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

Received for publication, May 22, 2006, and in revised form, July 12, 2006 Published, JBC Papers in Press, July 14, 2006, DOI 10.1074/jbc.M604907200

N. A. B. Nik Mahmood, Esther Biemans-Oldehinkel, Jason S. Patzlaff, Gea K. Schuurman-Wolters, and Bert Poolman

From the Membrane Enzymology Group, Department of Biochemistry, Groningen Biomolecular Science and Biotechnology Institute and Materials Science Centre, University of Groningen, Nijenborgh 4, 9747 AG Groningen, The Netherlands

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 (DIPDEVEEVEEIEKE-N). 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 (Mg2+/H11011OpuA and the anionic tail deletion mutant OpuA were completely inhibitory to the transporter even in the presence of 0.2 M KCl. 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 (Mg2+ and Ba2+) were more efficient in activating OpuA than NH4+/H11545, 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 molar RbCl was sufficient to completely inhibit the transporter even in the presence of 0.2 molar 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 NH4+ (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 CBS2 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

---

* This work was supported by “Top-subsidie” Grant NWO-CW 700-50-302, a fellowship (to N. A. B. N. M.) from the UT-Malaysian Government, and funding from the EU-FP6 Program E-Mep 504601 and Cost Action D22. The fellowship (to N. A. B. N. M.) from the UTM-Malaysian Government, 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 (DIPDEVEEVEEIEKE-N). 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 (Mg2+/H11011OpuA and the anionic tail deletion mutant OpuA were completely inhibitory to the transporter even in the presence of 0.2 M KCl. 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 (Mg2+ and Ba2+) were more efficient in activating OpuA than NH4+/H11545, 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 molar RbCl was sufficient to completely inhibit the transporter even in the presence of 0.2 molar 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 NH4+ (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 CBS2 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

---

2 The abbreviations used are: CBS, cystathionine β-synthase; DDM, n-dodecyl-β-D-maltoside; DOPG, 1-α-dioleoyl-phosphatidylglycerol; DOPE, 1-α-dioleoyl-phosphatidylethanolamine; DOPC, 1-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 OprA 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 OprA 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 oprA genes deleted (8)) was complemented in *trans* with plasmids (pNZOpuAHis and derivatives) carrying wild-type or mutant oprA genes. The strains were grown semi-aerobically at 30 °C in a medium containing 2% (w/v) gistex LS (Strik BV, Eemnes, Netherlands) and 65 mM potassium phosphate (potassium Pi), 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 A600 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 OprA**

Membrane vesicles were reuspended in buffer A (50 mM potassium Pi, 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 Ni2+–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 0.1% (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).

**Effect Assays**

-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/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 Pi, pH 7.0 (to lower the glycercol 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 Pi, 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 MgSO4 and 10 mM Na2ATP) 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 Pi, 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 Pi, 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 Pi, pH 7.0) or hypertonic medium (50 mM potassium Pi, 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 [14C]glycine betaine (Amersham Biosciences) to a final concentration of 45 μM (more than 10-fold above the Kd value for transport and the Kd 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 Pi, pH 7.0, or 50 mM potassium Pi, 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 [14C]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 Pi, pH 7.0, and preincubated for 3 min at 30 °C. Following addition of 10 mM MgATP (prepared from 10 mM MgSO4 plus 10 mM Na2ATP or 10 mM K2ATP) and incubation for another 2 min, [14C]glycine betaine efflux was initiated by diluting the mixture 2-fold with pre-warmed 10 mM potassium Pi, pH 7.0, supplemented with different concentrations (up to 250–350 mM) of KCl, NaCl, LiCl, RbCl, CsCl, NH4Cl, MgCl2, or BaCl2. The rate of efflux was estimated from the decrease in internal [14C]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
Regulation of ABC Transporter by Ionic Strength

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 freeze-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 [14C]glycine betaine, for subsequent analysis of glycine betaine efflux.

Ion Specificity of OpuA Regulation

Alkali Metals—Fig. 2A shows transport of glycine betaine via inside-out oriented OpuA at different concentrations of KCl added to the basal medium (10 mM potassium Pi, pH 7.0, 10 mM MgSO4 plus 10 mM Na2ATP or 10 mM K2ATP, both corre-
Corresponding 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. 2A). Maximal activity was reached around 250 mM KCl, corresponding to an ionic strength of ~0.32. The XCl concentration dependence of OpuA is plotted for X = K⁺, Na⁺, Li⁺, Rb⁺, and Cs⁺ in Fig. 2B; 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 (Amax) and ion activation constant (Kion) could be estimated (see “Materials and Methods”). Although the Amax was 2-fold higher with KCl and NaCl than with LiCl, the Kion values (~125 mM XCl plus basal medium components) and the threshold values for activation (Tion values) were similar (~75 mM XCl 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 NH₄⁺ as an ion that does not belong to the group 1 elements (alkali metals). Fig. 3A shows that NH₄Cl activates OpuA but much less effectively than KCl. Although NH₄⁺ will be membrane-impermeant on the time scale of the measurements, NH₃ is highly membrane-permeable, and the influx of NH₃ and subsequent protonation will alkalinize the proteoliposome lumen. To prevent build up of a pH gradient, the experiments of Fig. 3A were repeated in the presence of the protonophore 3,5-di-tert-butyl-4-hydroxy benzylidene-malonitrile (SF6847). Fig. 3B shows that in the presence of SF6847, NH₄Cl 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, Amax, Kion, n, and Tion values were very similar for NH₄Cl and KCl (Fig. 3C). 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. 4A shows that the group 2 alkaline earth metal ions Mg²⁺ and Ba²⁺ 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
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$^{2+}$/H$^{+}$ and Ba$^{2+}$/H$^{+}$, 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. 4B). The collective data conformed to a nearly unique relationship when plotted as a function of ionic strength (Fig. 4C).

**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. 2B). 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. 5A), 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
Regulation of ABC Transporter by Ionic Strength

The ion-sensing CBS module of OpuA is followed by a C-terminal tail with the sequence DIPDEDEVREEKEENEK (see also Fig. 6). We have recently shown that deletion of most of this sequence (deleted amino acid sequence is DIPDEDEVREEKEEN), resulting in 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. 2B 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 other ions (compare Fig. 2B and Fig. 7C). Comparison of the data of wild-type OpuA (Fig. 2B) 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 KCl concentrations when the KCl/NaCl ratio was increased (data not shown). These last experiments indicate that the differences...
Regulation of ABC Transporter by Ionic Strength

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 (NH₄⁺) and alkaline earth metals (Mg²⁺ and Ba²⁺) 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. 8C). This inhibition was no longer observed when most of the C-terminal anionic tail was deleted (Fig. 8; compare Figs. 2B and 7C). 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. 7C; Table 1), but at saturating concentrations (i.e. 350 mM; not shown) of K⁺, Rb⁺, and Cs⁺ the activities were equal (Fig. 8H). 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. 5B 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 (OpuAΔ12) is only weakly dependent on the type of ion, with the smallest (Li⁺) and...
 Regulation of ABC Transporter by Ionic Strength

![Figure 8](https://www.jbc.org/article/S1939-6261(20)-00449-8/)

**TABLE 1**

<table>
<thead>
<tr>
<th>Property of cations</th>
<th>Ionic radius (Å)</th>
<th>Enthalpy of hydration (kJ/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Name of ion</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mg²⁺</td>
<td>0.86</td>
<td>1920</td>
</tr>
<tr>
<td>Li⁺</td>
<td>0.90</td>
<td>520</td>
</tr>
<tr>
<td>Na⁺</td>
<td>1.16</td>
<td>405</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>1.49</td>
<td>1650</td>
</tr>
<tr>
<td>K⁺</td>
<td>1.52</td>
<td>321</td>
</tr>
<tr>
<td>NH₄⁺</td>
<td>1.61</td>
<td>301</td>
</tr>
<tr>
<td>Rb⁺</td>
<td>1.66</td>
<td>300</td>
</tr>
<tr>
<td>Cs⁺</td>
<td>1.81</td>
<td>277</td>
</tr>
</tbody>
</table>

* Effective ionic radii are from Refs. 31 and 32.

* 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 NH₄⁺.

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. 2B, Fig. 3C, 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
Regulation of ABC Transporter by Ionic Strength

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 Methanolphilus 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 CIC 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 CIC-1 are anionic, although this region is lacking in CIC-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 μM. 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.

Acknowledgments—We thank Klaas Dijkstra and Ruud Scheek for the NMR experiments and Guus Erkens for stimulating discussions.

REFERENCES


Regulation of ABC Transporter by Ionic Strength