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Functional Characterization of Amphipathic α-Helix in the Osmoregulatory ABC Transporter OpuA

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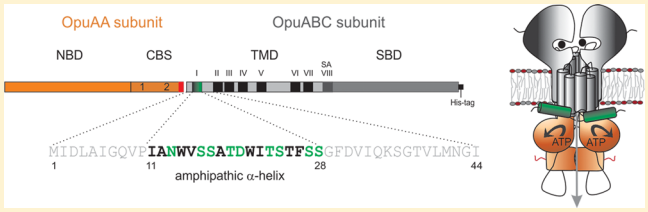
Supporting Information

ABSTRACT: The ATP-binding-cassette transporter OpuA from *Lactococcus lactis* is composed of two ATPase subunits (OpuAA) and two subunits (OpuABC) with the transmembrane domain fused to an extracellular substrate-binding protein. Of the almost 1900 homologues of OpuA known to date, a subset has an amino-terminal amphipathic helix (plus extra transmembrane segment) fused to the core of the transmembrane domain of the OpuABC subunit. FRET measurements indicate that the amphipathic α-helix is located close to the membrane surface, where its hydrophobic face interacts with the transport protein rather than the membrane lipids. Next, we determined the functional role of this accessory region by engineering the amphipathic α-helix. We analyzed the consequence of the mutations in intact cells by monitoring growth and transport of glycine betaine under normal and osmotic stress conditions. More detailed studies were performed in hybrid membrane vesicles, proteoliposomes, and bilayer nanodisks. We show that the amphipathic α-helix of OpuA is necessary for high activity of OpuA but is not critical for the biogenesis of the protein or the ionic regulation of transport.



Bacterial cell survival depends on the physicochemical conditions of the environment. Temperature, osmolality, and salinity are key factors having a direct influence on the cell's physiology. Hyperosmotic conditions represent a major environmental stress, which causes cells to lose turgor and to diminish their cell volume. Microorganisms react to this undesirable situation by accumulating so-called compatible solutes, which can be done either via uptake from the environment or *de novo* biosynthesis.[1]

Transport of compatible solutes across bacterial cell membranes is mediated by both ATP-binding cassette transporters (ABC) and ion-coupled transporters.[2,3] Of these the ABC transporter OpuA from *Lactococcus lactis*, the proton-coupled ProP system from *Escherichia coli*, and the sodium-coupled BetP from *Corynebacterium glutamicum* are best understood in terms of osmosensing and regulation.[4−6] OpuA, the transporter investigated here, belongs to the osmoprotectants taurine cyanate and nitrate (OTCN) subfamily within the ABC superfamily. OTCN-type ABC transporters mediate the translocation of a diverse group of substrates such as glycine betaine, choline, carnitine, nitrate, cyanate, *N*-alkylsulfonates, alkylphosphonates, and phosphites.[7,8] OpuA from *L. lactis*, however, is rather specific for glycine betaine and only accepts other substrates with orders of magnitude lower affinity.[9]

The core of typical ABC transporters is composed of two transmembrane domains (TMDs) and two nucleotide-binding domains (NBDs). OpuA is more complex than prototypical ABC transporters as it has tandem cystathionine-β-synthase (CBS) domains fused to each of the NBDs. These CBS domains are critical for transport by ensuring tight coupling between ATP hydrolysis and glycine betaine translocation [Karasawa, A., Swier, L., and Poolman, B., unpublished] and serve a role in the osmotic regulation of the protein.[10] In fact, the CBS module together with an anionic membrane surface is needed for sensing osmotic stress, i.e., changes in ionic strength. Deletion of the CBS module or lowering of the fraction of anionic lipids diminishes the ionic strength dependence of transport. A glycine betaine-specific binding domain, preceded by signal anchor sequence (SA VIII in Figure 1A), is fused to the C-terminus of each of the TMDs of OpuA. We note that in OpuA from *Bacillus subtilis* the substrate-binding domain (SBD) is not fused to the TMD but tethered to the membrane via a lipid moiety,[11] whereas in the homologous transporter (ProU) from *Escherichia coli* the SBD is a soluble protein (ProX) that is located in the periplasm.[12] The core of the TMD of OpuA from *L. lactis* is composed of five transmembrane segments that are relatively well conserved (III−VII in Figure 1A). Among the OpuA homologues (currently more than 1900 in the databases), 30 transporters are predicted to have an amphipathic plus transmembrane α-helix preceding the core of five transmembrane segments (I and II in Figure 1A); see Discussion for a more elaborate presentation of the OTCN family and the presence of the amphipathic α-helix among its members. The spatial arrangement of polar and nonpolar residues along the axis of the amphipathic α-helix divides the surface into a

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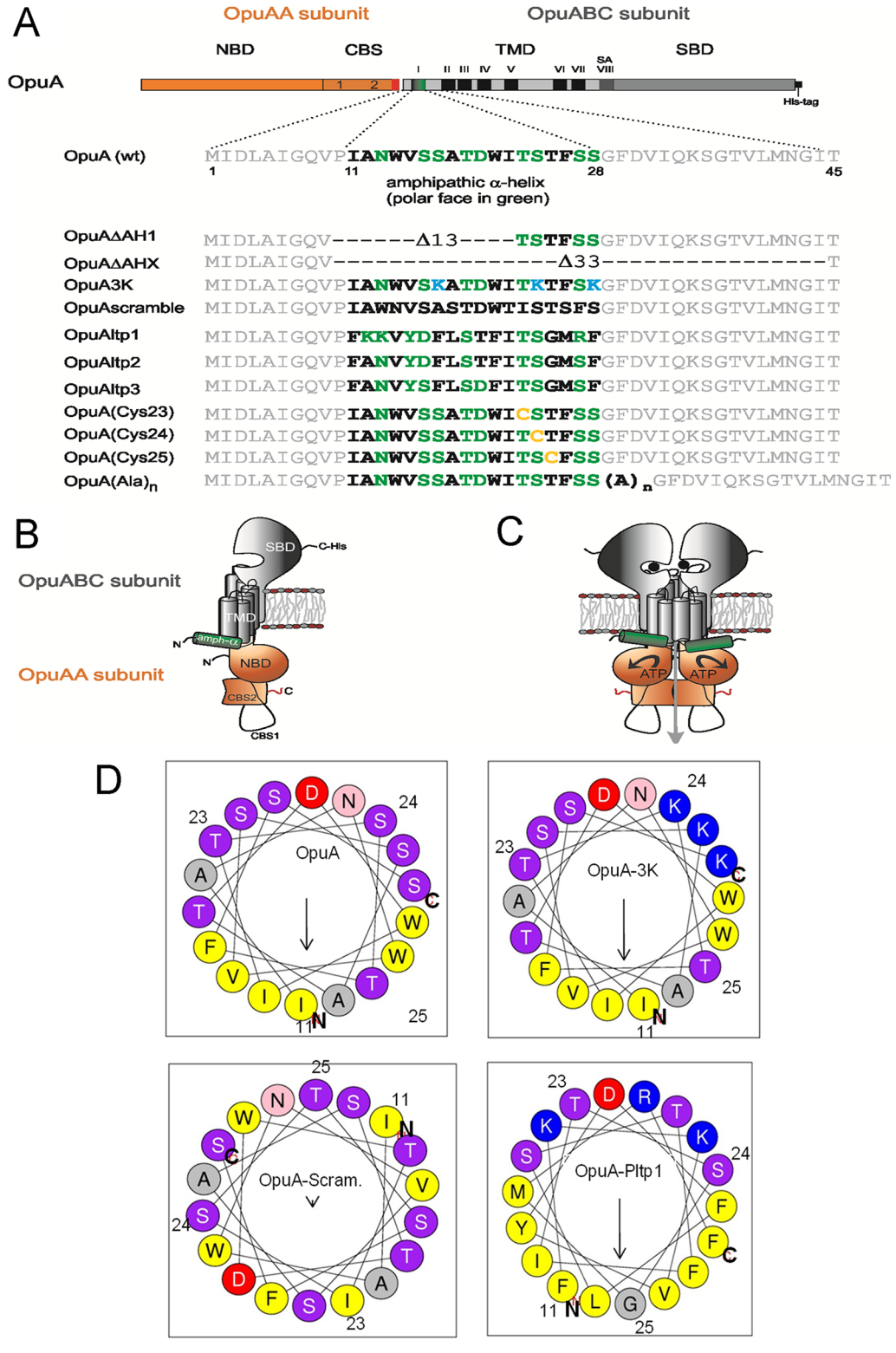

Figure 1. Schematic representation of OpuA and specification of the mutants. (A) Domain organization of OpuA from *L. lactis* and specification of the different constructs made and analyzed in this paper. OpuA is composed of two OpuAA (indicated in orange) and two OpuABC (indicated in gray/green) subunits. OpuAA contains the nucleotide-binding domain (NBD) and two CBS domains in tandem; OpuABC contains the predicted amphipathic helix I (in green), the transmembrane domain (TMD; putative α-helices II–VII), and the substrate-binding domain linked to the TMD via a signal anchor sequence (SA VIII). (B) Organization of the OpuA subunits relative to the membrane. (C) Schematic of the architecture of OpuA from *L. lactis* (dimeric complex). The gray arrow shows the translocation pathway for glycine betaine and the black arrows shows the hydrolysis of ATP. (D) Helical wheel representation of the amphipathic α-helix of OpuA (A), OpuA-3K (B), OpuA-Scramble (C), and OpuA Pltp1 (D), using http://heliquest.ipmc.cnrs.fr. Hydrophobic residues of the helix are shown in yellow and polar residues in other colors. The arrow indicates the hydrophobic moment.

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hydrophobic (nonpolar) and hydrophilic (polar) face and yields a large hydrophobic moment (μ_H).[13] Amphipathic helices have been classified in different categories depending on the angle subtended by the polar face and the distribution of charged residues on the polar face.[14] In many proteins, amphipathic N-terminal sequences function in the proper targeting and insertion (or stable integration) of the protein into the membrane.[15] Moreover, amphipathic α-helices may mediate regulatory functions via reversible membrane interactions as described for amphiphysin, endophilin, and RICH/nadrin.[16] Amphipathic α-helices in transporters have been recently exploited as potential drug targets.[17]

Here, we focus on the role of the amphipathic α-helix in the ABC transporter OpuA. We have made deletion constructs, modified the amphipathicity of the helix, and substituted it for one unrelated in sequence. We have analyzed the mutant proteins *in vivo*, in hybrid membrane vesicles, and in proteoliposomes with defined protein and lipid composition. Our data show that the amphipathic α-helix of OpuA is not critical for ion sensing but is required for maximal activity of the transporter.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions. For nisin A-inducible expression of wild-type and mutant OpuA, the *L. lactis* Opu401 was used as host strain. Opu401 is a derivative of *L. lactis* NZ9000 with the chromosomal *opuA* genes deleted.[18] Wild-type and mutant derivatives were cultivated semiaerobically in 2% (w/v) gistex LS (Eemnes, NL) and 65 mM sodium phosphate, pH 6.5, supplemented with 1% (w/v) glucose plus 5 μg/mL chloroamphenicol, using 2 L pH- and temperature-controlled bioreactors. Cells were cultivated to OD_{600} of ~2, after which 0.1% (v/v) nisin A (filter-sterilized culture supernatant from *L. lactis* NZ9700) was added to induce the nisin A promoter. After 2 h, the cells were harvested by centrifugation, washed with 50 mM potassium phosphate (KPi), pH 7.0, resuspended in the same buffer, and stored at −80 °C after flash-freezing of 40 mL aliquots of cells in liquid nitrogen. Membrane vesicles were prepared by lysing the cells in a cell disruption system (high pressure Constant Systems cell disrupters, UK) at a pressure of 39 000 psi at 4 °C. Subsequently, different (ultra)centrifugation steps were done to separate membrane vesicles from cell debris and cytosolic fractions as described.[19]

Construction of Amphipathic α-Helix Deletion Mutants. pNZOpuAhisΔAH1 and pNZOpuAhisΔAHX were constructed by using pNZOpuAhis (wild-type *opuAA* and *opuABC* genes) as template. The *opuABC* gene in this plasmid possesses a unique *Spe*I site at position 1291 of the amphipathic α-helix. A second *Spe*I site was created at position 1252, i.e., within the amphipathic α-helix. OpuAhisΔ*H*1 was constructed by deleting 39 nucleotides (corresponding to the amino acid sequence: PIANWVSSATDWI) upon digestion with *Spe*I. The same strategy was followed for the construction of OpuAhisΔAHX by forming a *Spe*I site at position 1351, resulting in deletion of 126 nucleotides (corresponding to 42 amino acids, that is, amino acids 10−22 and 25−44 of *OpuABC*).

Construction of OpuA Derivatives with Site-Specific Mutations, Scrambled Sequences, and Alternative Amphipathic α-Helix. Plasmid pREBackOpuAhis(*Aat*II−*Kpn*I) was constructed by engineering an *Aat*II recognition sequence at nucleotide position 1315 and a *Kpn*I site at nucleotide position 1248, using the Quik change site-directed mutagenesis kit. pREbackOpuAhis with the wild-type *opuAA* and *opuABC* genes was used as template, and two primers (**5′GATTTA**GCTATTGGACA**G**G**TACC**CATAGCAAAC3′; single substitution underlined and *Kpn*I site in boldface) and (**5′GTTCAGGA**TTT**GA**C**GT**C**A**TTCAAAAATCAGG3′; 2 substitutions underlined and *Aat*II site in boldface). pREBackOpuAhis(*Aat*II-*Kpn*I) was digested sequentially with *Aat*II and *Kpn*I, with plasmid purification steps in between the restriction digestions. For construction of pScramble, p3K, pltp1, pltp2, pltp3, and mutants with single Cys substitutions, a gene fragment was exchanged for complementary oligonucleotides of 67 nucleotides in length, containing *Aat*II and *Kpn*I compatible ends. The complementary oligonucleotides with the desired mutations (STable 1) were first annealed by slowly decreasing the temperature from 94 to 40 °C (over 3 min). After annealing, the double-stranded oligonucleotides were ligated into the plasmid (previously cut with *Aat*II and *Kpn*I) for 1 h at 20 °C and then transformed to *E. coli* MC1061. All mutants were confirmed by DNA sequencing analysis. The exchange of the vector backbone of pRE-OpuA to pERL, containing a *L. lactis* origin of replication, was performed as described earlier.[20]

Construction of Alanine Insertion Mutants. Alanine insertion mutants were created, using pNZOpuAhis as a template and touchdown PCR,[21] using Phusion polymerase. A unique restriction site, *BssH*II, was added in both the forward and reverse primers and extra AGC codons, specifying alanine residues, were introduced via the reverse primer (see STable 1). After PCR amplification, restriction digestion was done with *BssH*II, and the sticky ends produced by *BssH*II were ligated overnight at 20 °C. Ligase inactivation was done at 80 °C for 20 min, and subsequently, plasmid DNA was introduced into *L. lactis* Opu401 by electroporation.

Growth Experiments. To test for osmoregulatory properties *in vivo*, *L. lactis* Opu401 expressing wild-type or mutant OpuA was grown in chemical-defined medium (CDM) supplemented with 1% glucose plus 5 μg/mL chloroamphenicol (GCDM) and varying amounts of NaCl (from 0 to 0.8 M). Precultures in GCDM were grown to OD_{600} ~ 0.5, after which they were diluted 2-fold with prewarmed GCDM to yield a final nisin titer of 0.01%. Cells were allowed to induce OpuA for about 2 h, and at an OD_{600} of ~0.5, they were diluted 20-fold with prewarmed GCDM, nisin A plus NaCl at the indicated concentrations. The growth was monitored continuously at 30 °C in 96-well plates in a volume of 300 μL, using a BIOTEC apparatus Power wave 340.

Hybrid Membranes. Membrane vesicles were fused with preformed liposomes composed of 50% 1,2-dioleoyl-*sn*-glycero-3-phosphatidylethanolamine (DOPE), 38% 1,2-dioleoyl-*sn*-glycero-3-phosphatidylglycerol (DOPG) plus 12% 1,2-dioleoyl-*sn*-glycero-3-phosphatidylcholine (DOPC) (unless stated otherwise) in 1:10 ratio (on lipid basis). Synthetic lipids were obtained from Avanti Polar Lipids, Alabaster, AL. The hybrid membranes were obtained by two freeze−thaw cycles, followed by extrusion through a polycarbonate filter (200 nm pore size). The ATP regenerating system ARS (10 mM Mg-ATP, 24 mM phosphocreatine plus 2.4 mg/mL creatine kinase in 50 mM KPi) was enclosed during fusion of membrane vesicles with liposomes.[22]

PEGylation of OpuA in Membrane Vesicles. The reactivity and accessibility of the thiol groups in the Cys mutants were assessed by labeling with poly(ethylene glycol)

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maleimide (Mal-PEG). Membrane vesicles at 5 mg/mL in 50 mM KPi pH 7.0 were pretreated with 1 mM dithiothreitol (DTT; freshly prepared) for 1 h at 20 °C, followed by washing by centrifugation (15 min at 80 000 rpm) to remove excess of DTT. The membranes were then incubated with 0.5 mM Mal-PEG for 2 h at 4 °C. The reaction was stopped by addition of 5 mM DTT, and the extent of labeling was estimated from the mobility shift of OpuABC on SDS-PAGE and Western blots.

Labeling of Cysteine Mutants and Their Purification. Membrane vesicles (10 mg of total protein) bearing wild-type or mutant OpuA were treated with 0.5% DDM for 30 min after resuspension in buffer A (50 mM KPi, 200 mM KCl plus 20% glycerol) to a final concentration of 5 mg/mL of protein. Following ultracentrifugation at 80 000 rpm for 15 min, the solubilized material was incubated with Ni-Sepharose (GE Healthcare) (20 mg of protein/mL resin) for 1 h at 4 °C in the presence of 15 mM imidazole. Next, the resin was washed with 20 column volumes of buffer A with 0.05% DDM plus 50 mM imidazole. Labeling of cysteine mutants with 25 μM fluorescein-5 maleimide (F5M) (Molecular Probes) and 50 μM Mal-PEG was done 2 h at room temperature. Following washing with 10 column volumes of buffer A with 15 mM imidazole and 0.05% DDM, labeled OpuA was eluted in buffer A with 200 mM imidazole plus 0.05% DDM. To determine the efficiency of fluorophore labeling, the molar absorption coefficient of the dye was used (F5M: ε_{495} = 68 000 M^{-1} cm^{-1}) and calculated as described by Pierce (http://www.piercenet.com/files/0359dh5.pdf). For some experiments, the labeling reaction was done for 16 h rather than 2 h. After overnight incubation of Ni-Sepharose bound OpuA, the activity of the transporter was lower than when the protein was reconstituted after 2 h, presumably due to some instability of the protein complex.

Reconstitution of Purified OpuA in Proteoliposomes. For membrane reconstitution in proteoliposomes, preformed liposomes composed of synthetic lipids were used, that are in mole percent 50% DOPE, 38% DOPG plus 12% DOPC and 50% DOPE, 38% DOPC plus 12% DOPG. Purified OpuA was incorporated at a protein-to-lipid ratio (w/w) of 1:100 as described.[22]

Reconstitution of OpuA in Bilayer Nanodisks. The purification of MSP1D1 and the reconstitution of purified OpuA in lipid bilayer nanodisks were performed as described before with only a few modifications.[23] The required amount of preformed liposomes, composed of 38% DOPG, 12% DOPC, and 49% DOPE and including 1% of DOPE-Rhodamine, was thawed and extruded through a 400 nm filter to generate large unilamellar vesicles (LUVs). After extrusion, 12 mM DDM (a concentration well above the critical micellar concentration of ~0.2 mM) was added to the liposomes, and the solution was vortexed until it became optically clear. The solubilized lipids, purified MSP1D1, and purified F5M-labeled OpuA were mixed in a final volume of 700 μL, including 50 mM KPi, pH 7.0, 12 mM DDM, 4% (w/v) glycerol, 1.43 μM OpuA, 14.3 μM MSP1D1, and 1.43 mM lipid. This gave a molar ratio of OpuA:MSP1D1:lipid of 1:10:1000. The reconstitution mixture was incubated for 60 min at 4 °C with gentle agitation, after which 500 mg of Biobeads SM2 (Biorad) was added. The mixture was incubated overnight at 4 °C with gentle agitation. The Biobeads and aggregated protein were removed by transferring the mixture to a clean Eppendorf tube, using a syringe and centrifuged at 25000*g* for 10 min at 4 °C. The supernatant was stored at −80 °C after flash-freezing in liquid nitrogen.

***In Vivo* Uptake Experiments.** For glycine betaine uptake in whole cells, *L. lactis* OpuA, containing the appropriate plasmid, was grown in GCDM plus 5 μg/mL chloroamphenicol. At OD_{600} ~ 0.5, the cells were induced with 0.01% nisin A for ~2 h, and at OD_{600} ~ 1, the cells were washed twice and resuspended to 2.5 mg/mL (OD_{600}~ 1 = 0.2 mg/mL protein) in 50 mM HEPES-methylglucamine pH 7.3. For uptake assays, the cells were diluted to 0.2 mg/mL total cell protein and pre-energized with 10 mM glucose for 5 min at 30 °C. At t = 5 min, 50 mM HEPES-methylglucamine pH 7.3 supplemented with varying amounts of sucrose (0−800 mM) plus [^{14}C]glycine betaine (final concentration of 1 mM; the total reaction mixture was 500 μL), and uptake was monitored over time. To prevent protein synthesis, 50 μg/mL chloroamphenicol was present during the assay. At given time intervals, 80 μL samples were withdrawn and diluted with 2 mL of ice-cold assay buffer of equal osmolality and immediately filtered through 0.45 μm cellulose nitrate filters under high vacuum. After washing with 50 mM HEPES-methylglucamine pH 7.3 with the appropriate sucrose concentration, the filters were dried and dissolved in 2 mL of scintillation liquid and radioactivity was determined in a scintillation counter.

Glycine Betaine Uptake in Hybrid Membranes and Proteoliposomes. For ATP-driven uptake of glycine betaine into proteoliposomes or hybrid membranes, the vesicles were extruded and washed twice in 100 mM KPi pH 7.0, and prior to the start of the assay, they were diluted to a final lipid concentration of 5 mg/mL. The assay buffer was composed of 100 mM KPi pH 7.0 plus 50−250 mM KCl. Following 2 min of equilibration at 30 °C, the transport reaction was started by the addition of 30 μM [^{14}C]glycine betaine. At given time intervals, 40 μL samples were withdrawn and diluted with 2 mL ice-cold assay buffer (isotonic with the reaction conditions) and immediately filtered through 0.45 μm cellulose nitrate filters. Following washing of the filter with another 2 mL of ice-cold assay buffer, the radioactivity was determined as described above.

ATPase Activity in Bilayer Nanodisks. The activity of OpuA was determined by measuring the ATPase activity of the transporter as described with some modifications.[24] The coupled enzyme ATPase activity assay was performed at 30 °C in 96-well plates, using a Synergy MX 96-wells plate reader. A standard measurement solution of 200 μL of 50 mM KPi, pH 7.0, 1.13 μM OpuA incorporated in nanodisks, 62 μM glycine betaine, 300 mM KCl, 4 mM phosphoenol-pyruvate, 0.3 mM NADH, and 3.5 μL of pyruvate kinase/lactic dehydrogenase (PK/LDH enzymes from rabbit muscle (Sigma-Aldrich), in 50% glycerol) was added per well. After incubation of the assay mixture for 3 min at 30 °C, 10 mM Mg-ATP, pH 7.0 was added to each well, and the absorbance at 340 nm was followed for 7 min. The ATPase activity was estimated from the derivative of the reaction progress curve, that is, between 2 and 4 min after the addition of Mg-ATP.

Fluorescence Measurements. Fluorescence measurements were performed using a Varian Cary Eclipse spectrophotometer. Resonance energy transfer (RET) between F5M-labeled OpuA and rhodamine-labeled lipid was measured in nanodisks suspended in 50 mM KPi pH 7.0. Nanodisks (4.5 μg of OpuA in a total volume of 1 mL) were irradiated at 460 (slit width of 5 nm) to excite F5M. Fluorescence emission was recorded from 500 to 700 nm. For the solubilization of the

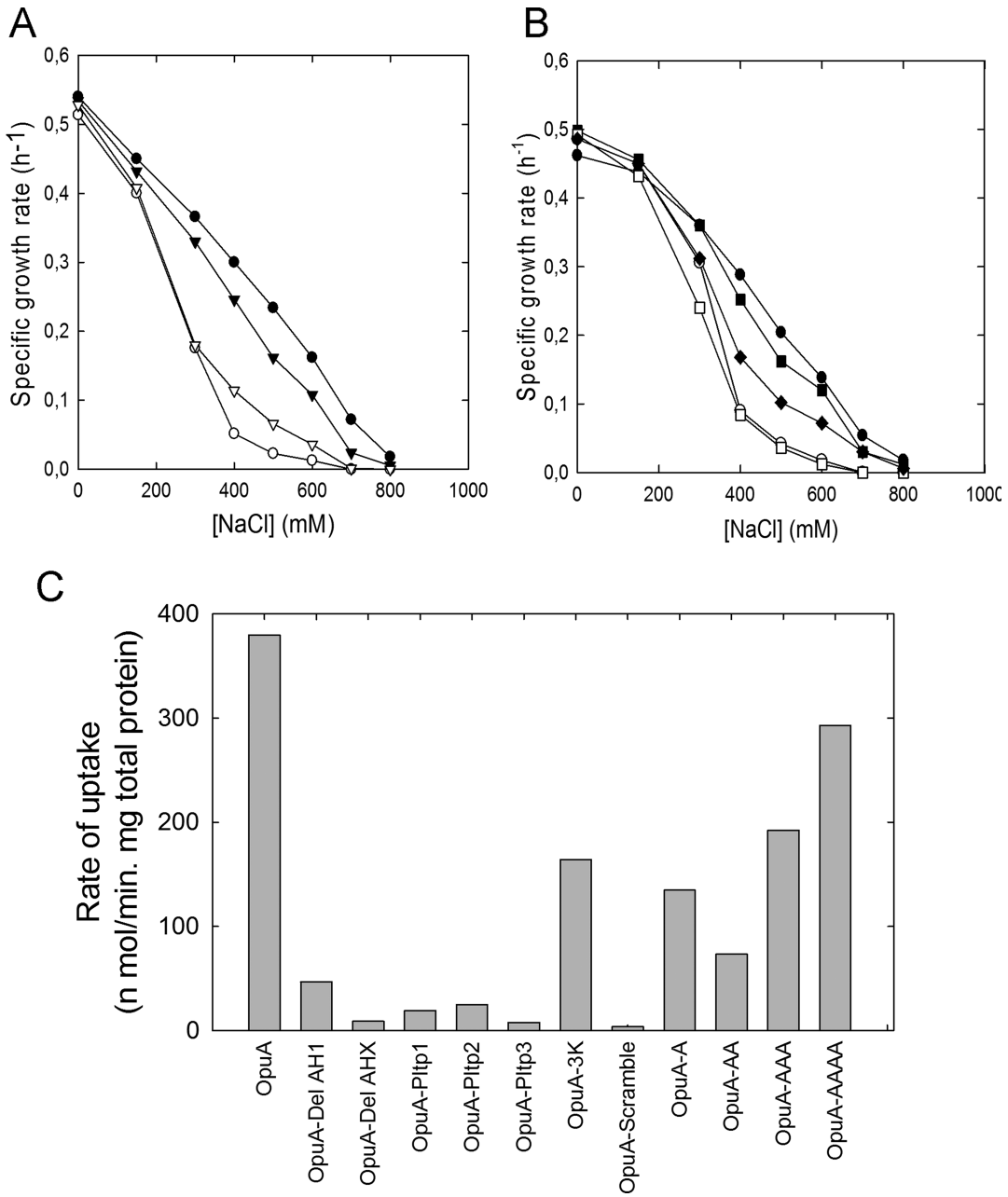

Figure 2. *In vivo* characterization of OpuA mutants. (A) Specific growth rate of *L. lactis* Opu401 carrying a control plasmid (○), pNZOpuAhis (wild-type OpuA) (●), pNZOpuAΔAH1 (▼), and pNZOpuAΔAHX (▽) grown in GCDM with different concentration of NaCl (0−800 mM) at 30 °C. Cells were cultivated to OD_{600} of ∼0.5 after which 0.01% (v/v) nisin A (filter-sterilized culture supernatant from *L. lactis* NZ9700) was added to induce the *opuA* genes; the cells were induced for 2 h before incubation with NaCl. (B) Specific growth rate of *L. lactis* Opu401 carrying pNZOpuAhis (wild-type OpuA) (●), Opu401 carrying a control, empty plasmid (○), pNZOpuA3K (■), pNZOpuAScramble (□), and pNZOpuApltp1 (♦). (C) *In vivo* uptake of glycine betaine. Cells were grown semiaerobically at 30 °C in GCDM supplemented with 5 μg/mL chloroamphenicol. For expression of OpuA, nisinA was added at 0.01% final concentration (see panel A) and cells were induced for 2 h. Uptake of [^{14}C]glycine betaine (1 mM, final concentration) was assayed in 50 mM HEPES pH 7.0 in the presence of 10 mM glucose plus 50 μg/mL chloroamphenicol and 600 mM sucrose. The concentration of cells in the assay was 0.2 mg of total protein/mL. The initial rates of uptake were determined from the linear parts of the progress curves (from 0 to 120 s); measurements were done in duplicate, and the variation in the data was within 10%.

nanodisks, 100 μL of 10% DDM (w/v) was added, and fluorescence was measured after 1 min.

To determine the ATP-binding properties of the various mutants, we used TNP-ATP as a probe (the excitation and emission wavelengths were 409 nm (slit width of 1 nm) and 540 nm (slit width of 5 nm), respectively, as described previously.[9]

Miscellaneous. Radiolabeled [*N*-methyl-^{14}C]choline chloride (55 mCi/mmol) was obtained from Amersham Biosciences, Piscataway, NJ. The [*N*-methyl-^{14}C]choline chloride was used as precursor for the synthesis of [*N*-methyl-^{14}C]glycine betaine as described.[25] Creatine kinase and creatine phosphate were obtained from Sigma Chemical Co. All other chemicals were of reagent grade and purchased from commercial sources, unless specified otherwise.

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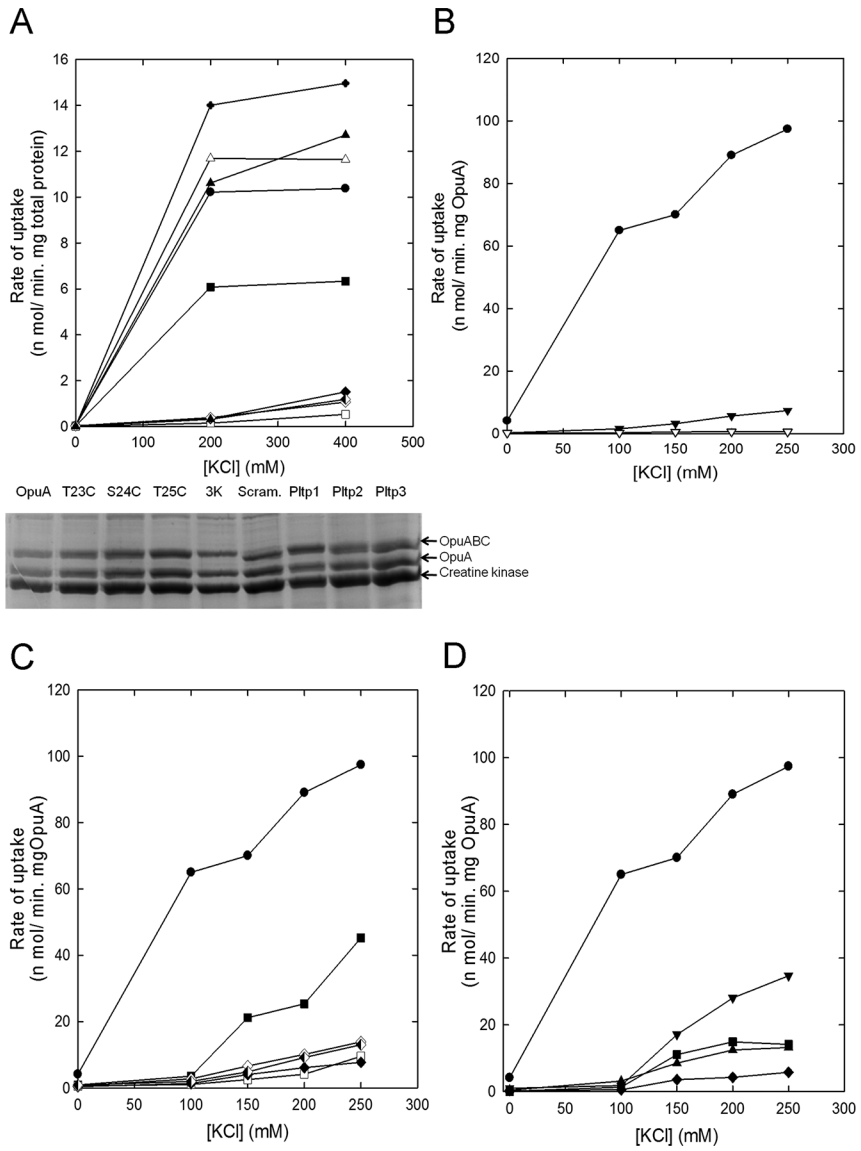

Figure 3. Effect of site-specific mutations, scrambling, or replacement of the amphipathic α-helix on OpuA-mediated transport. (A) Rate of [^{14}C]-glycine betaine uptake as a function of KCl concentration for wild-type OpuA(●), OpuA-T23C (△), OpuA-S24C (▲), OpuA-T25C (+), OpuA-3K (■), OpuA-Scramble (□) OpuA-Pltp1 (◆), OpuA-Pltp2 (◇), and OpuA-Pltp3 (half-filled diamond) in hybrid membranes prepared by fusing membrane vesicles with liposomes composed of 50 mol % DOPE, 38 mol % DOPG, and 12 mol % DOPC. The ATP regenerating system was enclosed inside the hybrid membranes. The final total protein concentration was 0.4 mg/mL. The expression levels are indicated below panel A. (B) Rate of glycine betaine uptake for wild-type OpuA (●), OpuAΔAH1 (▼), and OpuA-AHX (▽) in proteoliposomes composed of 50 mol % DOPE, 12 mol % DOPC, and 38 mol % DOPG and the ATP-regenerating system enclosed. Uptake of [^{14}C]-glycine betaine was assayed in 100 mM KPi, pH 7.0, at a final OpuA concentration of 0.04 mg/mL. (C) Rate of glycine betaine uptake for wild-type OpuA (●), OpuA-3K (■), OpuA-Scramble (□), OpuA-Pltp1 (◆), OpuA-Pltp2 (◇), and OpuA-Pltp3 (half-filled diamond) in proteoliposomes. (D) Rate of glycine betaine uptake for wild-type OpuA (●), OpuA-A (■), OpuA-2A (◆), OpuA-3A (▲), and OpuA-4A (▼) in proteoliposomes. Please note that the activity measurements in panel A are based on hybrid membranes and those of panels B–D on proteoliposomes.

RESULTS

Strategy for Engineering the Amphipathic α-Helix of OpuA. An overview of the various OpuA constructs and the domain organization is presented in Figure 1. To determine the importance of the amphipathic α-helix in the catalytic activity and regulation of OpuA, the following strategy was taken: (i) part [OpuAΔAH1] or the entire amphipathic α-helix [OpuAΔAHX] was deleted to determine whether this accessory sequence is essential; (ii) the sequence of the amphipathic α-helix was scrambled [OpuAscramble] to determine the importance of the amphipathicity; (iii) three consecutive serine residues on the polar face of the amphipathic α-helix were replaced by lysines to determine whether charge interactions (e.g., with the anionic surface of the membrane) play a role; (iv) the amphipathic α-helix was replaced with the one from the human phospholipid transfer protein PLTP[26] [OpuApltp1 and derivatives OpuApltp2 and OpuApltp3] to determine whether sequence specificity is important for function of the amphipathic α-helix; (v) up to four alanine residues were inserted C-terminal of the amphipathic α-helix [OpuA(Ala)$_n$] to influence the orientation in case the linker region forms a rigid structure; and (vi) single Cys residues were introduced at position 23, 24, and 25 of the amphipathic α-helix, yielding OpuA(T23C), OpuA(S24C), and OpuA(T25C),

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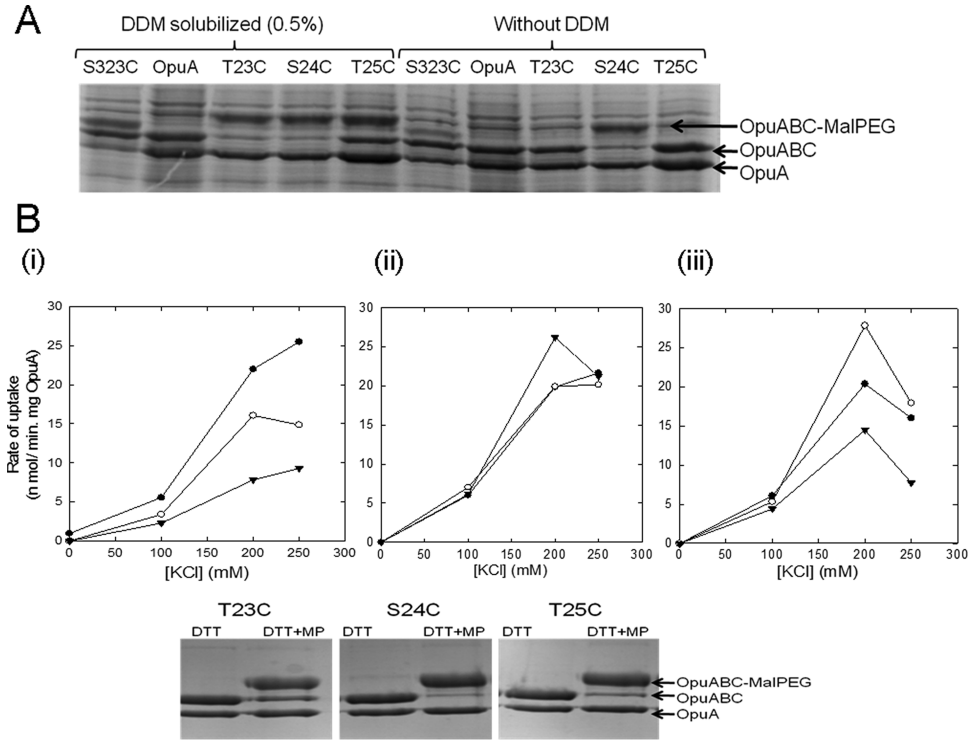

Figure 4. Accessibility of Cys residues in amphipatic α-helix. (A) Mal-PEG labeling of OpuA in membrane vesicles after and before solubilization with 0.5% DDM. Protein samples were separated on SDS-PAGE (12.5% PAA). (B) The effect of Mal-PEG labeling on glycine betaine transport activity of OpuA (i) OpuA(T23C), (ii) OpuA(S24C), and (iii) OpuA(T25C). Uptake of [^{14}C]-glycine betaine (30 μM, final concentration) by Cys mutant (●), DTT-treated Cys mutant (○), and Mal-PEG-treated Cys mutant (▼). The Mal-PEG labeling efficiency of OpuA is indicated below panel B.

to probe the accessibility of the different faces of the helix. Moreover, the single-Cys mutants labeled with fluorophore were used to determine by resonance energy transfer (RET) their position relative to the membrane surface.

The expression level of each of the variants was comparable to that of wild-type OpuA; typical gels are presented below. Membrane vesicles were isolated and each of the proteins could be purified in quantities similar to that of wild-type OpuA (SFigure 1A). Also, we did not notice diminished stability as assessed by turbidity measurements or SDS-PAGE (no signs of aggregation; SFigure 1B). TNP-ATP binding measurements yielded a dissociation constant of ~1−2 μM for wild-type and mutant OpuA. Taken together, these experiments demonstrate that mutations in the amphipathic α-helix do not have distant structural effects on the overall conformation of the transporter.

Growth Experiments. To assess the importance of the amphipathic α-helix in the osmoregulatory function of OpuA, the mutant proteins were expressed in *L. lactis* Opu401 (deletion of the chromosomal copy of the *opuA* operon). This strain is sensitive to hyperosmotic stress but can be complemented *in trans* by wild-type OpuA.[16] Because the GM17 medium contains several compatible solutes, masking the role of OpuA in protecting cells against osmotic stress, *L. lactis* Opu401/pNZOpuA and derivatives were grown in synthetic GCDM medium[2] without and with glycine betaine. The data show that deletion of the amphipathic α-helix, scrambling its sequence or replacing the helix for the one from the phospholipid-binding protein had severe impact on the ability of *L. lactis* to grow at increased osmolarity in the presence of glycine betaine (Figure 2A,B). On the contrary, substituting three of the Ser residues on the polar face for Lys or substituting the residues at position 23, 24, or 25 of the amphipathic helix for Cys had much less effect (Figures 2C and 3A). These data indicate that the amphipathic α-helix of OpuA is critical for growth of *L. lactis* in high osmolarity medium. The data also indicate that an amphipathic α-helix *per sé* is not sufficient but that the actual sequence matters. The amphipathic α-helix could determine the activity or modulate the ionic regulation of OpuA. The production of the OpuA was not severely affected by the mutations, ruling out the possibility that the amphipathic α-helix plays a role in targeting and insertion or stable integration of the protein into the membrane. To dissect the role of the amphipathic α-helix in the functioning of OpuA, we determined the uptake of glycine betaine *in vivo* and *in vitro* at various osmotic conditions. We used membrane vesicles fused with liposomes (hybrid membranes) and proteoliposomes to assess the effect of the mutations on the functionality of the OpuA transporter. The hybrid membranes were used to rule out possible artifacts due transporter complex dissociation, which happens more readily when the protein is exposed to detergents (needed for protein purification and membrane reconstitution). On the other hand, the proteoliposomal system is most defined in terms of lipid and protein composition.

Deletion of the Amphipathic α-Helix Inactivates OpuA. Consistent with the growth experiments, deletion of the amphipathic α-helix had major impact on the uptake of glycine betaine in whole cells (probed as a function of sucrose concentration; Figure 2C) and proteoliposomes (probed as a function of KCl concentration; Figure 3B). Deleting part of the helix but leaving the amino-terminus and linker region intact

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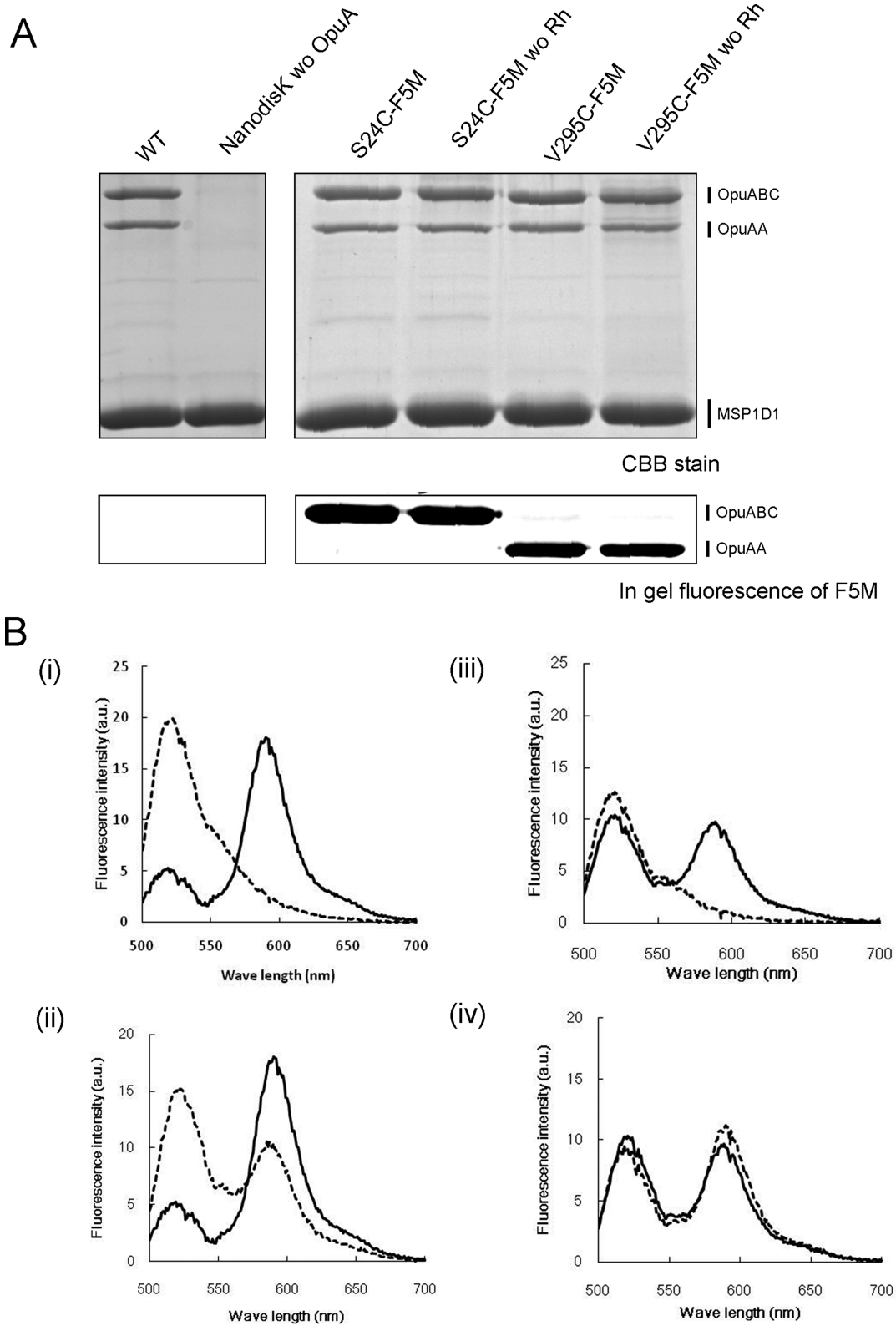

Figure 5. RET between F5M-labeled OpuA protein and rhodamine-labeled lipids. (A) Visualization by SDS-PAGE of the fluorescein-5-maleimide (F5M)-labeled proteins, reconstituted in bilayer nanodisks; 3 μg of OpuA in nanodisks was loaded. The CBB-stained gel is shown on the top; the in-gel fluorescence of F5M is shown below the CBB stained gel image. (B) Fluorescence spectra of nanodisks containing both OpuA-F5M and DOPE-Rh lipid (normal line), OpuA-F5M alone (dotted line); the excitation wavelength was 460 nm. S24C, (i); V295C, (iii). The fluorescence change of nanodisks containing both OpuA-F5M and DOPE-Rh lipid after adding 1% DDM (dotted line) was also measured. S24C, (ii); V295C, (iv).

yielded low activity; similar results were obtained in hybrid membranes (Figures 2C and 3A,B).

Engineering of the Amphipathic Sequence. Next, we scrambled the sequence of the amphipathic α-helix (OpuAs-

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cramble) or substituted its 18 amino acids for a similar helix from the human phospholipid-transfer protein PLTP (OpuAPltp1). Neither of these constructs displayed significant activity, in either intact cells (Figure 2C) or proteoliposomes (Figure 3C). On the contrary, substituting three Ser for Lys residues in the amphipathic α-helix of OpuA had only moderate impact on the activity of OpuA (Figures 3A and 4C). Taken together, it can be concluded that an amphipathic α-helix is critical for maximal activity but not for the ionic regulation of the transporter; e.g., the ionic activation profiles of wild-type OpuA, OpuA3K, and OpuA(T25C) are very similar (Figure 3A).

Positioning of the Amphipathic α-Helix. Although the amphipathic sequence of the phospholipid-transfer protein resembles that of OpuA, the direction of the hydrophobic moment is shifted by 30° (Figure.1). Depending on the rigidity of the downstream structural elements, this could affect the proper function of the amphipathic α-helix. We thus constructed OpuApltp2 and OpuApltp3, but also these transporters were inactive (Figure 3A,C). Next, we inserted one up to four alanine residues downstream of the amphipathic α-helix [OpuA(Ala)$_n$] and assayed the activity of the transporters in proteoliposomes. Clearly, inserting 2 alanines has a much more pronounced impact than 1, 3, or 4 residues (Figures 2C and 3D). Without a high-resolution structure, it is difficult to interpret these data in terms of orientation of the amphipathic α-helix, but clearly the positioning of the helix or linker region seems important.

Accessibility of Amphipathic α-Helix. The experiments described heretofore indicate that not only an amphipathic α-helix but also its particular sequence is important for OpuA activity, suggesting that the helix specifically interacts with another part of the protein, either the OpuAA or OpuABC subunit. It would also suggest that either the hydrophilic or hydrophobic face of the protein is more accessible for labeling. We thus introduced a Cys at position 23, 24, and 25 of the amphipathic helices and probed the reaction of the cysteine with the thiol-specific, membrane-impermeable probe Mal-PEG. Figure 4A shows the labeling of OpuA(T23C), OpuA(S24C), and OpuA(T25C) by Mal-PEG in membrane vesicles, either or not solubilized with DDM. The procedure for making the membrane vesicles (disruption by pressure cell at 39 000 psi) is such that the majority of the membranes is inside-out in terms of orientation of the protein in the membrane. In DDM-solubilized membranes, each of the mutants was efficiently labeled with Mal-PEG as observed from the mobility shift of the OpuABC subunit (Figure 4A). However, in the native membranes only OpuA(S24C), i.e., with the Cys on the most hydrophilic face, was labeled.

Next, we labeled each of the Cys mutants after purification in the DDM-solubilized state, while bound to Ni-Sepharose, and reconstituted the modified proteins in proteoliposomes. Unlike wild-type OpuA, each of the Cys mutants was quantitatively labeled with Mal-PEG. The effect of Mal-PEG labeling on uptake activity was determined. Glycine betaine uptake by OpuA(S24C) was not affected by the labeling, whereas that of OpuA(T23C) and OpuA(T25C) was significantly decreased, consistent with a disruption of the interaction of the amphipathic α-helix with the surface of the protein (Figure 4B).

Interaction between Membrane Lipids and Amphipathic Helix. To determine the position of the amphipathic α-helix relative to the membrane surface, we performed RET measurements and benchmarked the data against the distance between the CBS module and the membrane. The S24C residue in the amphipathic α-helix and V295C in the CBS1 domain were labeled with fluorescein-5-maleimide (F5M) and reconstituted in bilayer nanodisks, which contained 2 mol % of DOPE-Rhodamine (DOPE-Rh) as described in Materials and Methods. The labeling efficiency was over 80% in both cases. Indeed, the F5M fluorescence was uniquely present in OpuABC in the case of S24C and in OpuAA in the case of V295C (Figure 5A), indicating that labeling of the Cys mutants was very specific. The ATPase activity of OpuA(S24C) and OpuA(V295C) was comparable to that of wild-type OpuA (STable 2). We observed that the donor fluorescence of F5M at 520 nm was significantly lower in the presence of DOPE-Rh than in the absence of fluorescence acceptor, indicating a high RET signal for OpuA-S24C (Figure 5B(i)). This result indicates that the amphipathic α-helix is located in proximity of the membrane surface. Upon breaking of the nanodisk structure by 1% DDM, the RET signal disappeared (Figure 5B(ii)). Furthermore, a mixture of OpuA(S24C)−F5M reconstituted without Rhodamine lipids or empty DOPE-Rh nanodisks did not show a significant RET signal (data not shown), indicating that internanodisks RET does not occur. The RET signal was very low in the case of OpuA(V295C) (Figure 5B(iii,iv)), suggesting that the CBS1 moiety of the CBS module is spaced distantly from the membrane.

DISCUSSION

This research was aimed at the elucidation of the role of the N-terminal amphipathic α-helix in the functioning of the ABC transporter OpuA. We analyzed all the OpuA homologues present in the databases to date and found that 30 out of a total of ∼1900 are predicted to possess an amphipathic α-helix. The well-studied homologues ProU from *E. coli*, OpuA from *B. subtilis* or OpuC from *P. syringae* do not contain the amphipathic α-helix. In fact, the amphipathic α-helix is predominantly present in OpuA homologues in Gram-positive bacteria. We now show that the amphipathic α-helix is mandatory for proper functioning of OpuA from *L. lactis*. The various truncations and modifications made indicate that the ionic regulation of OpuA is not mediated by the amphipathic α-helix.

What have we learned from the engineering of the amphipathic α-helix of OpuA? First, not only is the amphipathicity important but also a specific sequence as an equivalent structural element from the human phospholipid-transfer protein could not replace the endogenous helix. Second, three Ser residues on the hydrophilic face of the amphipathic α-helix could be replaced by Lys, suggesting that the hydrophobic rather than hydrophilic face of the helix is making critical contacts with the protein. Consistent with this notion are the labeling studies with Mal-PEG, showing that a 5 kDa moiety only affects OpuA when coupled near the hydrophobic face. Third, introducing one up to four Ala residues in the region C-terminal of the amphipathic α-helix had variable effects, suggesting that the linker region is crucial for the orientation/positioning of the helix and its interaction with the protein. Fourth, in the detergent DDM, Cys residues on either face of the amphipathic α-helix are accessible for labeling with Mal-PEG, while only S24C is accessible for membrane-embedded OpuA. Most probably, the detergent is disrupting the apolar interactions of the amphipathic α-helix with the protein surface, on either the OpuABC or the OpuAA subunit. We do not expect the interaction to take place via the

membrane surface because of the sequence requirements; i.e., the amphipathic α-helix from the phospholipid-transfer protein could not substitute the endogenous helix, not even after tuning of its sequence (see constructs OpuApltp1, OpuApltp2, and OpuApltp3). Fifth, the RET measurements position the amphipathic α-helix much closer to the membrane surface than the CBS1 of the CBS module.

CONCLUSION

We show that specific interactions between the hydrophobic face of the N-terminal amphipathic α-helix and the core of OpuA are required for activity. The biosynthesis, membrane insertion, and ionic regulation of OpuA are not affected by the amphipathic α-helix. The amphipathic α-helix is mostly found in OpuA homologues in the phyla Firmicutes (class *Bacilli*), Actinobacteria (class *Actinobacteria*), and Proteobacteria (class *α-Proteobacteria*) and may be an adaptation to the specific membrane environment in which the transporter needs to function.

ASSOCIATED CONTENT

Supporting Information

Primers used for construction of the mutants, the labeling efficiency and ATPase activity of the Cys mutants, and the purification and stability of the OpuA derivatives. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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