glc} (incubation # 5). These data suggested that one or more lysine residues of IIA^{glc} might be involved in the interaction with lac permease.

Mutagenesis of Lysine Residues of IIA^{glc}. The Genbank sequence (ecoptshi.genbank) for *E. coli* IIA^{glc} indicates the presence of 17 lysine residues in the protein (see Figure 2). Visualization of the three-dimensional structure of the protein (*25*, *26*) indicates that these lysine residues are distributed around the surface of the molecule. The amino-terminal region of the structure is disordered; thus, the four Lys residues in that region are unlikely to be of any importance in regulatory interactions. To gain an insight into the lysine residue(s) important for the IIA^{glc}−lac permease interaction, a representative sampling (nine) of the lysine residues in different regions of the molecule, including all those near the active site His, was subject to mutagenesis (see Experimental Procedures and Table 1). The residues were mutated from lysine to glutamic acid, resulting in a charge change. Lys69 was also mutated to Leu. Some of the mutated Lys residues were in the vicinity of the active site His (H90) (Lys69, Lys99, and Lys132), some others were on the same surface as the active site (Lys130 and Lys168) and others faced away from the active site region (Lys53, Lys58, Lys104, and Lys114).

Characterization of Mutated Forms of IIA^{glc}. It was considered possible that the change of a residue from lysine to glutamic acid might have a drastic effect on the structure of IIA^{glc}, perhaps affecting the fold of the protein. If this occurred, then it might be expected that the binding reaction would be inhibited, but for an indirect reason. Consequently, all the mutated forms of IIA^{glc} were tested for their capability

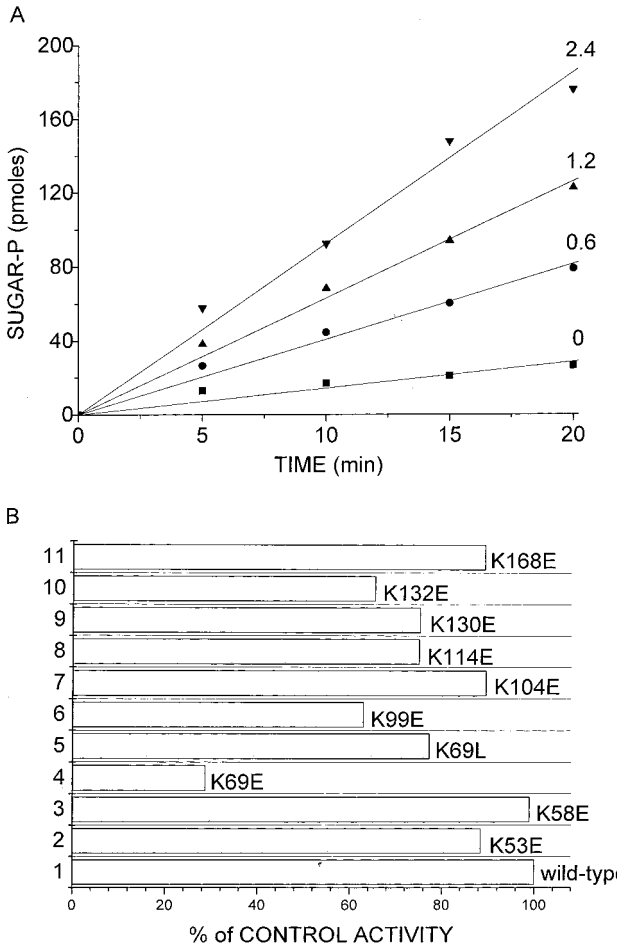


FIGURE 3: Relative activities of IIA^{glc} proteins in sugar phosphorylation. An *E. coli* strain deleted for the gene encoding IIA^{glc} (strain TP2865)(*20*) was cultivated in LB medium (500 mL) to $A_{600} \sim 0.7$. The washed cells were resuspended (1/10 w/v) in 10 mM Tris, pH 7.4, and the suspension was passed twice through a French pressure cell at 10 000 psi. An aliquot (10 μL) of the extract was added to each incubation mixture. Incubation mixtures contained, in a total volume of 250 μL: 0.05 M Tris buffer, pH 7.4; 2.5 mM $MgCl_2$; 25 mM KF; 10 μM [^{14}C]α-methylglucoside (42 cpm/pmole); 0.5 mM PEP; and the extract. The mixtures were equilibrated to 37 °C and then samples of IIA^{glc} proteins were added, as indicated. Samples (50 μL) were withdrawn at 5, 10, 15, and 20 min after initiation of the reaction and processed on Dowex-1 columns for separation of free and phosphorylated sugar as described (*28*). Panel A: Effect of wild-type IIA^{glc} concentration on phosphotransferase activity. The amount of wild-type IIA^{glc} added to each incubation mixture, in μg/mL, is shown above each curve. Panel B: Survey of wild-type and mutated IIA^{glc} proteins for stimulation of phosphotransferase activity. The indicated IIA^{glc} proteins were added to the incubation mixtures at a concentration of 0.6 μg/mL. The activities are expressed as % of control (i.e., wild-type) activity.

to serve as phosphoryl acceptors from the phosphocarrier HPr (see Experimental Procedures). The study (data not shown) established that wild-type as well as all the mutated IIA^{glc}s examined are able to be phosphorylated by phospho-HPr.

Many of the preparations of IIA^{glc} proteins showed the presence of two bands, corresponding to the previously described "fast" (a form in which the amino-terminal heptapeptide has been removed) and "slow" (the full-length protein) forms of IIA^{glc} (*27*). Both of these forms were phosphorylated by P-HPr (data not shown).

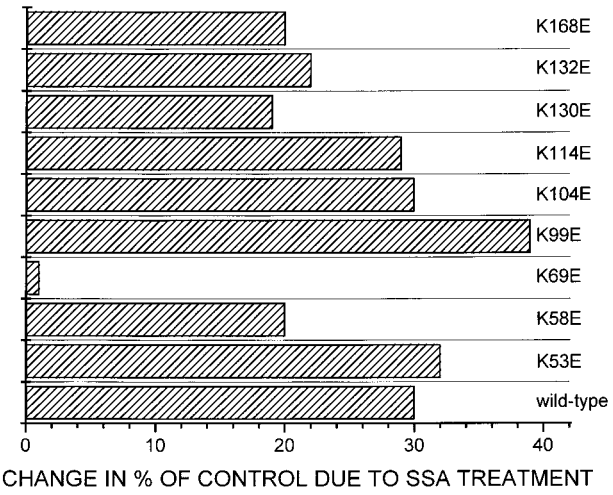


FIGURE 4: Effect of SSA on the binding of mutated IIA^{glc} proteins to lac permease. Wild-type and the indicated mutated IIA^{glc} proteins were treated with SSA or inactivated SSA as in incubation mixture # 6 or #3 of Figure 1. Binding of IIA^{glc} to lac permease was carried out as described in Experimental Procedures using 600 μg of membrane protein and 4 mM melibiose. The indicated preparation of wild-type or mutated IIA^{glc} was used at a ratio of unlabeled/labeled IIA^{glc} of 4/1. The melibiose-dependent cpm bound to lac permease in control (no added unlabeled IIA^{glc}) incubation mixtures was between 396 (presence of SSA) and 447 cpm (absence of SSA). The binding (calculated as % of the control) was determined for the various preparations of IIA^{glc}for both untreated (i.e., treated with inactivated SSA) and SSA-treated proteins. The value for the % of control for untreated IIA^{glc} was subtracted from the % of control value for SSA-treated IIA^{glc}.

The collection of IIA^{glc} proteins was also characterized for its ability to donate the phosphoryl group to the appropriate membrane-bound acceptor (enzyme IIBC^{glc}); in the test system, the phosphoryl group is ultimately transferred to [^{14}C]-methyl-α-glucoside (*28*). Extracts of an *E. coli* strain deleted for the gene encoding IIA^{glc} were incubated with [^{14}C]-methyl-α-glucoside and the preparation of IIA^{glc} (wild-type or mutant) to be evaluated for activity. Figure 3A shows a test of the proportionality of the activity to the amount of added wild-type IIA^{glc}. On the basis of this standardization test, a concentration of 0.6 μg/mL (in the proportional range) was chosen to evaluate the activities of the mutated IIA^{glc}s. The results (Figure 3B) indicate that almost all the mutated IIA^{glc} proteins are as active as wild-type IIA^{glc} as phosphoryl donors. Only the Lys69Glu mutant showed substantially reduced (to about 30% of the control) activity in this assay. It should be noted that mutagenesis of Lys69 to Leu (K69L) did not result in a drastic change of activity in this assay.

Effect of SSA on Binding of Mutated IIA^{glc}s to lac Permease. The data of Figure 1 showed that SSA treatment of IIA^{glc} reduced its capability to interact with lac permease. We reasoned that, if there was a single Lys residue in IIA^{glc}, important in the lac permease—IIA^{glc} interaction, mutagenesis of that residue would result in a IIA^{glc} that, on treatment with SSA, would not have a reduced capability to interact with lac permease. A competition approach, shown in Figure 4, was used for this analysis.

Incubation mixtures containing [^{3}H]-IIA^{glc} were assayed without and with wild-type or mutated IIA^{glc}s that were untreated (i.e., incubated with ethanolamine-inactivated SSA) or treated with SSA. Addition of unlabeled wild-type IIA^{glc} (ratio of unlabeled/labeled = 4:1) reduced the melibiose-

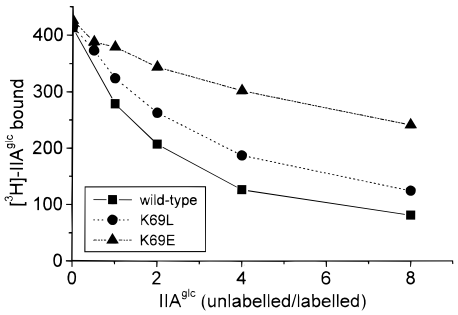


FIGURE 5: Concentration dependence for competition of [^{3}H]IIA^{glc} binding to lactose permease by wild-type and mutant proteins. Binding of IIA^{glc} was carried out as described in Experimental Procedures using 600 μg of membrane protein and 1.25 μg of [^{3}H]-IIA^{glc}. Some incubation mixtures were supplemented with unlabeled IIA^{glc} proteins at the indicated ratio of unlabeled/labeled protein. The data are corrected for the radioactivity bound to membranes in the absence of 4 mM TDG (93 cpm).

dependent radioactivity bound to lac permease from 447 to 115 cpm (26% of control). When the unlabeled wild-type IIA^{glc} was treated with SSA, the melibiose-dependent bound radioactivity was reduced from 396 to 222 cpm (56% of the control). The data in Figure 4 is shown as the change in % of control due to SSA treatment. For wild-type IIA^{glc}, this was 30% (56−26%). The results show that, with one exception, all the mutated IIA^{glc} samples showed a considerable decrease in capacity to compete with labeled IIA^{glc} for binding to lac permease when treated with SSA. Only the K69E protein was essentially competely resistant to SSA treatment. These findings suggest that the partial loss of binding capacity by treating wild-type IIA^{glc} with SSA seen in Figure 1 is the result of acetylating Lys69.

Effect of Different Replacements at Lys69 on Binding to lac Permease. The data of Figure 4 indicated that mutagenesis of Lys69 to Glu of IIA^{glc} resulted in a significant change in IIA^{glc}s capability to respond to SSA inactivation in the lac permease binding assay. The results of Figure 3 showed that, while IIA^{glc}(K69E) displayed diminished phosphocarrier activity to the IIB^{glc} domain, IIA^{glc}(K69L) was not defective in that assay. Therefore, a comparison of the K69E and K69L mutant forms of IIA^{glc} for competition with the binding of [^{3}H]-IIA^{glc} was carried out (see Figure 5). The competition profile for wild-type IIA^{glc} was consistent with a saturation of the binding site on lac permease by IIA^{glc}. The results showed that wild-type and the K69L IIA^{glc}s showed similar competition patterns, while IIA^{glc}(K69E) was relatively ineffective in the competition test. These data suggest that, while the interaction of IIA^{glc} with lac permease is not substantially affected by the change of Lys69 to Leu, the Lys69 region of IIA^{glc} is part of the interface region with lac permease (see Discussion).

Effect of Mutagenesis of Other IIA^{glc} Residues on Binding to lac Permease. To further explore the boundaries on IIA^{glc} for interaction with lac permease, mutagenesis of some other residues was carried out (see Table 1 and Experimental Procedures). Phe71 was converted to either Lys or Ser; Glu86 was converted to either Lys or Leu; and Asp94 was converted to Gly. The mutated proteins were expressed and purified as described in Experimental Procedures. All of these proteins were active in the test of sugar phosphorylation described in Figure 3 (F71S and K and D94G, 70% as active as wild-type IIA^{glc}; E86K and L, 90% as active as wild-type IIA^{glc}). The purified proteins were then tested for their capacity to compete with [^{3}H]-IIA^{glc} for binding to lac permease (see Figure 6). While wild-type IIA^{glc} reduced the binding of [^{3}H]-IIA^{glc} to 8% of the control, the mutated proteins were less effective in the competition (53−101% of the control value). The conclusion from this study was that Phe71, Glu86, and Asp94 are probably located within the contact region with lac permease.

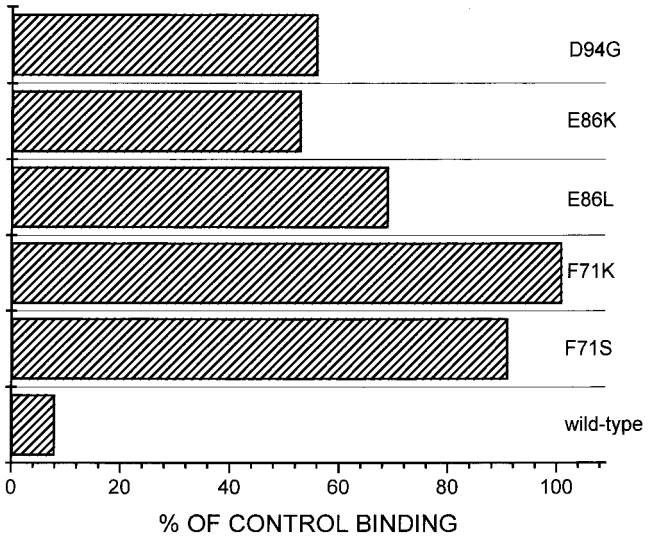


FIGURE 6: Competition for binding to lac permease by mutated IIA^{glc} proteins. The indicated mutated IIA^{glc} proteins were tested for competition for [^{3}H]-IIA^{glc} binding to lac permease as described in Figure 5, but using 4 mM melibiose. The radioactivity bound to membranes in the absence of added melibiose was 298 cpm and in the presence of melibiose was 715 cpm. Thus, the melibiose-dependent binding (100% of control) was 417 cpm. The ratio of unlabeled/labeled IIA^{glc} in the competition tests was 4:1.

Regulation of lac Permease by IIA^{glc} in Intact Cells. Some studies on the regulation by IIA^{glc} of permeases have been carried out in intact cells. There have been several reports (*29*, *30*) that the ratio of IIA^{glc} to a non-PTS protein, regulated by IIA^{glc}, influences the activity of the protein. When permeases for lactose or maltose were induced to high levels, they became less sensitive to inhibition by methyl-α-glucoside. Nelson et al. (*30*), studying glycerol uptake, showed that uptake was inhibited by methyl-α-glucoside at low levels of induction, but as the induction progressed, the sensitivity to methyl-α-glucoside disappeared.

Mitchell et al. (*29*) attempted to control the level of IIA^{glc} in intact cells by using plasmids encoding IIA^{glc}. They reported that the level of lac permease was essentially unchanged in cells carrying a plasmid that overproduced IIA^{glc} to twice the normal level, but there was about a 40% decrease in lac permease activity in a strain in which IIA^{glc} was overproduced to 10 times the normal level.

To further investigate our in vitro observations on the binding of mutated IIA^{glc} proteins to lac permease, we designed an in vivo system, which measured lactose uptake in intact cells, to study the regulation of lac permease by IIA^{glc}, (see Experimental Procedures). A wild-type strain of *E. coli* (TP2860 (*20*)) was cotransformed with pRK248 (*21*), encoding the temperature-sensitive λ repressor and derivatives of the expression vector pRE1 (*14*), which is controlled by the λ P_L promoter. Thus, a collection of transformants harboring various derivatives of pRE1 encoding wild-type

or mutant forms of IIA^{glc} was created. These strains were cultured overnight at 30 °C in lactose-supplemented medium to induce lac permease; these are conditions where the pRE1-derived vectors would be repressed. Cultures were then resuspended in fresh medium at a low density and exposed to temperatures appropriate for IIA^{glc} expression due to inactivation of the λ repressor. It was observed that, when IIA^{glc} was highly expressed, lac permease activity was inhibited approximately 50% in all the transformants, consistent with the previous studies of Mitchell et al. (*29*). Since all the mutant proteins (K53E, K69E, F71K, D94G, E86K, and K114E) tested under these conditions resulted in IIA^{glc}-dependent inhibition of lac permease activity, we concluded that mutation of a single site in the interface region was insufficient to totally eliminate the interaction between IIA^{glc} and lac permease.

To evaluate differences in the efficiency of the various mutant IIA^{glc} proteins to interact with lac permease, it appeared necessary to study lac permease activity as a function of the degree of expression of IIA^{glc}. In the case of expression of wild-type IIA^{glc} using a transformant harboring pPR3 (*10*), it was possible to titrate the expression of IIA^{glc} down to levels resulting in less than maximal inhibition of lactose uptake. However, in that range of IIA^{glc} accumulation, relatively small changes in the level of IIA^{glc} led to large differences in the degree of inhibition of lactose transport. In different experiments, it was not possible to relate a specific level of IIA^{glc} expression to a reproducible partial inhibition of lactose uptake. Consequently, it was not feasible, in this test system, to clearly quantitate a relationship between the cellular concentration of IIA^{glc} and its effect on lactose uptake.

It is believed that only the dephospho-form of IIA^{glc} is responsible for regulation of lac permease (*1*). The distribution between phospho- and dephospho-forms of this protein in intact cells may be influenced by numerous cellular processes. The specific mutations in IIA^{glc} studied here might show different distributions of the two forms of IIA^{glc} in intact cells. This might further compound the difficulty in comparing transformants containing different plasmid constructs expressing mutated IIA^{glc} proteins.

It is reasonable to assume that, if some of the amino acid residues in IIA^{glc} studied here are involved in the interaction with lac permease, mutations in these residues might affect the affinity of the association of the two proteins. If this were the case, then saturating levels of the mutated proteins would be expected to result in a similar level of inhibition of lactose transport as from the wild-type protein; as indicated above, that is the observed result. However, for the reasons described above, it was not reliable to study quantitative differences in lactose transport dependent on variations in levels of dephospho-IIA^{glc} from transformant to transformant.

An alternate approach to an in vivo evaluation of the regulation of lac permease by mutated IIA^{glc} proteins was undertaken. Strain GI698(Δpts) (*16*) was transformed with pRE1 (*14*) derivatives encoding the following IIA^{glc} mutant proteins: K69E, K69L, F71K, F71S, E86K, E86L, and D94G; tests of fermentation of these transformants on lactose-MacConkey plates indicated that all the transformants showed an inhibition of lactose fermentation. These observations were consistent with the results of the lactose transport experiments described above. We concluded that uncontrolled expression of IIA^{glc} mutant proteins masked differences in the affinity of those proteins for lac permease.

In summary, we concluded that the controlled in vitro studies reported here, using purified preparations of wild-type and mutant IIA^{glc} proteins, guaranteed to be completely in the dephospho-form, were a more reliable approach to evaluating the effect of mutations in IIA^{glc} on the interaction with lac permease.

DISCUSSION

SSA is a reagent specific for modification of the ϵ-amino groups of Lys residues and the amino-terminus of proteins. The finding that SSA treatment of IIA^{glc}, but not lac permease, resulted in a reduction of the binding to lac permease (Figure 1) was the stimulus for investigating the role of Lys residues of IIA^{glc} for interaction with lac permease.

There are seventeen Lys residues in *E. coli* IIA^{glc} (Figure 2 and 7). Of the nine Lys residues examined by mutagenesis, only Lys 69 (designated residue # 70 in the alignment of Figure 2) appears to be in the region that interacts with lac permease. Alignment of the sequences of IIA proteins specific for various sugars and from different bacteria may give an insight into the importance of these residues. Lys69 is present in *E. coli* IIA^{glc} as well as the IIA^{glc} proteins from *Salmonella typhimurium* and *Hemophilus influenzae*. Interestingly, it is also found in the IIA^{nag} proteins from *E. coli* and *Klebsiella pneumoniae*. It is noteworthy that *E. coli* IIA^{nag} has been shown to replace IIA^{glc} for inducer exclusion (*31*).

The IIA^{glc} proteins from *Bacillus subtilis*, *Bacillus stearothermophilus*, *Mycoplasma capricolum*, and *Mycoplasma genitalium* do not contain Lys69. It might be predicted from this examination that these IIA^{glc} proteins do not interact with permeases. This is consistent with the generalization that Gram-positive bacteria and mycoplasmas use a mechanism involving the phosphorylation of Ser46 of HPr to regulate transport (*32*). It should be noted that *E. coli* IIA^{glc} interacts with and inhibits not only the activity of lac permease but also the permeases for maltose, raffinose, and melibiose as well as cellular glycerol kinase (*1*).

The presumptive acetylation of Lys69 of IIA^{glc} results in a significant reduction of the binding of that protein to lac permease (Figures 1, 4, and 5). It is not clear from these findings whether the derivatization of Lys69 with SSA eliminates an electrostatic interaction with lac permease or creates a steric effect leading to inhibition of binding. It is interesting to note that mutation of Lys69 to Glu has a more pronounced inhibitory effect than does the mutation to Leu (Figure 5). Our proposed interpretation of these findings is that Lys69 is located in the region of the surface of IIA^{glc} that interacts with lac permease. When Lys69 is mutated to Leu, there is only a small change in the binding affinity (Figure 5), because the shape of the binding site is relatively unaffected. However, mutagenesis of Lys69 to Glu introduces a charge and steric effect that results in a drastic inhibition of the binding. The proximity of Lys69 to the active site His90 is noteworthy, since imposition of a negative charge by phosphorylation of His90 results in loss of IIA^{glc} binding to lac permease. Thus, the K69E mutation may mimic the physiological regulation associated with His90 phosphorylation.

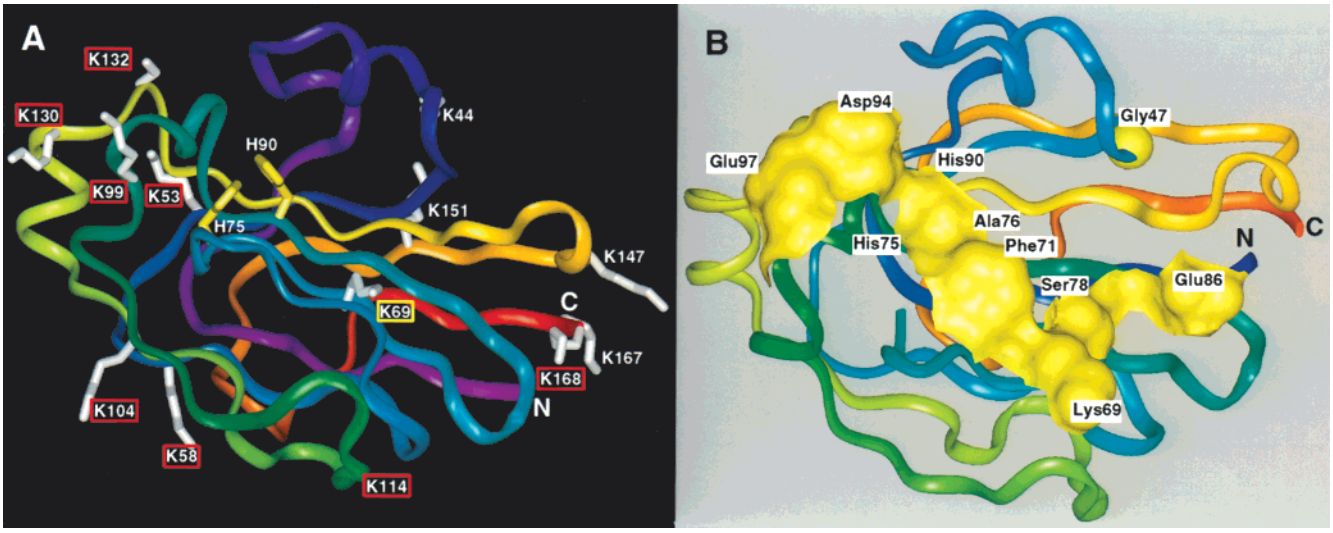


FIGURE 7: The structure of IIA^{glc}. The ribbon structure of *E. coli* IIA^{glc} was drawn with Insight II (MSI, San Diego, CA) using the coordinates from 1f3g in the Brookhaven Protein Data Bank. Panel A: The location of the Lys side chains. Those enclosed by boxes are the residues mutated in this study. Lys69, the residue that, when mutated, becomes defective in binding to lac permease, is enclosed in a yellow box. The side-chains of His residues 75 and 90 are shown in yellow. Panel B: The location of residues that, when modified, affect the binding to lac permease. The patch over the structure, created as a Connolly surface, corresponds to the proposed contact region with lac permease. The significance of Gly47 for the interaction with lac permease is alluded to in the Discussion.

Figure 7 (panel A) shows the placement of 13 of the Lys residues in IIA^{glc} as well as the active site His90 and the regulatory His75. The remaining four Lys residues are located in the amino-terminal region, which is disordered (*25*, *26*). It is clear from the data of Figure 4 that most of these Lys residues are outside the sphere of interaction with lac permease. Of the two Lys residues (Lys69 and Lys99) that are close to and on the same surface as the active site, only the region around Lys 69 appears to be important for the interaction with lac permease. Figure 7 (panel B) delineates the location of those residues in IIA^{glc}, which, when mutated, diminish the IIA^{glc}−lac permease interaction. His90, when phosphorylated, leads to loss of IIA^{glc} regulation (*7*). In the present studies, mutation of Lys69 to Glu was shown to diminish the IIA^{glc}−lac permease interaction (see Figure 5). Mutagenesis with hydroxylamine resulted in single site mutants in IIA^{glc} impaired in inducer exclusion (*8*). These were A76T, G47S, and S78F. Ala76 and Ser78, but not Gly47, are in the vicinity of the surface between His90 and Lys 69 (see Figure 7, Panel B). Other mutagenic studies (*9*) implicated Glu97 as important for inducer exclusion. Mutation of this residue to Lys eliminated the binding.

Two conserved residues (Phe71 and Asp94) have been implicated (*33*) in the IIA^{glc}−glycerol kinase interaction (see Figure 7B). Phe71 participates in a hydrophobic interaction and Asp94 is a partner in a long H-bond interaction. It is likely that these two residues also participate in the IIA^{glc}−lac permease interaction. Mutagenic studies on Phe71 and Asp94 (Figure 6) established the importance of these residues in the IIA^{glc}−lac permease interaction.

Glu86 has been proposed (*34*) to be involved in the interaction with glucose permease. Mutagenesis of this residue (Figure 6) provided evidence that the interaction with lac permease extends into that region.

It should be stressed that all the studies carried out in this work and other studies (*8*, *9*) involving mutants in IIA^{glc} do not necessarily identify the requirement for a unique residue in IIA^{glc} for binding to lac permease. The mutation might introduce a local conformational change or a steric hindrance that diminishes the protein−protein interaction. On the other hand, these studies do provide a localization of the interaction region.

On the basis of current information concerning residues that, when mutated or modified, affect the IIA^{glc}−lac permease interaction, a model for the contact surface between the two proteins (Figure 7B) can be proposed. The cartoon shown delineates a contact patch covering the region including residues Lys69, Phe71, His75, Ala76, Ser78, Glu86, His90, Asp94, and Glu97. Since Gly47 is in a different region of the structure, it is proposed that mutation of this residue to Ser results in a movement of the loop containing residue 47 in the direction of the contact region, resulting in a steric blockade to interaction with lac permease. Clearly the specific contacts between the two proteins are not delineated here. Crystallographic analysis of the complex would probably be required to deduce the precise interactions. While the structure of IIA^{glc} has been elucidated (*25*, *26*), that of the membrane-bound permease is significantly more challenging.

Substantial information has accumulated dealing with the binding surface of IIA^{glc} that interacts with lac permease ((*8*, *9*) and this work), glucose permease (*34*), glycerol kinase (*33*), and HPr (*35*, *36*). The interactions of IIA^{glc} with HPr and glucose permease are catalytic, involving phosphotransfer where there is a likely transition state intermediate involving a bridging trigonal bipyramidal phosphorus. On the other hand, the interactions of IIA^{glc} with glycerol kinase and lac permease are regulatory where the unphosphorylated protein binds effectively and phosphorylation eliminates the binding. Since all of these interactions either involve a phosphoryl transfer or are regulated by the state of phosphorylation of IIA^{glc}, the active site must be an important region for the interaction.

For *B. subtilis* HPr, it was suggested that Arg17 of HPr interacts with Asp31 and Asp87 of *B. subtilis* IIA^{glc} (*35*). For all species examined, the three-dimensional structures

of IIA^{glc} show a similar fold (*1*). Inspection of the three-dimensional structure of IIA^{glc} indicates that these Asp residues are in the vicinity of Lys69 of *E. coli* IIA^{glc}, suggesting a similarity in the binding region on IIA^{glc} for lac permease and HPr.

Glycerol kinase from *E. coli* has 10 contacts on IIA^{glc} (*33*). The hydrophobic interactions involve Val40, Phe41, Ile45, Val46, Phe71, Phe88, and Val96. In addition to salt bridges involving Glu43 and Asp38, there is a long H-bond involving Asp94. Residues 71, 88, 94, and 96 are in the vicinity of the active site. It is not obvious that glycerol kinase contacts IIA^{glc} in the Lys69 region.

E. coli glucose permease (*34*) binds to a region of IIA^{glc} on five adjacent β-strands and two short helices surrounding the active site His90 (*34*); this surface is similar to that described previously for binding to HPr (*36*). It is interesting to note that the NMR studies on glucose permease demonstrated that the amide signal of Lys69 of IIA^{glc} was one of the chemical shifts observed on complex formation with the IIB^{glc} subunit. In fact, every amide from Thr66−Glu80 showed a shift suggesting that the region around Lys69 is important for binding to IIBC^{glc}. Since the perturbations associated with formation of the IIA^{glc}−IIBC^{glc} complex are restricted to a small area on the surface of IIA^{glc}, it is improbable that a conformational change of IIA^{glc} is associated with the binding. It is worth noting that the contact area on IIA^{glc} is mainly populated by hydrophobic residues. However, it was suggested (*34*) that Lys69, Ser141, and Glu86 in glucose permease might be hydrogen bond partners in the complex, adding specificity to the orientation of the two proteins.

In summary, the present data and that of other workers (*8*, *9*) has allowed us to propose a topographic model for the interaction of lac permease with IIA^{glc}. The model suggests that the surface of IIA^{glc} that interacts with lac permease overlaps, but is not identical to, that for interaction with glucose permease, glycerol kinase, or HPr.

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