A sensor for intracellular ionic strength

Esther Biemans-Oldehinkel, Nik A. B. N. Mahmood, and Bert Poolman*

Department of Biochemistry, Groningen Biomolecular Sciences and Biotechnology Institute, University of Groningen, Nijenborgh 4, 9747 AG Groningen, The Netherlands

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Cystathionine-β-synthase (CBS) domains are found in >4,000 proteins in species from all kingdoms of life, yet their functions are largely unknown. Tandem CBS domains are associated with membrane transport proteins, most notably members of the ATP-binding cassette (ABC) superfamily; voltage-gated chloride channels and transporters; cation efflux systems; and various enzymes, transcription factors, and proteins of unknown function. We now show that tandem CBS domains in the osmoregulatory ABC transporter OpuA are sensors for ionic strength that control the transport activity through an electrostatic switching mechanism. The on/off state of the transporter is determined by the surface charge of the membrane and the internal ionic strength that is sensed by the CBS domains. By modifying the CBS domains, we can control the ionic strength dependence of the transporter: deleting a stretch of C-terminal anionic residues shifts the ionic strength dependence to higher values, whereas deleting the CBS domains makes the system largely independent of ionic strength. We present a model for the gating of membrane transport by ionic strength and propose a new role for CBS domains.

ATP-binding cassette transporter | cystathionine-β-synthase domains | cell volume regulation | ionic strength sensor | osmoregulation

CBS stands for cystathionine β-synthase, the enzyme that catalyzes the condensing of homocysteine and serine and plays a pivotal role in mammalian sulfur metabolism. CBS is composed of a heme-binding, a catalytic, and a regulatory domain (1); the latter domain is a small protein module, known as the "CBS domain." Point mutations in this domain cause homocystinuria in humans (1), whereas a number of patient-derived mutations in the catalytic domain of the enzyme have consequences that are alleviated by the deletion of the CBS domain (2). The CBS domains are not unique to CBS but can be identified in a wide variety of proteins (see ref. 3 and www.sanger.ac.uk/Users/agb/CBS/CBS.html), including ATP-binding cassette (ABC) transporters, voltage-gated chloride channels and transporters, a variety of other transporter families, and a number of enzymes, or exist as tandem domains without catalytic moiety (Table 1, which is published as supporting information on the PNAS web site). Inspection of published genome databases reveals >4,000 proteins that possess CBS domains, in most cases as tandem pairs with a N-β-α-β-β-α-C secondary structure (Fig. 7, which is published as supporting information on the PNAS web site). Although proteins with CBS domains are found in all kingdoms of life (Table 1), a regulatory role has been shown in a few cases, and 3D structures of the domains have been determined (4, 5), their regulatory mechanism(s) are unknown. Their importance in biology and medicine is highlighted by the fact that hereditary disease mutations have been found in CBS domains of various proteins (2, 6, 7). Recent work suggests that tandem pairs of CBS domains bind adenine nucleotides or *S*-adenosyl methionine (8). In the case of the skeletal muscle ClC-1 chloride channel, there is evidence that adenine nucleotides affect the gating of the channel (9), but the proposed regulatory role is still enigmatic, because ATP and AMP, but also adenosine, have similar effects.

The ABC transporter OpuA and other types of osmoregulatory transporters (10–12) mediate the uptake of compatible solutes, such as glycine betaine, in response to increasing extracellular osmolality, and thereby reverse the osmotic shrinkage of the cell. When reconstituted in proteoliposomes, the transporters are activated by increased concentrations of luminal ions (10, 13, 14). The activation of the ABC transporter is instantaneous both *in vivo* and *in vitro* and requires only threshold levels of ionic osmolytes. The threshold for activation by ions depends on the ionic lipid content (charge density) of the membrane, indicating that the signal is transduced to the transporter by critical interactions of protein domains with membrane lipids. The ABC transporter OpuA has two CBS domains in tandem at the C-terminal end of the ATPase subunit OpuAA (15). With two ATPase subunits per functional unit, a total of four CBS domains are present in OpuA. The C-terminal tail of the tandem CBS in OpuA is highly charged, with 10 acidic and 2 basic residues in a stretch of 15 amino acids. The charged C terminus of the CBS domains in OpuA and other ABC transporters is unusual, and the length and charge of the tail are highly variable among CBS domains (Fig. 7).

We now show that the activation of ABC transporter OpuA by increased concentrations of luminal ions reflects gating by internal ionic strength. We also demonstrate that the CBS domains of OpuA constitute the lipid-dependent ion-sensing modules of the transporter and describe a specific mechanism for regulation of protein function by these widespread protein domains. The sensing properties of the CBS domains are modulated by the anionic C-terminal tail.

Results

Sensing of Ionic Strength. To investigate the effect of osmotic stress on OpuA from *Lactococcus lactis*, the transporter was reconstituted into liposomes with varying luminal composition, and the activity was determined over a range of external osmolarities. Mg-ATP was present in the vesicle lumen to drive the translocation reaction, and the concentration of potassium phosphate (KPi) was varied from 10 to 120 mM. The concentration of the internal components was isotonic, with 30–140 mM KPi, pH 7.0, in the external medium (0 KCl in Fig. 1*A*). Fig. 1*A* shows the activity of OpuA as a function of external osmolality (KCl concentrations ranging from 0 to 300 mM) and varying luminal concentrations of KPi; similar results were obtained when the external osmolality was varied with KPi, NaCl, or sucrose (not shown). Because (proteo)liposomes behave osmometrically (water diffuses across the membrane in response to the osmotic difference between the internal compartment and the outside medium), their volume will decrease with increasing concentration of KCl (or any membrane-impermeant osmolyte, e.g., KPi, NaCl, or sucrose) on the outside. The internal osmolality and ionic strength will increase accordingly. The data demonstrate that a larger osmotic upshift was needed to activate OpuA when the internal KPi concentration was lower, irrespective of whether KCl (Fig. 1*A*) or another ionic or nonionic

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Abbreviations: CBS, cystathionine β-synthase; ABC, ATP-binding cassette; I_{in}, internal ionic strength; DOPG, phosphatidylglycerol; DOPE, phosphatidylethanolamine; DOPC, phosphatidylcholine.

*To whom correspondence should be addressed. E-mail: b.poolman@rug.nl.

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Fig. 1. The effect of osmotic stress, internal ionic strength, on the transport activity of OpuA. (*A*) Uptake of [^{14}C]glycine betaine by OpuA in 30 (△), 55 (■), 85 (□), 110 (●), or 140 (○) mM KPi, pH 7.0, with or without added KCl as indicated on the *x* axis. The proteoliposomes were composed of 50 mol % DOPE, 12 mol % DOPC, and 38 mol % DOPG. The lumen of the vesicles, isotonic with the external medium, consisted of 9 mM Na_2-ATP, 9 mM $MgSO_4$ plus 10 (△), 30 (■), 60 (), 90 (●), or 120 (○) mM KPi, pH 7.0. (*B*) The same data plotted as a function of internal ionic strength.

membrane-impermeant osmolyte was used to raise the external osmolality (not shown). Stated differently, the lower the internal ion concentration, the more the luminal contents had to increase (by shrinkage of the vesicles) to reach the same activity. Importantly, if the collective data were plotted as a function of internal ionic strength (I_{in}), a single sigmoidal relationship was observed (Fig. 1*B*). Activation of OpuA was largely independent of the type of salt (KPi, KCl, or NaCl) in the vesicle lumen and strongly correlated with I_{in}.

To study the effect of bivalent cations, we had to develop an alternative assay, because free Mg^{2+} or Ba^{2+} ions above a given concentration cause membrane fusion and aspecific substrate leakage from the vesicles. The sensitivity for free multivalent cations depends on the lipid composition of the membrane; with the proteoliposomes specified in the legend to Fig. 8, which is published as supporting information on the PNAS web site, we could use Mg^{2+} or Ba^{2+} ions up to 25–30 mM in the external medium (not taking into account the 9 mM that is complexed with ATP) before leakage of [^{14}C]glycine betaine occurred. Because a fraction of OpuA is reconstituted inside-out, that is, with the ATPase and CBS domains on the outside, one can study glycine betaine efflux, after the addition of MgATP, as a function of external salt concentration. Fig. 8 shows that the rate of glycine betaine efflux increased with increasing KCl, starting at a threshold of ≈75 mM. When 100 mM KCl plus 20 mM $MgCl_2$, in terms of ionic strength equal to 160 mM KCl, was used, the stimulation of transport was significantly greater than with the corresponding concentration of KCl alone. Although bivalent cations could be used only over a small concentration range, this and other experiments showed that Mg^{2+} and Ba^{2+} ions were more effective in activating OpuA than monovalent cations (K^+ or Na^+). Control experiments with MgADP instead of MgATP showed that the efflux was mediated by OpuA and not some aspecific leakage. The collective data of Figs. 1 and 8 (and data with other salts; unpublished results) are consistent with an electrostatic interaction-based activation mechanism and I_{in} the physical parameter being sensed by OpuA.

Engineering Strategy and Kinetic Characterization. To elucidate the role of the CBS domains in OpuA, we have constructed, expressed, and purified a series of OpuA CBS mutants and compared their activities and osmosensing properties after membrane reconstitution. The mutants were chosen to mimic homologues of OpuA that lack either the anionic tail and/or one or both CBS domains (Fig. 2*A*): (*i*) OpuAΔ12 lacks most of the anionic tail; (*ii*) OpuAΔ61 lacks CBS2 plus the anionic tail; and (*iii*) OpuAΔ119 lacks both CBS domains and the anionic tail (see *Supporting Text*, which is published as supporting information on the PNAS web site). Purified, *n*-dodecyl-β-D-maltoside-solubilized OpuA, OpuAΔ12, and OpuAΔ61 bound the fluorescent ATP analogue TNP-ATP with dissociation constants (K_d) of 0.9 ± 0.3 μM. Moreover, the affinity constant (K_m) for glycine betaine transport was 4.7 ± 0.7 μM and the maximal activities (V_{max}) under optimal electrolyte concentrations (see below) were similar, irrespective of the CBS mutation, showing that the mutant proteins were not intrinsically affected in their catalytic performance.

Ionic Strength and Anionic Lipid Dependence. The OpuA, OpuAΔ12, and OpuAΔ61 proteins were reconstituted into liposomes containing dioleoyl lipids with three different headgroups: phosphatidylcholine (DOPC; zwitterionic, bilayer forming), phosphatidylethanolamine (DOPE; zwitterionic, nonbilayer forming), and phosphatidylglycerol (DOPG; anionic, bilayer forming). An ATP-regenerating system was incorporated inside the vesicle lumen to maintain high ATP/ADP ratios and

	Modifications	C-terminus
OpuA	Wildtype	ALADIPDEDEVEEIEKEEENK
OpuA(PstI)	E402A, K403A	ALADIPDEDEVEEIAAEEENK
OpuAΔ12	Δ391-402, K403A	ALA - - - - - - - - - - - - AEEENK
OpuAΔ61	Δ342-402, K403A	Deletion of CBS2 plus anionic tail
OpuAΔ119	Δ284-402, K403A	Deletion of CBS1, CBS2 plus anionic tail

Fig. 2. Schematic of modifications of CBS domains. (*A*) Modifications made to the CBS moieties (in orange) linked to the transporter (in gray) are shown. OpuA(PstI) contains two amino acid substitutions, but its osmoregulatory properties are identical to wild-type OpuA (not shown). Details of the modifications and C-terminal sequences are specified in the table. Because the genes encoding the OpuA systems are translationally coupled, the sequence coding for the five C-terminal amino acids (EEENK) of OpuAA and containing the ribosome-binding site for *opuABC* were not modified. (*B*) Visualization of the expression and purification of OpuA CBS constructs: lane 1, OpuA(PstI); lane 2, OpuAΔ12; lane 3, OpuAΔ61; lane 4, OpuAΔ119; lane 5, OpuA(PstI); lane 6, OpuAΔ12; lane 7, OpuAΔ61. Vesicles (lane 1–4) and Ni-NTA purified proteins (lane 5–7) were analyzed on a Coomassie brilliant blue-stained SDS/PAGE gel (10% polyacrylamide); a total of 6.5 μg of total membrane protein and 2 μg of purified protein were loaded per lane.

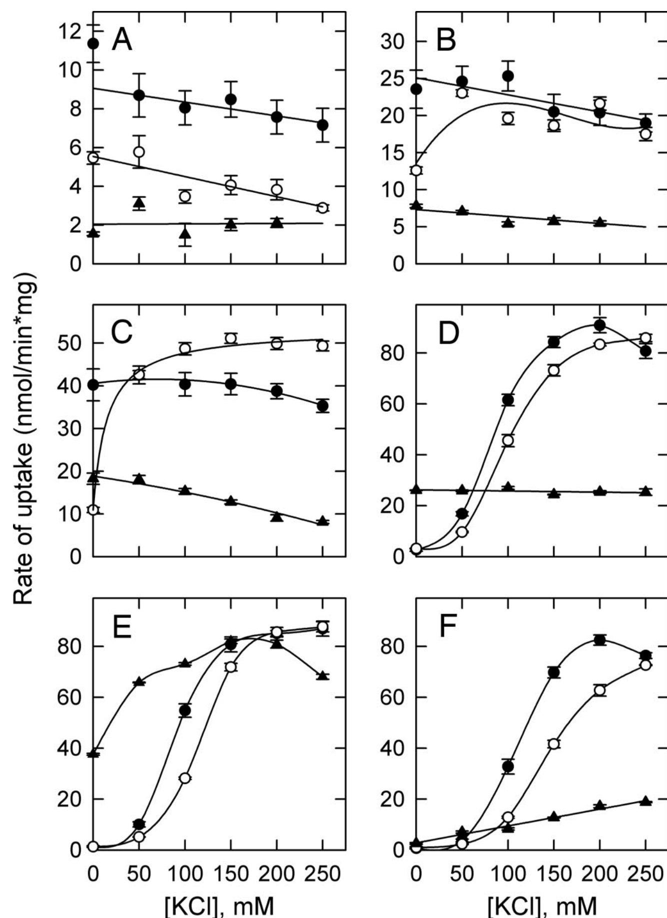

Fig. 3. *In vitro* activation profiles of OpuA and derivatives. Uptake of [14C]glycine betaine by OpuA (●), OpuAΔ12 (○), and OpuAΔ61 (▲) was assayed in 90 mM KPi, pH 7.0, with or without added KCl as indicated on the *x* axis. The proteoliposomes were composed of 50 mol % DOPE, 44 mol % DOPC, 6 mol % DOPG (*A*); 50 mol % DOPE, 37 mol % DOPC, 13 mol % DOPG (*B*); 50 mol % DOPE, 32 mol % DOPC, 18 mol % DOPG (*C*); 50 mol % DOPE, 25 mol % DOPC, 25 mol % DOPG (*D*); 50 mol % DOPE, 12 mol % DOPC, 38 mol % DOPG (*E*); or 50 mol % DOPE, 50 mol % DOPG (*F*). The ATP-regenerating system was enclosed inside the proteoliposomes. The average rates and standard deviations of at least four time points in the linear range (10–45 sec) of the uptake curve are shown.

to drive the translocation reaction for prolonged periods of time. With the ATP-regenerating system inside the vesicle lumen, the I_{in} was ≈0.2, and the concentration of the internal components was isotonic with 90 mM of KPi, pH 7.0, in the external medium. By increasing the external KCl concentration from 0 to 0.25 M, the I_{in} increases from ≈0.2 to ≈0.7. The anionic lipid dependence of OpuA was analyzed by varying the mol % of DOPG from 6 to 50 and adjusting the fraction of DOPC reciprocally. At or below 18 mol % of DOPG, OpuA was already maximally active at I_{in} ≈0.2 and not much affected when the external KCl concentration was increased from 0 to 0.25, and hence the I_{in} was raised from ≈0.2 to ≈0.7 (Fig. 3 *A–C*). It is important to emphasize that at I_{in} ≤0.1, sigmoidal activation profiles similar to those of Fig. 1 were recorded also with 18 mol % DOPG (data not shown). At or above 25 mol % of DOPG in the membrane, OpuA was inactive when I_{in} was low. Consistent with previous work (10), the higher the fraction of anionic lipid, the more salt was needed for activation (Fig. 3 *D–F*).

Contrary to wild-type OpuA, OpuAΔ12 was already inhibited at an I_{in} of ≈0.2 (0 KCl in Fig. 3 *B* and *C*) when the fraction of DOPG was 13 or 18 mol %. At these fractions of anionic lipid, OpuAΔ12 was stimulated by the addition of KCl and hence an increase in I_{in}. Compared to wild-type OpuA, OpuAΔ12 required a higher I_{in} to reach maximal activity; the open-circle curves (OpuAΔ12) in Fig. 3 *D–F* are shifter to higher salt concentrations relative to the closed-circle curves (OpuA). The activity of OpuAΔ61, on the other hand, was <2-fold different between low and high I_{in} when DOPG was varied from 6 to 38 mol % (Fig. 3).

The activities of wild-type OpuA, OpuAΔ12, and OpuAΔ61 as function of the fraction of anionic lipids in the membrane and at low (I_{in} ≈ 0.2) and high ionic strength (I_{in} ≈ 0.6) are shown in Fig. 4. The stronger inhibition by anionic lipids of OpuAΔ12 is evident at 10–20 mol % of DOPG (Fig. 4*B*) but, by varying the I_{in}, the differences between OpuA and OpuAΔ12 could be observed over the entire range of anionic lipids (Fig. 3 *B–F)*. OpuAΔ61 was largely independent of I_{in} in the 0.2–0.6 range and displayed a completely different DOPG dependence than wild-type OpuA and OpuAΔ12. The residual activation of OpuAΔ61 by salt was observed only at high DOPG and may not reflect the genuine electrostatic interaction-based sensing mechanism. The most critical observation is that, contrary to wild-type OpuA and OpuAΔ12, OpuAΔ61 is no longer inhibited when the fraction of DOPG is high (>25%) and I_{in} is low (<0.2). Clearly, the OpuAΔ61 mutant has lost the ionic switching mechanism that becomes operative in OpuA at ≈25% or higher fractions of anionic lipids in the membrane. Although the activity of OpuAΔ61 at 35–40 mol % of DOPG was comparable to that of wild-type OpuA, at <30 and >45 mol % of DOPG, the activity was low. The I_{in} and DOPG dependencies of OpuAΔ119 were very similar to those of OpuAΔ61 (Fig. 4*C Inset*), except that the measured activities were low because of at least a 10-fold reduced expression of the OpuAAΔ119 subunit. In our opinion, the moderate salt (I_{in}) dependence of OpuAΔ61 and OpuAΔ119 (Figs. 4*C* and 3) does not reflect ion sensing to control the transporter activity but some basic ionic strength dependence that any enzyme or transporter may have. Taken together, we propose that the deletion of CBS2 (OpuAΔ61) is sufficient to eliminate the ion switch, whereas ATP binding to the ABC subunit and transporter activity (under optimal conditions; Fig. 4*C*) are comparable to those of wild-type OpuA. The negatively charged C terminus of the CBS shifts the I_{in} dependence of the transporter to higher values but is not essential for ion sensing.

Osmotic Activation *in Vivo*. To assess the physiological relevance of the *in vitro* biochemical experiments, the properties of wild-type OpuA and CBS mutants were also determined in whole cells of *L. lactis*. The initial rate of glycine betaine uptake was measured as a function of external osmolality (Fig. 5; see Fig. 9 *A* and *B*, which is published as supporting information on the PNAS web site, and *Supporting Text* on *in vivo* experiments). The activity of wild-type OpuA increased sigmoidally with increasing osmotic stress, i.e., increasing sucrose concentration as shown in Fig. 5, but similar results were obtained with KCl. To activate OpuAΔ12, higher concentrations of sucrose (or KCl) were required, whereas OpuAΔ61 was largely independent of the applied hyperosmotic stress and displayed only 25% of the maximal activity of wild-type OpuA. Strikingly, these *in vivo* osmotic activation profiles of wild-type OpuA, OpuAΔ12, and OpuAΔ61 are qualitatively very similar to those of the purified proteins reconstituted in liposomes with 50% DOPG (Fig. 3*F*). The fraction of anionic lipids in *L. lactis* is ≈60 mol % and comprises ≈40 mol % of DOPG derivatives plus ≈20% of glycerophosphoglycolipids (16). The agreement between the two datasets indicates that the *in vitro* measurements are made under physiologically relevant conditions. Also, the osmosensitive phenotype of the *L. lactis* Opu401/pNZOpuA(Δ61)His cells *in vivo* (Fig. 9 *A* and *B*) is in full agreement with the low activity of OpuAΔ61 at >45 mol % of anionic lipid.

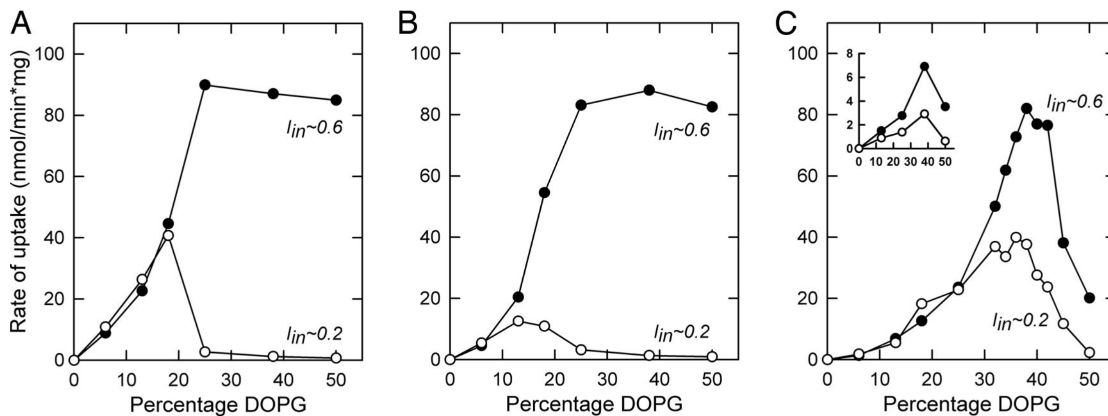

Fig. 4. The effect of anionic lipids on the ionic activation of OpuA and derivatives. The maximal (●) and iso-osmotic activity (○) of OpuA (*A*), OpuAΔ12 (*B*), and OpuAΔ61 (*C*) in proteoliposomes as a function of mol % DOPG. The proteoliposomes were composed of DOPE (50 mol %) DOPC (44–0 mol %) and DOPG (6–50 mol %). Maximal activities were obtained from plots such as those presented in Fig. 3 (each repeated two to three times) and correspond to rates of uptake at 200 mM KCl (I_{in} = ≈0.6); iso-osmotic activities correspond to 0 KCl (I_{in} = ≈0.2). *C Inset* shows the data for OpuAΔ119.

Discussion

Ionic Strength-Sensing Mechanism. Fig. 6 presents a model to explain the effects of ionic strength and anionic lipids on the activation of OpuA. The on/off states represent the active and inactive conformations, respectively. With a low fraction of anionic lipids in the membrane, here represented by 18 mol % DOPG, and an $I_{in} \approx 0.2$, we propose that the CBS domains do not interact with the membrane surface, and the transporter is in the "on" state. Under these conditions, the repulsion by the C-terminal anionic tail may be stronger than the attraction of the CBS domains. With $I_{in} \ll 0.2$, wild-type OpuA was (partly) inhibited at 18 mol % DOPG, and increasing I_{in} stimulated the activity to ≈40 nmol/(min × mg of OpuA). Thus, the on/off state of the transporter is a function of the fraction of anionic lipids and I_{in} over the entire range of DOPG.

At 38 mol % of DOPG, the CBS domains weakly interact with the membrane, and intermediate to high I_{in} is needed for activation. The OpuAΔ12 mutant behaves similarly, except that intermediate to high I_{in} is needed to activate the system at 18 mol % of DOPG, and only high I_{in} suffices to switch the system from "off" to "on" at 38 mol % of DOPG. Apparently, the anionic C terminus prevents the system from assuming an electrostatically locked state, because it is expected to be repelled from the negative surface of the membrane and thereby weaken the interaction of the ion sensor. Without the anionic C terminus, more salt is needed to switch the system from an inactive electrostatically locked state to an active state. Collectively, these experiments provide strong support for the contention that the C-terminal residues of the CBS tandem modulate the activity of the ion sensor.

The OpuAΔ61 mutant is no longer strongly regulated by I_{in}, because the remaining CBS1 is insufficient for interaction with the membrane surface. For the sake of simplicity, we ignore the

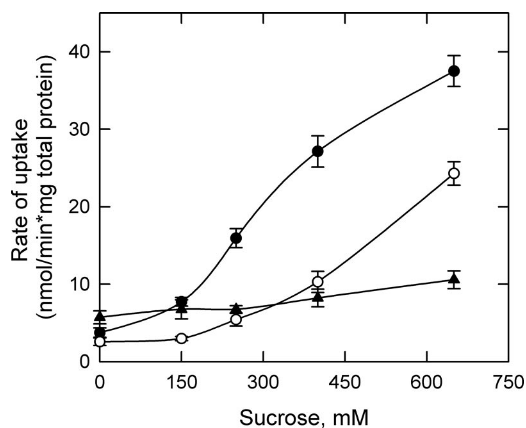

Fig. 5. *In vivo* activation profiles of OpuA and derivatives. *L. lactis* Opu401 cells carrying pNZOpuAHis, pNZOpuA(Δ12)His, or pNZOpuA(Δ61)His were grown in M17 supplemented with 0.5% glucose/5 μg/ml chloramphenicol. For the induction of OpuA (●), OpuAΔ12 (○), and OpuAΔ61 (▲), $1.3 \cdot 10^{-4}$% (vol/vol) culture supernatant of the nisin A producing strain NZ9700 was used. After induction, the cells were washed twice with ice-cold 50 mM Hepes, pH 7.3. Before initiation of transport, cells at 0.4 mg of total protein/ml were preenergized for 5 min with 10 mM glucose (at 30°C). Uptake of [^{14}C]glycine betaine was assayed in 50 mM Hepes, pH 7.3, supplemented with 50 μg/ml chloramphenicol and 10 mM glucose, with or without added sucrose as indicated on the *x* axis.

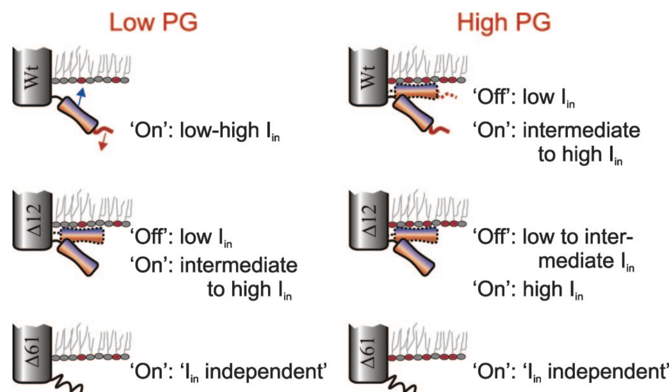

Fig. 6. Schematic representation of the activities of OpuA, OpuAΔ12, and OpuAΔ61 at low (18 mol %) and high (38 mol %) DOPG. "On" and "off" refer to the active and inactive state of transporter; the "off" state is represented by dotted lines. The ionic strength (salt) dependencies of the on/off switch are indicated. Low I_{in} refers to 0 KCl (as in Fig. 3) and corresponds to an internal ionic strength of ≈0.2. High I_{in} refers to 200 mM KCl, yielding an I_{in} ≈0.6, and intermediate I_{in} corresponds to a value of ≈0.4. The transporter (ligand-binding receptor, translocator including ABC) 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; the curled tail in Δ61 depicts the truncated CBS domain; anionic and neutral lipids are represented by red and gray headgroups, respectively. The attraction of the cationic CBS surface and the repulsion of the anionic C terminus by the anionic membrane are highlighted by blue and red arrows, respectively.

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residual activation at high DOPG concentrations (see also *Results*). The ionic switch is no longer functional, because tandem pairs of CBS domains, intimately associated by hydrophobic interactions between homologous β-sheets (4, 5), are most probably needed to make a stable structure.

Preliminary experiments indicate that soluble CBS protein indeed physically interacts with the membrane as a function of surface charge and ionic strength (Fig. 10, which is published as supporting information on the PNAS web site). Whether this electrolyte-dependent interaction forms the sole basis for the proposed ionic activation of OpuA needs to be established in future work. On the basis of the published crystal structures, one could predict the regions that would possibly be interacting with negatively charged membrane surfaces. CBS1 has an α-helical region (denoted H1 in Fig. 7) with cationic residues on the same face of the surface exposed helix. Another potential membrane-interaction site is the turn region (denoted T1) that connects two β-sheets in CBS1 (Fig. 11, which is published as supporting information on the PNAS web site). Irrespective of whether two CBS tandem domains would dimerize, as suggested by the TM0935 structure and confirmed by size-exclusion chromatography experiments with the purified tandem CBS of OpuA (unpublished results), both regions are surface-exposed, as shown from the surface charge distribution of a homology model of the tandem CBS domain (Fig. 11).

Implications of CBS Domains for Transport Mechanism. Because both polypeptides of OpuA are present twice in the functional unit, it is possible that the two tandem CBS pairs of the ATPase subunits also form a dimeric structure. In the catalytic cycle of ABC transporters, the nucleotide-binding domains (NBD) come close together (17), and dimerization of two CBS tandems might increase the affinity between the NBDs. This would be reminiscent of the situation in the maltose transporter from *Escherichia coli*, where the accessory regulatory domain linked to the ATPase unit accounts for the high affinity between the NBDs (18). We also emphasize that, unlike most other well studied ABC transporters, *in vitro* ATP hydrolysis and translocation activity are always tightly coupled in OpuA (19). In the presence of glycine betaine but without threshold levels of salt (or reduced levels of anionic lipids), ATP, bound to the NBDs, is not hydrolyzed. Ionic activation may thus involve a repositioning of the NBDs that allows the catalytic cycle to proceed (unlock the system) rather than alter the conformation of the transmembrane domains directly and thereby affect translocation.

Our data show that the C-terminal anionic tail of the CBS domains in OpuA modulates the ion-sensing properties of the transporter. This role in osmosensing is reminiscent to the coiled-coil structure that is present at the C terminus of the osmoregulatory ProP system (20). In ProP, the accessory α-helical coiled-coil structure is also not essential for osmotic activation of the transporter but tunes the regulation over a specific osmolality range. The sequence of the C-terminal anionic tail of the CBS domains is poorly conserved, and in ProV of the ProU systems from *E. coli* and *Aspergillus fulgidus*, this region is actually missing (Fig. 7). Other homologues of OpuA such as the ProV proteins in methanogenic archaea have extended tails with lengths of more than hundred residues (Fig. 7). The common denominator in many of these sequences are pairs of Glu and/or Asp residues, frequently followed by a Lys residue. We speculate that the C-terminal tails of the CBS domains have evolved to fine tune the ion sensor in response to the abundance of ionic lipids in the membrane and ionic strength of the cytoplasm, which is likely to vary strongly between the different organisms.

Biological Relevance of Electrostatic Switching Mechanism. We provide evidence for a relatively simple mechanism of osmosensing, involving protein modules (CBS domains) that are associated with a wide variety of enzymes and membrane proteins, present in species as diverse as microorganisms, plants, and mammals. The CBS domains of OpuA constitute the sensor that switches the transporter between an inactive electrostatically locked and an active thermally relaxed state by interacting (in)directly with the negatively charged membrane surface in response to the ionic strength. The switching mechanism is an effective means for cells to respond to osmotic shifts, because an increase in medium osmolality will result in a decrease in cell volume, and the accompanying increase in cytoplasmic ionic strength will activate the transporter. Glycine betaine accumulation, followed by water influx, will increase the cell volume and decrease the ionic strength in due time, and the transporter will ultimately be switched off. This inherent feedback mechanism prevents over-accumulation of glycine betaine and couples transporter activity with ionic strength in the cell. Whether other CBS-containing proteins are regulated similarly remains to be determined. It is highly possible that other CBS domains have evolved to sense specific ligands (e.g., adenosine ligands; refs. 8 and 9) rather than ionic strength. Although a high ionic strength activates OpuA (cell shrinkage), the switching logic could be reversed in other CBS-containing proteins, resulting in activation at low ionic strength (e.g., ClC channels activated by cell swelling).

Finally, the high crowding of the cytoplasm and the transient stabilization of macromolecules by screened electrostatic forces suggest that many enzymes and membrane-bound proteins are under the control of ionic strength (21). The CBS domains may be the first of a class of ionic strength sensors to be discovered and, in terms of functional role, it is tempting to make a comparison with the voltage-sensor paddle in the voltage dependent K^+ channels (22). Whereas voltage sensing results from a repositioning of charges in a hydrophobic protein segment within the membrane electric field, ion sensing results from electrostatic interactions between CBS protein segments and the membrane surface. In both cases, structural rearrangements in the sensor are linked to the activation of the transport device. The unraveling of the structural principles associated with the regulation of enzyme and transporter function by CBS domains represents a challenging task for the future.

Materials and Methods

Bacterial Strains, Growth Conditions, and Vesicle Preparations. *L. lactis* strain Opu401 (derivative of NZ9000 in which the *opuA* genes were deleted; see *Supporting Text* for a full description and characterization of the engineered strains) was cultivated semi-anaerobically at 30°C in a medium containing 2% (wt/vol) gistex LS (Strik BV, Eemnes, The Netherlands) and 65 mM Kpi, pH 6.5, supplemented with 1.0% (wt/vol) glucose and 5 μg/ml chloramphenicol when carrying pNZopuAHis or derivatives. For the isolation of membrane vesicles, cells were grown in a 2-liter pH-regulated fermentor to an A_{600} of 2, after which transcription from the *nisA* promoter was switched on by the addition of 0.1% (vol/vol) culture supernatant of the nisin A-producing strain NZ9700. The cells were harvested, and membrane vesicles were prepared according to standard procedures (23).

Purification and Membrane Reconstitution of OpuA. OpuA was purified from membrane vesicles by using Ni^{2+}-NTA chromatography as described (15). The his-tagged proteins were eluted in 50 mM KPi, pH 8.0/200 mM KCl/20% glycerol/0.05% (wt/vol) *n*-dodecyl-β-D-maltoside/200 mM imidazole. Purified OpuA and derivatives were reconstituted in liposomes composed of the desired lipids, essentially as described (23), with some modifications (24). The final protein to lipid ratio was 1:100 (wt/wt); synthetic lipids were obtained from Avanti Polar Lipids.

Ligand-Binding Assay. Binding of 2′(3′)-*O*-(2,4,6-trinitrophenyl) adenosine 5′-trisphosphate (TNP-ATP) was analyzed as described (24).

Transport Assay. ATP-driven uptake of glycine betaine by right-side-in reconstituted OpuA was performed as described (23), with some modifications. Briefly, proteoliposomes were loaded with 9 mM MgATP (prepared from 9 mM $MgSO_4$ plus 9 mM Na_2-ATP) or with an ATP-regenerating system, consisting of 2.4 mg/ml creatine kinase and 9 mM MgATP plus 24 mM creatine-phosphate (disodium salt; Sigma); unless specified otherwise, the standard proteoliposome lumen also contained 50 mM KPi, pH 7.0. After extrusion of the proteoliposomes through a polycarbonate filter (200-nm pore size), the proteoliposomes loaded with an ATP-regenerating system were washed twice with 90 mM KPi, pH 7.0, and resuspended in the same buffer unless specified otherwise (90 mM KPi, pH 7.0, is iso-osmotic with the standard luminal contents and corresponds to an osmolality of ≈220 mosmol/kg) to a concentration of 125 mg of lipids per milliliter. The proteoliposomes loaded with MgATP instead of the ATP-regenerating system were washed and resuspended in 70 mM KPi, pH 7.0 (osmolality of ≈170 mosmol/kg). For osmotically activated transport, the proteoliposomes were diluted to a lipid concentration of 12 mg/ml into assay buffer (90 mM KPi, pH 7.0, or 70 mM KPi, pH 7.0, supplemented with different concentrations of KCl). After incubation for 2 min at 30°C, the transport reaction was initiated by the addition of $[^{14}C]$glycine betaine (Amersham Pharmacia Biosciences) to a final concentration of 31 μM (>10-fold above the K_m for transport and K_d for binding; ref. 15); for kinetic experiments, the $[^3H]$glycine betaine or $[^{14}C]$glycine betaine concentration was varied from 0.06 to 40 μM. At given time intervals, 40-μl samples were taken and diluted with 2 ml of ice-cold isotonic assay buffer. The samples were filtered rapidly through 0.45-μm pore-size cellulose nitrate filters and washed twice with 2 ml of assay buffer. The radioactivity on the filters was determined by liquid scintillation counting.

Calculations. The osmotic pressure of the media was measured by freezing-point depression with an Osmomat 030 (Gonotec, Berlin). Linear relationships between the freezing-point depression and concentrations of the osmolytes were observed. Plots were made to relate osmotic pressure (osmoles per killigram) to the concentration of external osmolytes (different concentrations and ratios of KPi and KCl) and to the concentration of luminal components (including KPi, ATP, $MgSO_4$, and others). By combining the osmotic pressure data, osmotic changes of the external medium were related to changes in the concentration of luminal components. The concentrations of luminal ions were subsequently used to calculate the internal ionic strength (I), according to $I = \frac{1}{2}\sum c_i z_i^2$, 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^+, creatine-HPO_4^-, and creatine-PO_4^{2-}; and assuming that Mg^{2+} is largely complexed with ATP^{4-} (dissociation constant ≈0.1 mM).

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