Aerobic *sn*-glycerol-3-phosphate dehydrogenase from *Escherichia coli* binds to the cytoplasmic membrane through an amphipathic α-helix

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sn-Glycerol-3-phosphate dehydrogenase (GlpD) from *Escherichia coli* is a peripheral membrane enzyme involved in respiratory electron transfer. For it to display its enzymic activity, binding to the inner membrane is required. The way the enzyme interacts with the membrane and how this controls activity has not been elucidated. In the present study we provide evidence for direct protein–lipid interaction. Using the monolayer technique, we observed insertion of GlpD into lipid monolayers with a clear preference for anionic phospholipids. GlpD variants with point mutations in their predicted amphipathic helices showed a decreased ability to penetrate anionic phospholipid monolayers. From these data we propose that membrane binding of GlpD occurs by insertion of an amphipathic helix into the acyl-chain region of lipids mediated by negatively charged phospholipids.

Key words: binding sites, enzyme activation, glycerol 3-phosphate, membrane insertion, protein–lipid interaction.

INTRODUCTION

In *Escherichia coli*, *sn*-glycerol 3-phosphate (G3P) is either used as precursor in the biosynthesis of phospholipids or as a carbon source for energy supply [1]. Several sources of G3P are utilized by *E. coli*. The metabolic system serves as a salvage pathway for glycerol derived from breakdown of phospholipids and triacylglycerol. In the periplasm glycerophosphodiesters are hydrolysed to G3P by the phosphodiesterase GlpQ [2] and transported into the cytoplasm by the permease GlpT [3]. Uptake of glycerol from the medium occurs by facilitated diffusion across the cytoplasm through the facilitator GlpF [4,5] followed by internal phosphorylation to G3P by GlpK [6,7]. G3P enters glycolysis via oxidation to dihydroxyacetone phosphate. Under aerobic conditions, oxidation of G3P is catalysed by the homodimeric *sn*-glycerol-3-phosphate dehydrogenase (GlpD) with subsequent transfer of two electrons and two protons to the electron-transfer chain [8].

The structural genes for glycerol metabolism make up the *glp* regulon, which is negatively regulated by the common repressor protein GlpR [9]. Transcription of GlpD is tightly controlled due to the existence of four repressor-binding sites [10]. As a consequence low basal levels of GlpD prevent wasteful degradation of endogenous glycerol phosphate needed for phospholipid synthesis. Intracellular depletion of G3P due to constitutive expression of the *glpD* gene results in an imbalance in the levels of membrane phospholipids [11].

In addition to transcriptional control, the cellular localization of the enzyme affects its activity. *In vitro* GlpD is fully active when either associated with the cytoplasmic membrane or reconstituted with phospholipids or amphipaths [12,13]. The mechanism of its association with the membrane is obscure. Competition of GlpD with other peripheral-membrane respiratory enzymes for attachment sites at the membrane has been reported [14]. The finding that endogenous GlpD is purified from the membrane fraction after solubilization with deoxycholate and salt implies strong lipid–protein interaction [15]. However, sequence data reveal no transmembrane domain [8]. Instead, GlpD was suggested to associate with the membrane by binding to a membrane-anchoring subunit, as was found for other respiratory enzymes [16–18].

The aim of the present study was to investigate the possibility of direct GlpD–lipid interaction. Membrane penetration would stably anchor the protein at the cytoplasmic membrane, where it is functionally active. To test this hypothesis, we characterized the interaction of GlpD with lipid monolayers spread at the air/water interface and determined its lipid specificity. Lipid monolayers, when used at sufficiently high surface pressure, mimic the lipid-packing properties of biomembranes and are a reliable model system to study the mechanism whereby membrane-active proteins insert into cell membranes [19].

Our data demonstrate that GlpD penetrates the lipid monolayer. The penetrative power of GlpD was strongly increased in the presence of anionic phospholipids. Mutants with alterations in a predicted basic amphipathic α-helix spanning residues 355–370 showed decreased ability to insert into lipid monolayers. We propose that attachment of GlpD to the membrane is mediated by the exposure of a basic amphipathic helix that inserts into the hydrophobic-core regions of membrane lipids.

MATERIALS AND METHODS

Materials

Chemicals were from Sigma (Munich, Germany) and Roth (Karlsruhe, Germany). Nickel nitriloacetate (Ni^{2+}-NTA)-agarose and the pQE30 vector were from Qiagen (Hilden, Germany). Restriction endonucleases were from MBI Fermentas (St. Leon-Rot, Germany) or New England Biolabs (Schwalbach, Germany). 1,2-Dioleoyl-*sn*-glycero-3-phosphocholine (DOPC),

Abbreviations used: BCA, bicinchoninic acid; CL, cardiolipin; DOPC, dioleoyl-*sn*-glycero-3-phosphocholine; DOPE, dioleoyl-*sn*-glycero-3-phosphoethanolamine; DOPG, dioleoyl-*sn*-glycero-3-phosphoglycerol; GlpD, *sn*-glycerol-3-phosphate dehydrogenase; G3P, glycerol 3-phosphate; LUVETs, large unilamellar vesicles; MTT, 3-(4,5-dimethylthiazolyl-2-)-2,5-diphenyltetrazolium bromide; Ni^{2+}-NTA, nickel-nitriloacetate; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PMS, phenazine methosulphate; R359E, $Arg^{359} \rightarrow Glu$ etc.; TLE, total phospholipid extract from *Escherichia coli*.

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1,2-dioleoyl-*sn*-glycero-3 phosphoethanolamine (DOPE), 1,2-dioleoyl-*sn*-glycero-3-phosphoglycerol (DOPG) and cardiolipin (CL; diphosphatidylcholine) from ox heart were purchased from Avanti Polar Lipids (Alabaster, AL, U.S.A.). BCA (bicinchoninic acid) Protein Assay Kit was from Pierce (Rockford, MD, U.S.A.). Total phospholipid extract of *E. coli* (TLE) was isolated from wild-type strain W3899 by Bligh and Dyer extraction [20] followed by column chromatography on silica gel. Neutral lipids and other contaminants were eluted with chloroform and the phospholipids with chloroform/methanol (50:50, v/v). Polyclonal rabbit antibodies to GlpD were raised against purified recombinant GlpD as described by Havell [21]. Peroxidase-conjugated anti-rabbit IgG was purchased from Dianova (Hamburg, Germany).

Plasmid constructs and site-directed mutagenesis

The GlpD open reading frame was amplified from chromosomal *E. coli* MC4100 DNA by PCR using the following primers: *glpD*-fwd (5′-CGCGGATCCGAAACCAAAGATCTGATTG-TG-3′) and *glpD*-rev (5′-GCGCCTAGGCTTTGGTTTCTAG-ACTAACAC-3′), which include the restriction sites for *Bam*HI and *Hin*dIII respectively. Expression plasmid pAW4, which encodes wild-type polyhistidine-tagged GlpD, was constructed by digesting pQE30 with *Bam*H1 and *Hin*dIII and ligating with the amplified *glpD* gene digested by *Bam*HI and *Hin*dIII.

Site-directed mutagenesis was performed using the PCR gene fusion technique [22]. In brief, mutagenic primers complementary to each other were designed with the desired mutation [for R359E-GlpD: R359E-fwd (forward) (5′-cggtaagctgaccacctacga-aaaactggcggaacatgcg-3′); R359E-rev (reverse) (5′-cgcatgttccgcc-agtttttcgtaggtggtcagcttacc-3′); for K360E: K360E-fwd (5′-ctgac-cacctaccgagaactggcggaacatgcg-3′); K360E-rev (5′-cgcatgttccgcc-agttctcggtaggtggtcag-3′); for the double mutant A362E/H364D (where Ala^{362} is replaced by Glu and His^{364} by Asp): A362E/H364D-fwd (5′-cctaccgaaaactggacgaagatgcgctggaaaaactaacgc-3′), A362E/H364D-rev (5′-gcgtaagtttttccagcgcatcttcctccagttttc-ggtagg-3′)] (note: R359E means $Arg^{359} \rightarrow Glu$ etc.). The first fragment was amplified with the *glpD*-fwd and the mutagenic-rev primer; the second was amplified with the mutagenic-fwd and the *glpD*-rev primer using 10 ng pAW4 as template. In a third PCR reaction, these fragments were mixed together and amplified using *glpD*-fwd and *glpD*-rev primers. Extension of the overlap by DNA polymerase created the full-length mutant with the desired point mutation. The PCR-products were digested with *Bam*H1 and *Hin*dIII and ligated with digested pQE30 vector. The nucleotide sequences of all cloned genes were confirmed by DNA sequencing. The resulting plasmids encode wild-type or mutant proteins with an N-terminal extension of six histidine residues. For overexpression of wild-type and mutant GlpD the plasmids were transformed into *E. coli* M15, which was purchased from Qiagen.

Expression and purification of wild-type and mutant GlpD

Cultures were grown overnight at 28 °C in Luria-broth medium supplemented with 100 μg/ml ampicillin and 50 μg/ml kanamycin, diluted 100-fold into fresh broth and grown to an attenuance (D_{600}) of 0.4–0.5. Expression of N-terminal polyhistidine-tagged protein was induced by addition of 1 mM isopropyl β-D-thiogalactoside for 3 h at 28 °C before centrifugation (8000 ***g***, 30 min, 4 °C). Harvested cells were resuspended in 1:20 culture volume of ice-cold lysis buffer (50 mM Na_2HPO_4/500 mM NaCl/10 mM imidazole/10 mM β-mercaptoethanol, pH 8.0) and subjected to two lysis cycles (82.8 kPa, 4 °C) in a French pressure cell (American Instrument Co., Silver Spring, MD, U.S.A.). Unbroken cells and debris were separated from the lysate by centrifugation for 30 min at 18000 ***g***. The supernatant was applied to a Ni^{2+}-NTA column equilibrated in lysis buffer (5 ml/min flow rate), washed with washing buffer (50 mM Na_2HPO_4/1 M NaCl/30 mM imidazol/10 mM β-mercaptoethanol, pH 7.0), and eluted with elution buffer [50 mM Na_2HPO_4/250 mM NaCl/250 mM imidazole/10 % (v/v) glycerol, pH 7.4]. Purified protein was dialysed three times for 24 h against a 1000-fold excess of 50 mM Tris/HCl/75 mM NaCl/10 % glycerol, pH 7.4, and stored at a concentration of 0.3 mg/ml at −80 °C.

Monolayer experiments

The (platinum) Wilhelmy plate method [19] was used to measure protein-induced changes in the surface pressure of a monomolecular layer of phospholipids at a constant surface area. Surface pressure was measured at 28 ± 1 °C using a Cahn microbalance while continuously stirring the subphase with a magnetic bar. Unless stated otherwise measurements were performed with the following parameters. A subphase buffer (50 mM Tris/HCl/75 mM NaCl, pH 7.4) filtered through a 22 μm-pore-size filter prior to use was placed in a Teflon trough (5 ml volume, 8.81 cm² surface area). The monolayer was spread from a chloroform or chloroform/methanol (75:25, v/v) solution of lipids to give an initial surface pressure between 25 and 35 mN/m. Lower initial surface pressures were not used, since all proteins studied gave rise to surface pressure of 24 mN/m in the absence of a lipid monolayer. In all experiments saturating amounts of protein (3 μg/ml) were added to the subphase through a hole in the edge of the dish. The pressure changes were followed until the surface pressure increase had reached a maximum, usually within 40–50 min.

Vesicle-binding assay

Large unilamellar vesicles (LUVETs) were prepared by means of extrusion through a polycarbonate filter (Whatman; Anotop No. 10; 0.2 μm pore size) of a rehydrated (50 mM Tris/HCl/75 mM NaCl, pH 7.4) lipid film. The concentrations of phospholipids were determined as P_i after destruction with $HClO_4$ [23]. LUVETs were incubated with purified protein in 400 μl of 50 mM Tris/HCl/75 mM NaCl, pH 7.4, for 2 h at room temperature. Vesicles were pelleted by centrifugation for 60 min at 436000 ***g*** at 4 °C in a TLA 100.2 rotor (Beckman Instruments Inc., Palo Alto, CA, U.S.A.). Pelleting efficiencies of the vesicles were calculated after phosphorus determination on supernatant and pellet and were always above 95 %. Co-sedimentation of GlpD with the vesicles was analysed by SDS/PAGE followed by immunoblotting with anti-GlpD serum. Protein determination of soluble and pelleted protein was done (after protein precipitation with 10 % trichloroacetic acid) as described by Smith et al. [24] by using the BCA Protein Assay Kit. Control experiments without vesicles showed that more than 99 % of the added protein was recovered in the supernatant. Each value of the specific activity presented is the average for three different experiments.

Enzyme activity assay

The enzymic activity of GlpD was measured after preincubation with or without LUVETs for 2 h by monitoring the phenazine

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methosulphate (PMS)-coupled reduction of 3-(4,5-dimethylthiazolyl-2-)-2,5-diphenyltetrazolium bromide (MTT) at 570 nm and room temperature as previously described [25]. The assay mixture (250 μl) contained 50 mM Tris/HCl, pH 7.4, 75 mM NaCl, 0.5 mM MTT, 0.2 mM PMS, 10 μM FAD and 75 ng of GlpD, and the reaction was initiated with 20 mM DL-glycerol 3-phosphate. Each value of the specific activity presented is the average for three different experiments.

RESULTS

Direct GlpD–lipid interaction

To investigate the possibility that the association of GlpD with membranes occurs via direct lipid–protein interactions, we examined the ability of GlpD to insert in between phospholipids by using the monolayer technique. Total phospholipid extract from *E. coli* was spread at the air/water interface to an initial surface pressure of 27 mN/m. Injection of GlpD under the monolayer gave rise to a surface pressure increase which stabilized after 40 min (Figure 1A). Increase in surface pressure reflects insertion of part of the protein between the headgroups or between the acyl chains of the phospholipids and is not merely due to a peripheral attachment to the lipid head groups [19]. Therefore the observed surface pressure increase is interpreted to be an efficient insertion of GlpD into monolayers of TLE. The lipid specificity of GlpD insertion was investigated by using pure lipid monolayers. The kinetics and extent of surface-pressure increase were very sensitive to the composition of the lipid monolayer (Figure 1B). GlpD inserted into monolayers of all three lipid types. GlpD evoked an approximately 3-fold higher surface-pressure change in CL monolayers and a 2.2-fold higher pressure change in DOPG monolayers than in DOPE monolayers. This indicates a higher amount of insertion of GlpD

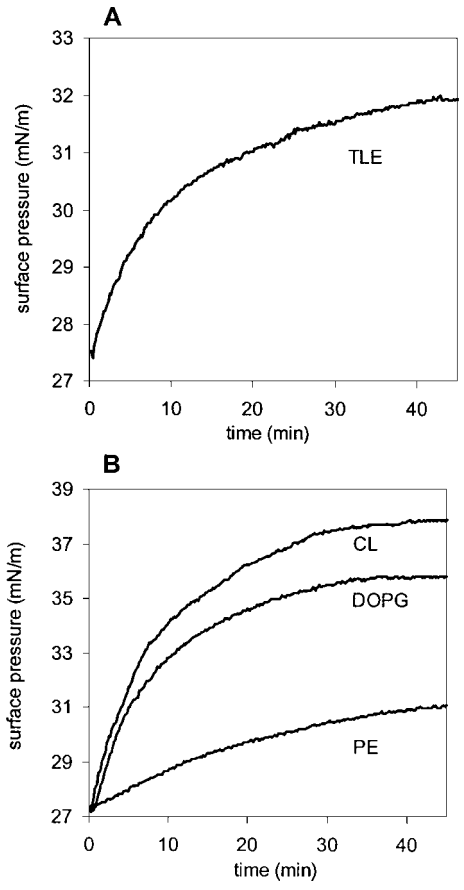

Figure 1 Interaction of GlpD with monolayers

After injection of the protein, changes in surface pressure were followed. (**A**) Insertion profile of GlpD into monolayers of TLE, which was spread at 27 mN/m initial surface pressure. (**B**) Insertion profile of GlpD into ox heart CL, DOPG and DOPE monolayers which were spread at 27 mN/m initial pressure.

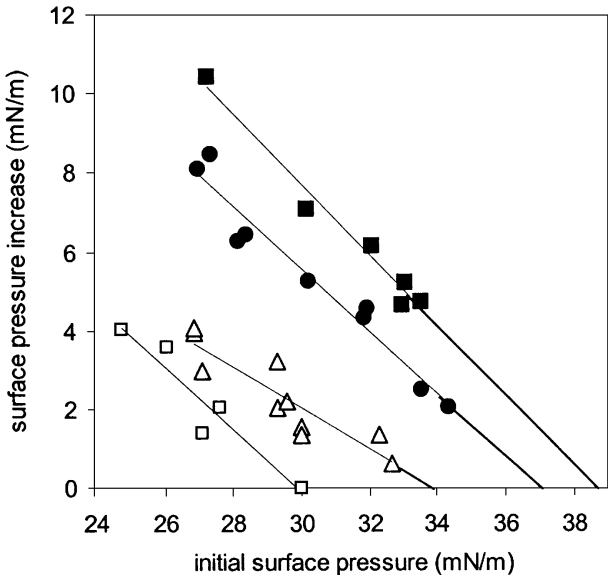

Figure 2 GlpD insertion into different phospholipid monolayers

The increase in surface pressure upon injection of the protein into the subphase was measured as a function of the initial surface pressure. Monolayers of DOPC (□), DOPE (△), DOPG (●) and ox heart cardiolipin (■) were used.

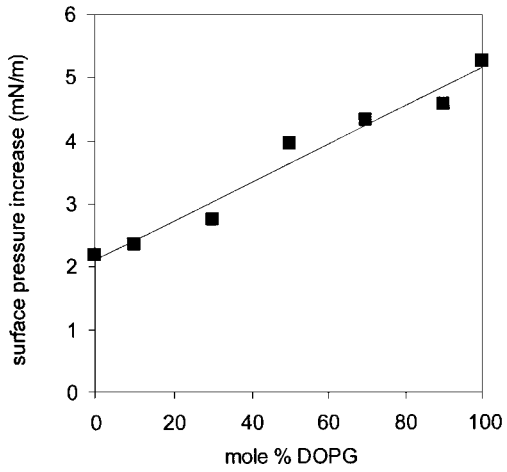

Figure 3 Insertion of GlpD as a function of the amount of negatively charged lipids in the monolayer

Mixed DOPG/DOPE monolayers were spread at 30 mN/m initial surface pressure.

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Wild-type	L_{355}	T	T	Y	**R_{359}**	**K_{360}**	L	**A_{362}**	E	**H_{364}**	A	L	E	K	L	T_{370}
R359E	L_{355}	T	T	Y	**E_{359}**	K	L	A	E	H	A	L	E	K	L	T_{370}
K360E	L_{355}	T	T	Y	R	**E_{360}**	L	A	E	H	A	L	E	K	L	T_{370}
AH362/4DE	L_{355}	T	T	Y	R	K	L	**D_{362}**	E	**E_{364}**	A	L	E	K	L	T_{370}

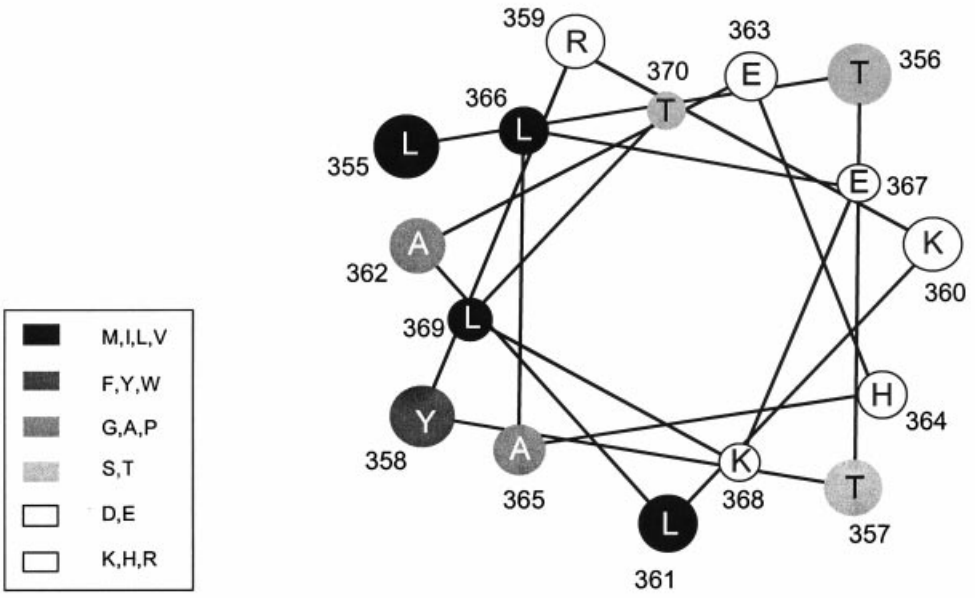

Figure 4 Amino acid sequence of the predicted amphipathic helix in GlpD and its helical-wheel projection

Amino acids altered by site-directed mutagenesis are indicated below the sequence.

into a monolayer of negatively charged lipids and demonstrates the lipid specificity of GlpD membrane insertion.

Lipid specificity of GlpD insertion

To gain insight into the penetrative power of GlpD, the protein was tested for insertion into monolayers of pure DOPG, DOPE, DOPC and CL as a function of the initial surface pressure. Higher initial surface pressures of the monolayer correlate with higher packing densities of the lipids and consequently reduce the penetrative power of the protein [19]. Indeed, the GlpD-induced surface-pressure increase was progressively reduced with higher packing densities (Figure 2). Insertion of GlpD into monolayers of negatively charged lipids was clearly higher than insertion into zwitterionic phospholipids, independently of the initial surface pressure. Thus the penetrative power of GlpD is remarkably dependent on the type of lipid. Although both are zwitterionic phospholipids, insertion into DOPE monolayers was clearly more efficient than into a DOPC monolayer, illustrating the specificity of the interaction with *E. coli* phospholipids, which do not contain phosphatidylcholine (PC). Moreover, there are important differences in the properties of these zwitterionic phospholipids. Phosphatidylethanolamine (PE) can hydrogen-bond through its ionizable amine [26] and the small headgroup size of PE results in a lower packing density at the membrane interface, allowing insertion of proteins in between the PE headgroups [27].

Extrapolation of the data yielded maximal initial pressures beyond which the proteins could not insert. Calculated maximal initial pressures were 38.5 mN/m for CL, 37.2 mN/m for DOPG, 33.5 mN/m for DOPE and 30 mN/m for DOPC monolayers. Since the packing density of biomembranes is equivalent to surface pressures of monolayer lipids between 31 and 35 mN/m [19], the data suggest that, *in vivo*, GlpD has a strong potency to insert into the lipid part of the membrane via its interaction with negatively charged phospholipids.

Using mixed monolayers of DOPG and DOPE we sought to find a correlation between penetration ability and the presence of anionic phospholipids. The insertion of GlpD showed a linear dependence on the fraction of DOPG present in a DOPE monolayer (Figure 3). These data demonstrate that insertion into lipid monolayers is promoted by anionic phospholipids.

Identification of the domain involved in membrane penetration

Membrane association through basic amphipathic α-helices has been established for many proteins interacting with the membrane [28–31]. Analysing the amino acid sequence of GlpD by means of secondary-structure prediction programs such as PHD [32], SOPM [33], PREDATOR [34] and GORIII [35], an α-helix with amphiphilic properties [36] including residues 355–370 is predicted. When the sequence is represented in a helical-wheel projection (Figure 4), a cluster of hydrophobic residues on one side of the wheel and a cluster of hydrophilic residues on the other become

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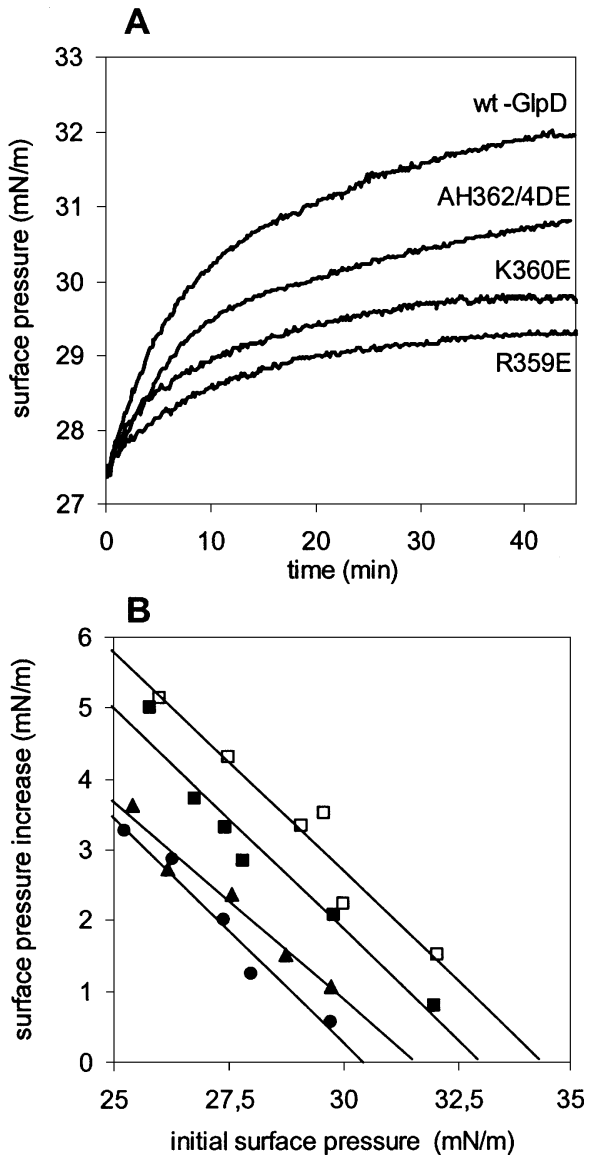

Figure 5 Insertion of wild-type and mutant proteins into TLE monolayers

(**A**) Time course of the insertion of wild-type and mutated proteins into TLE. (**B**) Insertion of wild-type (wt-GlpD, □), A362E/H364D-GlpD (■), R359E-GlpD (▲) and K360E-GlpD (●) in TLE as a function of the initial surface pressure.

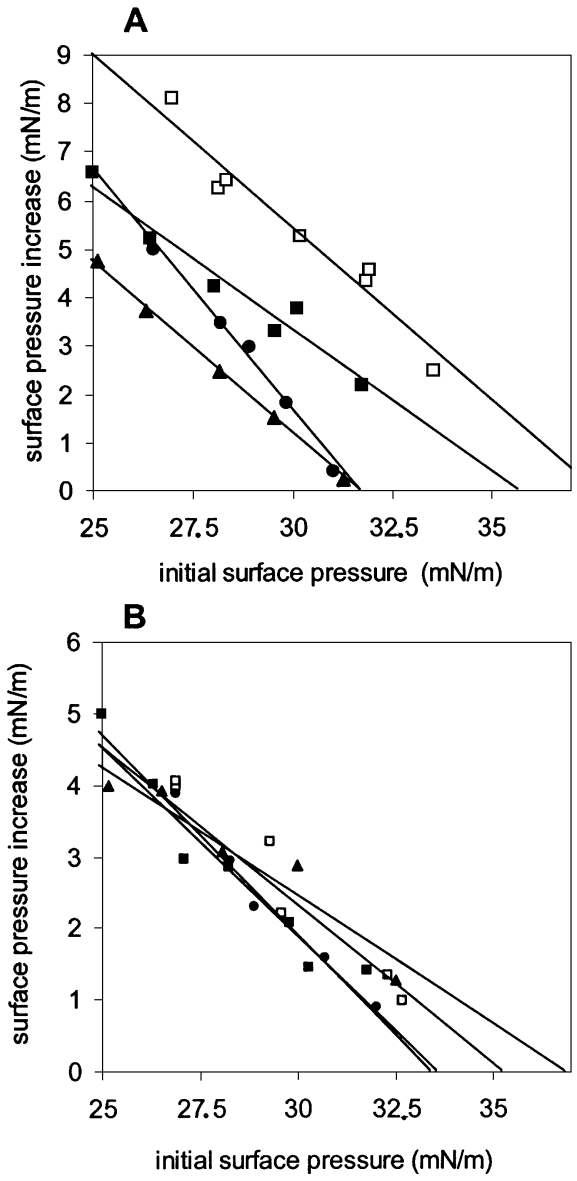

Figure 6 Lipid-specific insertion of wild-type and mutant proteins

Increases in surface pressure upon injection of wild-type GlpD ('wt-GlpD', □), A362E/H364D-GlpD ('AH362/4DE', ■), K360E-GlpD ('K30E', ●) and R359E-GlpD ('R359E', ▲) into the subphase were measured as a function of the initial surface pressure of DOPG (**A**) and DOPE monolayers (**B**).

evident. Two positively charged residues (Arg^{359} and Lys^{368}) are located at the boundary of the hydrophobic and hydrophilic faces. Such features are characteristic for the class A amphipathic α-helix in apolipoproteins which interacts with anionic lipids [37]. To test whether the basic amphipathic α-helix is involved in membrane insertion, we generated mutants which were electronegatively charged in this domain, whereas the wild-type is electropositively charged (Figure 4). Site-directed mutagenesis yielded two mutants (R359E and K360E) with one basic residue (Arg^{359} or Lys^{360}) exchanged against glutamic acid, presenting an excess of electronegative residues in the hydrophilic domain of the α-helix. To test whether the charge distribution in the alpha helical region rather than its net charge is crucial for membrane penetration we generated a double mutant (A362E/H364D) with an electronegative residue in the hydrophobic and a neutral residue in the hydrophilic region. Under physiological conditions (pH 7.4) the helical net charge of the wild-type helix was calculated to be +1, whereas the net charge of all of the mutants was −1.

Electrostatic forces are crucial for insertion into anionic phospholipids

To investigate the importance of the basic α-helix for membrane penetration we compared insertion of wild-type and mutant proteins into TLE. With respect to the kinetics and extent of surface-pressure increase, an obvious difference was observed between wild-type and mutant proteins (Figure 5A). To compare

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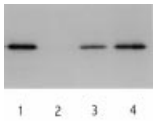

Figure 7 Liposome binding studies with purified GlpD and LUVETs composed of TLE

Purified GlpD (250 ng) was incubated in the absence (lanes 1 + 2) or in the presence (lanes 3 + 4) of LUVETs corresponding to 500 nmol of phospholipids. After ultracentrifugation, samples were divided into supernatant (lanes 1 + 3) and pellet (lanes 2 + 4) fractions. Samples were analysed by SDS/PAGE followed by immunoblotting, using 1:50000 diluted polyclonal anti-GlpD antibodies.

the penetrative power of the mutants with that of the wild-type, we measured surface pressures as a function of the initial surface pressure (Figure 5B). The highest maximal surface pressure was found for wild-type protein (34.2 mN/m) followed by the A362E/H364D double mutant (33 mN/m). The penetrative power of the R359E (31.3 mN/m) and of the K360E mutants (30.2 mN/m) was clearly reduced. The higher insertion potential of the A362E/H364D mutant protein compared with the R359E and K360E mutant proteins can be explained by the different arrangement of the negatively charged residues. The hydrophilic face of the basic amphipathic α-helix of the wild-type protein is charged positively, whereas in the A362E/H364D mutant protein it is neutral, and in both the R359E and K360E mutant proteins it is electronegative. The reduced insertion abilities observed for the mutant proteins indicate that electrostatic forces play a major role in membrane insertion of GlpD.

To gain an insight into the lipid specificity of the mutants, wild-type and mutant proteins were compared for insertion into monolayers of pure DOPG and pure DOPE as a function of varying initial surface pressure. The ability to penetrate negatively charged DOPG was clearly reduced in all of the mutants (Figure 6A), but no difference was observed when DOPE monolayers were used (Figure 6B), in agreement with an important role of R359 and K360 in anionic lipid-mediated insertion. Compared with the wild-type protein, with a calculated maximal initial pressure of 37.2 mN/m for DOPG, both the R359E and the K360E mutant proteins showed considerably diminished penetrative power with 31.5 mN/m. By contrast, the maximal initial surface pressure of the A362E/H364D double mutant was only slightly reduced (35.5 mN/m). These data suggest that the charge distribution of the hydrophilic face of the helix, rather than its net charge, is crucial for the penetrative capability of GlpD.

GlpD binding to liposomes

Direct interaction of GlpD with a phospholipid bilayer was confirmed by vesicle-binding experiments. In contrast with the results of the monolayer experiments, the association of GlpD with lipid vesicles can be caused by binding to the surface as well as by insertion of part of the protein between the lipids. Purified wild-type and mutant proteins were incubated with LUVETs composed of TLE. After ultracentrifugation, pelleted and soluble GlpD was analysed by SDS/PAGE followed by immunoblotting (Figure 7). In the absence of liposomes, GlpD was quantitatively recovered in the supernatant after ultracentrifugation (Figure 7, lane 1). When GlpD was incubated with vesicles prior to centrifugation, a substantial fraction of the protein sedimented

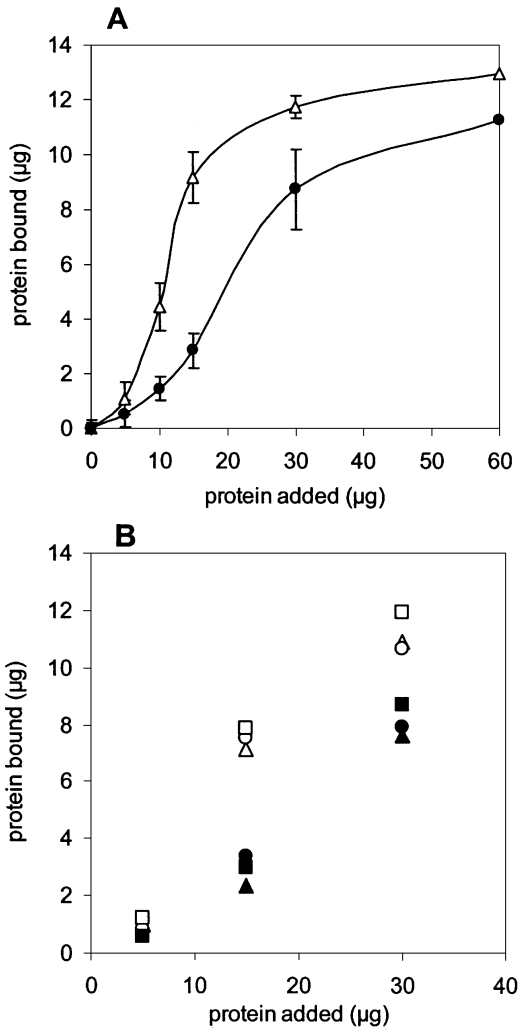

Figure 8 Liposome binding studies with wild-type and mutated proteins

(**A**) Dependence of GlpD-liposome binding on lipid composition. GlpD bound to LUVETs corresponding to 200 nmol phospholipids composed of DOPE/PC (△) and DOPG (●). (**B**) R359E (▲, △), K360E (○, ●) and A362E/H364D (■, □) bind to DOPE/PC LUVETs (□, ○, △) and to DOPG-LUVETs (■, ●, ▲) with the same affinities as wild-type protein. Values are means ± S.D. for at least three separate determinations.

with the vesicles (Figure 7, lane 4). Thus GlpD binds to phospholipid bilayers composed of the target membrane lipids.

Lipid specificity of GlpD–liposome binding

To investigate the lipid specificity for GlpD–membrane binding, we incubated the protein with DOPG or DOPE/DOPC vesicles (molar ratio 6:4; note that pure DOPE vesicles cannot be prepared, since it is a non-bilayer-forming lipid). We detected a considerable difference in the amount of pelleted protein when small amounts of protein (5–15 μg) were added (Figure 8A). Addition of 15 μg of protein resulted in 3-fold higher amounts of co-sedimented GlpD with DOPE/PC than with DOPG liposomes. The higher affinity for DOPE indicates a specific interaction between GlpD and the zwitterionic lipid, possibly due to hydrogen-bonding to the lipid headgroup. We observed only small differences with saturating amounts of bound protein for DOPE/PC and DOPG liposomes. This is probably due to

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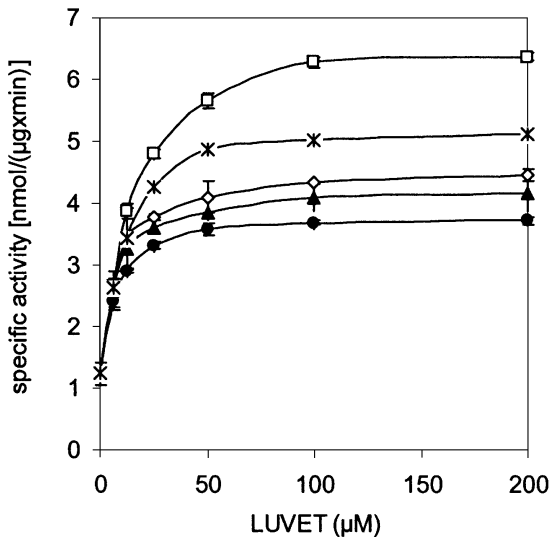

Figure 9 Effect of phospholipids on the PMS-coupled MTT-reducing activity of GlpD

Purified GlpD (75 ng) was preincubated with increasing amounts of liposomes for 2 h. Dose-dependent activity of GlpD stimulated by liposomes composed of DOPE/PC (6:4 molar ratio, □), TLE (◇), DOPE/PG (7:3 molar ratio, ▲), DOPG (●) and DOPC (✱) was measured. Values are means ± S.D. for at least three separate determinations.

unspecific binding to the membrane surface. Since the mutant proteins bound to the liposomes with similar affinities (Figure 8B), mutations in the α-helical domain had no influence on liposome binding.

Stimulation of enzyme activity

The effects of phospholipids on the enzyme activity of GlpD were examined. After preincubation of purified GlpD with increasing amounts of LUVETs, enzyme activity was measured (Figure 9). Enhanced GlpD activity after addition of various liposomes was observed. LUVETs composed of DOPE/PC (6:4) were most effective (520 % stimulation). The lowest stimulation (300 %) was obtained with pure DOPG liposomes. DOPG/PE (3:7 molar ratio, 340 % stimulation) enhanced GlpD activity to the same extent as TLE liposomes (360 % stimulation). Both types of liposomes showed approximately the same molar ratio of anionic to zwitterionic lipids, since TLE of wild-type strain W3899 contains 76 % PE, 14 % phosphatidylglycerol (PG) and 10 % CL [38]. Higher stimulation of enzyme activity by DOPE/PC vesicles is mainly due to the presence of DOPE, since pure DOPC liposomes resulted in 4-fold stimulation only (results not shown).

DISCUSSION

The present study analyses the mechanisms of interaction of the peripheral protein GlpD with the membrane. Evidence for direct lipid–protein interaction was obtained using two different approaches – studies on lipid monolayers and studies on bilayer membranes. The results reveal two different modes of interaction with GlpD and phospholipids.

Using the monolayer technique we showed that GlpD is a membrane-active protein capable of penetrating in between membrane lipids. When used at high initial surface pressures, lipid monolayers are a reliable model system to study protein insertion. For example, packing of the lipids in the outer layer of erythrocyte membranes is comparable with monolayers at surface pressures of 31–35 mN/m [39]. The penetrative power of GlpD is remarkably dependent on the type of lipid. Insertion into monolayers of negatively charged phospholipids was detected for high initial surface pressures above 37 mN/m. Thus GlpD has the potency to penetrate biological membranes. Experiments with mixed monolayers demonstrated that insertion into lipids is promoted by anionic phospholipids. Since all of the proteins analysed carry the same histidine tag, yet showed remarkable differences in their penetrative power, it is unlikely that the positive charge of the hexahistidine tag influences lipid interaction. In agreement with this, previous studies addressing the lipid interaction of nisin demonstrated the same lipid-binding properties of the native and histidine-tagged protein [40].

Which part of GlpD inserts into anionic phospholipids? Membrane attachment of several peripheral membrane proteins such as DnaA [41], enzyme $IIA^{Glucose}$ [31] and RGS4 [30] is mediated by amphipathic α-helical structures. Mutations which alter the originally electropositive charge of the predicted amphipathic α-helical region of GlpD to electronegative resulted in a diminished ability to insert into TLE monolayers. Thus electrostatic forces are involved in membrane insertion of GlpD. Although the net charge of the α-helix was −1 for all the mutants, differences in their penetrative power was observed. In contrast with the A362E/H364D mutant protein, the R359E and K360E mutant proteins failed to insert at initial surface pressures comparable with the lipid packing density in biological membranes. Both mutants are negatively charged in the hydrophilic face of the α-helix, whereas the A362E/H364D mutant is neutral. Consequently, the position of negatively charged residues in the basic amphipathic helix appears to be crucial for the penetrative power. Experiments with pure DOPG and DOPE monolayers confirmed the critical role of the basic amphipathic α-helix in membrane insertion of GlpD. For DOPG monolayers the penetrative ability of all three mutants was significantly reduced. Insertion into DOPE monolayers was not affected. These results suggest that the α-helical domain is important for insertion of GlpD into the hydrophobic core region of anionic phospholipids. We propose that GlpD displays an electropositive surface composed of the basic amphiphilic α-helix spanning residues 355–370. Anionic phospholipids promote exposure of this α-helix, which penetrates one leaflet of the membrane and stably anchors the protein at the cytoplasmic face of the membrane.

Liposome binding experiments confirmed direct GlpD–lipid interaction. We show GlpD binding to liposomes composed of *E. coli* lipids. Although we measured higher affinity for binding to neutral than to negatively charged liposomes, the maximal amount of bound protein was the same in both cases. Association to PE is presumably strong enough to permit co-sedimentation. These data suggest that GlpD specifically interacts with the polar headgroups of DOPE, possibly via hydrogen-bonding or by filling 'insertion sites' caused by its small headgroup [27]. Vesicle-binding studies with the mutant proteins revealed the same behaviour. Thus alteration in the α-helical domain did not influence protein–lipid binding to negatively charged liposomes, unlike insertion into monolayers.

Addition of LUVETs composed of various phospholipids resulted in enhanced enzyme activity of wild-type GlpD. Highest stimulation was obtained with DOPE-containing liposomes, probably due to preferential DOPE–GlpD interaction. TLE and DOPE/PG (7:3 molar ratio) liposomes enhanced activity to the same extent. In accordance with the observation that GlpD

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activity is stimulated by a broad range of amphipaths [12], these data suggest that addition of PE phospholipids increases hydrophobicity of the protein with concurrent enhancement of enzymic activity. Association of PE stimulates the activity of the protein more than insertion into PG. The mechanism by which this occurs is obscure. It is possible that association takes place in a perfectly oriented way and that this promotes proper exposure of the catalytic site. We assume that association to PE might be more important for enzymic function of GlpD than insertion of its amphipathic helix into PG. Furthermore, our results so far do not allow us to address the possibility of conformation changes of the protein in the presence of different phospholipids. This important question could be analysed in further studies by applying biophysical techniques such as CD or IR spectroscopy.

The striking insertion ability of GlpD and its strong lipid specificity are expected to have important implications for the biological activity of GlpD. Limiting availability of binding sites for GlpD on the cell membrane *in vivo* [14] thus might regulate its activity. Competition of GlpD and D-lactate dehydrogenase attaching to anionic phospholipids for these membrane sites suggests that GlpD is targeted to anionic phospholipids [14]. Anionic phospholipids are involved in a wide range of bacterial processes, such as protein translocation and chromosomal replication, by influencing the functional properties of membrane-associated proteins such as SecA [42,43], FtsY [44] or DnaA [45]. Recently, the bacterial cytoplasmic membrane was found to contain microdomains enriched in anionic lipids and proteins [46,47]. Formation of membrane domains was proposed to control bacterial division [48]. We propose that *in vivo* localization of the peripheral respiratory enzyme GlpD to microdomains containing anionic lipids may serve to fuel other enzymes associated with these domains.

We conclude that there are two different kinds of GlpD–lipid interaction: (i) vesicle binding assays suggest that soluble GlpD binds randomly to the membrane surface; (ii) monolayer experiments suggest that, in the presence of anionic phospholipids, an amphipathic α-helical domain is formed which is able to insert into the acyl-chain region of the membrane lipids and stably anchors the protein at the cytoplasmic membrane.

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