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Biochemical and Structural Analysis of the *Bacillus subtilis* ABC Transporter OpuA and Its Isolated Subunits

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B. subtilis, osmoprotection · *B. subtilis*, re-association of intact OpuA transporter · OpuA, in vivo studies · OpuA, in vitro analysis · OpuAA, motor domain · SBP OpuAC, from specificity to recognition

Abstract
Adaptation of microorganisms to changing osmotic conditions is a prerequisite for survival and cellular vitality for most microorganisms. In the Gram-positive soil bacterium *Bacillus subtilis*, five transport systems catalyze the uptake of compatible solutes across the plasma membrane that allow the growth of *B. subtilis* over a wide range of osmotic conditions. Focus of this review is the osmoprotectant uptake A (OpuA) transporter, a member of the family of substrate-binding protein (SBP)-dependent ATP-binding cassette (ABC) transporters that mediates the uptake of the compatible solutes glycine betaine and proline betaine. OpuA is composed of three subunits: a nucleotide-binding domain (OpuAA) located in the cytosol, a transmembrane domain (OpuAB), and a SBP (OpuAC), which binds glycine betaine and proline betaine with high specificity and targets it to OpuAB for ATP-dependent translocation across the plasma membrane. After a brief introduction in the field of bacterial osmoadaptation, we will summarize our recent findings about the biochemical and structural analysis of the components of the OpuA systems. Our studies covered both the isolated subunits of the OpuA transporter and initial investigations of the whole transporter in vitro.

General Introduction

Cell volume regulation [Strange, 2004] in the face of sudden osmotic changes in the water content of the environment is of crucial importance to survival and normal cellular function for most microorganisms [Record et al., 1998a, b; Wood, 1999]. Since water can diffuse freely across a cell membrane, any change in the extra- or intracellular solute concentrations will result in the immediate flow of water across the membrane until a steady-state condition is restored [Miller and Wood, 1996; Wood, 1999]. Microorganisms lack active water transport systems to control cell volume [Calamita, 2000]. Furthermore, all *Bacteria* sustain a positive cell turgor with reported values of 3–10 bar for Gram-negative *Bacteria* and 20 bar for Gram-positive *Bacteria* [Csonka and Epstein, 1996; Whatmore and Reed, 1990]. This positive turgor, which is thought to be the driving force for cell expansion, is achieved by maintaining gradients of solute concentrations between the inside and outside of the cell. Following

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the direction of the gradient, the water net flux will cause swelling of the cell under hypotonic conditions or dehydration of the cytoplasm under hypertonic conditions [Miller and Wood, 1996; Wood, 1999]. Thus, microorganisms have to actively cope with such sudden changes in the osmotic conditions of the environment. The discovery of transmembrane proteins acting as water channels, the aquaporins in eukaryotic cells and several microbial species [Agre and Kozono, 2003; Calamita et al., 1998] suggests that dedicated water channels might actively participate in turgor control in some microorganisms. However, not all bacterial species do contain aquaporins.

Whatever their molecular nature, the mechanisms used by microorganisms to maintain cell volume and turgor are generally summarized as 'osmoadaptation', 'osmoregulation' or 'osmoprotection' [Record et al., 1998a]. Since *active* transport systems for water are missing in many microorganisms, the only possibility to regulate turgor is the control of the concentration of osmotically active compounds in the cytoplasm. In the past, two major response pathways have been identified. First, the accumulation of ions, in particular K^+, in the cytosol and, second, the uptake of so-called 'compatible solutes'. The first strategy [Galinski and Trüper, 1994; Ventosa et al., 1998], is normally the initial response of microorganisms to sudden increases in environmental osmolarity. For example, the soil bacterium *Bacillus subtilis* reacts by an immediate uptake of K^+ via high- and low-affinity potassium transport systems [Holtmann et al., 2003; Whatmore et al., 1990; Whatmore and Reed, 1990]. With the exception of certain halophilic *Archea* and halotolerant *Bacteria*, the massive accumulation of potassium interferes with vital cellular processes such as protein function, protein-DNA interactions and DNA replication in most *Bacteria*. Consequently, *B. subtilis* and many other *Bacteria* switch to the second response pathway and start to accumulate compatible solutes either via *de novo synthesis* or *active uptake* [Bremer, 2002; Csonka and Epstein, 1996; Galinski and Trüper, 1994; Kempf and Bremer, 1998; Miller and Wood, 1996]. These compatible solutes not only protect against loss of turgor, they also allow the cell to release the accumulated but detrimental potassium via K^+-specific efflux systems (Kef in *E. coli*) and nonspecific channels (the small and large mechanosensitive channel, MscS and MscL) [Booth and Louis, 1999; Csonka and Epstein, 1996; Stumpe et al., 1996].

Compatible solutes within the domain of *Bacteria* are certain sugars, polyols, amino acids and their derivatives, quaternary amines or their sulfonium counterparts, sulfate esters, N-acetylated amino acids, and small peptides [Welsh, 2000]. All of these molecules are highly soluble under physiological conditions and, most importantly, bear no net charge at neutral pH. In *Archea* on the other hand, it is often found that negatively charged compatible solutes are used for osmoprotective purposes. Since the topic of this review is the osmoprotectant uptake A (OpuA) transporter from the bacterium *B. subtilis*, we will not discuss this interesting difference further. In general, compatible solutes, which bear no net charge at physiological pH, are accumulated to high internal concentration in *Bacteria* without interfering with cellular processes such as DNA replication, cell proliferation, DNA- and RNA-protein interaction, metabolism, or the function of the respiratory chain [Record et al., 1998a, b; Strøm and Kaasen, 1993; Wood, 1999; Yancey, 1994]. Most of the osmoprotectants used by *Bacteria* are metabolically inert and fulfill a dual function in cellular osmostress responses. Their accumulation in the cytosol equilibrates concentration gradients across the cellular membrane and thus maintains cell turgor [Wood, 1999]. Furthermore, they also serve as protein stabilizers [Arakawa and Timasheff, 1985]. This function is not completely understood, but according to the 'volume-exclusion model', compatible solutes are excluded from the immediate vicinity of proteins, thereby increasing the hydration shell around a protein. This effect seems to stabilize the native state of a protein, a function of vital interest in times of stress [Yancey, 1994].

Microorganisms normally use a wide spectrum of compatible solutes and do not limit the uptake to a single type of osmoprotectant [Bremer, 2002; Galinski and Trüper, 1994]. Thus, even variations in the availability of a particular compatible solute does not diminish the effectiveness of compatible solute accumulation as an osmoprotective strategy, since it is highly unlikely that the whole spectrum of compatible solutes used by a single microorganism becomes a limiting factor in natural settings. In addition, the spectrum and the overlapping substrate specificity of the transport systems go in hand with the amount and availability of certain compatible solutes. This is exemplified by glycine betaine (GB), a compatible solute produced by plants and one of the most abundant osmoprotectants found in nature. Many microorganisms have responded to this availability by evolving multiple systems for acquiring GB. For instance, the soil bacterium *B. subtilis* possesses three systems that can transport GB and two that can transport choline, which serves as the precursor for de novo synthesis of GB (fig. 1) [Boch et al., 1994; Bremer, 2002; Bremer and Krämer, 2000; Kappes et al., 1999; Kempf and Bremer, 1998].

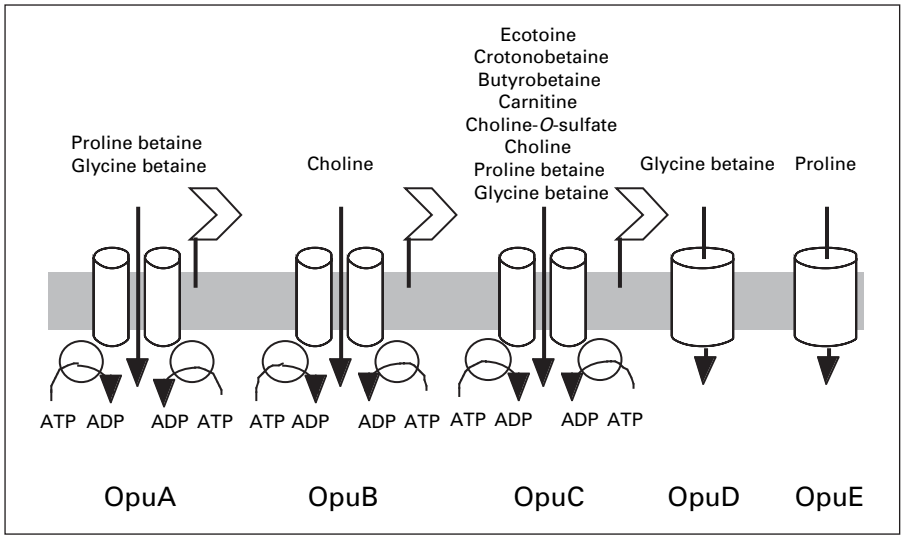

Fig. 1. Osmostress response systems of *B. subtilis*.

Osmoprotection in *B. subtilis*

Five osmoprotectant uptake (Opu) systems have been identified in *B. subtilis* that are specific transporters for compatible solutes (fig. 1) [Bremer, 2002; Kempf and Bremer, 1998]. Three of these, OpuB [Kappes et al., 1999], OpuD, and OpuE [von Blohn et al., 1997], are specific for a single substrate, whereas the other two are rather promiscuous with respect to the nature of the substrate and transport compatible solutes such as GB, ectoine, caritine, choline-*O*-sulfate and proline betaine (PB) but also very rare osmoprotectants such as arsenobetaine [Bremer, 2002].

The Opu systems belong to the families of primary and secondary transporters. OpuD and OpuE are composed of a single component and belong to the family of secondary transporters. OpuE, specific for proline, is a member of the sodium-solute symporter (SSF) subfamily [Spiegelhalter and Bremer, 1998; von Blohn et al., 1997], while OpuD, specific for GB, belongs like BetP from *Corynebacterium glutamicum* and BetT and CaiT from *Escherichia coli* to a growing subfamily of trimethylammonium uptake systems (BCCT) [Kappes et al., 1996; Peter et al., 1996]. OpuA, OpuB, and OpuC are members of the ATP-binding cassette (ABC) transporter family, which can be found in all three kingdoms of life and use the energy of ATP hydrolysis for active transport of an enormous variety of substances across biological membranes [Higgins, 1992]. By definition, these transmembrane proteins are composed of two nucleotide-binding domains or subunits (NBD) and two transmembrane domains or subunits (TMD), which can be arranged in any possible fashion [Schmitt and Tampé, 2002]. In *Archea* and *Bacteria*, all four domains are normally encoded on separate genes, whereas in *Eukaria* all four domains are generally fused on a single polypeptide chain. These systems are referred to as 'full-size transporters', while the fusion of an NBD and a TMD corresponds to a 'half-size transporter'. Thus it is clear that a functional transporter consists of an NBD_2-TMD_2 architecture. The classification of the ABC transporter family relies solely on certain conserved sequence motifs, which are all located within the NBD. These conserved motifs are the Walker A and B motifs (consensus sequence GXXGX(G)KST (Walker A) and ΦΦΦΦD (Walker B), respectively, where X denotes any amino acid and Φ any hydrophobic amino acid) [Walker et al., 1982]; the C-loop or signature motif (consensus sequence LSGGQ), located 20–30 amino acids upstream of the Walker B motif, and the D-loop motif (consensus sequence SALD), placed C-terminal of the Walker B motif [Zaitseva et al., 2005a]. These motifs and the presence of a TMD are the foundation of ABC transporters (see fig. 2). In principle, ABC transporters can perform both the import and export of substrates. However, while in *Archea* and *Bacteria* ABC systems are found that catalyze the vectorial transport in and out of a cell, only exporters of the ABC transporter family have been described in *Eukaria*. In the case of archeal and bacterial importers such as OpuA, OpuB, and OpuC, a fifth component is necessary for functionality, the substrate-binding protein (SBP) [Ames, 1986]. Although it does not belong to the ABC transporter based on the above given definition, it

Fig. 2. Sequence alignment of OpuAA from *B. subtilis* with OpuAA from *L. lactis*, GlcV from *Sulfolobus solfataricus*, MalK from *E. coli*, MalK from *T. litoralis* and CysA from *Alicyclobacillus acidocaldarius*. The latter three NBDs have been chosen because crystal structures are available [Chen et al., 2003; Diederichs et al., 2000; Scheffel et al., 2005]. Conserved motifs within the sequences, which are specific for ABC NBDs are given in bold and labeled. A gray box highlights the two CBS domains of OpuAA in *B. subtilis* and *L. lactis*. The sequence motif specific for ABC NBDs of the CUT1 subfamily (carbohydrate uptake transporter 1) [Saier, 2000; Schneider, 2001] is highlighted by a black box (consensus sequence GI/VRPE [Schneider, 2001]).

is absolutely required for substrate import, since it binds the substrate in the periplasm of Gram-negative *Bacteria* or directly from the extracellular medium in the case of Gram-positive *Bacteria* [Tam and Saier, 1993]. In Gram-negative *Bacteria*, SBPs diffuse freely in the periplasmic space, while in Gram-positive *Bacteria*, SBPs exist as a lipid-anchored form. One of the best-known examples of SBPs is the maltose-binding protein (MalE) [Sharff et al., 1992], which like all other members of this family is composed of two domains or lobes linked by a different number of connecting elements [Fukami-Kobayashi et al., 1999]. Based on structural work, a 'Venus-fly trap' model has been proposed describing the transition between the open (ligand-free) and closed (ligand-bound) form of the receptor in terms of a rigid body motion [Mao et al., 1982]. Thus, as shown in figure 1, the three ABC transporters in *B. subtilis* that confer osmoprotection via the uptake of compatible solutes are clearly members of a subfamily of ABC transporters, the SBP-dependent ABC transporters [Ames, 1986]. Following common nomenclature, the NBD of the OpuA transporter is referred to as OpuAA, OpuAB corresponds to the TMD, and OpuAC is the SBP.

For SBP-dependent ABC transporters, two further complications arise. First, the NBD of some of these importers contain an additional domain not present in ca-

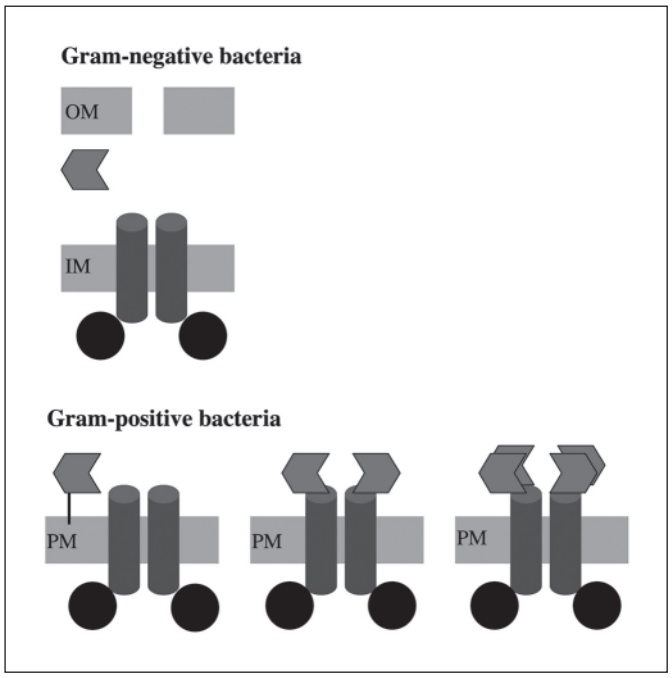

Fig. 3. Architecture of bacterial SBP-dependent ABC transporters. Black circles indicate the NBD, gray cylinders the TMD, and the 'Pacman-like' symbol the SBP. OM = Outer membrane; IM = inner membrane; PM = plasma membrane. The interrupted OM indicates the presence of porins, through which the substrates of SBP-dependent ABC transporters can diffuse into the periplasm.

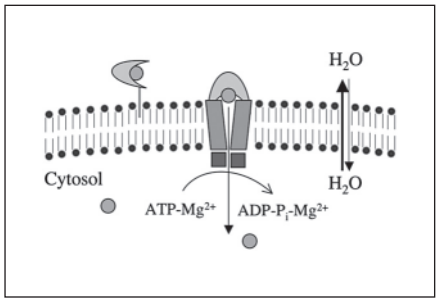

Fig. 4. Schematic architecture of the osmotically regulated ABC transporter OpuA. The circles represent GB or PB, the square OpuAA, the rectangles OpuAB, and the 'Pacman-like' symbol OpuAC.

nonical NBDs [Chang, 2003; Chang and Roth, 2001; Chen et al., 2003; Gaudet and Wiley, 2001; Hung et al., 1998; Locher et al., 2002; Reyes and Chang, 2005; Scheffel et al., 2005; Schmitt et al., 2003; Verdon et al., 2003; Yuan et al., 2001; Zaitseva et al., 2005a]. Examples for these 'non-classic' are MalK from *E. coli* [Davidson, 2002], *Salmonella typhimurium* [Morbach et al., 1993], or *Thermococcus litoralis* [Greller et al., 1999], and GlcV from *Sulfolobus solfataricus* [Verdon et al., 2003]. Rather, these NBDs contain a C-terminal extension of roughly 150 amino acids [Chen et al., 2003; Diederichs et al., 2000; Verdon et al., 2003] (fig. 2). In the maltose-specific ABC transporter from *E. coli*, it was shown that the C-terminal extension interacts with the positive regulatory protein of the maltose regulon, MalT [Panagiotidis et al., 1998] thereby controlling the expression of genes involved in maltose uptake and metabolism. However, such a regulatory function has not been identified in *B. subtilis* for OpuAA. MalK from *E. coli* and *S. typhimurium* belongs to the CUT1 subfamily of ABC transporters [Saier, 2000; Schneider, 2001]. Members of this family contain a diagnostic motif within the additional domain (fig. 2), which is not present in OpuAA. From sequence comparisons, it is evident that OpuAA contains two cystathionine β-synthase (CBS) domains [Banerjee and Zou, 2005]. These domains are involved in the binding of small molecules such as S-adenosine methionine or AMP, thereby regulating the activity of the enzyme. So far, there is no experimental evidence showing whether this is true for OpuAA. Second, it has been shown that SBP, which have long been considered an important but independent part of SBP-dependent ABC transporters, are in certain cases fused to the TMD [Obis et al., 1999; van der Heide and Poolman, 2002]. Based on the analysis of Poolman and co-workers, one, two, or even four SBP can be fused to the TMDs of the cognate ABC transporter (fig. 3). The most prominent example of this subfamily is OpuA from *Lactococcus lactis* [Obis et al., 1999; van der Heide and Poolman, 2000a, b, 2001]. Here, the SBP (OpuAC) is fused to the TMD (OpuAB), while the NBD is encoded on a separate gene. By contrast, in *B. subtilis*, OpuA, like OpuB and OpuC (fig. 1) are classical SBP-dependent ABC transporters: all components, the NBD, the TMD, and the SBP, are encoded on separate genes.

In vivo Studies of OpuA

The architecture of the osmotically regulated ABC transporter OpuA is shown schematically in figure 4. In analogy to other SBP- and non-SBP-dependent ABC transporters it is assumed that the stoichiometry of the complex is OpuA (A_2B_2C). However, the recent finding of Poolman and co-workers [van der Heide and Poolman, 2002], that in certain cases more than one SBP is fused

to the TMD, raises the question whether the above made assumption, which is based on mutational studies performed on the histidine [Ames et al., 2001] and maltose permeases [see for example Hor and Shuman, 1993; Treptow and Shuman, 1985], applies to all SBP-dependent ABC transporters.

The *B. subtilis* OpuA systems was discovered by Kempf and Bremer through functional complementation of an *E. coli* mutant defective in GB uptake [Kempf and Bremer, 1995]. It is composed of the SBP OpuAC (molecular weight 30.5 kDa), the TMD OpuAB (molecular weight 24 kDa), and the ABC subunit OpuAA (molecular weight 47 kDa). The *opuA* locus (3.4 kb) consists of three open reading frames *(opuAA, opuAB, opuAC)* that are genetically organized as an operon whose transcription is induced under high osmolarity growth conditions [Kempf and Bremer, 1995]. The osmotic control of the OpuA operon allows the cell to sensitively adjust the number of the OpuA transporter to the physiological need of the cell – increased uptake of GB – under high osmolarity growth conditions. The OpuA system is related to the ProU osmoprotectant ABC uptake system from *E. coli* [Dattananda and Gowrishankar, 1989; Gowrishankar, 1989; May et al., 1986]. For example, the transmembrane proteins OpuAB and ProW show a sequence identity of 47% over their entire length and they can be aligned without a single amino acid gap. However, OpuAB is considerably smaller than ProW. Most of the reduced size can be ascribed to the N-terminal part of the protein, where the long, periplasmically located N-terminal tail of ProW is missing [Haardt and Bremer, 1996]. A small segment of OpuAB (residues 183–203) display little homology to ProW and it has been speculated that the interaction site for OpuAA is located within or around these residues [Doige and Ames, 1993]. Nevertheless, one can expect a similar topological organization (seven α-helices) for ProW and OpuAB due to the high sequence identity. In the case of the maltose transporter, Ehrmann et al. [1998] proposed eight α-helices for MalF and six helices for MalG. In the crystal structure of the vitamin B_{12} importer BtuCD [Locher et al., 2002], two times 10 α-helices form the TMD. These results clearly suggest that variability in the topological organization within the TMD exists. The functional consequences of such variability within the TMDs are an open question and deserve further studies.

Alignment of OpuAA with ProV, the corresponding protein of the *E. coli* ProU transporter, reveals a sequence identity of 58% and only a single gap needs to be introduced over the entire length. The conservation between these proteins is particularly apparent in the N-terminal half, especially around the Walker A and B motifs and the C-loop, which is a common observation for ABC domains [Schmitt et al., 2003]. However, OpuAA like MalK or ProV from *E. coli* contains a C-terminal extension with respect to other ABC domains (see above). Finally, OpuAC exhibits the features of a secreted protein and contains the characteristic signature of a lipoprotein signal sequence in its first 20 N-terminal amino acids [Kempf et al., 1997]. The positively charged N-terminus is followed by a hydrophobic stretch of amino acids and the sequence LAAC that conforms to the consensus sequence recognized by signal peptidase II. Cys constitutes the N-terminus of the processed protein, and is modified through covalent attachment of lipids, which serve as an anchor to the cytoplasmic membrane in Gram-positive *Bacteria* [Kempf et al., 1997]. OpuAC and the *E. coli* homologue ProX show the least sequence conservation (33% identity) among the components of the OpuA and ProU system. However, Kempf et al. [1997] succeeded in characterizing an OpuAC variant lacking the N-terminal Cys residue; it displays the same binding affinity and kinetic parameters as the wild-type protein but lacks the lipid anchor [Kempf et al., 1997]. Instead, this lipidless mutant OpuAC protein is anchored in the cytoplasmic membrane via its very inefficiently processed signal sequence [Kempf et al., 1997]. In analogy to the well-characterized ABC transporters for maltose [Davidson and Chen, 2004; Ehrmann et al., 1998] and histidine [Ames et al., 2001], and to the recently determined crystal structures of ABC transporters [Chang, 2003; Chang and Roth, 2001; Locher et al., 2002; Reyes and Chang, 2005], one can assume that the OpuAA/ProV and OpuAB/ProW proteins function as homodimers in the *B. subtilis* OpuA and *E. coli* ProU transport systems.

Over the years, the Opu systems from *B. subtilis* have been intensively characterized at the genetic and functional level (for reviews, see Bremer [2002], Bremer and Krämer [2000], and Kempf and Bremer [1998]). The different characteristics of the OpuA, OpuB, and OpuC system with respect to specificity, affinity (K_M = 2–13 μ*M* for different substrates), and transport velocities (V_{max} = 60–280 nmol/min·mg protein for different substrates) permit a flexible adaptation of *B. subtilis* to high osmolality ecological niches with a different spectrum of compatible solutes [Bremer, 2002; Kappes et al., 1999; Kempf and Bremer, 1998]. The kinetic parameters and properties of these ABC transporters allow an efficient scavenging of minute quantities of compatible solutes from environmental sources and their high level intracellular accumulation by *B. subtilis* for osmoprotective purposes

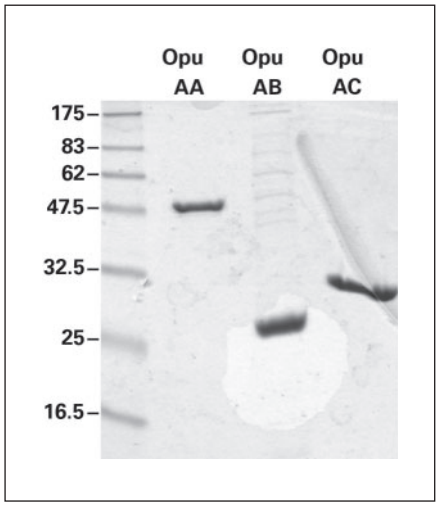

Fig. 5. SDS-PAGE (Coomassie-blue stained) of the individual components of the OpuA transport system. From left to right: Marker proteins (molecular weight are given to the left), OpuAA, OpuAB, and OpuAC. For further details see text.

(>1 *M* [S. Moses, E. Bakker and E. Bremer, unpubl. results]). It must be pointed out that with the exception of proline, compatible solutes used by *B. subtilis* are metabolically inert and are hence only accumulated as osmostress-protective compounds [Boch et al., 1994; Bremer, 2002].

The generation of single and multiple deletion mutants of the individual transporter systems supported the functional in vivo characterization of the osmostress transporter systems in *B. subtilis*. Due to the overlapping substrate specificity of the individual transporters (see fig. 1), these genetically modified *B. subtilis* strains were of paramount importance in the detailed in vivo physiological studies. In addition to the function of the various Opu systems (fig. 1) for the acquisition of compatible solutes for osmoprotective purposes [Bremer, 2002], recent studies revealed a physiological role of compatible solutes in the cellular defense of *B. subtilis* against chill (growth at 15°C) and heat stress (growth at 52°C) [Brigulla et al., 2003; Holtmann and Bremer, 2004]. The OpuA transporter contributes significantly to GB uptake by cells grown either at low or high temperature. Deletion of the various Opu transport systems prevents thermoprotection by compatible solutes thereby ascribing a physiological function to the Opu transporters that goes well beyond their well-established role in osmoprotection. The beneficial effect of GB for *B. subtilis* cells subjected to temperature stress probably stems from the fact that this compatible solute can aid the correct folding and stabilization of proteins, and it is unlikely to be connected to its role in the control of turgor that is vital under osmostress conditions.

Towards an in vitro Analysis of OpuA

Obviously, one of the prerequisite for in vitro studies is the overexpression and functional purification of the OpuA transporter. We followed a strategy, in which all subunits were individually overexpressed in *E. coli* (fig. 5) [Horn et al., 2003, 2005; Kempf et al., 1997]. While purification of the SBP OpuAC was already described [Kempf et al., 1997], new expression protocols were established for the NBD and the TMD, OpuAA and OpuAB, respectively. OpuAA was cloned as an N- or C-terminal His-tagged fusion protein and overexpression was achieved rather straightforwardly in *E. coli* BL21 (DE3) under the control of an arabinose-inducible promoter [Horn et al., 2003]. In contrast, overexpression of the isolated TMD, OpuAB, was only possible in the so-called 'Walker strains', BL21(DE3)C41 and C43 [Arechaga et al., 2000; Miroux and Walker, 1996] under the control of an IPTG- or arabinose-inducible promoter as N- and C-terminal His- and Strep-tagged versions [Horn et al., 2005]. All proteins, OpuAA, OpuAB and OpuAC, could by purified by a two-step purification scheme using the affinity tag as a first step and size exclusion chromatography (SEC) (fig. 5) [Horn et al., 2003, 2005].

Analysis of the Motor Domain OpuAA

As pointed out above, OpuAA was overexpressed as an N- and C-terminal His-tagged fusion protein. During purification, a dynamic monomer dimer equilibrium of the isolated motor domain was observed (fig. 6) [Horn et al., 2003]. Using SEC as a readout for the interconversion, a K_D of dimerization of the nucleotide-free form of OpuAA was calculated to 6 ± 1 μM. The appearance of dimers in the nucleotide-free state is rather unusual for isolated NBDs of ABC transporters. Thus far, dimerization of isolated NBDs by biochemical means has been demonstrated only in the presence of ATP [Janas et al., 2003; Moody et al., 2002; Zaitseva et al., 2005b]. Such an ATP-induced dimerization is further supported by the available crystal structures [Chen et al., 2003; Smith et al., 2002; Zaitseva et al., 2005a, b]. However, MalK from *E. coli* also formed dimers in the absence of nucleotides

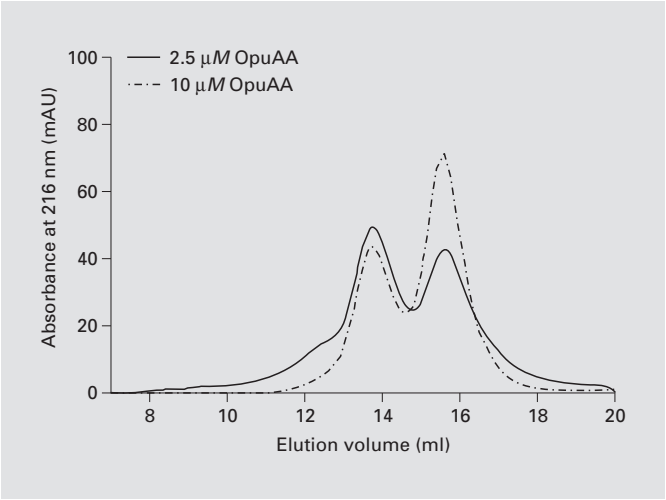

Fig. 6. Size exclusion chromatogram of OpuAA at two different protein concentrations [adopted from Horn et al., 2003].

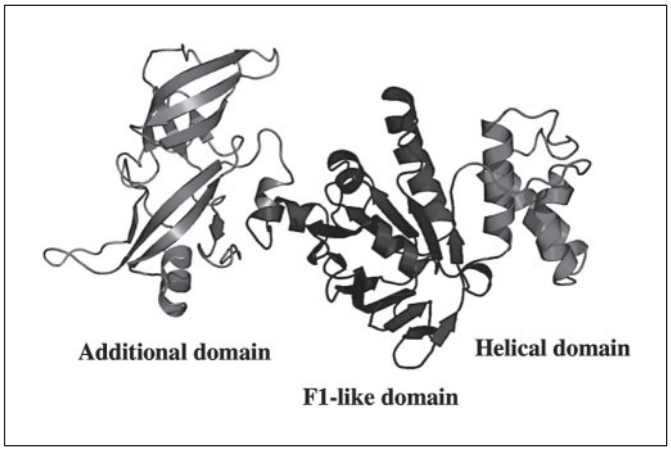

Fig. 7. Molecular model of OpuAA derived from the crystal structure of MalK from *T. litoralis*. Modeling was performed using the web interface of SwissModel (swissmodel.expasy.org) employing standard settings.

[Sharma and Davidson, 2000]. As shown by cross-linking studies [Samanta et al., 2003], dimer stabilization occurs entirely via the additional C-terminal 150 amino acids, which form the regulatory domain of MalK. OpuAA also contains an additional domain at its C-terminus with unknown function. A structural model of OpuAA (fig. 7) displays a monomeric organization similar to that of MalK from *E. coli* [Chen et al., 2003] and *T. litoralis* [Diederichs et al., 2000]. Thus, one can easily envision that dimer formation in the nucleotide-free state of the isolated OpuAA also occurs via interactions of the additional domain. However, no stable dimers were detected for GlcV from *S. solfataricus* [Verdon et al., 2003] and MalK from *T. litoralis* [Greller et al., 1999] in the nucleotide-free form, although both NBDs also contain an additional domain. This raises the obvious question of whether the simple presence of such an additional domain is sufficient for dimerization in the nucleotide-free state or whether certain structural and/or functional requirements of so far unknown nature have to be encoded within the additional domain to allow dimer formation. In summary, experimental results derived from certain NBDs support the statement that the additional domain in NBDs stabilizes a dimeric NBD form. However, this particular dimer is different from the catalytically active dimeric NBD form, in which a composite dimer of the Walker A motif of monomer 1 and the C-loop of monomer 2 with ATP sandwiched in between is formed [Fetch and Davidson, 2002], and a clear distinction has to be made. Rather, the dimer induced by the presence of the additional form allows a 'tweezers-like motion' [Chen et al., 2003] around the additional domain, which obviously plays a pivotal role in this movement. However, one has to keep in mind that the crystal structure of MalK was determined in the absence of the TMD [Chen et al., 2003] and that although the vanadate cleavage data establishing the composite dimer arrangement in MalFGK2 [Fetch and Davidson, 2002] supports the crystal structure of the isolated NBD, the possibility still exists that the MalK architecture in the fully-assembled maltose transporter might look different from the one seen in the crystal structure. Further details for the interested reader are given in Chen et al. [2003] and Davidson and Chen [2004].

A dynamic monomer-dimer equilibrium will hamper any detailed functional analysis, because the contribution of each molecular species to, for example, the apparent ATPase activity cannot be separated in a straightforward manner. As pointed out above, the initial response of *B. subtilis* to hyperosmotic conditions is an uptake of potassium ions, which increases the ionic strength of the cytosol [Kempf and Bremer, 1998; Whatmore and Reed, 1990]. In search of conditions that stabilized the monomeric and dimeric species, e.g. that would slow down the interconversion rate between both species, it was observed that increasing ionic strength increased the half-time of interconversion. For example, at physiological salt conditions (150 m*M* NaCl), a half-time of interconversion of 1.5 ± 0.2 days was calculated for the in vitro situation. In contrast, the half-time increased to 16 ± 0.5 days in 1 *M* NaCl [Horn et al., 2003]. The interconver-

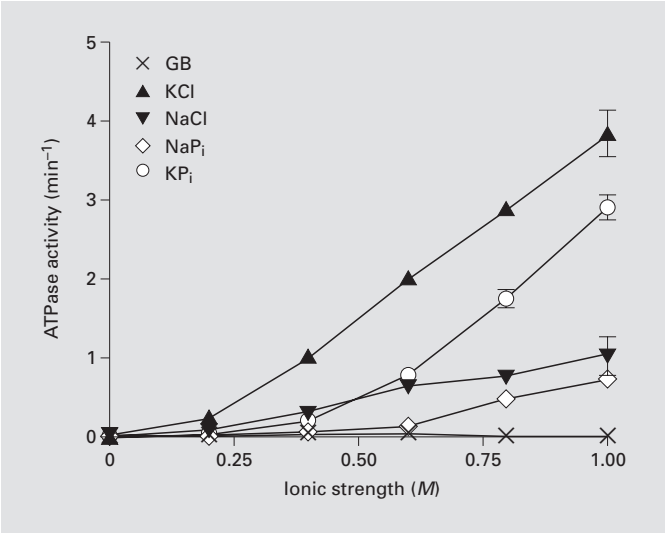

Fig. 8. Influence of ionic strength and nature of the compounds on the ATPase activity of isolated OpuAA [adopted from Horn et al., 2003].

Table 1. ATPase activity of monomeric and dimeric OpuAA

Oligomeric species	Salt	K_M, m*M*	k_{cat}, min^{-1}
Monomer	1 *M* KCl	5.4 ± 0.6	15.5 ± 1.10
Monomer	1 *M* NaCl	0.45 ± 0.06	2.3 ± 0.1
Dimer	1 *M* NaCl	1.3 ± 0.5	0.24 ± 0.04
Monomer and dimer	0.15 *M* NaCl	0.25 ± 0.03	0.25 ± 0.01

Data were taken from Horn et al. [2003].

sion rates of monomeric and dimeric OpuAA will not effect substrate transport in vivo but represent a challenge for any in vitro study, but this increase was sufficient to separate both molecular species to near homogeneity (>95%, data not shown) and therefore to determine the catalytic parameters of monomeric and dimeric OpuAA under different conditions separately (table 1, fig. 8). ATPase activity was dependent on ionic strength. Increasing concentrations of GB, one of the two main substrates of the OpuA transporter, did not influence the kinetic parameters of ATP hydrolysis (data not shown; for further details, see fig. 8 and Horn et al. [2003]). However, it was evident that the dimer of OpuAA displayed a much lower ATPase activity than the monomeric species of OpuAA. This result was quite surprising, since it is now generally accepted that a dimeric NBD is the catalytically active species during ATP hydrolysis (see below). On the other hand, potassium ions used as a first response to osmotic stress also modified ATPase activity of the isolated NBD (table 1) [Horn et al., 2003]. In light of the in vivo situation, a K^+ modulation of ATPase activity would reflect a potential mechanism to control the initial steps in the OpuA-mediated GB uptake by *B. subtilis*. Furthermore, this modulation also suggested the presence of a K^+-binding site within OpuAA, a situation reminiscent of the signal kinase KdpD of *E. coli* [Jung et al., 2000] or the osmoreactive betaine carrier BetP from *C. glutamicum* [Kramer and Morbach, 2004; Schiller et al., 2004].

Activation of ATPase activity of the close relative OpuA from *L. lactis* occurs via negatively charged lipids and phosphate ion-containing buffers [van der Heide and Poolman, 2000a, b; van der Heide et al., 2001]. However, stimulation of the ATPase activity of OpuAA from *B. subtilis* was rather insensitive to the nature of the anion (fig. 8). Rather, the nature of the cation had a more pronounced effect. Here again, K^+ produced a roughly 4-fold higher stimulation than Na^+, whereas GB did not produce any stimulation [Horn et al., 2003]. This implies that the OpuA transporters from *L. lactis* and *B. subtilis* use different modes of activation and regulation of their activity despite their high sequence similarity, their osmoprotective function, and the common function as GB transporters.

In the past, fluorescence-modified nucleotides such as trinitrophenyl (TNP)-modified nucleotides have been used for nucleotide-binding proteins [Faller, 1989, 1990] to determine kinetic and thermodynamic parameters of the respective complexes. However, in the case of OpuAA and other ABC systems, such fluorescence-modified nucleotides are not as powerful, since the additional fluorophore moiety resulted in a 10- to 100-fold increase in affinity (table 2) [Horn et al., 2003]. To obtain the real affinity constants of the OpuAA/ATP and OpuAA/ADP complexes, competition experiments were performed. The results obtained revealed that the cofactor Mg^{2+} was essential for effective binding. This is in clear contrast to for example the NBD of the ABC transporter HlyB, where the cofactor had no detectable influence on nucleotide affinity [Zaitseva et al., 2005b]. Second, affinities decreased with increasing ionic strength and one has to keep in mind that the OpuA transporter has to import GB under high ionic strength conditions.

NBDs of ABC transporters energize the translocation of the substrate and adopt different functional states during the catalytic cycle. The K_D of dimerization of OpuAA was determined to 6 ± 1 μ*M* in the nucleotide-free state.

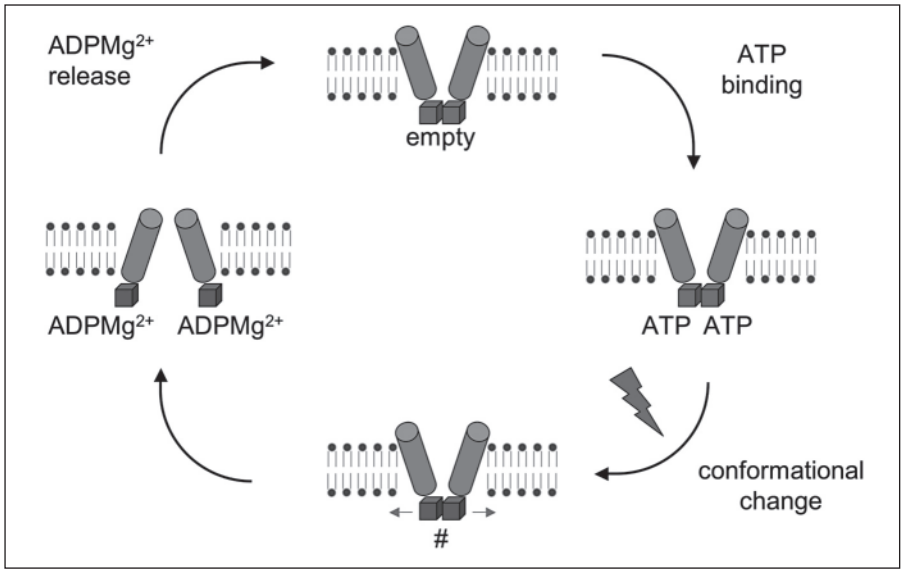

Fig. 9. Proposed model of OpuAA and the coupling of mechanical movement of the NBD with conformational changes of the TMD [adopted from Horn et al., 2003]. The third state (highlighted by an #) of the model represents the moment of ATP hydrolysis, which drives dimeric OpuAA apart. In the model, the unknown conformational change indicated by the symbol, occurs due to NBD:TMD interaction as proposed for example for BtuCD [Locher et al., 2002].

Interestingly, the K_D of dimerization was dependent on the functional state of OpuAA. These data suggested that the capability of dimerization can be used to generate mechanical movement of the NBDs, which can be transmitted to the TMDs as suggested in figure 9. Addition of AMP-PNP/Mg^{2+}, which is thought to mimic the moment of ATP hydrolysis, raised the K_D of dimerization to 50 $\pm$ 4 μM, while no dimer was detectable after the addition of ADP or ADP/Mg^{2+} (K_D >200 μM). Thus, according to our model, ATP hydrolysis drives the NBD dimer apart and dimerization is prevented in the ADP-bound state. This movement is transmitted to the TMD, and the mechanical stress imposed induces conformational changes within the TMD. According to the model proposed for the maltose permease [Davidson, 2002], conformational changes within the TMD open and close the translocation pathway for the substrate. Obviously, the tight coupling between dimerization and the functional state of the NBD is used to control these changes and allow efficient energy coupling and transmission, which ensures efficient substrate translocation across the membrane [Davidson and Chen, 2004].

Table 2. Dissociation constants of OpuAA/ATP and OpuAA/ADP complexes

Oligomeric species	NaCl *M*	$MgCl_2$ m*M*	Ligand	K_D, m*M* AXP	K_D, μM TNP-AXP
Monomer	0.15	0	ATP	2.8 ± 0.3	9.5 ± 0.1
Dimer	0.15	0	ATP	3.5 ± 0.5	5.1 ± 0.1
Monomer	0.15	0	ADP	5.0 ± 0.2	9.5 ± 0.1
Dimer	0.15	0	ADP	4.3 ± 0.4	5.1 ± 0.1
Monomer	0.15	5	ATP	0.45 ± 0.05	7.8 ± 0.5
Dimer	0.15	5	ATP	1.8 ± 0.3	34 ± 1
Monomer	0.15	5	ADP	0.09 ± 0.01	7.8 ± 0.5
Monomer	1	5	ATP	0.054 ± 0.004	7.0 ± 1.5
Dimer	1	5	ATP	>10	20 ± 1
Monomer	1	5	ADP	0.02 ± 0.002	7.0 ± 1.5

For comparison, K_D values of the corresponding complexes using TNP-modified nucleotides are also given. Data were taken from Horn et al. [2003].

Re-Association of the Intact OpuA Transporter from *B. subtilis*

The best-studied systems of SBP-dependent ABC transporters are the histidine and maltose importers (for detailed reviews, see Ames [1986] and Davidson and Chen [2004]). For both systems, it was demonstrated that the TMDs, HisM and HisQ in the case of the histidine importer and MalF and MalG in the case of the maltose importer, respectively, could only be overexpressed in the presence of the NBD [Liu and Ames, 1998; Sharma et al., 2005]. This raised the possibility that the NBD acts as a 'chaperone' that supports the functional folding of the TMD components. Nevertheless, overexpression of the intact ABC transporter allowed a specific exchange of the NBDs in membrane vesicles enriched in the correspond-

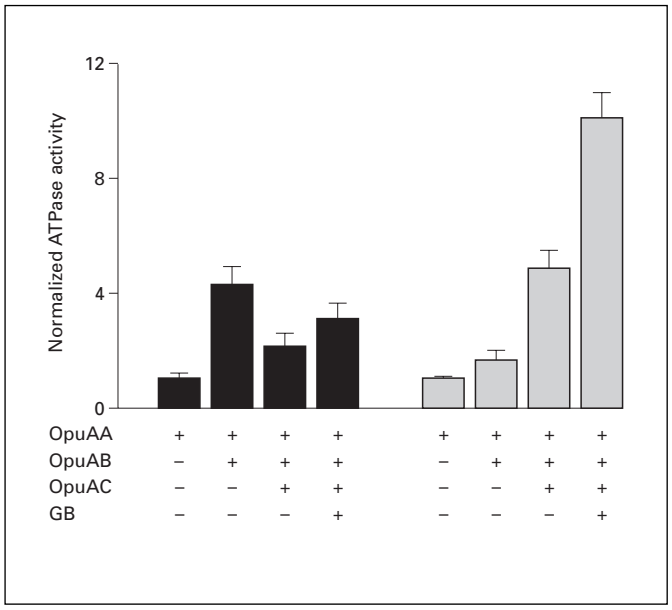

Fig. 10. 'Mixing experiments' [Horn et al., 2005]. The individual components of the OpuA transporter were mixed in detergent solution as indicated and the ATPase activity of OpuAA was used as readout. Monomeric (black bars) and dimeric OpuAA (gray bars) were separated prior to the experiments [reproduced from Horn et al., 2005, with permission].

ing permease. This was possible for the wild-type NBD of the histidine permease, HisP, but only for a His-tagged version of the NBD of the maltose importer, MalK. In contrast, the TMD of the OpuA transporter, OpuAB, was successfully overexpressed in the so-called 'Walker strains' [Arechaga et al., 2000; Miroux and Walker, 1996] even in absence of its cognate NBD (see above) [Horn et al., 2005].

OpuAB was readily solubilized from the membrane of overproducing *E. coli* strains (data not shown) using a minimal detergent screens and could be easily extracted in dodecylmaltoside (DDM) or Triton X-100. After a two-step purification procedure employing the affinity tag (either Strep or His tag) and a SEC step, OpuAB was obtained with a purity greater than 90% as judged from a Coomassie-stained SDS gel (see fig. 5). Furthermore, SEC revealed that detergent solubilized OpuAB (DDM or Triton X-100) eluted in a higher oligomeric form (data not shown). However, size estimates of membrane proteins based on SEC have to be interpreted with caution and further experiments are necessary to determine the oligomeric state of detergent-solubilized OpuAB beyond any doubt. Nevertheless, the fact that OpuAB eluted in a non-aggregated form implied that the protein adopted a native-like structure in a detergent micelle. This is to our knowledge next to RbsC, the TMD of the ribose importer [Zaitseva et al., 1996], the only TMD of ABC transporter, which was overexpressed and purified in the absence of the cognate NBD [Horn et al., 2005].

Despite the rather promising SEC data, a final proof for functional folding of OpuAB was still missing. Therefore, the OpuA transporter was 're-associated' in detergent solution from its individual components. Here, formation of the OpuA transporter from its subunits should result in ATPase stimulation of OpuAA if a functional competent transporter was formed. To evaluate this proposal, monomeric and dimeric OpuAA were separated (fig. 10) [Horn et al., 2003]. The highest degree of stimulation for monomeric OpuAA was detected for the OpuAA-OpuAB pair (4-fold). However, a different picture emerged for dimeric OpuAA. Here, hardly any stimulation was detected for the OpuAA/OpuAB combination. In contrast, a 5-fold stimulation was detected in the case of OpuAA/OpuAB/OpuAC in the absence of GB and a 10-fold stimulation for the same combination in the presence of GB. This situation reflects what is expected for SBP-dependent ABC transporters and was already described for the maltose and histidine systems [Liu et al., 1997; Sharma et al., 2005]. Furthermore, it indicates that all three components, although purified in isolated form, adopt a functional fold and are able to form at least transiently the fully assembled OpuA transporter in detergent solution [Horn et al., 2005].

From Specificity to Recognition – the SBP OpuAC

In all SBP-dependent ABC transporters whether from *Archea*, Gram-positive, or Gram-negative *Bacteria*, the SBP primes the ABC transporter with specificity, because the SBP recognizes and delivers the substrate to the TMD for further, ATP-dependent translocation [Ames, 1986]. In the case of OpuA, OpuAC achieves this task. After translocation via the Sec machinery, the first 20 amino acids of the precursor protein are cleaved by signal peptidase II and the mature protein becomes lipidated at residue Cys20, which tethers the protein to the plasma membrane. As pointed out above, OpuAC was overexpressed heterologous in *E. coli* as a MBP-OpuAC fusion protein without the first 20 N-terminal amino acids and with a mutation at position 20 (Cys20Ala) to prevent OpuAC lipidation [Kempf and Bremer, 1995]. OpuAC

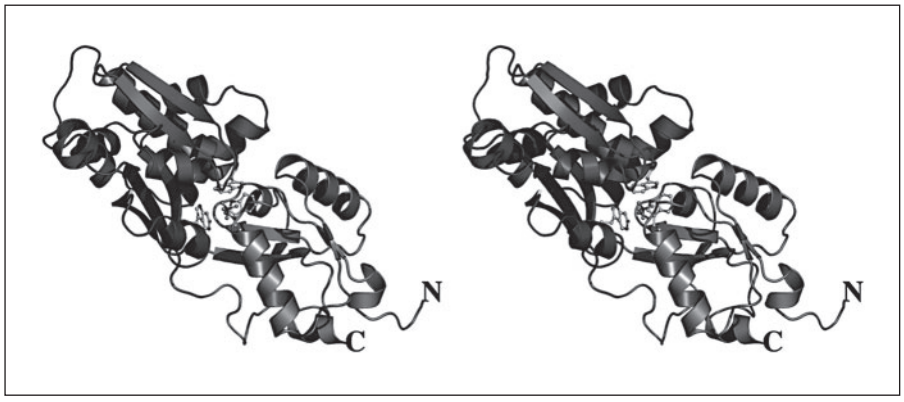

Fig. 11. Stereoview of the overall crystal structure of the OpuAC/GB complex at 2.0-Å resolution. The three Trp residues forming the Trp prism are highlighted in stick representation.

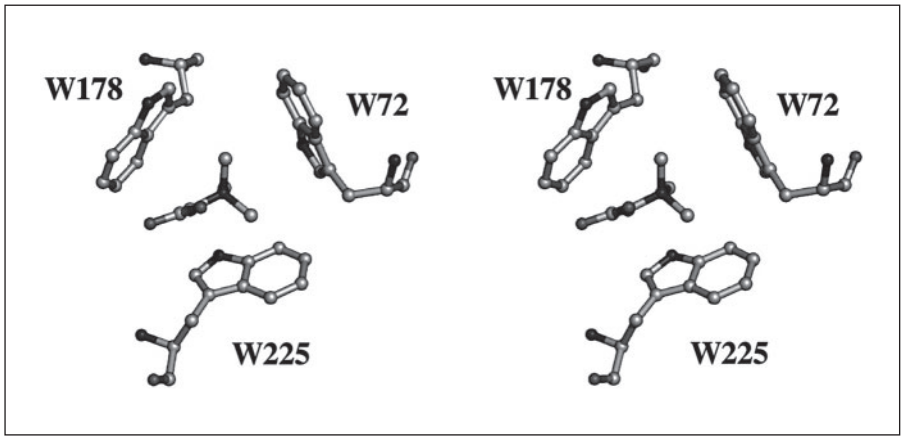

Fig. 12. Stereoview of the architecture of the ligand-binding site. The three Trp's form a 'Trp prism', which coordinates the delocalized positive charge of the quaternary trimethylammonium moiety of GB.

was liberated from the fusion protein by proteolytic cleavage and then purified to homogeneity (fig. 5).

As an initial step in characterizing the function of OpuAC, the affinities of GB and PB were determined by intrinsic Trp quenching. Interestingly, the affinity of these two substrates differed by an ordered of magnitude. The affinity of OpuAC to GB was determined to 19 ± 3 μ*M*, while PB was bound at an affinity of only 219 ± 30 μ*M*. This fact was quite surprising because both substrates are rather similar in their chemical structure: a quaternary ammonium group and a carboxylate moiety. Both GB and PB are recognized by the *E. coli* solute-binding protein ProX with similar affinities: 1 μ*M* for GB and 5 μ*M* for PB [Haardt et al., 1995; May et al., 1986] and are bound by ProX within the same binding pocket [Schiefner et al., 2004]. To further elucidate the molecular rules dictating the different affinities of OpuAC for GB and PB, the crystal structures of the OpuAC/GB and OpuAC/PB complexes were determined at a resolution of 2.0 and 2.8 Å, respectively [Horn et al., J Mol Biol, in press].

The structure of OpuAC resembles the well-known overall structure of SBPs [Fukami-Kobayashi et al., 1999; Quiocho and Ledvina, 1996], which are composed of two domains or lobes, which are connected by two hinges (fig. 11). Each domain is composed of a central five-stranded β-sheet flanked by several α-helices. According to the topology of the central β-sheet, OpuAC belongs to class II of the SBP superfamily [Fukami-Kobayashi et al., 1999]. The ligands, either GB or PB, are bound in a deep cleft between the domains, which according to the 'Venus-fly trap' model [Mao et al., 1982] is thought to convert from an open to a closed conformation upon ligand binding. In this ligand-closed conformation, the quaternary trimethylammonium group of GB is coordinated via cation-π interactions [Ma and Dougherty, 1997] between GB and three highly conserved Trp residues of OpuAC (highlighted in sticks in fig. 11). Here, the three Trp residues adopt a 'Trp prism'-like architecture (fig. 12), which accommodates the delocalized positive charge of the trimethylammonium group of GB [Horn et al., J Mol Biol, in press]. This arrangement is slightly different from the

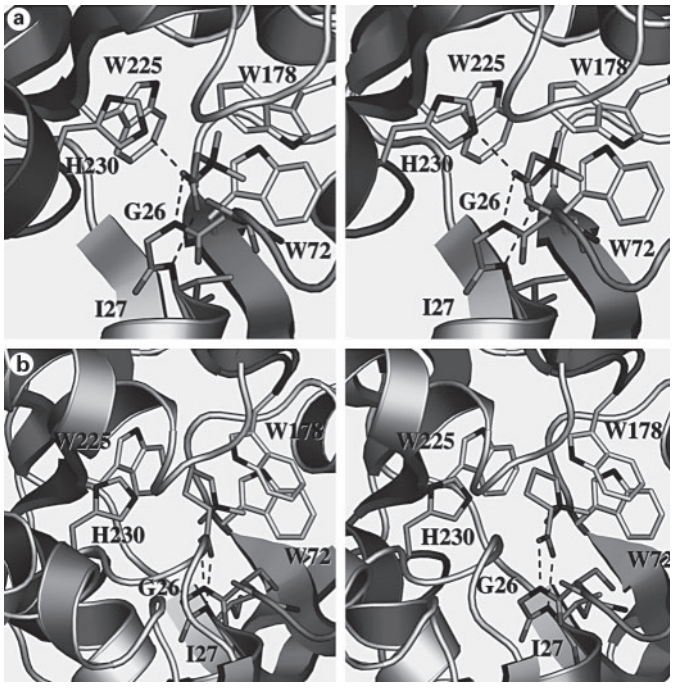

Fig. 13. Stereoview of the geometry of the ligand binding sites: OpuAC/GB (**a**) and OpuAC/PB (**b**).

architecture observed for a homologue of OpuAC, ProX from *E. coli* [Schiefner et al., 2004]. This SBP is also specific for GB and PB, but the three aromatic amino acids, which are important for ligand binding, form a 'Trp box' rather than the prism-like arrangement observed here. Thus, cation-π interactions, which are an important determinant in many ligand recognition processes [Ma and Dougherty, 1997], can adopt multiple conformations such as the Trp box or the Trp prism to stabilize and efficiently bind the ligand GB.

The ligand-binding sites of the OpuAC/GB and OpuAC/PB complexes are depicted in figure 13. Cation-π interactions fix the delocalized positive charge of the substrates, while hydrogen bonds with two backbone amide and with His230 coordinate the negative charge of the carboxylate of GB. However, the hydrogen bond between His230 and GB is not observed in the PB complex, while all other distances were preserved within experimental error. Actually, the distance is increased from 2.6 Å for the GB complex (distance of a strong hydrogen bond), to 4.7 Å in the PB complex, a distance that does not allow hydrogen bonding [Horn et al., J Mol Biol, in press]. Based on energetic arguments, loss of a single hydrogen bond (4–20 kJ/mol per hydrogen bond [Fersht, 1997]) might easily explain the 10-fold increase in affinity of the OpuAC/GB complex over the OpuAC/PB complex ($\Delta\Delta G$ = 6.7 kJ/mol). Additionally, this missing hydrogen bond in the OpuAC/PB complex changes the interactions across the domain-domain interface in a rather subtle manner (data not shown). Thus, the two crystal structures provided valuable insight into the binding mode for the two main substrates of OpuAC, GB and PB. Most important, they provide a structural explanation for the drastic difference in affinity despite the similar chemical structure of both compatible solutes.

SBP are structurally rather conserved, although the primary sequences might diverge. Obviously, three-dimensional structures of members of SBP subfamilies serve as templates to derive structural models for other members of the same subfamily without having to determine each individual crystal structure. However, in all these efforts, domain dislocation [Fukami-Kobayashi et al., 1999] and other evolutionary changes might influence the outcome of sequence alignments. In the case of OpuAC, examination of the complete primary sequence revealed a number of close homologues such as the known GB-binding protein GbuC from the human pathogen *Listeria monocytogenes* [Ko and Smith, 1999]. After taking domain dislocation, e.g. exchange of domains 1 and 2 into account, for example the GB/PB-binding protein ProX from *E. coli* could readily be aligned in terms of primary sequence as well as three-dimensional structure. However, many more protein sequences (around 40 so far, data not shown) could be aligned with OpuAC after splitting the protein into two halves. This split did not coincide with the domain boundaries of SBPs. Rather a cut within the central β-sheet of domain provided a perfect template for sequence-based alignment. Based on this 'in-domain' cut [Horn et al., J Mol Biol, in press], all three Trp residues forming the 'Trp prism' were absolutely conserved in all SBPs used in the alignment. Furthermore, residues essential for ligand binding and domain-domain interactions were either retained or exchanged conservatively. The crystal structure of OpuAC might thus serve as a suitable template to predict the structures of many putative GB/PB ligand-binding proteins from a variety of microbial species. In summary, the crystal structures not only provided a molecular explanation for the different affinities of the *B. subtilis* OpuAC protein for GB and PB, which did not occur in ProX from *E. coli* [Schiefner et al., 2004], but also raised the question of the evolutionary origin of a SBP ancestor [Fukami-Kobayashi et al., 1999].

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