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Ion Specificity and Ionic Strength Dependence of the Osmoregulatory ABC Transporter OpuA*

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The ATPase subunit of the osmoregulatory ATP-binding cassette transporter OpuA from *Lactococcus lactis* has a C-terminal extension, the tandem cystathionine β-synthase (CBS) domain, which constitutes the sensor that allows the transporter to sense and respond to osmotic stress (Biemans-Oldehinkel, E., Mahmood, N. A. B. N., and Poolman, B. (2006) *Proc. Natl. Acad. Sci. U. S. A.* 103, 10624–10629). C-terminal of the tandem CBS domain is an 18-residue anionic tail (DIPDEDEVEEIEKEEENK). To investigate the ion specificity of the full transporter, we probed the activity of inside-out reconstituted wild-type OpuA and the anionic tail deletion mutant OpuAΔ12; these molecules have the tandem CBS domains facing the external medium. At a mole fraction of 40% of anionic lipids in the membrane, the threshold ionic strength for activation of OpuA was ~0.15, irrespective of the electrolyte composition of the medium. At equivalent concentrations, bivalent cations (Mg^{2+} and Ba^{2+}) were more effective in activating OpuA than NH_4^+, K^+, Na^+, or Li^+, consistent with an ionic strength-based sensing mechanism. Surprisingly, Rb^+ and Cs^+ were potent inhibitors of wild-type OpuA, and 0.1 mM RbCl was sufficient to completely inhibit the transporter even in the presence of 0.2 M KCl. Rb^+ and Cs^+ were no longer inhibitory in OpuAΔ12, indicating that the anionic C-terminal tail participates in the formation of a binding site for large alkali metal ions. Compared with OpuAΔ12, wild-type OpuA required substantially less potassium ions (the dominant ion under physiological conditions) for activation. Our data lend new support for the contention that the CBS module in OpuA constitutes the ionic strength sensor whose activity is modulated by the C-terminal anionic tail.

Bacteria respond to osmotic stress by adjusting the pools of cellular osmolytes, thereby keeping the volume, crowding of the cytoplasm, and the turgor within certain limits (1). Following an osmotic upshift of the external medium, cells accumulate and/or synthesize compatible solutes to compensate the loss of water and decrease in cell volume. How an enzyme or transporter senses osmotic stress and transduces the signal into an activity change represents one of the major challenges in the field of cellular osmoregulation. Sensing of osmotic stress is best understood for a few osmoregulatory transporters and mechanosensitive channel proteins (2). The osmoregulatory transporters protect cells against hyperosmotic stress and ultimately plasmolysis by accumulating compounds like glycine betaine or proline against large concentration gradients. The mechanosensitive channel proteins protect cells against hypoosmotic stress, and ultimately lysis, by rapid efflux down the concentration gradient of these and other osmolytes. The mechanism of osmosensing and the regulation of the mechanosensitive channels are entirely different from that of the osmoregulatory transporters and are discussed elsewhere (2).

The best understood osmoregulatory transporters are the ProP protein from *Escherichia coli*, the BetP protein from *Corynebacterium glutamicum*, and the OpuA system from *Lactococcus lactis* (3–5). Whereas ProP and BetP are driven by the electrochemical proton and sodium gradient across the membrane, respectively, OpuA is driven by ATP. Despite the differences in energy coupling mechanisms and protein structures (the polypeptides belong to entirely different protein families), there is a common denominator in the osmosensing mechanisms. When reconstituted in proteoliposomes, each of the systems is activated by an increase in concentration of luminal ions. For BetP, the larger alkali ions K^+, Rb^+ and Cs^+ are more effective in activating than Na^+ or NH_4^+ (5, 6), whereas ProP and OpuA seem largely ion-aspecific (3, 7), but extensive titration experiments have not been reported. For the ATP-binding cassette transporter OpuA, there is strong evidence that the protein senses cytoplasmic ion concentrations (8). This sensing is mediated by a regulatory domain, known as the CBS[2] module, whose activity is dependent on the fraction of charged lipids in the membrane and the ionic strength. At physiologically relevant fractions of anionic lipids and low ionic strength, the transporter is inactive but can be "switched on" rapidly by increasing the ionic strength (*i.e.* following a decrease in cell or vesicle volume in response to an osmotic upshift). The activation-deactivation process is entirely reversible.

The OpuA transporter is composed of two substrate-binding translocator subunits and two ATPase subunits (9, 10). Covalently linked to the ATPase subunit are two CBS domains, here referred to as the tandem CBS domain. The functional transporter complex is a dimer, giving OpuA a total of four CBS

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[2] The abbreviations used are: CBS, cystathionine β-synthase; DDM, *n*-dodecyl-β-D-maltoside; DOPG, L-α-dioleoyl-phosphatidylglycerol; DOPE, L-α-dioleoyl-phosphatidylethanolamine; DOPC, L-α-dioleoyl-phosphatidylcholine.

domains. From x-ray crystallography studies (11, 12), it is clear that two CBS domains combine to form a stable structure. Moreover, two of these tandem CBS structures have a strong tendency to dimerize (the 2 × 2 module (8)). Although the role of dimerization of two tandem CBS domains in OpuA has not been established, it seems likely that for ionic strength sensing the CBS 2 × 2 module is needed.

In this study, we report on the ion specificity of OpuA by precisely monitoring the activity of inside-out reconstituted transporter molecules and titration of the external ion composition and ionic strength. We demonstrate that the species-specific C-terminal tails of the CBS module tune the ionic sensitivity of the transporter.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

L. lactis strain Opu401 (derivative of NZ9000 with chromosomal *opuA* genes deleted (8)) was complemented in *trans* with plasmids (pNZOpuAHis and derivatives) carrying wild-type or mutant *opuA* genes. The strains were grown semi-anaerobically at 30 °C in a medium containing 2% (w/v) gistex LS (Strik BV, Eemnes, Netherlands) and 65 mM potassium phosphate (potassium P_i), pH 6.5, supplemented with 1.0% (w/v) glucose and 5 μg/ml chloramphenicol. Cells were grown in a 2-liter pH-regulated fermentor to an A_{600} of 2.0, followed by induction with 0.1% (v/v) culture supernatant of the nisin A-producing strain NZ9700. The cells were harvested, and membrane vesicles were prepared according to standard procedures (9).

Purification and Membrane Reconstitution of OpuA

Membrane vesicles were resuspended in buffer A (50 mM potassium P_i, pH 8.0, 200 mM KCl, 20% glycerol) to a final concentration of 5 mg/ml protein and solubilized with 0.5% (w/v) *n*-dodecyl-β-D-maltoside (DDM) for 30 min on ice. After centrifugation, the solubilized material was incubated with Ni^{2+}-nitrilotriacetic acid resin (0.5 ml of resin per 10 mg of membrane protein) 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 supplemented with 0.05% (w/v) DDM and 15 mM imidazole. The His-tagged proteins were eluted from the column with 3 column volumes of buffer A supplemented with 0.05% (w/v) DDM and 200 mM imidazole.

Purified OpuA and derivatives were reconstituted in liposomes composed of the desired lipids (Avanti Polar Lipids, AL) as described (9, 13) with some modifications. Briefly, preformed liposomes (4 mg/ml; DOPG/DOPE/DOPC at mol % of 40:40:20) were destabilized with Triton X-100, and the physical state of the liposomes was monitored by measuring the turbidity of the suspension at 540 nm. Unless stated otherwise, liposomes were destabilized to a point just beyond "detergent saturation" (14) and mixed with purified protein in a 100:1 ratio (w/w). The mixture was incubated for 30 min at 4 °C under gentle agitation. To remove the detergent, 40 mg/ml wet weight polystyrene beads (Bio-beads SM2) were added, followed by 15 min of incubation at 4 °C. Bio-beads SM2 were added four times, and the incubation times were 15 and 30 min, overnight, and 1 h, respectively, all at 4 °C. After 10× dilution with 50 mM potassium P_i, pH 7.0 (to lower the glycerol concentration), the proteoliposomes were collected by centrifugation for 1.5 h at 150,000 × *g* and 4 °C, resuspended to 20 mg/ml of lipid in 50 mM potassium P_i, pH 7.0, flash-frozen, and stored in liquid nitrogen.

ATP-regenerating System

In a typical experiment (final concentrations are indicated), MgATP at 10 mM (prepared from 10 mM $MgSO_4$ and 10 mM Na_2ATP) and adjusted to pH 7.0 together with creatine kinase (2.4 mg/ml; Roche Diagnostics) and creatine phosphate (24 mM, disodium salt; Sigma) in 50 mM potassium P_i, pH 7.0, were mixed together with the proteoliposomes (20 mg of lipid/ml) and frozen in liquid nitrogen. The mixture was slowly thawed (tubes with proteoliposomes were placed in contact with the wall of a styrofoam block) at room temperature. The freeze-thaw cycles were repeated five times, after which proteoliposomes were made homogenous by extrusion through polycarbonate filters (200 nm pore size). Subsequently, the external components were removed by centrifugation at 300,000 × *g* for 15 min, and the proteoliposomes were washed two times before resuspension in isotonic media, in most experiments 100 mM potassium P_i, pH 7.0.

Transport Assays

Uptake Assay—ATP-driven uptake of glycine betaine in proteoliposomes was conducted as described before (9), with some modifications. Briefly, concentrated proteoliposomes (80 mg/ml of lipid) were diluted 3.2-fold into isotonic (100 mM potassium P_i, pH 7.0) or hypertonic medium (50 mM potassium P_i, pH 7.0, plus 0.45 M sucrose); hypertonic media were used to osmotically activate the transporter. Following incubation for 2 min at 30 °C, the transport reaction was initiated by addition of [^{14}C]glycine betaine (Amersham Biosciences) to a final concentration of 45 μM (more than 10-fold above the K_m value for transport and the K_D value for glycine betaine binding) (15). At given time intervals, 8-μl samples were taken and diluted with 2 ml of ice-cold isotonic medium (100 mM potassium P_i, pH 7.0, or 50 mM potassium P_i, pH 7.0, supplemented with 0.45 M sucrose). The samples were filtered rapidly through 0.45-μm pore-size cellulose nitrate filters (Schleicher & Schuell) and washed twice with 2 ml of assay medium. The radioactivity on the filters was determined by liquid scintillation counting. The uptake reaction was followed for 12–13 min, and part of the reaction mixture (preloaded proteoliposomes) was kept on ice for subsequent efflux experiments (see under "Efflux Assay").

Efflux Assay—The proteoliposomes, preloaded with [^{14}C]glycine betaine (50–100 nmol/mg OpuA, corresponding to 0.50–1 nmol/mg lipid or 0.5–1 mM concentration), were diluted five times into 10 mM potassium P_i, pH 7.0, and preincubated for 3 min at 30 °C. Following addition of 10 mM MgATP (prepared from 10 mM $MgSO_4$ plus 10 mM Na_2ATP or 10 mM K_2ATP) and incubation for another 2 min, [^{14}C]glycine betaine efflux was initiated by diluting the mixture 2-fold with pre-warmed 10 mM potassium P_i, pH 7.0, supplemented with different concentrations (up to 250–350 mM) of KCl, NaCl, LiCl, RbCl, CsCl, NH_4Cl, $MgCl_2$, or $BaCl_2$. The rate of efflux was estimated from the decrease in internal [^{14}C]glycine betaine concentration, which was linear for a period of up to 1.5–2 min (4–5 time points). At given time intervals, 75-μl samples were withdrawn and diluted with 2 ml of ice-cold isotonic assay medium. Rapid

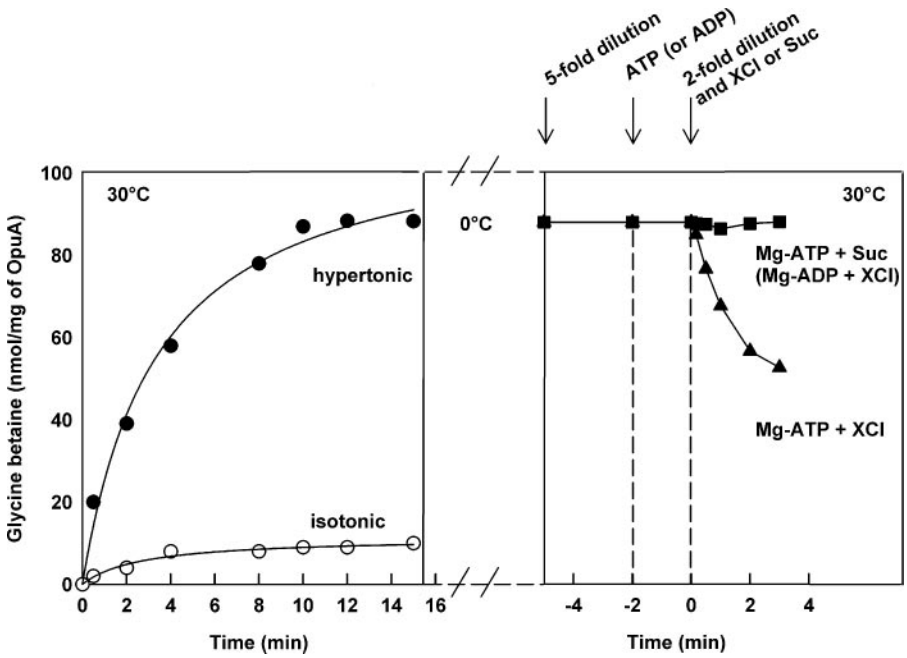

FIGURE 1. **Setup of a typical experiment and demonstration of glycine betaine uptake and efflux by OpuA.** Uptake at hypertonic (●) and isotonic (○) conditions are shown in the *left panel*. Following preloading with [^{14}C]glycine betaine and assaying for actual uptake, part of the proteoliposome sample was stored on ice for subsequent measurements of glycine betaine efflux via inside-out reconstituted OpuA (*right panel*). Following storage on ice, samples were diluted and equilibrated for 3 min at 30 °C, and then Mg-ATP (or Mg-ADP, negative control) was added (*2nd arrow*) to energize inside-out oriented OpuA. After 2 min, efflux was initiated by the addition of salt, *X*Cl (activating conditions, ▲), or the equivalent osmolality of sucrose (*Suc*) (nonactivating conditions, ■); the addition was done by a 2-fold dilution with pre-warmed medium of the appropriate composition.

filtration, washing of the filter, and counting of radioactivity were done as described under "Uptake Assay."

Data Analysis and Calculations

The reported average rates of glycine betaine efflux plus the standard error were calculated from n independent experiments, as specified in the figure legends, and were based on activity assays with protein purified and reconstituted on different days. The rate of glycine betaine efflux (v) as a function ion concentration ([ion]) generally followed a sigmoid function (Equation 1) from which the maximal activity (A_{max}), the ion activation constant (salt concentration required to attain half-maximal activity, K_{ion}), and a constant n (related to the slope of the curve and reflecting the cooperativity for salt) were obtained,

$$v = A_{max} \cdot \left(1 + \frac{K_{ion}^{n}}{[Ion]^{n}}\right)^{-1} \quad \text{(Eq. 1)}$$

This analysis is similar to the one used by Wood and co-workers (16) for the ProP system, except that here only the response to salt concentration is considered. Moreover, we empirically define the threshold ion concentration (T_{ion}) for activation as the point where the tangent of the sigmoid intersects the x axis (2).

The ionic strength (I) was calculated from the concentrations of ions, according to $I = \frac{1}{2}\Sigma\, c_i z_i^2$, where c_i and z_i are the concentration and valence of the ith ion in the solution. Ions taken into account include K^+, HPO_4^{2-}, $H_2PO_4^-$, ATP^{4-}, Mg^{2+}, SO_4^{2-}, Na^+ and assuming that Mg^{2+} is largely complexed with ATP^{4-} (dissociation constant ~0.1 mM). Thus, Mg-ATP^{2-} rather than ATP^{4-} was taken as the prevailing ionic species in the solution.

RESULTS

Experimental Setup

Previous work has shown that for maximal activity of OpuA, proteoliposomes should contain 25–40 mol % of anionic lipids (3, 8). We have now used 40 mol % of L-α-dioleoylphosphatidylglycerol (DOPG) to observe full osmoregulatory activity (maximal activity at high salt; less than 1% activity below threshold levels of salt) and to be within the physiological range of anionic lipid concentrations for *L. lactis* (17). The reconstitution procedure yielded both right-side-in and inside-out oriented OpuA molecules, as described previously (18). In fact, by freezing-thawing the proteoliposomes multiple times, the orientation became random as could be inferred from the initial rates of uptake and efflux. The basal medium in which the transport assays were carried out contained 100 mM potassium phosphate, pH 7.0 (isotonic medium), or 50 mM potassium phosphate, pH 7.0, plus 450 mM sucrose (hypertonic medium). Hypertonic conditions caused the proteoliposomes to shrink and the luminal ionic strength to increase. The increase in internal ion concentration activates right-side-in OpuA molecules (8), and accumulation of glycine betaine proceeds at the expense of luminal ATP. After 10–15 min of uptake, the glycine betaine level plateaus because of the decrease in ATP and the build up of ADP; the right-side-in oriented OpuA molecules are inactivated under these conditions (19). When Mg-ATP is now added to the external medium, glycine betaine exits the vesicles via the inside-out oriented OpuA molecules, provided the external ionic strength is sufficiently high (see below); the setup of a typical experiment and the activities of right-side-in and inside-out oriented OpuA are shown in Fig. 1. Because the ion-sensing CBS module is on the cytoplasmic face of OpuA, the inside-out oriented molecules allow direct access of the sensor from the external medium. Proteoliposomes pre-loaded with glycine betaine via right-side-in oriented OpuA could be stored on ice for several hours without measurable leak of substrate or loss of activity of inside-out oriented OpuA (data not shown). This enabled us to stockpile batches of proteoliposomes, pre-loaded with [^{14}C]glycine betaine, for subsequent analysis of glycine betaine efflux.

Ion Specificity of OpuA Regulation

Alkali Metals—Fig. 2*A* shows transport of glycine betaine via inside-out oriented OpuA at different concentrations of KCl added to the basal medium (10 mM potassium P_i, pH 7.0, 10 mM $MgSO_4$ plus 10 mM Na_2ATP or 10 mM K_2ATP, both corre-

sponding to an ionic strength of ~0.07). Addition of 50 mM KCl, resulting in a total ionic strength of ~0.12, or less was insufficient to activate OpuA (▼ in Fig. 2*A*). Maximal activity was reached around 250 mM KCl, corresponding to an ionic strength of ~0.32. The *X*Cl concentration dependence of OpuA is plotted for $X = K^+$, Na^+, Li^+, Rb^+, and Cs^+ in Fig. 2*B*; the results were the same for ATP as sodium or potassium salt (not shown). The data were fitted to a sigmoid function from which the maximal activity (A_{max}) and ion activation constant (K_{ion}) could be estimated (see "Materials and Methods"). Although the A_{max} was 2-fold higher with KCl and NaCl than with LiCl, the K_{ion} values (~125 mM *X*Cl plus basal medium components) and the threshold values for activation (T_{ion} values) were similar (~75 mM *X*Cl plus basal medium components). Also, the apparent cooperativity for salt (*n* in Equation 1) did not differ significantly for KCl, NaCl, and LiCl and varied between 3.5 and 4.5. Surprisingly, Rb^+ and Cs^+ ions were unable to activate OpuA.

Non-metal Ions—Next, we tested NH_4^+ as an ion that does not belong to the group 1 elements (alkali metals). Fig. 3*A* shows that NH_4Cl activates OpuA but much less effectively than KCl. Although NH_4^+ will be membrane-impermeant on the time scale of the measurements, NH_3 is highly membrane-permeable, and the influx of NH_3 and subsequent protonation will alkalinize the proteoliposome lumen. To prevent build up of a pH gradient, the experiments of Fig. 3*A* were repeated in the presence of the protonophore 3,5-di-*tert*-butyl-4-hydroxy benzylidene-malonitrile (SF6847). Fig. 3*B* shows that in the presence of SF6847, NH_4Cl was much more effective than in the absence of the protonophore, indicating that the increase in internal pH was inhibiting the transporter. In fact, in the presence of SF6847, A_{max}, K_{ion}, *n*, and T_{ion} values were very similar for NH_4Cl and KCl (Fig. 3*C*). As expected SF6847 had no effect on the activation of OpuA by KCl, because a pH gradient is not formed under these conditions.

Alkaline Earth Metals—For an ionic strength-based sensing mechanism, one expects bivalent cations to be more effective than monovalent ions. However, bivalent cations also cause membranes to aggregate and fuse, and ultimately leakage of solutes occurs. Fig. 4*A* shows that the group 2 alkaline earth metal ions Mg^{2+} and Ba^{2+} at concentrations above ~25 mM caused glycine betaine to aspecifically leak from the proteoliposomes; 10 mM Mg-ADP instead of Mg-ATP was added to

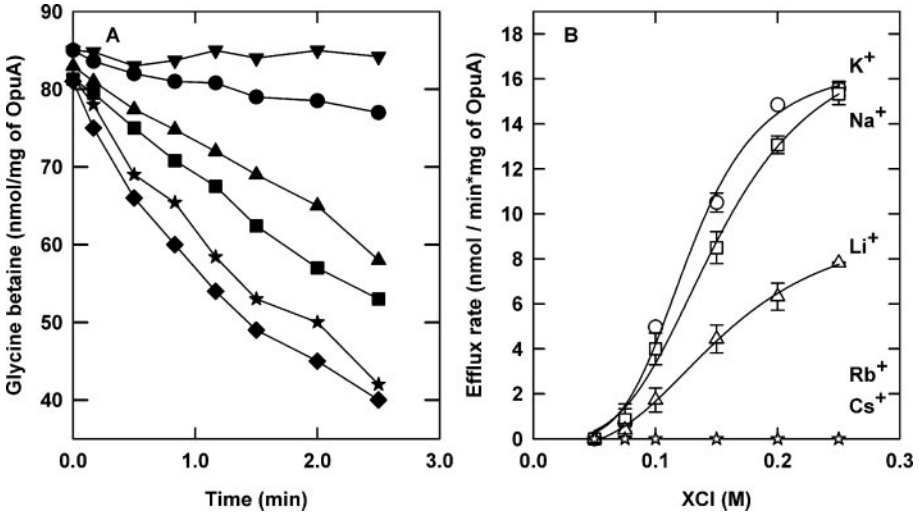

FIGURE 2. **Salt dependence of inside-out reconstituted OpuA.** *A,* time course of glycine betaine efflux at different concentrations of KCl (50 mM, ▼; 75 mM, ●; 100 mM, ▲; 150 mM, ■; 200 mM, ★; 250 mM, ♦). *B,* initial rate of glycine betaine efflux as a function of cation chloride (*X*Cl) concentration. The *error bars* indicate the S.E. of $n = 6$ independent experiments, which are six independent protein purifications-membrane reconstitutions-activity assays, carried out on different days.

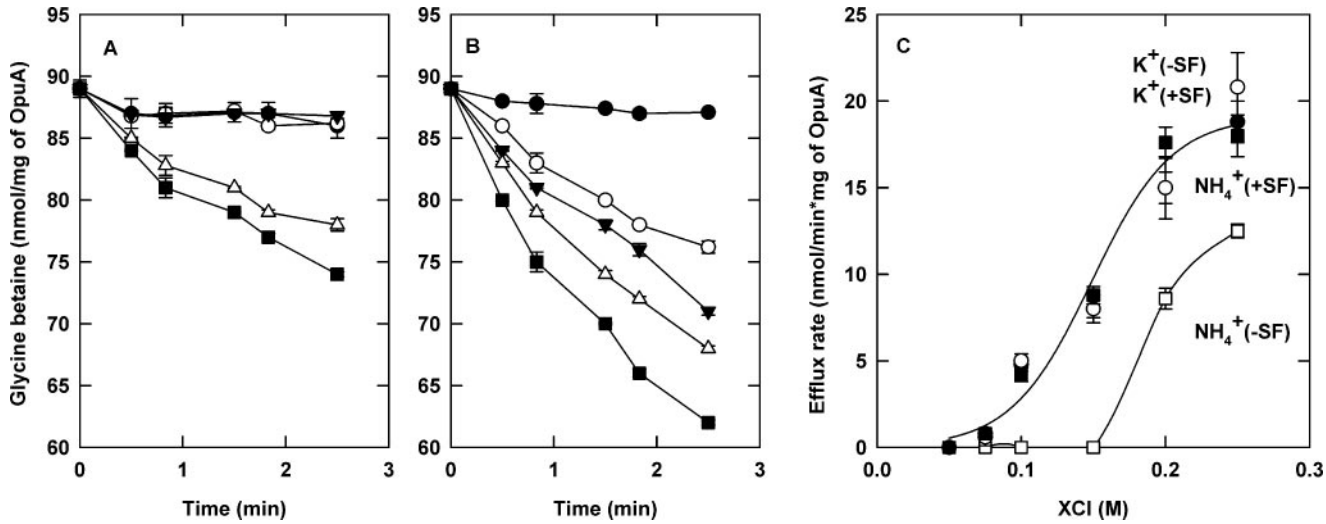

FIGURE 3. **NH_4Cl activation of inside-out reconstituted OpuA.** Time course of glycine betaine efflux with increasing NH_4Cl (75 mM, ●; 100 mM, ○; 150 mM, ▼; 200 mM, △; 250 mM, ■) concentration in the absence (*A*) or presence of the protonophore SF6847 (2 μM) (*B*); in the figure, SF6847 is indicated by *SF*. The initial rate of glycine betaine efflux as a function of KCl (●, ○) or NH_4Cl (■, □) concentration and in the absence (*open symbols*) and presence (*closed symbols*) of SF6847 (2 μM) is presented in *C*. The *error bars* indicate the S.E. of $n = 2$ experiments, as defined in the legend to Fig. 2.

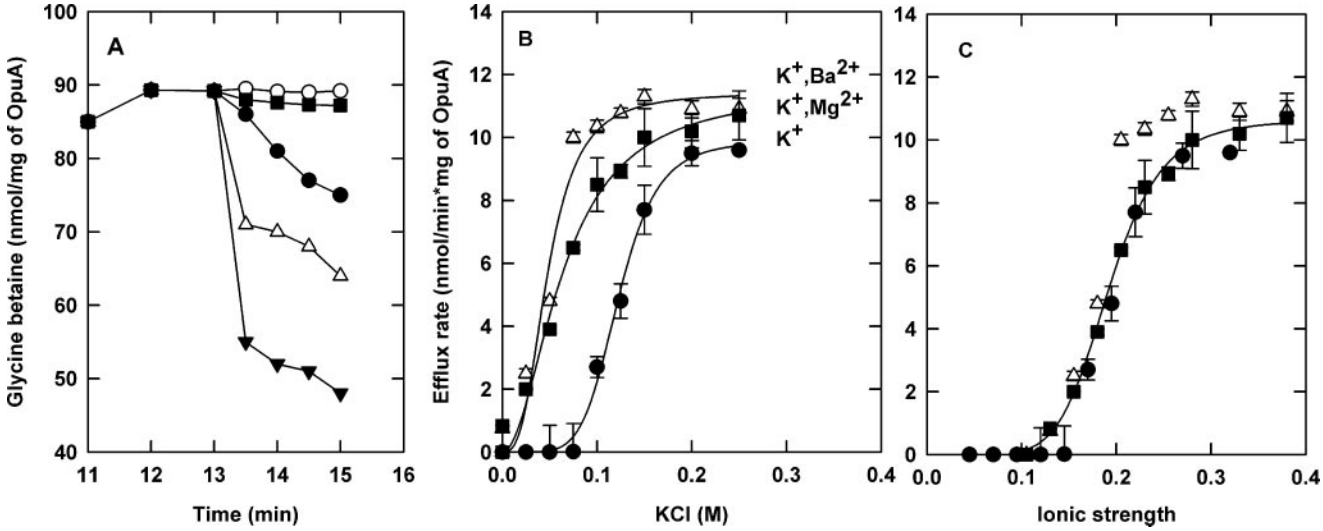

FIGURE 4. **Effect of alkaline earth metals on the activation of OpuA.** *A,* time course of glycine betaine efflux with 10 mM MgADP plus 20 mM $MgCl_2$ (or $BaCl_2$) (○), 10 mM MgATP plus 20 mM $MgCl_2$ (●), 10 mM MgADP plus 25 mM $MgCl_2$ (■), 10 mM MgADP plus 30 mM $MgCl_2$ (△) and 10 mM MgADP plus 50 mM $MgCl_2$ (▼). *B,* initial rate of glycine betaine efflux as a function of KCl concentration in the absence (●) or presence of 20 mM $MgCl_2$ (■) or 20 mM $BaCl_2$ (△). The initial rate of glycine betaine efflux as function of ionic strength is shown in *C*. The *error bars* indicate the S.E. of $n = 2$ experiments (see legend to Fig. 2).

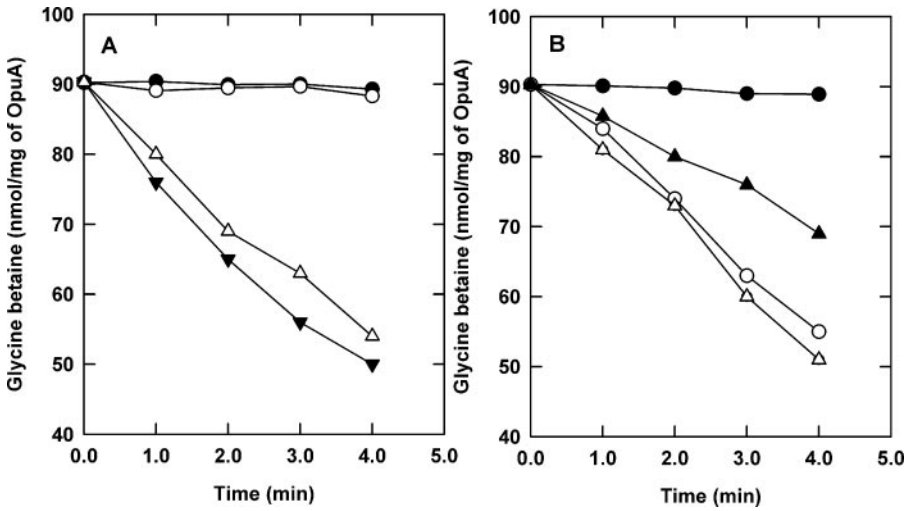

FIGURE 5. **Rb^+ ions inhibit OpuA.** *A,* ATP-driven glycine betaine efflux was triggered by the addition of 1.2 mM tetracaine in the absence (▼) or presence (○) of 0.1 mM RbCl or absence (△) or presence of 0.2 M KCl (●). For each condition, the external medium was made of the same osmolality by adjusting with sucrose. *B,* ATP-driven glycine betaine efflux with 0.2 M KCl (●, ○) or with 0.25 M KCl (▲, △) in the absence (*open symbols*) or presence (*closed symbols*) of 0.1 mM RbCl.

prevent transport via OpuA and to keep the ionic composition of the assay medium similar otherwise. These and other control experiments indicated that the integrity of the proteoliposomes was maintained up to at least 20 mM of Mg^{2+} and Ba^{2+}, which in terms of ionic strength would correspond to 80 mM of monovalent ions. By keeping $MgCl_2$ and $BaCl_2$ at 20 mM and varying the concentration of KCl from 0 to 250 mM, we could show that the bivalent cations were more effective in activating OpuA than KCl (Fig. 4*B*). The collective data conformed to a nearly unique relationship when plotted as a function of ionic strength (Fig. 4*C*).

Rb^+ and Cs^+ Ions Inhibit Wild-type OpuA

Given the wide spectrum of ions capable of activating OpuA, it was puzzling that Rb^+ and Cs^+ had no effect (Fig. 2*B*). This could imply that ionic strength is not the principal parameter that is sensed and through which the activity of the transporter is controlled. Alternatively, it is possible that Rb^+ and Cs^+ do activate but, in addition, exert a secondary inhibitory effect that is distinct from the sensing of ionic strength. To discriminate between these two scenarios, an alternative means of OpuA activation was explored. We have shown previously that the activation profile of OpuA is dependent on the fraction of anionic lipids in the membrane. The higher the fraction of anionic lipids, the more salt was needed for activation. Most importantly, the activation profiles shifted to lower salt concentrations when cationic amphipaths, like tetracaine, were employed (3). These membrane-active compounds insert into the bilayer, slowly flop-flip from the outer to the inner leaflet of the membrane, and "neutralize" part of the negative surface charge originating from the anionic lipids. The flop-flip to the inner membrane leaflet was a prerequisite for the shift in the activation profiles of right-side-in oriented OpuA. More importantly, we now show that inside-out oriented OpuA, reconstituted in membranes with 40% of anionic lipid (DOPG), was instantaneously activated by tetracaine (Fig. 5*A*), providing further support for the notion that lowering of the negative surface charge at the cytoplasmic face of OpuA is required for activation. Furthermore, this assay allows one to activate the transporter in the absence of salt, simply by adding low concentrations of a cationic amphipath. In fact, 1.2 mM tetracaine proved equally effective as 0.2 M KCl. Tetracaine is effective at this apparent low

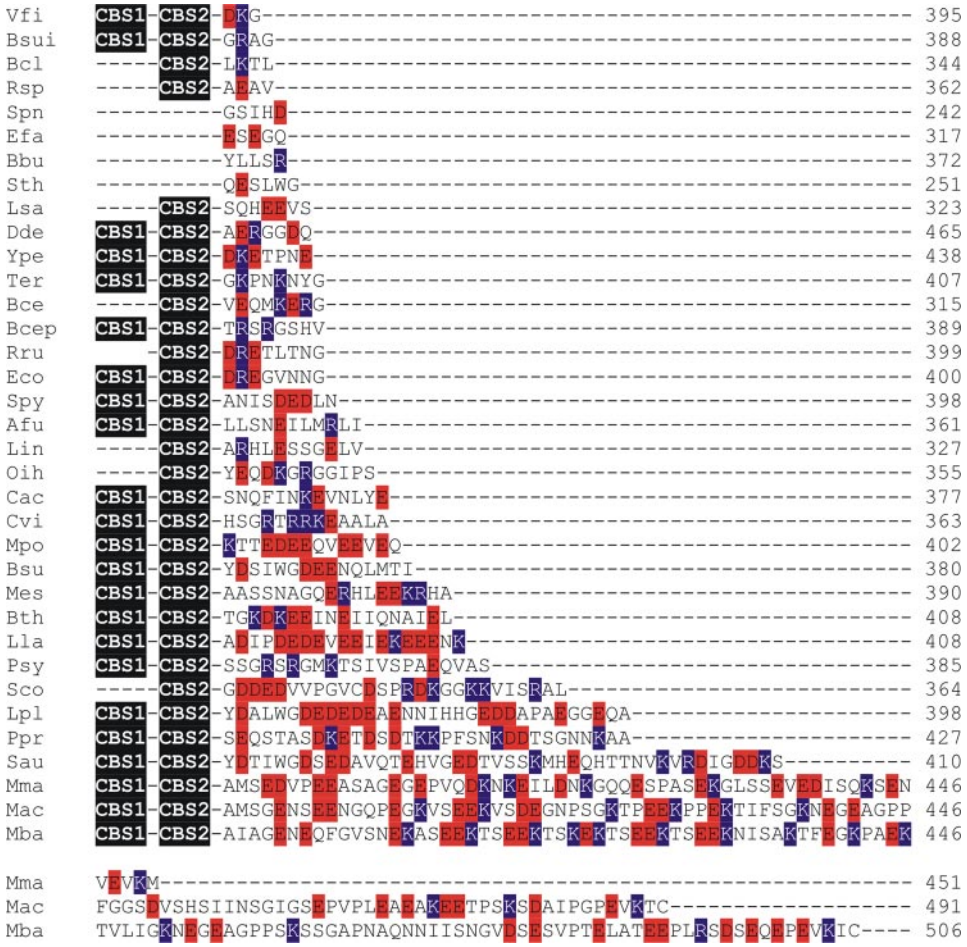

FIGURE 6. **Alignment of the C-terminal ends of the ATPases of a subset of ABC transporters that belong to the OTCN family.** Of a total of more than 260 homologues, 35 were chosen to emphasize the variation in length of the C-terminal tail and the high fraction of anionic residues in this part of the protein. Shading is as follows: *red*, negatively charged residues; and *blue*, positively charged residues. *Black boxes* indicate conserved CBS domains 1 and 2. Organisms are as follows: *Vfi, Vibrio fischeri* (GenBank™ accession number YP_204170); *Bsui, Brucella suis* (AAN33922); *Bcl, Bacillus clausii* (YP_175488); *Rsp, Rhodobacter sphaeroides* (ZP_00006677); *Spn, Streptococcus pneumoniae* (NP_359270); *Efa, Enterococcus faecalis* (NP_814425); *Bbu, Borrelia burgdorferi* (AAC66523); *Sth, Streptococcus thermophilus* (YP_139755); *Lsa, Lactobacillus salivarius* (YP_163805); *Dde, Desulfovibrio desulfuricans* (ZP_00130123); *Ype, Yersinia pestis* (NP_668546); *Ter, Trichodesmium erythraeum* (ZP_00326994); *Bce, Bacillus cereus* (YP_083651); *Bcep, Burkholderia cepacia* (ZP_00213477); *Rru, Rhodospirillum rubrum* (ZP_00270086); *Eco, E. coli* (BAB36963); *Spy, Streptococcus pyogenes* (YP_059521); *Afu, Archaeoglobus fulgidus* (NP_069814); *Lin, Listeria innocua* (NP_470796); *Oih, Oceanobacillus iheyensis* (NP_692611); *Cac, Clostridium acetobutylicum* (AAK80793); *Cvi, Chromobacterium violaceum* (AAQ58872); *Mpo, Methanohalophilus portucalensis* (M.-C. Lai and S.-C. Chen, unpublished data), *Bsu, Bacillus subtilis* (O34992); *Mes, Mesorhizobium* sp. *BNC1* (ZP_00194953); *Bth, Bacteroides thetaiotaomicron* (AAO76858); *Lla, Lactococcus lactis* (AAF37878), Psy: *Pseudomonas syringae* (NP_794326), Sco: *Streptomyces coelicolor* (CAB59474); *Lpl, Lactobacillus plantarum* (CAD64047); *Ppr, Photobacterium profundum* (YP_133447); *Sau, Staphylococcus aureus* (BAB58610); *Mma, Methanosarcina mazei* (NP_632064); *Mac, Methanosarcina acetivorans* (NP_617063); *Mba, Methanosarcina barkeri* (ZP_00295317).

concentration, because the lipophilic molecule accumulates in the membrane. The membrane partition coefficient for charged tetracaine is at least 100-fold as can be deduced from its log*P* value of 3.2 (20) and the correlation between octanol/water and membrane/water partition coefficients of membrane-active compounds (21). Thus, the membrane electrostatic potential may be affected similarly by (sub)millimolar concentrations of tetracaine and 0.2 M KCl or NaCl. Contrary to KCl, RbCl completely inhibited the amphipath-induced activation of OpuA, even at concentrations as low as 0.1 mM. Fig. 5*B* shows that 0.1 mM of RbCl completely inhibited the activation elicited by 200 mM of KCl (with or without tetracaine). At 250 mM KCl part of the inhibition by Rb^+ ions was overcome, possibly because K^+ interacts at the same site but with a much lower affinity than Rb^+. Similar results were obtained with Cs^+ ions except that ~10-fold higher concentrations of this alkali ion were required to achieve the same degree of inhibition (data not shown). These experiments indicate that Rb^+ and Cs^+ ions inhibit OpuA, and somewhere in the protein complex there must be a site with unusually high affinity for Rb^+ (and Cs^+) ions and low affinity for K^+. Importantly, RbCl and CsCl (tested up to 200 mM) did not inhibit uptake of glycine betaine via right-side-in reconstituted OpuA, demonstrating these ions only inactivate by interacting with the cytoplasmic face of the protein.

Rb^+ and Cs^+ Ions No Longer Inhibit OpuA When the C-terminal Tail Is Deleted

The ion-sensing CBS module of OpuA is followed by a C-terminal tail with the sequence DIPDEDEVEEIEKEEENK (see also Fig. 6). We have recently shown that deletion of most of this sequence (deleted amino acid sequence is DIPDEDEVEEIEK), yielding OpuAΔ12, shifted the activation profiles of right-side-in reconstituted transporter to higher potassium salt concentrations (8). Stated differently, OpuAΔ12 was more strongly inhibited by anionic lipids than wild-type OpuA, possibly through a stronger electrostatic interaction of the CBS module with the membrane surface. This effect could be reproduced for inside-out oriented OpuAΔ12 (compare Fig. 2*B* and Fig. 7, *A* and *B*); the K_{ion} for K^+ was increased by at least 0.1 M. Importantly, OpuAΔ12 was no longer inhibited by Rb^+ or Cs^+ ions (compare Fig. 2*B* and Fig. 7*C*). Comparison of the data of wild-type OpuA (Fig. 2*B*) with those of OpuAΔ12 (Fig. 7, *A* and *B*) suggested that, in contrast to K^+, activation by Na^+ and Li^+ was very similar (n = 6). We also investigated the salt dependence of wild-type OpuA and OpuAΔ12 at varying ratios of KCl and NaCl. For wild-type OpuA, all the curves were superimposable, whereas for OpuAΔ12 the activation curves shifted to higher *X*Cl concentrations when the KCl/NaCl ratio was increased (data not shown). These last experiments indicate that the differences

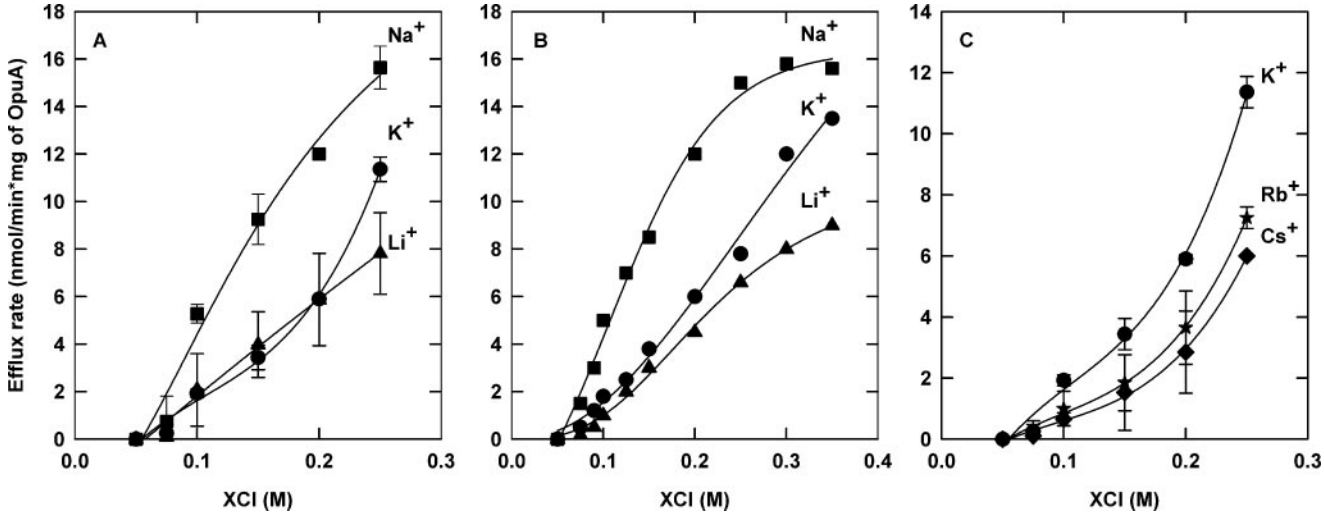

FIGURE 7. **Salt dependence of glycine betaine efflux by inside-out reconstituted OpuAΔ12.** *A*, KCl (●), NaCl (■), and LiCl (▲); *B* shows the activity over an extended concentration range, presented as $n = 1$ experiment (*symbols* as in *A*); *C*, KCl (●), RbCl (★), and CsCl (◆). The *error bars* indicate the standard error of $n = 6$ experiments (see legend to Fig. 2).

between K^+ and Na^+ are not caused by a specific effect exerted by either K^+ or Na^+ at low (millimolar or less) concentration, rather the differential effects are additive from 0 to 350 mM. Thus, in OpuAΔ12, the activation by K^+ is shifted to higher salt concentrations, and the inhibition by Rb^+ and Cs^+ is no longer observed, but the dependence of transporter on Na^+ or Li^+ ions is not affected by the deletion of the anionic C-terminal tail.

Collectively, these data show that the ion-sensing properties of the OpuA are modulated by the anionic tail C terminus of the tandem CBS domain. This region of the protein may interact with the surface of the CBS or other parts of OpuA and/or membrane surface. The interaction may form a site that recruits the larger alkali ions, because activation by Na^+ and Li^+ ions is not significantly different in wild-type OpuA and OpuAΔ12. The interactions with the larger alkali ions must be highly cation-specific as K^+ and Rb^+ (and Cs^+) exerted very different effects on the transporter; K^+ shifted the ionic strength dependence to lower values, whereas Rb^+ and Cs^+ ions completely abolished the transport activity. NMR spectroscopy of a synthetic peptide with the sequence formyl-DIPDEDEVEEIEKEEENK-COO^-, corresponding to the C-terminal extension of the tandem CBS, has shown that the peptide by itself is unstructured both at high and low ionic strength (with KCl or RbCl) (data not shown).

DISCUSSION

By assaying the activity of inside-out reconstituted OpuA and elaborating on previous work (8), we provide new information on the role of the CBS module in the ionic activation of the transporter. We now show that the largest (Cs^+) to the smallest (Li^+) alkali metal ions activate the transporter. Also, non-metal ions (NH_4^+) and alkaline earth metals (Mg^{2+} and Ba^{2+}) activate in a manner that is consistent with ionic strength being the physical parameter that is sensed. We also show that the differences of ions in activating OpuA can to a large extent be explained by the presence of the anionic tail, C-terminal to the second CBS domain. Our interpretation of the ionic strength and ion-specific effects are depicted in Fig. 8; the properties of the different cations are summarized in Table 1. The basic premise of the model is that a high fraction of anionic lipids stabilizes the inactive conformation of OpuA. In Fig. 8, *A* and *B* indicate that relatively high salt concentrations activate OpuA by breaking the electrostatic interactions between the CBS module and the anionic membrane. Contrary to most salts, Rb^+ and Cs^+ ions inhibited wild-type OpuA even in the presence of a 1000-fold excess of Na^+ or K^+ ions (Fig. 8*C*). This inhibition was no longer observed when most of the C-terminal anionic tail was deleted (Fig. 8; compare Figs. 2*B* and 7*C*). Although a peptide with the sequence corresponding to the anionic tail is unstructured (formyl-DIPDEDEVEEIEKEEENK-COO^-), it is possible that this region becomes structured in the presence of Rb^+ or Cs^+, provided it is associated with the CBS domains. The data indicate that the largest alkali metals inactivate the wild-type transporter, albeit reversibly (addition of a large excess of K^+ or removal of Rb^+ restored the activity). The effectiveness of K^+, Rb^+, and Cs^+ to activate OpuAΔ12 decreased with increasing ionic radius (Fig. 7*C*; Table 1), but at saturating concentrations (*i.e.* 350 mM; not shown) of K^+, Rb^+, and Cs^+ the activities were equal (Fig. 8*H*). Thus, ion sensing *per se* is not affected by the deletion of the anionic tail. One other important point concerns the observation that deletion of the anionic tail shifts the activation by K^+ ions to higher salt, whereas the activation by Na^+ (and Li^+) is not affected (schematically depicted in Fig. 8, *E versus B* and *G*). This suggests that the ion-sensing module of the wild-type transporter discriminates K^+ from the smaller alkali metal ions, presumably at a site that also recognizes Rb^+ and Cs^+ (see Fig. 5*B* for experimental support of the contention that K^+ and Rb^+ seem to compete with each other). We propose that binding of K^+ and Rb^+ (or Cs^+) triggers different protein conformations; with K^+ the transporter is more responsive to ionic strength, whereas with Rb^+ (or Cs^+) the wild-type OpuA is inactive. Finally, if one ignores the ion-specific effects imposed by the C-terminal anionic tail, the ionic strength dependence of the transporter (OpuΔ12) is only weakly dependent on the type of ion, with the smallest (Li^+) and

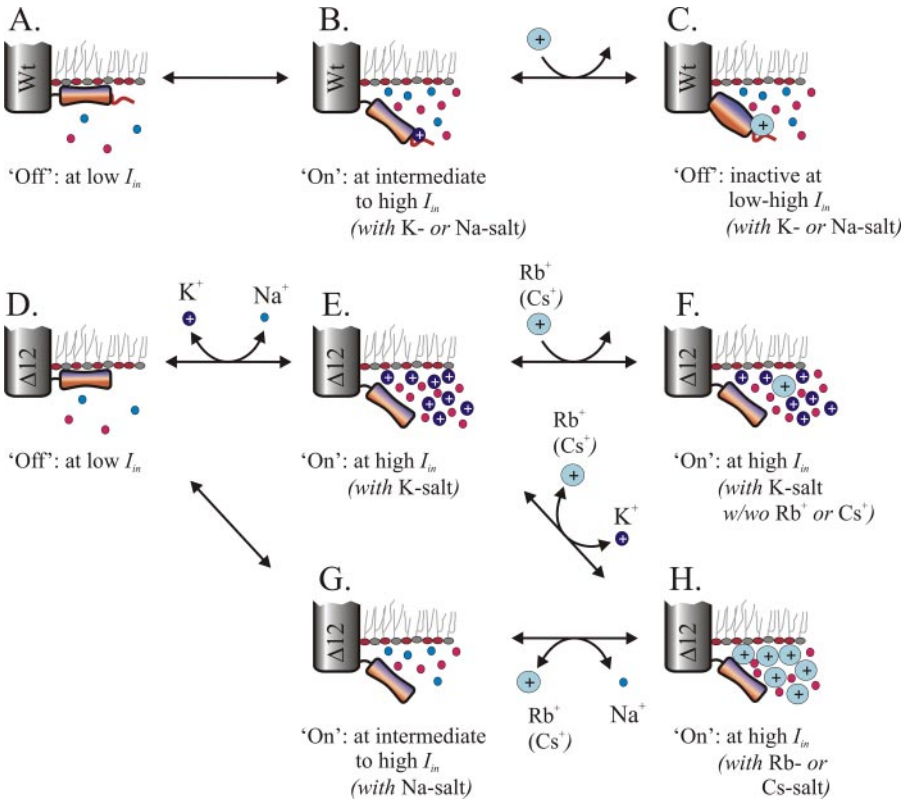

FIGURE 8. **Schematic of the effects of salts on the activity of OpuA.** Wild-type OpuA and OpuAΔ12 are indicated by *Wt* and *Δ12*, respectively. *On* and *off* refer to the active and inactive state of the transporter. The transporter (ligand binding receptor, translocator includes ATP-binding cassette) is depicted by the *gray cylinder*; the CBS moiety is in *orange-blue* (*blue* depicts the cationic surface possibly interacting with the anionic membrane); and the anionic C terminus is in *red*; anionic and neutral lipids are represented by *red* and *gray* head groups, respectively. The *small blue circles* depict small cations (Na^+ or Li^+); the *medium size blue circles* depict K^+; and the *large blue circles* depict (Rb^+ or Cs^+); the counterion is depicted as *small red circle*.

TABLE 1
Properties of cations

Name of ion	Ionic radius[a]	Enthalpy of ion hydration, $\Delta H_{hydration}$[b]
	Å	*kJ/mol*
Mg^{2+}	0.86	−1920
Li^+	0.90	−520
Na^+	1.16	−405
Ba^{2+}	1.49	−1650
K^+	1.52	−321
NH_4^+	1.61	−301
Rb^+	1.66	−300
Cs^+	1.81	−277

[a] Effective ionic radii are from Refs. 31 and 32.
[b] Enthalpies of hydration are from Ref. 33.

largest (Rb^+ and Cs^+) ions being only somewhat less effective in activating than the ions with intermediate ionic radii (and enthalpies of hydration), which are Na^+, K^+, and NH_4^+.

How could the electrostatic energy of interaction of the tandem CBS domains with the membrane control the activity of the transporter? As illustrated in Fig. 8, we postulate that the transporter is activated when the salt concentration (ion strength) reaches a threshold value and overcomes the binding energy for the electrostatic interaction of the CBS module with the membrane. Because the membrane electrostatic potential decreases exponentially with increasing salt concentration, the fraction of molecules interacting electrostatically with the membrane will also decrease exponentially. For the simple case where the membrane-bound and nonbound state reflect the inactive and active conformations, each additional cation will have a larger effect on the membrane adsorption equilibrium, and the activation profile will be a sigmoid function of the salt concentration (as shown in Figs. 2*B*, Fig. 3*C*, and Fig. 7). To obtain an estimate of the electrostatic energy of interaction of proteins and membrane surfaces, we embroider the studies of Ben-Tal *et al.* (22, 23). For small cationic peptides and proteins, each basic residue has been shown to contribute about −1 kcal/mol binding energy when the membrane contains 33% of anionic lipid, and the salt concentration is 0.1 M KCl. For pentalysine (22) or proteins with an equivalent stretch of cationic residues (23), the binding energy for interaction with the membrane decreased from approximately −7 to approximately −2 when the KCl concentration was increased from 0.05 to 0.4 M. The corresponding reduction in the apparent membrane association constant was from 80,000 to 25 M^{-1}. Similarly, the membrane binding energy increased with an increasing number of basic residues and an increasing mole fraction of anionic lipids in the membrane. The ranges of anionic lipids (10–50 mol %) and salt concentrations studied by Ben-Tal *et al.* (22, 23) are very similar to the parameters used to investigate OpuA. Moreover, homology modeling of the OpuA CBS domains, using the available crystal structures (see the supplemental material of Biemans-Oldehinkel *et al.* (8)), reveals a surface-exposed region with 5–6 cationic residues that might be the determinant for interaction with the membrane. For a membrane with 40 mol % of anionic lipids, and by using the data from Ben-Tal *et al.* (22, 23), this would correspond to a binding energy of the tandem CBS domain of approximately −6 kcal/mol at threshold concentrations of salt ($=T_{ion}$ and corresponds to an ionic strength of ~0.15). One important difference in respect to the studies of Ben Tal *et al.* (22, 23) is that the tandem CBS domains are tethered to the membrane and restricted in their mobility to a two-dimensional space. The entropy increase upon dissociation from the membrane will thus be lower than for a protein moving freely in a three-dimensional solution. Consequently, for the OpuA transporter to be switched from an inactive to an active conformation (increase in ionic strength from ~0.15 to ~0.32 for the lipid composition used in this paper), a higher binding energy may have to be overcome to free the domains from the membrane than for peptides and proteins in solution and using classical electrostatics. Possibly the overall

anionic nature of the tandem CBS domains, in particular that of the C-terminal anionic tails, contributes to a lower binding energy with the membrane and allows the system to respond at physiological salt concentrations.

Assay Strategy—In previous papers (3, 8, 15), we have monitored the uptake of glycine betaine via right-side-in reconstituted OpuA. The internal osmolality of the proteoliposomes was varied by incorporating varying amounts of nonionic or ionic osmolytes during the reconstitution procedure. Alternatively, the internal osmolality was increased by imposing osmotic stress to the proteoliposomes, thereby increasing the internal osmolality and ionic strength but keeping the composition constant. To screen a wide range of ions at different concentrations, it was advantageous to use the fraction of inside-out oriented OpuA molecules. In this way, we had direct access to the ion-sensing CBS module, simply by varying the composition of the assay medium. This was possible because transport via OpuA is strictly unidirectional, and re-uptake can be prevented by limiting the amount of internal ATP and taking advantage of the build up of ADP (19). It has been reported previously that proteoliposomes composed of *E. coli* lipids are very sensitive to bivalent cations such as Mg^{2+} and Ca^{2+}, and concentrations as low as 10 mM were sufficient to completely permeabilize the vesicles, resulting in leakage of internal solutes (24). Although the sensitivity of *E. coli* lipid extract membranes to bivalent cations varied from batch to batch, the observations of Ames and co-workers (24) could be reproduced by us. By using synthetic lipid mixtures (DOPG/DOPE/DOPC at mol % of 40:40:20), we were able to use free concentrations of Mg^{2+} and Ba^{2+} of up to 20 mM without detectable loss of integrity of the proteoliposomes. This enabled us to vary the ionic strength, albeit over a small range, by using salts with bivalent cations.

The Ionic Strength Sensing CBS Module—OpuA belongs to the OTCN family of the ABC superfamily, transporters involved in glycine betaine, carnitine, proline, and/or choline uptake. Homologues of OpuA such as ProU from *E. coli*, OpuA from *Bacillus subtilis*, and OtaA from *Methanohalophilus portucalensis* have been reported to function in cellular osmoregulation, and it seems likely that these systems and possibly other OTCN members (a total of 280 members; May, 2006) all use the CBS module as sensor. For the ATPase of the OpuA transporter from *B. subtilis*, it has been shown that the monomer-dimer equilibrium is nucleotide- *and* salt concentration-dependent (25). These important observations have been interpreted in terms of association-dissociation of the catalytic domain but, most likely, have included contributions from interacting CBS domains. Further research on the *B. subtilis* OpuA is required to establish the role of the CBS domains in ion sensing in this system. A feature of the CBS module of the transporters of the OTCN family that is highly variable among its members is the anionic C-terminal tail (Fig. 6 for a subset of the OTCN members). The C-terminal anionic tail is absent in homologues of OpuA, such as ProU from *E. coli*, and can be as long as 113 amino acids in homologues present in halophilic Archaea. This sequence and length variation together with our data of the OpuAΔ12 mutant suggest that the C-terminal tails tune the activation of the transporters to the ionic strength of the cytoplasm and/or specific lipid composition of the membrane, which is likely to vary among microbial species. Finally, the majority of chloride channels of the ClC family also have two CBS domains in tandem (26), and also for these systems one can observe a variation in C-terminal tails similar to that depicted in Fig. 6 for the ABC transporters (*e.g.* 17 of the 40 C-terminal residues in ClC-1 are anionic, although this region is lacking in ClC-0).

How do the data on OpuA relate to those of other osmoregulatory transporters? Both ProP and BetP lack CBS domains but contain C-terminal extensions that play a role in osmosensing. In the case of ProP, the sensing moiety is not an easily recognizable domain. The C-terminal extension of ProP forms a homodimeric, antiparallel, α-helical coiled-coil structure and is not essential for osmotic activation of the transporter but instead tunes the regulation over a specific osmolality range (27). This is similar to the role we now propose for the anionic C-terminal tail of OpuA. ProP and OpuAΔ12 respond similarly to increasing concentrations of Li^+, Na^+, K^+, NH_4^+, Rb^+ (the latter two tested for OpuA only), and Cs^+ with phosphate, sulfate (tested for OpuA only), or phosphate and Cl^- as anions. Thus, the signals sensed by OpuA and ProP may be similar, and the structural basis for ion sensing is different. It should be pointed out that ProP is not only responding to ionic strength as proposed for OpuA, but macromolecular crowding has been also implicated in osmosensing (7).

Although OpuAΔ12 seems to act as a pure ionic strength sensor, the presence of the anionic tail in wild-type OpuA tunes the activity to the physiologically most important cation, *i.e.* K^+. Contrary to OpuA and ProP, BetP displays strong cation specificity. BetP can be activated with K^+, Rb^+, or Cs^+ (and is not anion-specific), but smaller alkali ions such as Na^+ are much less effective (6). Krämer and co-workers (5) have proposed that BetP is a K^+ sensor (a chemosensor rather than an osmosensor), implying the existence of a binding site with a K^+ affinity of ~0.2 M. Even though the details of osmosensing by BetP might be different from those of OpuA and ProP, for each of the systems there is a strong case for ion(ic) (strength)-dependent electrostatic interactions underlying the sensing mechanism. On the basis of mutagenesis studies (28, 29), the putative K^+ sensor has been proposed to reside in a stretch of 25 amino acids of the C-terminal extension of BetP. Although the higher order structure of this domain is not known, its primary sequence and predicted secondary structure are different from the CBS domains and the anionic C-terminal tail of OpuA and the α-helical coiled-coil structure of ProP. This is not surprising as it is readily imaginable that an electrostatics-based sensing/switching mechanism has evolved independently several times, resulting in protein modules differing in sequence and structure but with similar ionic strength-dependent protein-lipid or protein-protein interactions (2). In a previous paper (30), we have described that electrostatic forces may play principal roles in the interaction of proteins not only with membranes but also with other macromolecules (proteins, DNA, and/or RNA) in the cytoplasm, and we proposed an electrochemical model of cell structure. This study provides further support for a role of electrostatic forces in the conformational states of enzymes, and it also illustrates that proteins may evolve with properties (*e.g.* differential effects of K^+ and Rb^+)

that are not readily predicted from simple physicochemical principles.

In conclusion, the anionic C terminus modulates the ion sensor and lowers the threshold for activation by K^+ ions, possibly by forming a potassium-binding site in conjunction with other parts of the protein and/or membrane lipids. This putative binding site may also accommodate Rb^+ and Cs^+ but then assume a conformation that completely (albeit reversibly) abolishes transport. The binding of Rb^+ and Cs^+ seems to occur with orders of magnitude higher affinity than K^+, which is intriguing but in our opinion physiologically not relevant. The main physiological function of the C-terminal anionic tail may be the tuning of the ion sensitivity of the CBS module.

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REFERENCES

1. Wood, J. M. (1999) *Mol. Biol. Rev.* **63,** 230–262
2. Poolman, B., Spitzer, J. J., and Wood, J. (2004) *Biochim. Biophys. Acta* **1666,** 88–104
3. van der Heide, T., Stuart, M. C., and Poolman, B. (2001) *EMBO J.* **20,** 7022–7032
4. Racher, K. I., Culham, D. E., and Wood, J. M. (2001) *Biochemistry* **40,** 7324–7333
5. Rübenhagen, R., Morbach, S., and Kramer, R. (2001) *EMBO J.* **20,** 5412–5420
6. Schiller, D., Kramer, R., and Morbach, S. (2004) *FEBS Lett.* **563,** 108–112
7. Culham, D. E., Henderson, J., Crane, R. A., and Wood, J. M. (2003) *Biochemistry* **42,** 410–420
8. Biemans-Oldehinkel, E., Mahmood, N. A. B. N., and Poolman, B. (2006) *Proc. Natl. Acad. Sci. U. S. A.* **103,** 10624–10629
9. van der Heide, T., and Poolman, B. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97,** 7102–7106
10. van der Heide, T., and Poolman, B. (2002) *EMBO Rep.* **3,** 938–943
11. Zhang, R., Evans, G., Rotella, F. J., Westbrook, E. M., Beno, D., Huberman, E., Jochiamiak, A., and Collart, F. R. (1999) *Biochemistry* **38,** 4691–4700
12. Miller, M. D., Schwarzenbacher, R., von Delft, F., Abdubek, P., Ambing, E., Biorac, T., Brinen, L. S., Canaves, J. M., Cambell, J., Chiu, H. J., Dai, X., Deacon, A. M., DiDonato, M., Elsliger, M. A., Eshagi, S., Floyd, R., Godzik, A., Grittini, C., Grzechnik, S. K., Hampton, E., Jaroszewski, L., Karlak, C., Klock, H. E., Koesema, E., Kovarik, J. S., Kreusch, A., Kuhn, P., Lesley, S. A., Levin, I., McMullan, D., McPhillips, T. M., Morse, A., Moy, K., Ouyang, J., Page, R., Quijano, K., Robb, A., Spraggon, G., Stevens, R. C., van den Bedem, H., Velasquez, J., Vincent, J., Wang, X., West, B., Wolf, G., Xu, Q., Hodgson, K. O., Wooley, J., and Wilson, I. A. (2004) *Proteins* **57,** 213–217
13. Knol, J., Veenhoff, L., Liang, W. J., Henderson, P. J., Leblanc, G., and Poolman, B. (1996) *J. Biol. Chem.* **271,** 15358–15366
14. Knol, J., Sjollema, K., and Poolman B. (1998) *Biochemistry* **37,** 16410–16415
15. Biemans-Oldehinkel, E., and Poolman, B. (2003) *EMBO J.* **22,** 5983–5993
16. Culham, D. E., Hillar, A., Henderson, J., Ly, A., Vernikovska, Y. I., Racher, K. I., Boggs, J. M., and Wood, J. M. (2003) *Biochemistry* **42,** 11815–11823
17. Driessen, A. J., Zheng, T., In't Veld, G., Op den Kamp, J. A., and Konings, W. N. (1988) *Biochemistry* **27,** 865–872
18. Poolman, B., Doeven, M. K., Geertsma, E. R., Biemans-Oldehinkel, E., Konings, W. N., and Rees, D. C. (2005) *Methods Enzymol.* **400,** 429–459
19. Patzlaff, J. S., van der Heide, T., and Poolman B. (2003) *J. Biol. Chem.* **278,** 29546–29551
20. Avdeef, A., Box, K. J., Comer, J. E., Hibbert, C., and Tam, K. Y. (1998) *Pharmacol. Res.* **15,** 209–215
21. Sikkema, J., de Bont, J. A. M., and Poolman, B. (1995) *Microbiol. Rev.* **59,** 201–222
22. Ben-Tal, N., Honig, B., Peitzsch, R. M., Denisov, G., and Mclaughlin, S. (1996) *Biophys. J.* **71,** 561–575
23. Ben-Tal, N., Honig, B., Miller, C., and Mclaughlin, S. (1997) *Biophys. J.* **73,** 17117–17127
24. Liu, C. E., Liu, P. Q., and Ames, G. F. L. (1997) *J. Biol. Chem.* **25,** 21883–21891
25. Horn, C., Bremer, E., and Schmitt, L. (2003) *J. Mol. Biol.* **334,** 403–419
26. Bennetts, G., Rychkov, G. Y., Ng, H. L., Morton, C. J., Stapleton, D., Parker, M. W., and Cromer, B. A. (2005) *J. Biol. Chem.* **280,** 32452–32458
27. Tsatskis, Y., Khambati, J., Dobson, M., Bogdanov, M., Dowhan, W., and Wood, J. M. (2005) *J. Biol. Chem.* **280,** 41387–41394
28. Schiller, D., Kramer, R., and Morbach, S. (2004) *Biochemistry* **43,** 5583–5591
29. Schiller, D., Ott, V., Kramer, R., and Morbach, S. (2006) *J. Biol. Chem.* **281,** 7737–7746
30. Spitzer, J., and Poolman, B. (2005) *Trends Biochem. Sci.* **30,** 536–541
31. Shannon, R. D. (1976) *Acta Crystallogr. Sect. A* **32,** 751–767
32. Cotton, F. A., and Wilkinson, G. (1988) *Advanced Inorganic Chemistry*, 5th Ed., pp. 1385–1388, Wiley-Interscience, New York
33. Bockris, J. O. M., and Reddy, A. K. N. (1970) *Modern Electrochemistry*, 1st Ed., Plenum Publishing Corp., New York