Topography of the Surface of the Signal-transducing Protein EIIAGlc That Interacts with the MalK Subunits of the Maltose ATP-binding Cassette Transporter (MalFGK₂) of Salmonella typhimurium*

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The signal-transducing protein EIIAGlc, a component of the phosphoenolpyruvate-glucose phosphotransferase system, plays a key role in carbon regulation in enteric bacteria, such as Escherichia coli and Salmonella typhimurium. The phosphorylation state of EIIAGlc governs transport and metabolism of a number of carbohydrates. When glucose as preferred carbon source is transported, EIIAGlc becomes predominantly unphosphorylated and allosterically inhibits several permeases, including the maltose ATP-binding cassette transport system (MalFGK₂) in a process termed “inducer exclusion.” We have mapped the binding surface of EIIAGlc that interacts with the MalK subunits by using synthetic cellulose-bound peptide arrays like pepscan and subtitutional analyses. Three regions constituting two binding sites were identified encompassing residues 69–79 (I), 87–91 (II), and 118–127 (III). Region III is MalK-specific, whereas residues from regions I and II partly overlap but are not identical to the binding interfaces for interaction with glycerol kinase and lactose permease. These results were fully verified by studying the inhibitory effect of purified EIIAGlc variants carrying mutations at positions representative of each of the three regions on the ATPase activity of the purified maltose transport complex reconstituted into proteoliposomes. Moreover, a synthetic peptide encompassing residues 69–91 was demonstrated to partially inhibit ATPase activity. We also show for the first time that the N-terminal domain of EIIAGlc is essential for inducer exclusion.

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2 The abbreviations used are: PTS, phosphoenolpyruvate carbohydrate phosphotransferase system; Ni-NTA, nickel nitrilotriacetic acid; TBS, Tris-buffered saline.

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is not identical to that for interaction with glycerol kinase (4, 5) and lactose permease (9), whereas the other is unique for MalK.

**EXPERIMENTAL PROCEDURES**

**Plasmids**—Plasmid pBB04 was constructed by ligating a PCR fragment encompassing the ___ gene (encoding enzyme IIAGlc) as an Ndel-BamHI fragment with expression vector pET15b (Novagene). Derivatives of pBB04 carrying single point mutations were obtained by site-directed mutagenesis using Stratagene’s QuikChange kit. Plasmid pBB06 harboring a ___ allele that lacks codons 1–17 was constructed similarly by using a PCR primer introducing an Ndel site (comprising an ATG codon) upstream of codon 17. As a consequence, the translated protein (ΔG1-D16) contains a methionine residue fused to the N terminus of Thr17 of native EIIAGlc. Plasmid described elsewhere (17).

The average specific radioactivity was 5 × 10^5 cpm/mg MalK.

**Preparative Procedures**—MalK (17), MalFGK2 (13), and MalE (18) of *S. typhimurium* were purified as described. MalE/maltose-loaded proteoliposomes were prepared as in Ref. 13. Enzyme IIAGlc (wild type and variants) was purified from the cytosolic fraction of *E. coli* strain BL21 (Δpts43crr::kan^R^) (16) harboring plasmid pBB04 or derivatives by Ni-NTA affinity chromatography. Subsequent removal of the His tag was carried out by incubation with thrombin according to the manufacturer’s instructions (Novagene). Samples were then passed through PD10 followed by a second passage through Ni-NTA. Tagless enzyme IIAGlc was collected in the flow through.

**Peptide Synthesis on Cellulose Membranes (SPOT Synthesis)**—Cellulose-bound peptide libraries were prepared by semi-automated SPOT synthesis using a SPOT robot (INTAVIS AG, Köln, Germany). SPOT synthesis was carried out as described in the SPOT synthesis protocol (19), and arrays were synthesized on modified Whatman 50 cellulose membranes (Whatman, Maidstone, UK). Sequence files and a design of the arrays were generated with the in house software LISA. Peptides derived from *S. typhimurium* EIIAGlc (accession number AAL 213277) (Gly₂–Lys^{169}) were used for pep scan analyses. To this end, three peptide arrays consisting of 13-mer, 16-mer, or 31-mer peptides, overlapping by 12, 15, and 30 amino acids, respectively, were synthesized. Complete substitutitional and length analyses of the interacting 16-mer peptides were generated using the software LISA and subsequently synthesized as described (21, 22).

**Screening of Cellulose Membrane-bound Peptide Arrays**—Before screening, the dried membranes were washed for 10 min in ethanol, 3 × 10 min in TBS (50 mM Tris-HCl, pH 8, 137 mM NaCl, 27 mM KCl) and subsequently incubated in TBS, supplemented with 5% blocking buffer (Sigma) and 5% sucrose, for 3 h at room temperature. After washing with TBS peptide arrays were incubated with 35S-labeled MalK (10^5 cpm) in blocking buffer, supplemented with 20% (v/v) glycerol for 6 h at room temperature with gentle shaking. Unbound MalK was removed with TBS, and peptide-bound MalK was visualized and quantified using a phosphoimager and associated software (Fuji, Japan).

**Peptide Synthesis**—Soluble peptides encompassing β-strands 5–7 (T66-IGKIFETNHAFSDESGLFLVFHGDIT-V96) or a non-binding region of enzyme IIAGlc (T136-PVVISMEIKIKLSGVTYGET-PVIR-I166) were prepared by automatic solid phase peptide synthesis on a Tentagel-SRam resin (Rapp Polymere, Tübingen, Germany) using Fmoc (N-(9-fluorenyl) methoxycarbonyl) chemistry. Chemical reactions were performed in plastic syringes at room temperature on a multipepptide synthesis robot (Syro2000, MultiSynTech, Witten, Germany) according to the manufacturer’s protocol. The final peptides were deprotected and cleaved off the resin using a mixture of 10 ml of trifluoroacetic acid, 0.75 g of phenol, 0.5 ml of water, 0.5 ml of methylphenyl sulfide, and 0.25 ml of 1,2-ethanediol. After incubation for 3 h at room temperature, the cleavage solution was collected, and the crude peptides were precipitated with dry ether at 0 °C. Purification of the peptides was achieved by high-pressure liquid chromatography on a RP-18 column using a linear solvent gradient (A, 0.05% trifluoroacetic acid in water; B, 0.05% trifluoroacetic acid in acetonitrile; gradient 5–60% B over 30 min). The identity of the purified peptides was validated by mass spectrometry using matrix-assisted laser desorption ionization time-of-flight (Voyager®LD, Applied Biosystems, Weiterstadt, Germany) or electrospray ionization-mass spectrometry (Q-TOFmicro™, micromass, Manchester, UK).

**Determination of the Phosphorylation State of Enzyme IIAGlc**—The phosphorylation state of enzyme IIAGlc was determined as described (23). The assay takes advantage of the observation that phosphorylation causes a shift of the apparent molecular weight of EIIAGlc on SDS gels. A cytosolic fraction of *E. coli* strain LS20 (Δcrr::kan) lacking EIIAGlc but containing EII and HPr was prepared from cells grown in minimal medium E (19) supplemented with glucose (0.4%), thiamine (2 mg/ml) and kanamycin (25 mg/ml) at 37 °C to an optical density of 1.2. Cells were subsequently harvested and disintegrated by ultrasonication. After centrifugation for 30 min at 200,000 × g the supernatant (cytosol) was stored at −80 °C until use. Assay mixtures (final volume: 30 μl) contained 50 mM Tris-HCl, pH 7.5, 20% glycerol (v/v), 0.15 mM NaCl, 0.1 mM phenylmethylsulfonyl fluoride, 5.7 mM phosphoenolpyruvate, 67 μM MgCl₂, and purified EIIAGlc variants (1 μg) as indicated. The reactions were started by the addition of cytosolic fraction (40 μg of protein) and terminated after 0 and 20 s, respectively, by adding 4 μl of 10X SDS-PAGE sample buffer. Subsequently, the solutions were loaded onto 14% SDS-polyacrylamide gels. After transfer to nitrocellulose, the blots were incubated with polyclonal anti-EIIAGlc antibody (generous gift of K. Jahreis, Universität Osnabrück, Germany) and a horseradish peroxidase-conjugated secondary antibody. Antigen-antibody interactions were visualized using the Western blot chemiluminescence reagent plus system (PerkinElmer Life Science products).

To determine the time dependence of EIIAGlc phosphorylation, the mixtures (140 μl) contained 200 μg of cytosolic fraction of LS20 and 5 μg of EIIAGlc variants. Aliquots (15 μl) were taken at the indicated times and further treated as described above.

**Analytical Procedures**—ATPase assay, protein determination, SDS-PAGE, and immunoblotting were performed as described elsewhere (16).
RESULTS

Screening of EIIA^Glc-derived Peptide Arrays for Binding to MalK—To determine the binding motif within EIIA^Glc recognized by MalK, we screened cellulose-bound peptide arrays synthesized by SPOT-synthesis and representing the complete EIIA^Glc sequence (Gly1-Lys168) for MalK binding. According to the convention adopted for E. coli IIA^Glc in the literature (2, 3), the N-terminal methionine, which can be hydrolyzed, is numbered as zero throughout the manuscript.

The peptide arrays consisted of 13-mers, 16-mers, or 31-mers that overlapped with adjacent peptides by 12, 15, and 30 amino acids, respectively. The cellulose membranes were incubated with 35S-labeled MalK and washed to remove excess label, and the retained radioactivity was visualized by phosphoimaging. Signals were consistently obtained with all three arrays in four rows but with different intensities (Fig. 1). Strongest signals were observed in rows 2 and 3, whereas the signal in row 1 was rather weak. The reliability of the data was further confirmed by probing three independently synthesized peptide arrays consisting of 16-mers. Each membrane gave the same signals with similar intensities (data not shown).

Substitutional and Length Analyses—To identify those amino acid residues within each peptide that are indispensable for binding of MalK, substitutional analyses of the peptides from signal rows 1–3 were performed (signal row 4 was suspected to result from an unspecific reaction possibly due to the positive charges at the C-terminal end of the protein and was thus not further studied, but see Fig. 7B). In these experiments every position was substituted one-at-a-time by all other genetically encoded amino acids. Thus, all possible single site substitution analogs were synthesized and screened. The results shown in Fig. 2 represent data obtained for spots 64 (A), 80 (B), and 112 (C) of the initial 16-mers array (see Fig. 1, center panel). Basically the same discrete substitution patterns were identified with 31-mers (not shown). The signal intensities obtained with the wild type sequence of each peptide (Fig. 2, left columns) correlated with those seen in Fig. 1. Thus, at least in case of Fig. 2A, the spots were somewhat difficult to detect on a printout. However, close inspection of the images on the computer screen clearly revealed individual residues on each membrane that could not be replaced by any other amino acid without affecting binding of MalK.

We also determined the minimal length of a peptide required to bind MalK. To this end, 16-mers and 31-mers were shortened sequentially from the N- and C-terminal end down to 10-mers and 16-mers, respectively (not shown).

The combined results identified the peptides comprising amino acids 69–79 (KIFETNHAFSI) (region 1), 87–91 (LFVHF) (region 2), and
118–127 (PVIFDFLPLL) (region 3) as being crucial for MalK binding (Fig. 3). None of the hydrophobic residues (underlined) can be replaced by amino acids with charged or polar side chains without loss of binding, suggesting that the binding interface is largely hydrophobic. Regions 1 and 2 basically encompass \(\beta\)-strands 5–7 of enzyme IIAGlc, which have also been implicated in recognizing other target proteins (4, 5, 7–9) (Fig. 3). However, the binding sites are not identical and, most importantly, region 3, located on the opposite face of the \(\beta\)-sandwich (Fig. 4), appears to be unique for binding MalK. Lys69 represents the only critically charged residue for interaction with MalK. Interestingly enough, hydrophobic residues substituted for Lys69 are tolerated, whereas negatively charged and some polar uncharged residues (Gln, Pro, Asn, Met) caused a loss of binding (Fig. 2A).

Mutational Analysis of Selected Residues of EIIAGlc—The results described above suggested that amino acid residues encompassing part of \(\beta5\), \(\beta6\), and \(\beta7\) as well as of \(\alpha1\) and \(\alpha2\) of EIIAGlc are crucial for binding of MalK. To verify these predictions in the context of the folded protein we monitored ATP hydrolysis catalyzed by the reconstituted MalFGK2 complex in the presence of purified EIIAGlc variants. To this end, variants of EIIAGlc carrying substitutions of residues from all three peptide regions involved in binding were constructed by site-directed mutagenesis (see “Experimental Procedures”). In particular, residues representing regions 1 (Lys69, Phe71) and 2 (Phe88, Phe91) were replaced by glutamine and phenylalanine, predicted by substitutional analyses (Fig. 2) to either eliminate or allow binding of MalK. Similarly, residues representing region 3 were changed to isoleucine and glutamine (Phe122) or threonine (Leu127). Moreover, Arg165 and Lys167 from the C-terminal end (signal row 4, Fig. 1) were also included. Each variant could be overproduced in soluble form comparable to wild type and was purified accordingly. Furthermore, after purification the N-terminal His tag was removed from each mutant protein to exclude possible artifacts by unspecific interaction (Fig. 5A).

As shown in Figs. 6 and 7, and in agreement with a previous study (13), wild type EIIAGlc when added in a 20–25-fold molar excess substantially inhibited the MalE-maltose-stimulated ATPase activity of MalFGK2-containing proteoliposomes. In comparison, the degree of inhibition displayed by the variants exactly confirmed the above predictions. Glutamine substituting for residues from regions 1 and 2 (Lys69, Phe71, Phe88, Phe91) caused a loss of inhibition (Fig. 6A), whereas...
replacement by tyrosine (where tested) had no or only little effect on the inhibitory potential (Fig. 6B). Similar results were obtained with variants carrying mutations in region 3. Although the F122I mutant protein still caused some inhibition, variants carrying F122Q or L127T mutations completely failed to inhibit the ATPase activity of MalFGK2 (Fig. 7A). Furthermore, mutations of the C-terminal residues Arg^165 and Lys^167 had no effect on the capability of the variants to inhibit ATPase activity (Fig. 7B).

This result is consistent with the above notion that the C-terminal portion of the protein is not involved in the interaction with MalK.

To exclude that the mutations, in particular those that caused loss of inhibition, might have affected EIIA^Glc integrity, we analyzed the ability of the variants to accept a phosphoryl group from HPr in vitro. The assay is based on the observation that phosphorylated EIIA^Glc is migrating slower in SDS gels than its unphosphorylated form (23). As shown in Figs. 6 and 7 (C and D) all variants behaved like the wild type, whereas a control carrying a mutation of the catalytic His^90 residue (→A) failed to become phosphorylated. Thus, we conclude that none of the mutations affected the structural integrity of EIIA^Glc. Interestingly, the H90A variant also failed to inhibit the ATPase activity of the maltose transporter (not shown), but here the possibility of a structural alteration of the protein must be taken into account. Together, these data not only nicely confirm the results from substitutional analyses (Fig. 2) but support the newly discovered hydrophobic patch constituted by /H9252^11 and /H9251^2 as being involved in interaction with MalK.

A Peptide Encompassing β5–β7 Partially Inhibits ATPase Activity of MalFGK2—Next, we wished to examine whether one of the two identified binding sites would be sufficient to allow at least partial inhibition of maltose transporter activity. To this end, a soluble peptide (β5–7) was synthesized encompassing residues Thr^66–Val^96. Monitoring the ATPase activity of MalFGK2 in proteoliposomes in the presence of the β5–7 peptide revealed a moderate but consistently observed inhibition by 20% as compared with the control (Fig. 8C). In contrast, a control peptide from the C-terminal region of the protein (residues Thr^136–Ile^166) that was not identified by the pep scan approach to be involved in MalK binding (see Fig. 1) was uneffective, thereby making an unspecific reaction rather unlikely. Thus, the data suggest that a functional interaction of the β5–β7 peptide with the MalK subunits had occurred, which is in further support of the above conclusions.
The N-terminal Region of EIIAGlc Is Required for Inhibition of Maltose Transporter Activity—The N-terminal region of EIIAGlc is not required to accept a phosphorous from the donor protein HPr but is essential for effective phosphotransfer to the membrane-bound enzyme IIBCGlc (24, 25). Structural studies suggested that the N-terminal tail confers amphitropism to the protein, allowing EIIAGlc to shuttle between the cytoplasm and the membrane (26). Whether the N-terminal peptide is crucial for binding to target proteins in the context of inducer exclusion is unknown. Thus, we examined the inhibitory potential of a purified EIIAGlc variant lacking amino acids Gly1-Asp16 (Fig. 5).

Figure 8. Inhibition of substrate (MalE-maltose)-stimulated ATPase activity of MalFGK2-containing proteoliposomes by EIIAGlc variants representing binding region 3 (A) and residues from the C-terminal end (B). C and D, phosphorylation of purified tagless EIIAGlc variants. See legend to Fig. 6 for details.
functional state of the protein because time-dependent phosphorylation of the protein comparable to wild type could be demonstrated (Fig. 8B).

Interestingly, a naturally occurring variant of EIIAGlc that lacks only the N-terminal residues Gly1–Lys7 (24) was previously shown to be less inhibitory on lactose transport in everted membrane vesicles than the native protein (27). Thus, we also monitored ATPase activity of the maltose transporter in the presence of purified recombinant EIIAGlc (Δ1–7). The result clearly revealed that even this variant had completely lost its capability to inhibit ATP hydrolysis catalyzed by MalFGK2 (data not shown).

Together, we conclude that binding of EIIAGlc to complex-assembled MalK in vivo requires association with the membrane via the N-terminal peptide. This finding might also explain why the ATPase activity of soluble MalK is only poorly inhibited by EIIAGlc (13).

The above observation that the β5–β7 peptide moderately affects ATPase activity in the absence of the N-terminal tail might thus be explained by better access of the small peptide to the target site as compared with the intact protein. Unfortunately, the question whether a fusion of β5–β7 to a peptide encompassing the N-terminal domain would increase the inhibitory potential could not be addressed because attempts to synthesize such a peptide were unsuccessful.

**DISCUSSION**

The signal-transducing protein EIIAGlc interacts with multiple target proteins within the context of glucose uptake, catabolite repression, and inducer exclusion (1). These interactions are either catalytic involving phosphotransfer to or from EIIAGlc (in the case of HPr and EIIBGlc) or regulatory, being dependent on the phosphorylation state of EIIAGlc (in the case of glycerol kinase, lactose permease, and the maltose ATP-binding cassette transporter). In the work presented here we provide first evidence for the structural requirements of EIIAGlc to recognize the MalK subunits of the maltose ATP-binding cassette transporter.

In the absence of co-crystals between EIIAGlc and the maltose transporter we used knowledge-based peptide arrays synthesized on coherent membranes to map the potential binding site(s) between EIIAGlc and MalK. Over the past decade peptide arrays have become a powerful tool to study molecular recognition events (28), and the reliability of the data has been shown (29, 30). The approach is superior over conventional mutational analyses or chemical modification, as employed in the case of lactose permease (9), because peptide arrays and subsequent substitutional analyses provide a complete data set on the residues in question for interaction with the target. Moreover, as demonstrated in this study, the results can perfectly guide a subsequent mutational analysis of functions in the context of the folded protein. By combining both approaches we have identified two putative binding sites located on opposite sites on the surface of EIIAGlc (Fig. 4). One comprises residues from β-strands 5–7, whereas the other involves residues located on β1 and α2 (Fig. 3). The latter region has not been implicated yet in binding of other target proteins and might thus be unique for interaction with the MalK subunits of the maltose transporter.

Structural analyses of complexes between EIIAGlc and several target proteins have revealed that among others the region encompassing β-strands 5–7, in particular, residues Lys69, Phe71, His90, Asp94, and Glu97 are crucial for binding of HPr (8), EIIBGlc (6, 7), and glycerol kinase (5). Moreover, in a study that combined chemical modification with mutational analysis it was suggested that β5–β7 is also required for interaction of EIIAGlc with lactose permease (9).

The data presented here (see Figs. 2A and 6) also indicate a key role of Lys69, although the positive charge seems not to be required as substitution by hydrophobic residues still allowed binding of MalK. This finding rather excludes the possibility that Lys69 forms a salt bridge with a negatively charged residue in MalK. Our results are also in agreement with data reported for lactose permease. Although the K69E variant was impaired in binding lactose permease, the K69L mutant protein exhibited binding activity comparable to wild type (9). These observations led the authors to speculate that a negative charge at position 69, which is in close proximity to the catalytic histidine 90 might mimic the phosphorylated and, thus, inactive state of the protein with respect to inducer exclusion (9). Our finding that substitution of Lys69 by polar but uncharged residues also eliminated binding, and, consequently, as shown for K69Q, inhibition of ATPase activity of MalFGK2 is not in support of this notion.

Phe71 is also crucial for binding of MalK in that it can be replaced only by residues with other aromatic side chains. Again, this result is consistent with the observation that variants F71K and F71S had lost their capacity to bind to lactose permease (9). In contrast, other residues from region β5–β7 that when mutated affect interaction with lactose permease like Ala76 (T), and Ser78 (F) (31) seem not to be required for contacting MalK.

Similarly, no interaction was found with the peptide region around α-helix 1, which adds to the binding of EIIAGlc to HPr, EIIBGlc, and glycerol kinase (see Figs. 1 and 3). In particular, Asp38 and Glu43 form ion pairs with Arg279 and Arg402, respectively, of glycerol kinase (5). In addition, Asp38 together with Asp94 is involved in salt bridges formed with Arg282/Arg401 of EIIBGlc (7). Asp94 was included in the complete substitutional analyses presented here and could be replaced by any other amino acid without eliminating MalK binding (see Fig. 2B). Also in the case of lactose permease, a D94G variant of EIIAGlc still displayed 60% binding activity compared with wild type.

Together, these observations clearly suggest that the binding sites of EIIAGlc for different target proteins overlap but are not identical. In particular, the structural requirements with respect to the catalytic activity of EIIAGlc in the context of glucose transport are in part distinct from those that promote regulatory interactions.

Can we draw any conclusion from these results on the interacting residues on MalK? Previous analyses have revealed that mutations conferring resistance to EIIAGlc inhibition are located in two regions in the helical domain and the C-terminal domain of MalK, respectively (12, 14, 15). Although additional evidence for the C-terminal domain being involved in EIIAGlc binding was subsequently obtained by competition experiments employing monoclonal antibodies, the same approach did not confirm the involvement of the two residues (Glu119, Ala124) from the helical domain (13). However, Samanta et al. (32) noticed that both clusters lie on the same face of the MalK dimer and might nonetheless form a binding site for EIIAGlc. The authors speculated that EIIAGlc might prevent the two N-terminal domains of MalK to move into closer proximity as a consequence of ATP binding (33), thereby arresting the transport cycle. The identification of two binding sites for MalK on EIIAGlc is consistent with this attractive hypothesis but clearly requires further proof. Thus, experiments addressing this question are underway in this laboratory.

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