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A Single Glutamate Residue Controls the Oligomerization, Function, and Stability of the Aquaglyceroporin GlpF†

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ABSTRACT: Like many other α-helical membrane proteins, the monomeric *Escherichia coli* aquaglyceroporin GlpF associates within cellular membranes and forms higher-order oligomeric structures. A potential impact of the oligomeric state on the protein function remains enigmatic. We have analyzed the role of residues W42 and E43 in the oligomerization of the *E. coli* GlpF protein in vitro and in vivo. In contrast to W42, the polar glutamate residue at position 43 appears to be critical for oligomerization. While other polar residues can substitute for the function of E43, replacement of E43 with alanine results in a greatly reduced GlpF oligomerization propensity. The reduced interaction propensity of GlpF E43A correlates with an impaired in vivo function as well as a decreased in vivo stability. Therefore, E43 is critical for the proper oligomerization of GlpF, and protein oligomerization appears to be crucial for the channel function as well as for the in vivo stability of the protein.

Approximately 30% of all genes in pro- and eukaryotes encode membrane proteins (*1*), and more than 50% of all drugs used today target membrane proteins. Many such membrane-bound or -associated proteins form supramolecular complexes and fulfill important cellular functions by mediating the transfer of energy, of chemical compounds, or of signals across biological membranes. Although membrane proteins are of fundamental biological importance, many basic aspects of proteins in biological membranes, such as their biosynthesis, membrane integration, folding, oligomerization, or function, are not well-understood (*2*−*5*).

Almost 20 years ago, a simple model was proposed, which describes folding of α-helical membrane proteins in two steps (*6*). According to this model, individual transmembrane (TM)[1] helices integrate independently into a biological membrane, and in a second step, single helices associate and assemble into higher-order oligomeric structures. On the basis of this model, interactions between α-helices constitute a key aspect in the folding of integral membrane proteins. While in the recent years oligomerization of individual TM helices has been studied to some extent, oligomerization of larger polytopic TM protein monomers was studied only sparsely. In several cases, functional membrane protein monomers appear to from higher-order oligomers within a biological membrane (*7*−*9*).

Like many other α-helical membrane proteins, aquaporin monomers associate within cellular membranes and form higher-order oligomers. Aquaporins facilitate the flux of water across different cellular membranes, and they occur in all kingdoms of life from bacteria to humans (*10*). Interestingly, all aquaporins characterized so far appear to form tetramers within cellular membranes. Human aquaporin 1 (Aqp1) is one of the best studied aquaporins, and the unusual folding pathway of Aqp1 has been investigated in detail (*11*, *12*). A motif consisting of three amino acids has been identified, which is responsible for tetramerization of Aqp1 (*13*).

In other members of the aquaporin family, homologous residues have been suggested to be involved in oligomerization, albeit without experimental proof.

In contrast to aquaporins, aquaglyceroporins facilitate not only the flux of water over cellular membranes but also the flux of polyalcohols like glycerol (*14*), and the glycerol facilitator GlpF from *Escherichia coli* is still the best studied aquaglyceroporin to date. GlpF facilitates the flux of linear polyalcohols (*15*) and antimonite (*16*) over the *E. coli* inner membrane and interacts with the tetrameric water-soluble glycerol kinase GlpK, whose activity is influenced by this interaction (*17*). The crystal structures for both *E. coli* aquaporins, the orthodox aquaporin AqpZ and the aquaglyceroporin GlpF, have been determined recently (*18*, *19*).

Over the past decade, the oligomeric state of GlpF was controversially discussed. GlpF has initially been found to exist as monomers in detergent (*20*), whereas an equilibrium between monomers and oligomers has also been described in the same detergent (*21*). While GlpF was tetrameric in cryoelectron micrographs of the detergent-solubilized protein (*22*, *23*), it was identified as a monomer in oocyte membranes (*24*). Thus, the oligomeric state of GlpF in vivo and the potential importance of the oligomeric state for protein function remain enigmatic. In the crystal structure of the tetrameric GlpF complex, two Mg^{2+} ions have been identified in the center of the tetramer near the periplasmic face of the protein (*18*). The most external Mg^{2+} ion is coordinated octahedrally by the side chains of four

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[1]Abbreviations: Aqp1, aquaporin1; GlpF, glycerol facilitator protein from *Escherichia coli*; GPCR, G protein-coupled receptor; TM, transmembrane; GpA, glycophorin A; PDB, Protein Data Bank.

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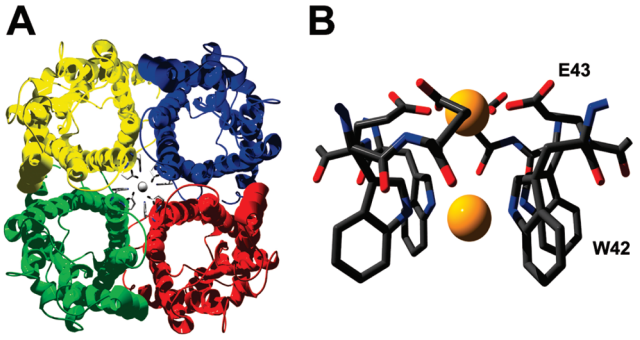

FIGURE 1: Crystal structure of GlpF [PDB entry 1LDA (*18*)]. (A) The GlpF tetramer as viewed from the periplasm with individual glycerol channels in differently colored monomers. In the center of the tetramer, a tryptophan residue at position 42 and a glutamate residue at position 43 from each subunit could be involved in binding two magnesium ions. (B) Residues 42 and 43 from each subunit are shown in a side view with the two magnesium ions. Other residues have been omitted for the sake of clarity.

glutamate residues (E43) and by water, whereas the more internal localized Mg^{2+} ion is ligated by four indol rings of a tryptophan residue (W42) and water (compare Figure 1). However, since the GlpF crystals were grown in the presence of high Mg^{2+} concentrations, it is not clear if binding of these ions has any physiological relevance. Interestingly, in the central channel of the aquaporin Aqy1 tetramer from *Pichia pastoris* (*25*), a single chloride ion was found to be bound by one tryptophan residue from each subunit.

While it would be possible that Mg^{2+} binding drives oligomerization of GlpF and/or is involved in stabilization of the tetrameric structure, it is also possible that a direct interaction of the polar side chains results in GlpF tetramerization, or that the polar residue is not important for the GlpF oligomerization and function at all. For model peptides, it has been shown that single polar residues can drive oligomerization of individual TM helices within membranes (*26*−*29*), and acidic amino acid residues have been shown to facilitate strong helix−helix interactions. Oligomerization of higher-order membrane protein monomers mediated by single polar or charged residues has, however, not been demonstrated.

In this study, we have analyzed the role of W42 and E43 in oligomerization of the *E. coli* GlpF protein. While the polar glutamate residue at position 43 appears to be critical for oligomerization, other polar residues can substitute for the function of E43, whereas replacement of E43 with alanine results in a greatly reduced GlpF oligomerization propensity. The reduced interaction propensity of the GlpF E43A variant correlates with an impaired in vivo function as well as with a decreased in vivo stability. Therefore, E43 is critical for the proper oligomerization of GlpF, and oligomerization appears to be crucial for stability and function.

EXPERIMENTAL PROCEDURES

Cloning and Mutagenesis. The *E. coli glpF* gene was amplified from genomic *E. coli* DNA by PCR and subsequently cloned into plasmid pRSET-His (*30*), pLexA (*31*), pMalp2 (New England Biolabs, Frankfurt am Main, Germany), or pASKIBA5 (IBA, Goettingen, Germany). Mutagenesis was performed using the Quick Change site-directed mutagenesis kit (Stratagene, La Jolla, CA). The sequences of the cloned and mutated *glpF* gene were confirmed by DNA sequencing (Eurofins MWG Operon, Ebersberg, Germany).

Bacterial Protein Expression and Purification. *E. coli* BL21(DE3) cells were transformed with plasmid pRSET-His-GlpF and plated on selective LB agar plates. Expression from this plasmid results in production of the GlpF protein with an N-terminally added decahistidine tail. Upon plasmid transformation, single colonies were used to inoculate LB medium with 100 μg/mL ampicillin, and after growing for 15 h, cells were harvested by centrifugation. Cells were resuspended in 20 mM Hepes buffer (pH 7.6) and lysed by tip sonification. Cellular debris was removed by centrifugation at 3000*g* (15 min at 4 °C), and membranes were subsequently separated from soluble proteins by centrifugation at 140000*g* for 1 h. Precipitated membranes were solubilized in 50 mM phosphate buffer (pH 8.0), 300 mM NaCl, and 10% glycerol. Membrane proteins were extracted by addition of 50 mM octyl glycoside (Biomol, Hamburg, Germany). After incubation for 1 h at room temperature, undissolved material was removed by centrifugation and solubilized proteins were mixed with Ni-NTA agarose (Qiagen, Hilden, Germany) and incubated for 1 h at room temperature. The Ni-NTA agarose was washed with 20 column volumes of solubilization buffer with 20 mM imidazole. His-tagged proteins were eluted with 1 mL of solubilization buffer containing 200 mM imidazole. *n*-Dodecyl β-D-maltoside (DDM, 10 mM) was added to the purified proteins, and samples were subsequently dialyzed against 10 mM Tris buffer (pH 8.0) with proteinase inhibitor cocktail from Sigma. After dialysis, potential protein aggregates were removed by ultracentrifugation (30 min at 100000*g* and 4 °C). Protein concentrations were determined using the BCA protein assay (Fisher Scientific GmbH, Schwerte, Germany). For SDS−PAGE analyses of purified GlpF proteins, 1 μg of protein was incubated in sample buffer at room temperature for 30 min, and the proteins were subsequently separated on a 16% SDS−PAGE gel.

GALLEX Measurements. GALLEX measurements were performed as described in detail elsewhere (*31*). Briefly, *E. coli* SU101 cells were transformed with plasmid pLexA-GlpF expressing the wild-type GlpF protein or variants fused to the *E. coli* LexA DNA-binding domain, and single colonies were used to inoculate LB medium with 100 μg/mL ampicillin. Cultures grown overnight were diluted 40-fold in fresh medium, and expression of the fusion proteins was induced by addition of IPTG. Cells grew to an OD_{600} of ≈0.6, and β-galactosidase activities were measured as described previously (*32*).

Stopped-Flow Measurements. GlpF and AqpZ deficient SK46 *E. coli* cells were a kind gift from E. Bremer (University of Marburg, Marburg, Germany). Cells were transformed with an empty control plasmid, the pGlpF and pGlpF-E43A plasmids, respectively. The latter plasmids encode the full length wild-type or E43A GlpF proteins without further modifications. Single colonies were used to inoculate LB medium with 100 μg/mL ampicillin, and cells were grown to an OD_{600} of ≈0.6. The cell density was then adjusted to an OD_{600} of exactly 0.6. Stopped-flow measurements were performed with an SX20 stopped-flow device (Applied Photophysics, Leatherhead, U.K.) as described in detail previously (*33*). Cells were rapidly mixed with an equal volume of LB medium containing 400 mM ribitol. Since the inner *E. coli* membrane is impermeable for ribitol, the activity of GlpF can be determined by measuring light scattering at 650 nm to follow shrinking and reswelling of the cells (*33*). Upon addition of ribitol, the cells shrink because of water efflux along the osmotic gradient toward the outside of the cells (increase in light scattering). As ribitol is moving through GlpF along its gradient

to the inside of the cells, water is following, resulting in a reswelling of the cells (decrease in light scattering). The rate constants are dependent on the GlpF concentration in the membrane, the osmotic gradient, and the ability of GlpF to serve as a conduit for the polyalcohol. The expressed protein amount of GlpF was quantified by Western blotting using isolated membranes from equal amounts of cells. Rate constants were calculated by fitting the stopped-flow data with a double-exponential decay.

Protein Stability in the E. coli Membrane. GlpF and AqpZ deficient SK46 *E. coli* cells were transformed with pASKIBA5 (empty plasmid, control), pASK-GlpF, or pASK-GlpF-E43A. Expression from the pASKIBA5-based plasmids is controlled by the tightly regulated *tet* promoter, and the plasmids encode the full-length proteins without further modifications. Single colonies were used to inoculate LB medium with appropriate antibiotics. At an OD_{600} of 0.6, protein expression was induced by addition of 200 μg/mL anhydrotetracycline. After 30 min, cells were washed three times with LB medium without anhydrotetracycline. Cells were allowed to continue growing for 24 h, and at different time points, samples with equal amounts of cells (measured as OD_{600}) were collected, precipitated, and frozen at −20 °C. The cells were subsequently resuspended in 10 mM Tris buffer (pH 8.3) and broken by sonication. Cellular debris was removed by centrifugation, and the supernatant was subjected to SDS−PAGE and Western blotting. An anti-GlpF antibody directed against C-terminal peptide VVEEKETTTP-SEQKASL of GlpF (Gramsch Laboratories, Schwabhausen, Germany) was used to detect GlpF by Western blot analyses. A peroxidase-coupled anti-rabbit antibody was used as a secondary antibody.

RESULTS AND DISCUSSION

E43, but Not W42, Is Critical for GlpF Oligomerization. In recent years, it has frequently been observed that membrane proteins form oligomers, which are sometimes even stable in SDS, but the impact of the oligomeric state on the protein function is typically rather enigmatic. Why do many membrane proteins form oligomers within membranes when a monomeric protein could essentially be functional? Several members of the aquaporin family are able to form tetramers, although the individual monomers contain the actual water channel. Except for junction- and array-forming aquaporins, it is largely unclear why oligomerization of aquaporins could be beneficial. For human Aqp6, formation of a central ion channel due to tetramerization of the monomeric subunits has been shown (*34*), which could indicate that tetramerization is critical for formation of this additional central channel. However, while in the structure of the *E. coli* GlpF protein polar residues are located in the center of the tetramer at its periplasmic side, most residues in the central cavity of the tetramer are hydrophobic, and formation of an ion channel is rather unlikely (*18*). Although GlpF from *E. coli* is probably the best studied aquaglyceroporin to date, many basic aspects of folding and function of this protein remain unanswered. The oligomeric state of GlpF has caused much dispute over the past decade, and oligomerization of the protein has been studied by various in vitro techniques. However, oligomerization of the protein has never been followed in vivo, and individual residues involved in oligomeric assembly have not been identified or described yet.

On the basis of the studies of Skach and co-workers, the oligomerization of Aqp1 depends on an intersubunit interaction of a lysine of one monomer (K51) with an aspartate of another monomer (D185) (*13*). The authors have determined the impact of these residues on protein oligomerization, and a variant of Aqp1 with nonconservative amino acid exchanges at positions 51 and 185 was impaired in oligomer formation.

Residues K51 and D185 form together with a third residue (N49) a tripartite motif, which is critical for maturation and oligomerization of the protein. Furthermore, a presence and possible influence of such a motif on the oligomerization of other aquaporin family members was discussed. The residue corresponding to Aqp1 D185 in GlpF is a proline, which is, on the basis of the GlpF crystal structure, not forming interactions with a neighboring subunit, whereas the residue corresponding to N49 of Aqp1 is the glutamate at position 43 in GlpF.

In the GlpF crystal structure, two Mg^{2+} ions have been identified in the central cavity formed by the four monomers (Figure 1A). These Mg^{2+} ions are ligated by four E43 residues or by four W42 residues (Figure 1B). The physiological impact of these ions remains, however, enigmatic. Recent observations have demonstrated that binding of ions appears not to be essential for in vitro oligomerization of GlpF (*35*), and it is likely that the observed Mg^{2+} ions were bound to GlpF because of the high Mg^{2+} concentration in the crystallization buffer (300 mM). However, it is possible that the polar side chains are directly involved in a hydrogen bonding network and thereby stabilize a tetrameric GlpF structure. To study a potential role of the two residues, W42 and E43, in the tetramerization of GlpF, we have analyzed oligomerization of purified wild-type GlpF and of GlpF mutants. As one can see in Figure 2A, wild-type GlpF forms stable dimers in SDS as well as an higher-order oligomer, which corresponds in its molecular mass to a trimeric or tetrameric GlpF species. Since the GlpF oligomers appear to have preserved secondary structures and tertiary contacts, the exact oligomeric state cannot be deduced from the migration behavior on SDS gels. Still, since different GlpF oligomers are clearly distinguishable, oligomerization of GlpF can be followed on SDS gels. After mutation of W42 to alanine, the oligomerization propensity of GlpF on SDS gels was not reduced, and it appears possible that the GlpF W42A oligomers are even slightly more stable on SDS gels than the wild-type protein. In contrast, mutation of E43 to alanine greatly disrupted oligomerization in SDS, and the protein was present only as a monomer. Thus, while W42 is not critically involved in GlpF oligomerization, E43 appears to have a prominent function in the formation of a stable tetrameric GlpF structure, at least in SDS.

While formation of the GlpF oligomer can be followed on SDS gels, and SDS gels are frequently used to follow oligomerization of TM proteins (e.g., refs (*36*−*38*)), an SDS micelle is a rather poor membrane mimic and the observations described above have to be discussed with great caution. In some cases, it has already been shown that mutations of polar residues can result in dramatic changes in the migration behavior of membrane proteins on SDS gels (*39*−*41*). To study in vivo oligomerization of wild-type GlpF and of variants in their native environment, the *E. coli* inner membrane, we have used the GALLEX system, which has originally been developed to study interactions of single TM helices within the *E. coli* inner membrane in vivo (*31*, *42*). In the GALLEX system, a TM helix of interest is fused to the C-terminus of the *E. coli* LexA DNA-binding domain, and interaction of two TM helices brings two DNA-binding domains together, which results in repression of a reporter gene (*lacZ*) activity. Here we have taken this approach one step further and

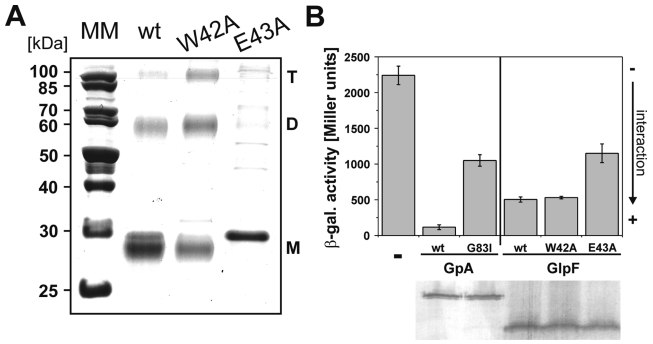

FIGURE 2: Oligomerization of wild-type GlpF and GlpF variants in vitro and in vivo. (A) SDS−PAGE analysis of purified wild-type GlpF and mutants with amino acid exchanges at positions 42 and 43 to alanine. Wild-type GlpF and the W42A variant form oligomers in SDS, whereas the GlpF variant E43A is impaired in its ability to form oligomers. One microgram of protein was loaded in each lane. M, D, and T denote the monomeric, dimeric, and tetrameric GlpF species, respectively. MM is the molecular mass standard. The results of the GALLEX measurements are shown in panel B. β-Galactosidase activities are given in Miller units and were obtained by three independent measurements with the indicated standard deviations. As controls, β-galactosidase activities were measured for cells transformed with the empty expression plasmid (−), a plasmid encoding the TM domain of GpA, or a plasmid encoding the mainly monomeric GpA variant G83I (left panel). Within the *E. coli* inner membrane, wild-type GlpF has a significant interaction propensity whereas GlpF E43A displays a significantly lowered interaction propensity. Equal expression levels of the chimeric proteins incorporated into the *E. coli* inner membrane were confirmed by Western blot analyses (bottom panel). Only the region of the monomeric proteins is shown since under the chosen experimental conditions no higher-order oligomers were detected on Western blots. While GpA and GlpF proteins are fused to the LexA DNA-binding domain, the GpA proteins are also fused to the MalE domain (*31*). Because of this, these proteins migrate slower on SDS gels.

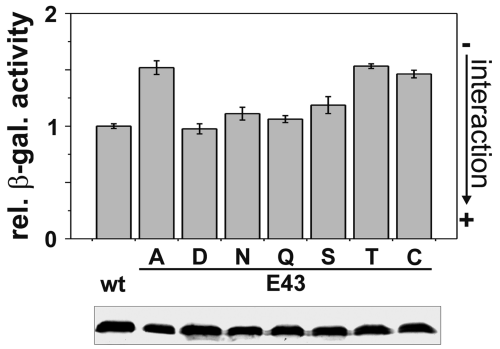

FIGURE 3: In vivo oligomerization of wild-type GlpF and of GlpF variants with amino acid exchanges at position 43. In vivo interaction propensities of wild-type GlpF and the variants where residue E43 was replaced with the indicated amino acids were measured with the GALLEX assay. Interaction tendencies are shown relative to that of wild-type GlpF (set to 1). Membrane integration of equal protein amounts was confirmed for all GlpF variants by Western blot analysis (bottom panel).

have fused the full-length GlpF protein to the LexA DNA-binding domain to follow interactions of the GlpF monomers within the *E. coli* inner membrane (Figure 2B). As a control, we have also measured the interaction propensity of the TM helix of human glycophorin A (GpA), which forms very stable TM homodimers, and of the GpA variant G83I, where the dimerization tendency is diminished (*31*, *36*). When compared to GpA G83I, the wild-type GlpF protein has a strong oligomerization tendency, although the GlpF protein does not form TM oligomers which are as stable as the GpA wild-type TM helix dimer (Figure 2B). In line with the observations on SDS gels, replacement of W42 with alanine does also not result in a changed oligomerization propensity in the *E. coli* membrane whereas replacement of E43 with alanine significantly lowered the oligomerization propensity in a membrane, and the degree of oligomerization of the GlpF E43A proteins was lowered to a level similar to that of the weakly oligomerizing GpA G83I variant.

Taken together, these results indicate that the tryptophan residue at position 42 is not critical for GlpF oligomerization in vitro or in vivo, whereas the glutamate at position 43 appears to be of special importance for proper oligomerization of GlpF monomers.

A Polar Residue at Position 43 Is Sufficient but Critical for GlpF Oligomerization. The observation described above indicated that E43 is essential for proper GlpF oligomerization. However, it appeared possible that other residues at this position also mediate and/or stabilize GlpF tetramerization. To further test if a glutamate residue is absolutely required at position 43, we have mutated this residue to other polar residues and have followed oligomerization of the various proteins in the *E. coli* inner membrane. As one can see in Figure 3, replacement of E43 with most of the other tested polar residues still resulted in wild-type-like GlpF oligomerization within a membrane. Only the mutations of E43 to threonine and to cysteine abolished oligomer formation, which has been observed before and after replacement of E43 with alanine. Noteworthy is the fact that in the GALLEX experiments the expression and membrane integration level was very similar for all proteins analyzed.

On the basis of these observations, we conclude that a polar residue at position 43 is important for proper tetramerization of GlpF. Other strongly polar amino acids, such as aspartate, glutamine, and asparagine, can replace the function of the naturally occurring glutamate residue. Also, the less polar residue serine is still able to promote oligomerization of GlpF in vivo. There appears to be no special requirement for a negatively charged residue, and most likely, all the polar residues form direct interactions to the respective residues on the neighboring subunits.

On the basis of the described observations, threonine and cysteine appear not to be sufficiently hydrophilic to promote proper oligomerization of GlpF within the *E. coli* inner membrane.

The Oligomerization Propensity of the GlpF E43A Mutant Is Greatly Reduced. To characterize the oligomerization of the GlpF E43A variant in more detail, we have used the GALLEX assay and have analyzed the interaction propensities of the wild type and the E43A variant in the *E. coli* inner membrane at increasing protein concentrations. In the GALLEX system, an inducible *lac* promoter controls the expression of the chimeric protein (*31*). The *lac* promoter can be gradually induced, which allows the expression level of a protein to be progressively controlled (*43*). A gradual increase in the IPTG concentration results in an increased level of protein expression (Figure 4B), and such a titration experiment can be used to estimate association constants of membrane proteins within a biological membrane (*41*, *43*). As one can see in Figure 4A, the interaction propensity of wild-type GlpF increases with increasing IPTG concentrations up to an IPTG concentration of 0.5 mM. At this concentration, the promoter appears to be completely induced and further addition of IPTG does not increase the amount of expressed protein.

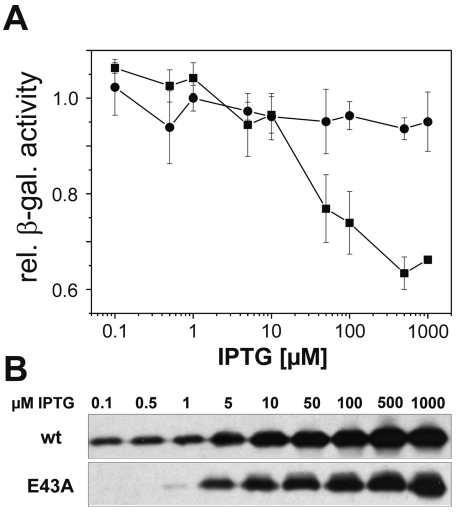

FIGURE 4: In vivo interaction propensity of wild-type GlpF and GlpF E43A at different protein concentrations. (A) GALLEX measurements were performed for wild-type GlpF (■) and GlpF E43A (●) at different IPTG concentrations. The β-galactosidase activities at 0 mM IPTG was set to 1. Each data point represents at least three independent measurements with the indicated standard deviations. (B) The amount of GlpF incorporated into the *E. coli* inner membrane was determined by Western blot analysis.

To define the maximal β-galactosidase activity, which will be reached when GlpF is essentially exclusively monomeric, the β-galactosidase activity of a strain containing an empty expression plasmid, where no LexA DNA-binding domain is encoded, was measured at 0 mM IPTG. Here the LexA-controlled promoter/operator was 100% active in the reporter strain, and the measured β-galactosidase activity was set to 1. This activity represents the activity at 0% repression, which is, for example, reached when GlpF is completely monomeric. Noteworthy is the fact that in strains containing wild-type GlpF or E43A mutant expression plasmids, the β-galactosidase activities measured at 0 mM IPTG also reach essentially the level of the control strain, indicating that the proteins are effectively monomeric.

In contrast to the wild type, addition of increasing amounts of IPTG to the GlpF E43A-expressing strain did not result in a significantly increased interaction propensity when measured with the GALLEX assay, which further indicates that the E43A protein is dramatically impaired in its ability to form stable oligomers within a membrane.

As one can see in Figure 4B, the expression level of the wild-type protein was higher at most IPTG concentrations when compared to the expression level of the E43A variant, although expression was similar at high IPTG concentrations (which has been used in the experiments shown in Figures 2 and 3). However, since the E43A variant has a very weak interaction at any IPTG (and protein) concentration with $< 10\%$ changes in the measured oligomerization propensity, normalization of the apparent interaction propensities to the actual protein concentration within the *E. coli* membrane does not influence the observation that oligomerization of the GlpF E43A protein is strongly impaired.

As outlined in ref *43*, the data presented in Figure 4A can be used to estimate the change in the apparent $\Delta\Delta G$ of tetramerization. The relative β-galactosidase activities of GlpF at the various IPTG concentrations equal the fraction monomeric protein under the assumption that full activity corresponds to 100% monomer and that the activity linearly correlates with fraction monomer, as outlined in detail in ref *43*. To properly compare the measured interaction propensities and the fractions of monomeric GlpF, we determined the densities of individual GlpF bands on the Western blots using Scicon Image (Scion Corp., Frederick, MD). On the basis of these measurements, the GlpF wild-type protein concentration after expression in the presence of 100 μM IPTG corresponds to the GlpF E43A protein concentration after expression in the presence of 500 μM IPTG (Figure 4B).

While the data shown in Figure 2A indicate that the GlpF tetramer forms from monomers via transient dimer formation, we here only consider the monomer−tetramer equilibrium $4M \longleftrightarrow T$, where M and T represent the monomeric and tetrameric GlpF species, respectively. We can use the measured interaction propensities at 100 and 500 mM IPTG and the corresponding fractions of monomeric GlpF, which are equal to the determined relative β-galactosidase activities (Figure 4A), to calculate the apparent ΔG^{app} values according to $\Delta G = -RT \ln K$. For the wild type we calculate a ΔG^{app} of −3.4 kJ/mol and for the mutant a ΔG^{app} of −10.2 kJ/mol. Therefore, replacement of E43 with alanine destabilizes the GlpF tetramer with a $\Delta\Delta G^{app}$ of ∼7 kJ/mol.

A Monomeric GlpF Does Not Facilitate Polyalcohol Flux across the E. coli Inner Membrane to the Same Extent as the Wild-Type Protein. The results presented above clearly suggest that E43 is critical for oligomerization of GlpF, and replacement of E43 with alanine results in a dramatically lowered oligomerization tendency. While these observations describe a role of E43 in GlpF tetramerization, the physiological impact of GlpF oligomerization and a potential correlation between tetramerization and function are still uncharacterized. To analyze how the propensity to form tetramers influences the function of the protein, we have measured the flux of the polyalcohol ribitol across the *E. coli* inner membrane in cells expressing either wild-type GlpF or the E43A variant. The proteins were expressed in *E. coli* strain SK46, in which both endogenous aquaporin *glpF* and *aqpZ* genes are disrupted (*44*), and thus, the GlpF protein expressed from the plasmid is the only aquaporin present in the *E. coli* inner membrane. As one can see in Figure 5A, upon addition of ribitol *E. coli* cells shrink immediately due to water efflux, resulting in increased light scattering (phase 1). In phase 2, ribitol diffuses into the *E. coli* cells via the expressed wild-type GlpF protein, resulting in reswelling of the cells caused by the flux of water into the *E. coli* cytoplasm. Expression of the GlpF E43A variant (dark gray line) results in a significantly decreased reswelling rate, which indicates an impaired ribitol flux across the membrane in these cells. However, as shown above, the E43A protein is eventually not incorporated into the *E. coli* inner membrane to the same extent as the wild-type protein. Because of this, we have followed the activity of the wild-type and E43A mutated GlpF proteins in intact *E. coli* cells upon expression of increasing amounts of GlpF into the membranes. As one can see in panels B and C of Figure 5, addition of > 10 μM IPTG results in expression of significant amounts of GlpF into the *E. coli* inner membrane and in rapid ribitol influx. Consequently, the reswelling rate continuously increases with increasing IPTG concentrations. However, expression of the oligomerization-impaired E43A GlpF variant results in a strongly reduced ribitol influx, indicating an impaired function of the protein.

To properly compare the measured rate constants, we determined the densities of individual GlpF bands on the Western blots using Scicon Image (Scion Corp.). On the basis of these measurements, the amount of membrane-incorporated wild-type

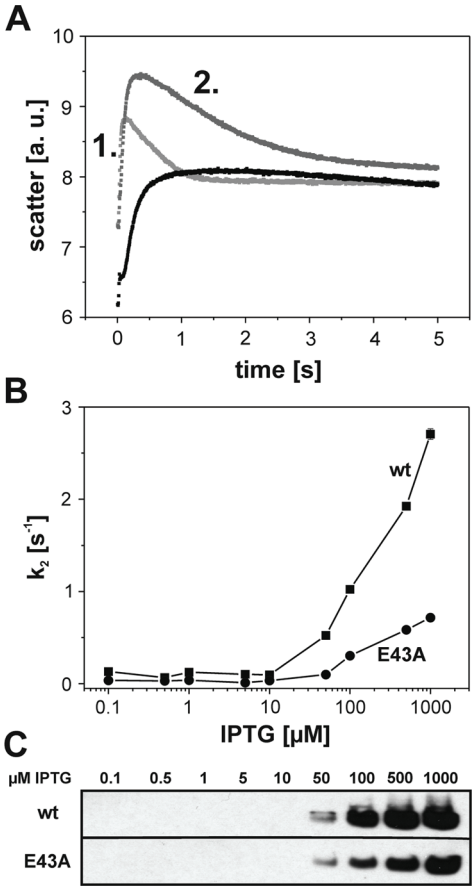

FIGURE 5: Functional measurement of wild-type GlpF and GlpF E43A in vivo activity with a ribitol assay. (A) Swelling and shrinking of cells were assessed by monitoring light scattering of *E. coli* SK46 cells in a stopped-flow device. When cells were rapidly mixed with a solution containing 400 mM ribitol, cells expressing wild-type GlpF (light gray line) shrink rapidly, resulting in an increase in light scattering (1.). A ribitol flux toward the cytoplasm results in reswelling of the cells and in a decrease in light scattering (2.). If no GlpF is expressed, the cells remain shrunken since ribitol is unable to permeate the *E. coli* membrane in the absence of GlpF (black line). Expression of GlpF E43A (dark gray line) results in a decreased reswelling rate. Each curve is the average of 10 measurements. (B) Ribitol assay performed with increasing amounts of membrane-integrated GlpF. Since the ability to facilitate rapid ribitol flux depends on the protein concentration, the ribitol flux assay was performed at increasing IPTG concentrations, and thus increasing amounts of GlpF incorporated into the *E. coli* inner membrane. The wild-type protein (■) facilitates rapid ribitol flux, whereas the E43A variant (●) is impaired in its ability to facilitate rapid ribitol flux. Each data point is the average of 10 repetitions, and all measurements were performed independently at least five times. Standard deviations are given but hidden by the used symbols. (C) Amount of GlpF protein incorporated into the *E. coli* membrane determined at all IPTG concentrations using Western blot analyses for both the wild type and the E43A GlpF protein.

GlpF and E43A protein is almost exactly equal at IPTG concentrations of 100 and 1000 μM, respectively. The measured rate constants (k_2) at these IPTG concentrations are 1.02 and 0.71 s^{-1} for the wild type and the E43A mutant, respectively. Thus, after alignment of the measured rate constants at the respective IPTG concentrations to the actual amount of membrane-incorporated protein, the E43A variant exhibits ~30% less activity than the wild-type protein.

Taken together, these results suggest that the oligomerization-impaired GlpF variant has a reduced in vivo activity. Thus, oligomerization of GlpF appears to be important for the GlpF channel function. It is, however, not evident how the flux rate of a polyalcohol through GlpF is increased by the formation of oligomers. The mechanism by which this functional enhancement is achieved through oligomerization merits further investigation, and it will be interesting to see whether a connection between the oligomeric state and the function and stability can be observed for other aquaporins as well. In the case of other membrane proteins, like the potassium channel KcsA (*45*), which forms a single TM channel by tetramerization, the functional implications are obvious. The recently determined crystal structure of the *E. coli* diacylglycerol kinase revealed that oligomerization of the protein is functionally important since the catalytic center is formed by two monomers (*46*). However, such a possibility can be ruled out in the case of the GlpF protein since the channel pore is located within the monomeric GlpF protein.

Oligomerization of membrane proteins where the functional unit seems to be the monomer has been only sparsely studied, although many hypotheses exist ranging from energetic considerations to protein stability (for a recent review, see ref *47*). Indeed, oligomerization enhances the stability of bacteriorhodopsin (*48*); a potential influence of the oligomerization on the function of each monomer is, however, not known. An increased activity of membrane proteins in an oligomeric state has recently been observed in a study on mitochondrial F_1F_O-ATP synthase supracomplexes (*49*). While general oligomerization of specific G protein-coupled receptors (GPCRs) is still heavily debated, it seems clear that at least some GPCRs form stable dimers within membranes (for a recent review, see refs *50* and *51*), although the physiological function of GPCR dimerization is also still mystic.

The in Vivo Stability of a Monomeric GlpF Variant Is Impaired. In several of the experiments described above, we have observed that less GlpF E43A protein is incorporated into the *E. coli* inner membrane when compared to the wild-type GlpF protein. Since the stability of membrane proteins can depend on the oligomeric state of the protein (*52*), we next studied the lifetime of the wild-type and the E43A mutated GlpF proteins within the *E. coli* inner membrane. To this end, we expressed the proteins from a plasmid in *E. coli* SK46 cells, and expression was under the control of the tightly controlled *tet* promoter (*53*). In the absence of the inducer anhydrotetracycline, the cells did not express the proteins (Figure 6A, −). Protein expression was induced when the culture had reached an OD_{600} of 0.6, and cells were grown for 30 min in the presence of the inducer. Afterward, the inducer and the antibiotic ampicillin were removed from the cells by washing the cells three times with medium, and at various time points after induction, the concentration of GlpF in *E. coli* membranes were determined by Western blot analyses. As one can see in Figure 6, the concentration of the wild-type GlpF protein within the *E. coli* inner membrane initially increased and thereafter gradually decreased 2 h after removal of the inducer, and the GlpF level within the membranes further decreased dramatically after 3 h. Thereafter, the wild-type GlpF protein level in the membrane remained approximately constant for up to at least 24 h. While the overall expression level was slightly decreased for the GlpF E43A protein, the protein was also degraded much faster than the wild-type protein. Two and three hours after removal of the inducer, the GlpF level in the membrane was similar to that of the wild-type protein. After 4 h, the protein was almost not visible in *E. coli* membranes, and after 8 h, no protein was detected by Western blot analyses (Figure 6). Noteworthy is the fact that the growth rates were

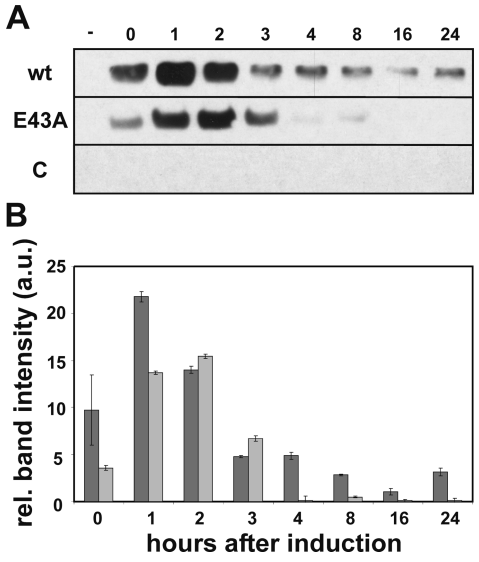

FIGURE 6: Stability of wild-type GlpF and GlpF E43A in the *E. coli* inner membrane. (A) An aliquot of cells corresponding to 1 equiv with an OD_{600} of 0.6 was taken at the indicated time points, and Western blots were performed with isolated membrane fractions. Protein stability was followed for 24 h in cells containing the empty expression plasmid (C), a plasmid containing wild-type *glpF* or E43A *glpF*. Before addition of the inducer anhydrotetracycline (−), no protein is expressed due to the tightly regulated *tet* promoter. Thirty minutes after addition of the inducer, cells were carefully washed and the amount of membrane incorporated GlpF protein was determined by Western blot analyses at different time points. At time zero, cells were analyzed directly after removal of the inducer. The experiment was performed two times with independent cultures, and a representative blot is shown. (B) Band intensities of the blots shown in panel A measured using Scicon Image (Scion Corp.). The relative intensities of the wild type (dark gray bars) and the E43A mutant (light gray bars) are shown for comparison.

approximately identical for the strains expressing the wild-type and E43A GlpF proteins.

Taken together, these observations indicate that the stability of the GlpF E43A protein within *E. coli* membranes is severely reduced. Thus, oligomerization of GlpF affects not only the function of the protein (as shown above) but also its in vivo stability.

Recently, it has been shown that the GlpF tetramer can be very stable in detergent, and there is essentially no exchange between free monomers and monomers as part of a tetramer (*21*). On the basis of the presented in vivo interaction propensities of wild-type GlpF (Figure 4), we conclude that a significant part of the GlpF population could be present as monomers within the *E. coli* membrane. The results shown in Figure 6 indicate that the oligomerization-impaired GlpF is rapidly metabolized whereas part of the (tetrameric) wild-type protein population is highly stable in the *E. coli* membrane. Therefore, it appears possible that formation of a stable tetramer not only is functionally important but also increases the stability of a protein in the membrane.

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