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Structural analysis of the choline-binding protein ChoX in a semi-closed and ligand-free conformation

Christine Oswald[1], Sander H.J. Smits[1], Marina Höing[2], Erhard Bremer[2] and Lutz Schmitt[1,*]

[1] Institute of Biochemistry, Heinrich Heine University Düsseldorf, Universitätsstr. 1, D-40225 Düsseldorf, Germany

[2] Laboratory for Microbiology, Department of Biology, Philipps University Marburg, Karl-von-Frisch Str. 8, D-35032 Marburg, Germany

* Corresponding author
e-mail: lutz.schmitt@uni-duesseldorf.de

Abstract

The periplasmic ligand-binding protein ChoX is part of the ABC transport system ChoVWX that imports choline as a nutrient into the soil bacterium *Sinorhizobium meliloti*. We have recently reported the crystal structures of ChoX in complex with its ligands choline and acetylcholine and the structure of a fully closed but substrate-free state of ChoX. This latter structure revealed an architecture of the ligand-binding site that is superimposable to the closed, ligand-bound form of ChoX. We report here the crystal structure of ChoX in an unusual, ligand-free conformation that represents a semi-closed form of ChoX. The analysis revealed a subdomain movement in the N-lobe of ChoX. Comparison with the two well-characterized substrate binding proteins, MBP and HisJ, suggests the presence of a similar subdomain in these proteins.

Keywords: ABC transporter; choline-binding protein; conformational change; membrane transport.

Introduction

Binding-protein dependent ABC (ATP binding cassette) transporters are widely distributed within the domains of the *Bacteria* and *Archaea* where they serve for the import of nutrients, ions, peptides, and osmolytes (Davidson et al., 2008). The functioning of these systems depends on an extracellular substrate-binding protein (SBP) that captures the ligand with high affinity and subsequently delivers the bound ligand to the cognate transmembrane domains (TMDs) of the ABC system for ATP-dependent import. The substrate specificity of a given ABC importer is established by the SBP; the SBP determines the directionality of the overall transport reaction and its interaction with the TMD regulates the ATPase activity of the nucleotide-binding protein (Davidson et al., 2008). As a consequence, the SBP is not only critical for capturing the substrate from the environment but it is also a key player for the communication process between the subunits of the ABC importer during the overall transport reaction (Orelle et al., 2008). Recently, the crystal structures of complete binding protein-dependent ABC importers have been determined together with their cognate SBP (Hollenstein et al., 2007; Hvorup et al., 2007; Oldham et al., 2007; Pinkett et al., 2007; Gerber et al., 2008). These structures have greatly fostered our insights into the interactions between the SBP and the TMD and the structural rearrangements that occur within a given ABC transporter during different stages of its transport cycle (Khare et al., 2009).

Structural studies of a substantial number of SBPs revealed a common fold and bilobal organization in which the 'N-lobe' and the 'C-lobe' of the SBP are connected via a flexible linker region (Wilkinson et al., 2003). In the ligand-free conformation, the two lobes are well separated from each other, thereby forming a deep, solvent exposed cleft, the so-called open conformation. The substrate-binding site lies within this cleft and binding of the ligand triggers a hinge-bending motion, and sometimes also a rotation, of the two lobes towards each other. As a consequence, residues originating from both lobes of the SBP dynamically form the architecture of the ligand binding site (Wilkinson et al., 2003). In the absence of the ligand, the two states of the SBP, unliganded-open and unliganded-closed, are in equilibrium and the function of the ligand is to shift this ratio towards the liganded-closed state. This sequence of events has been coined the 'Venus-fly trap mechanism' (Mao et al., 1982; Sack et al., 1989; Quiocho and Ledvina, 1996). The 'Venus-fly trap' model is supported by a substantial number of X-ray structures of SBPs in the absence and presence of ligands (Oh et al., 1993; Oswald et al., 2008a) and many biophysical techniques, including NMR, fluorescence spectroscopy, and small angle X-ray scattering (Wilkinson et al., 2003).

One of the best-characterized SBPs is the maltose binding protein (MBP) from the maltose/maltodextrin ABC transporter from *Escherichia coli* (Davidson et al., 2008) Recently, NMR spectroscopy of MBP in solution revealed that the ligand-free form of MBP is represented by a predominantly open species (95%) and a minor species (5%) that corresponds to a partially closed state of MBP. Both forms coexist in rapid equilibrium (Tang et al., 2007). The open form of MBP observed by NMR resembles the one detected in the crystal structure of the open, ligand-free conformation of MBP (Sharff et al., 1992). However, the partially closed species of MBP detected by NMR (Tang et al., 2007) does not correspond to the ligand-bound, fully closed form of MPB found in crystallographic studies (Spurlino et al., 1991). The partially closed sate of MBP is probably formed by flexible subdomains located in either of the two lobes of MBP (Tang et al., 2007). In support of these NMR studies are data reported recently that monitor the unfolding of MBP

by atomic force microscopy (AFM). Here, four different unfolding domains, the so-called unfoldons, were detected (Bertz and Rief, 2008). This study thus suggests that the individual lobes of MBP are not rigid; rather the N-lobe and the C-lobe of MBP probably contain individual building blocks.

The substrate-inducible ChoVWX ABC transporter from *Sinorhizobium meliloti* serves for the uptake of choline as a nutrient. ChoX is the SBP of this transport system and is located as a soluble protein in the periplasmic space (Dupont et al., 2004). Recently, the crystal structure of ChoX in complex with either choline or acetyl-choline was solved, and in addition a substrate-free but closed structure of ChoX was reported (Oswald et al., 2008a).

During our crystallographic analysis of ChoX, we detected a form of this binding protein that is distinct from the reported choline-bound closed and ligand-free closed forms of ChoX (Oswald et al., 2008a). This species represents a ligand-free, semi-closed form of ChoX. Our analysis of this unusual ChoX structure revealed a separately folded subdomain within the N-lobe that seems to undergo conformational changes that are independent of the N-lobe as one entity. Furthermore, our inspection of other SBP structures suggests that ChoX is not the only SBP containing such a subdomain.

Results and discussion

Structure of ChoX in a semi-closed ligand-free conformation

During our efforts to solve the crystal structure of the choline-binding protein ChoX from *S. meliloti* (Oswald et al., 2008a), we used the technique of microseeding (Oswald et al., 2008b) to obtain ChoX crystals in the ligand-free form. One of the X-ray datasets of ChoX obtained during this analysis revealed a ligand-free structure that was different from those that were expected for the ligand-free closed and open forms of SBPs described so far (Wilkinson et al., 2003; Davidson et al., 2008). Instead, ChoX was present in a ligand-free form for which the overall fold resembled that of the unliganded but fully closed ChoX structure (Oswald et al., 2008a). However, this new ChoX structure represented a more open state of the protein. The unit cell dimensions reflect the conformational change. The b-axis is almost 35 Å larger when compared to the unliganded-closed crystal form of ChoX: 196.2 Å for unliganded closed (Oswald et al., 2008a) and 232.2 Å for the semi-closed state, respectively. In the following sections, we will refer to this structure as the 'semi-closed' form for reasons outlined below. The crystal diffracted beyond 2.7 Å and was a perfect pseudo-merohedral twin (α=0.497) as observed for all crystals obtained by the microseeding technique (Oswald et al., 2008b). For refinement purposes, the dataset was cut at 2.9 Å owing to conservative judgment of the X-ray data (Table 1).

The overall fold of the semi-closed ChoX structure is the same as the unliganded closed ChoX structure (Oswald et al., 2008a). As seen in many SBPs, the two globular domains are connected via two strands (Figure 1A and B). This places ChoX in family II of ligand-binding proteins (Wilkinson et al., 2003) and the presence of a flexible linker segment connecting the two domains strongly suggests that ChoX is capable of undergoing the typical 'Venus-fly trap motion' of SBPs (Mao et al., 1982; Sack et al., 1989; Quiocho and Ledvina, 1996). When we compared the choline bound form of ChoX (yellow) and the unliganded semi-closed form of ChoX (magenta) reported here, small but significant differences were observed (Figure 2A). Protein-protein interactions or crystal contacts do not account for these differences as they are identical in the semi-closed and the unliganded closed ChoX structures. A superimposition of the closed liganded, closed unliganded, and unliganded semi-closed forms of ChoX revealed clear differences in domain I of the protein. For clarity, Figure 2A shows only the comparison between the closed liganded and semi-closed unliganded forms of ChoX. ChoX binds its substrate by an aromatic box consisting of residues originating from both the N- and C-lobes (Trp43, Trp90, Trp205, Tyr119, as well as Asp45). Furthermore, the indole moieties of Trp43 (N-lobe) and Trp205 (C-lobe) are stabilized via a water network and the side chain of Asp45. These additional interactions further stabilize the ligand-binding site in the ligand-bound, closed state of

Table 1 X-ray crystallography parameters.

Data collection	Unliganded semi-closed
Space group	$P2_1$
Cell dimensions	
a, *b*, *c* (Å)	34.1, 232.5, 47.3
β (°)	90.2
Wavelength (Å)	0.90
Resolution (Å)	20–2.9 (3.0–2.90)
R_{sym}	11.6 (42.1)
$I/\sigma I$	11.0 (2.8)
Completeness (%)	98.9 (99.4)
Redundancy	3.4 (3.4)
No. of unique reflections	15 055
Refinement	
Resolution (Å)	20–2.9
R_{work}/R_{free} (%)	24.4/28.4
Ramachandran (%)	
Favored	84.3
Allowed	15.1
Disallowed	0.6
No. atoms	
Protein	4352
Ligand/ion	–
Water	0
B-factors (Å^2)	
Protein	30.6
Ligand/ion	–
Water	–
rms	
Bond lengths (Å)	0.008
Bond angles (°)	1.161

Data in parentheses correspond to the highest resolution shells. Crystal parameters and data collection statistics are derived from XDS (Kabsch, 1993). Refinement statistics were obtained from REFMAC (Murshudov et al., 1997). To calculate R_{free}, 5% of the reflections were excluded from the refinement.

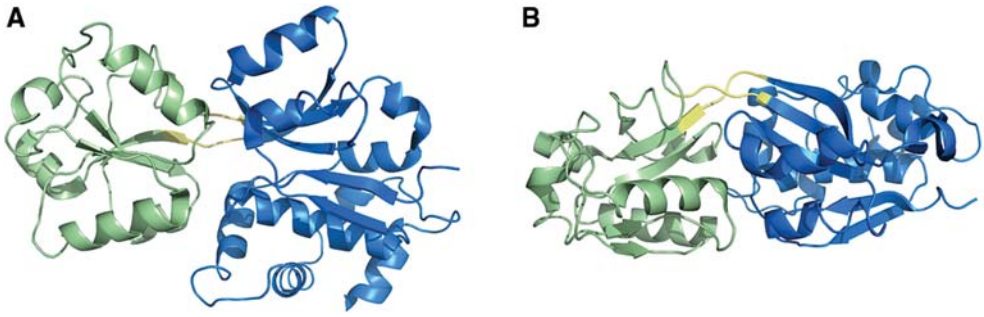

Figure 1 Overall representation of the ChoX structure in the semi-closed conformation.
Domains I and II are depicted in green and blue, respectively. The linker region is shown in yellow. (A) View from the membrane. (B) This view represents the orientation of ChoX when it is bound to the TMD of its cognate ABC transporter.

ChoX (Oswald et al., 2008a). Owing to altered interactions across the interfaces of both lobes (data not shown), the substrate-binding site is asymmetrically laid open in the semi-closed structure (Figure 2B). Whereas the ligand-binding residues originating from domain II of ChoX (shown in orange and yellow) superimpose well in the two structures, residues originating from domain I in the semi-open state (colored in light magenta) are displaced with regard to the liganded-closed state (colored in magenta).

When comparing the closed-liganded with the semi-closed unliganded ChoX structure all residues forming the ligand-binding site have an rmsd of 0.8 Å, whereas the liganded closed and unliganded closed have an rmsd of 0.2 Å (Oswald et al., 2008a). However, the asymmetric displacement of the N-lobe of ChoX results in a movement of the ligand-interacting residue Asp45 by ~2 Å (Figure 2B). As a consequence, Asp45 can no longer form the salt-bridge to the head group of choline that is observed in the choline-bound form of ChoX (Oswald et al., 2008a).

Deducing motions of ChoX from its crystal structures

The data and observations outlined above raise the question whether the detected semi-closed form of ChoX represents an intermediate conformation between the fully open and fully closed (either liganded or free) state of this SBP. To obtain further insights into the semi-closed state of ChoX, we analyzed the motions occurring in ChoX (unliganded-closed and semi-closed) and compared these to the Venus-fly type motions underlying the opening and closing movements of the maltose- (MBP) and the ribose- (RBP) binding proteins from *E. coli* (Spurlino et al., 1991; Sharff et al., 1992; Bjorkman et al., 1994;

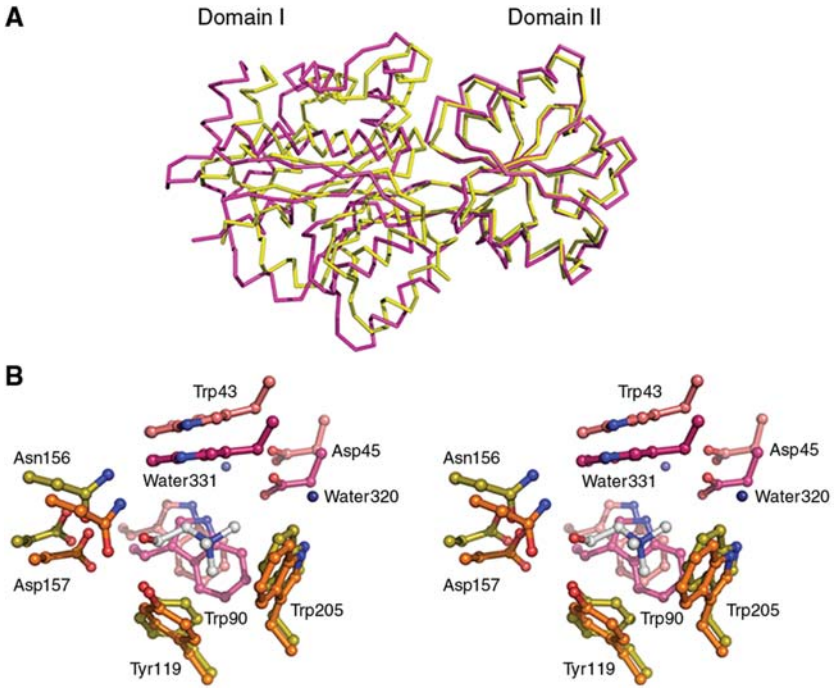

Figure 2 Comparison of the semi-closed and liganded-closed structures of ChoX.
(A) Structural superimposition of the semi-closed and the liganded-closed state of ChoX. The liganded-closed state is shown in yellow and the semi-closed state in magenta. Both structures were aligned using domain II as anchoring point. (B) Stereo view of a structural superimposition of the ligand binding sites of the liganded-closed (orange and magenta; choline is shown in ball-and-sticks representation) and the semi-closed (light magenta and yellow) states.

Bjorkman and Mowbray, 1998). The rmsd of Cα atoms of MBP, while transitioning from its ligand-free open state to the substrate-bound closed state, were mapped onto the crystal structure of the respective closed state of this protein (Figure 3A, left panel). The blue color in Figure 3 indicates no or little motion, whereas the red color indicates large movements in the proteins, as deduced from the appropriate crystal structures. To visualize these changes, the two states of MBP were superimposed. A quantitative analysis of this movement is provided in the right panel of Figure 3A. It is evident that domain II of MBP (indicated by the horizontal black line in the right panel) moves as one unit towards domain I during the ligand-induced closing reaction. However, the assessment of the 'Venus-fly trap'-type movement of MBP is complicated by the fact that the ligand-induced conformational changes in MBP also involve a rotational movement of both globular domains towards each other (Sharff et al., 1992).

We also applied this type of analysis to RBP, where no rotational movement component underlies the motion of the two domains (Bjorkman and Mowbray, 1998). As shown in Figure 3B, domain II of RBP moves as one unit towards domain I during the ligand-induced closing reaction. Thus, in both cases the 'Venus fly trap' mechanism conducts movements of both domains towards each other as a single entity.

When we conducted the same type of analysis of the movements for the closed and semi-closed state of ChoX, however, a different scenario became apparent (Figure 3C). Domain I of ChoX did not move as a single unit. Instead, only parts of domain I were affected in their position in the course of the transition of ChoX between semi-closed to the fully closed state (Figure 3C). A quantitative description of this movement is provided in the right panel of Figure 3C where the location of domain I of ChoX is indicated by the black, horizontal line, whereas the residues moving are highlighted by the blue, horizontal line. This representation of the movements in ChoX clearly shows that the motion is restricted to a specific subregion within domain I, comprising residues 44–63 (~helix 2) and residues 256–318 (Figure 3C). Hence, the movement of ChoX from the semi-closed to the closed state is not related to a typical Venus-fly trap type motion. In addition, the semi-closed unliganded structure we observed in ChoX does not correspond to a twist motion which has been observed in other SBPs (Ravindranathan et al., 2005, and data not shown).

Movement of a subdomain in ChoX upon ligand-binding

The subdomain movement that we have detected in ChoX prompted us to further analysis. To this end, we searched for the presence of possible hinge regions in ChoX by employing the Hingeprot server (Emekli et al., 2008). This search revealed a high score not only for lobes I and II of ChoX as rigid groups but also for a subdomain within domain I. This subdomain comprises residues 44–63 and residues 256–318, further called subdomain Ia (Figure 4A). The movement of subdomain Ia is accompanied by the loss of an extensive H-bonding network positioned along the interface of the subdomains (data not shown). Particularly important is the loss of a H-bond formed between two residues taking part in the binding of the substrate as observed in the choline bound structure (Oswald et al., 2008a), namely Asp45 originating from subdomain Ia and Trp90 (Figure 2B). The latter residue is not present in the observed subdomain. The distance between the two ligand-interacting residues Asp45 and Trp90 increases from 3.4 Å to 4.6 Å.

As a consequence of the absence of the substrate choline in the semi-closed ChoX structure, these residues (Asp45 and Trp90) are less tightly packed and thereby generate more flexibility in the ligand-binding pocket. Choline binds inside an aromatic box that coordinates the positively charged trimethylammonium headgroup of the substrate (Oswald et al., 2008a). Asp45 additionally binds the bulky cation of choline, and this interaction induces a rotational inward movement of subdomain Ia which can be observed in the ligand-bound form of ChoX. As a consequence, subdomain Ia can rotate outwards as observed in the semi-closed structure.

Interestingly, in the choline bound closed state of ChoX two water molecules bridge Asp45 with Trp20 (domain II) and Trp43 (domain I) and thereby further stabilize the binding site of the ligand. These water molecules were not detected in the unliganded-closed state despite good resolution (1.6 Å) of the ChoX structure (Oswald et al., 2008a). It can thus be safely assumed that this water network contributes to a further fixation of subdomain Ia in its closed conformation acting in concert with Asp45.

Comparisons with other SBPs

To test whether other SBPs also contain subdomain(s) as observed by us in ChoX, we decided to focus our analysis on two structurally and biochemically well-characterized SBPs, the maltose/maltodextrin binding protein MBP from *E. coli* (Spurlino et al., 1991; Sharff et al., 1992) and the histidine binding protein HisJ from *S. typhimurium* (Wolf et al., 1995) (Figure 4B and C).

Analysis of possible hinges and rigid parts within HisJ identified a possible subdomain composed of residues 10–33 and 215–238 (Figure 4B), which could undergo a rotational movement (sketched in Figure 4B). Biochemical investigations of the *S. typhimurium* HisJ protein indicated an important role for Tyr14 (Wolf et al., 1995). This residue performs stacking interactions with the imidazole ring of the substrate.

The Tyr14/His variant of the HisJ protein can adopt a closed conformation in the presence of the ligand; however, the mutant shows only poor recognition by a conformation-specific, monoclonal antibody that recognized the closed conformation of HisJ. Therefore, the closed conformation formed by the Tyr14/His variant was regarded as abnormal (Wolf et al., 1995). However, in view of the considerations and data presented above, this abnormal closed conformation of the mutant HisJ protein could occur owing to the movement of the subdomain as proposed for HisJ, which is similar to subdomain movement.

An analysis of potential subdomains in MBP (Figure 4C) reveals a subdomain that structurally coincides with subdomain Ia of ChoX (Figure 4A). This subdomain of

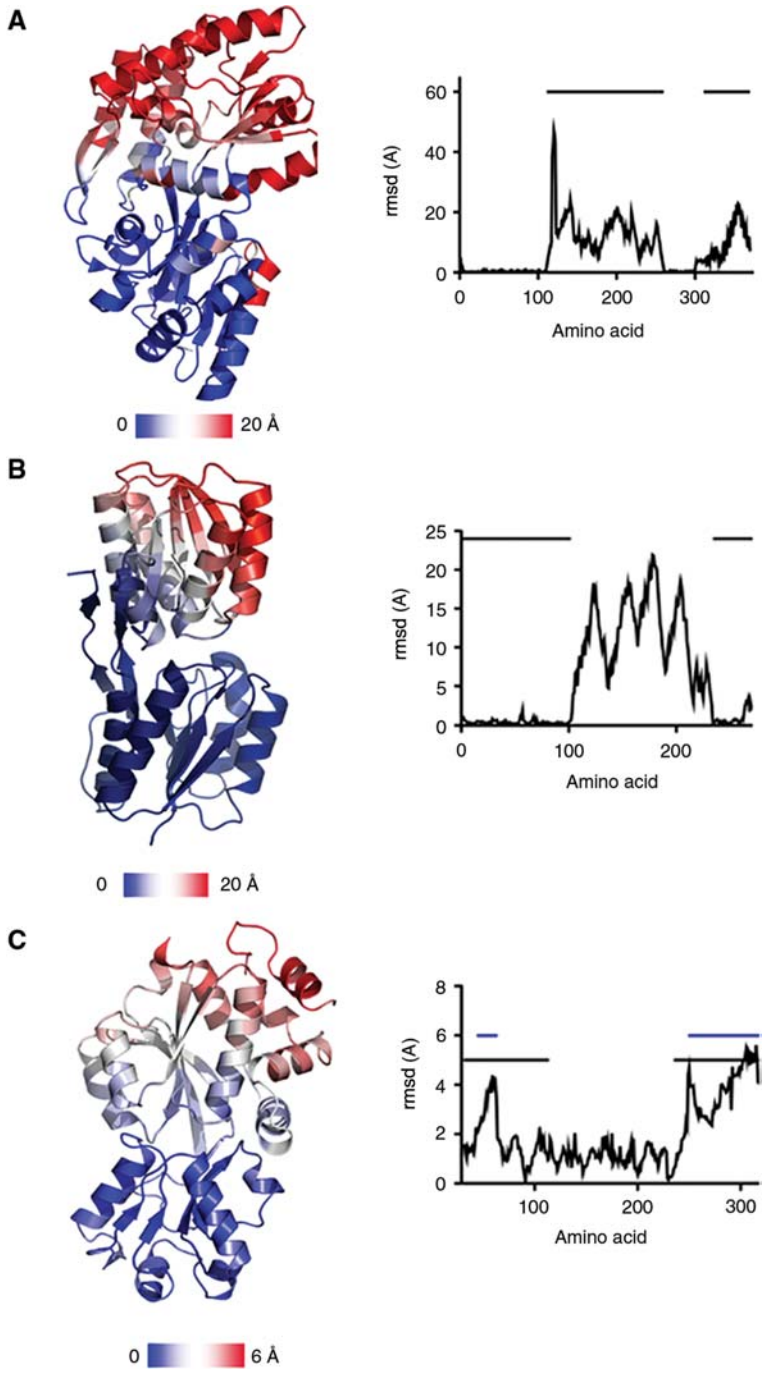

Figure 3 Visualizing movements in SBPs.
(A, left panel) Structure of the maltose binding protein (MBP) in the liganded-closed conformation. rms differences between the liganded-closed and the unliganded-open structures are mapped on the sketch representation of the liganded-closed structure colored-coded by a blue-white-red gradient as indicated below the sketch representation. (A, right panel) Quantitative analysis of rms differences of the liganded-closed and unliganded-open structures of MBP. The two black lines indicate the amino acids forming domain I. (B, left panel) Structure of the ribose binding protein (RBS) in the liganded-closed conformation. rms differences between the liganded-closed and the unliganded-open structures are mapped on the sketch representation of the liganded-closed structure colored-coded by a blue-white-red gradient as indicated below the sketch representation. (B, right panel) Quantitative analysis of rms differences of the liganded-closed and unliganded-open structures of RBS. The two black lines indicate the amino acids forming domain I. (C, left panel) Structure of the choline binding protein ChoX in the liganded-closed conformation. rms differences between the liganded-closed and the semi-closed structures are mapped on the sketch representation of the liganded-closed structure colored-coded by a blue-white-red gradient as indicated below the sketch representation. (C, right panel) Quantitative analysis of rms differences of the liganded-closed and semi-open structure of ChoX. The two black lines indicate the amino acids forming domain I. The blue lines highlight those amino acids which form subdomain Ia. It should be noted that the maximal displacement is 20 Å in parts (A) and (B) but only 6 Å in part (C). The same color-coding was used to measure the motions occurring in ChoX superimposed on domain II; therefore, domain II is displayed in blue indicating that no movement occurs.

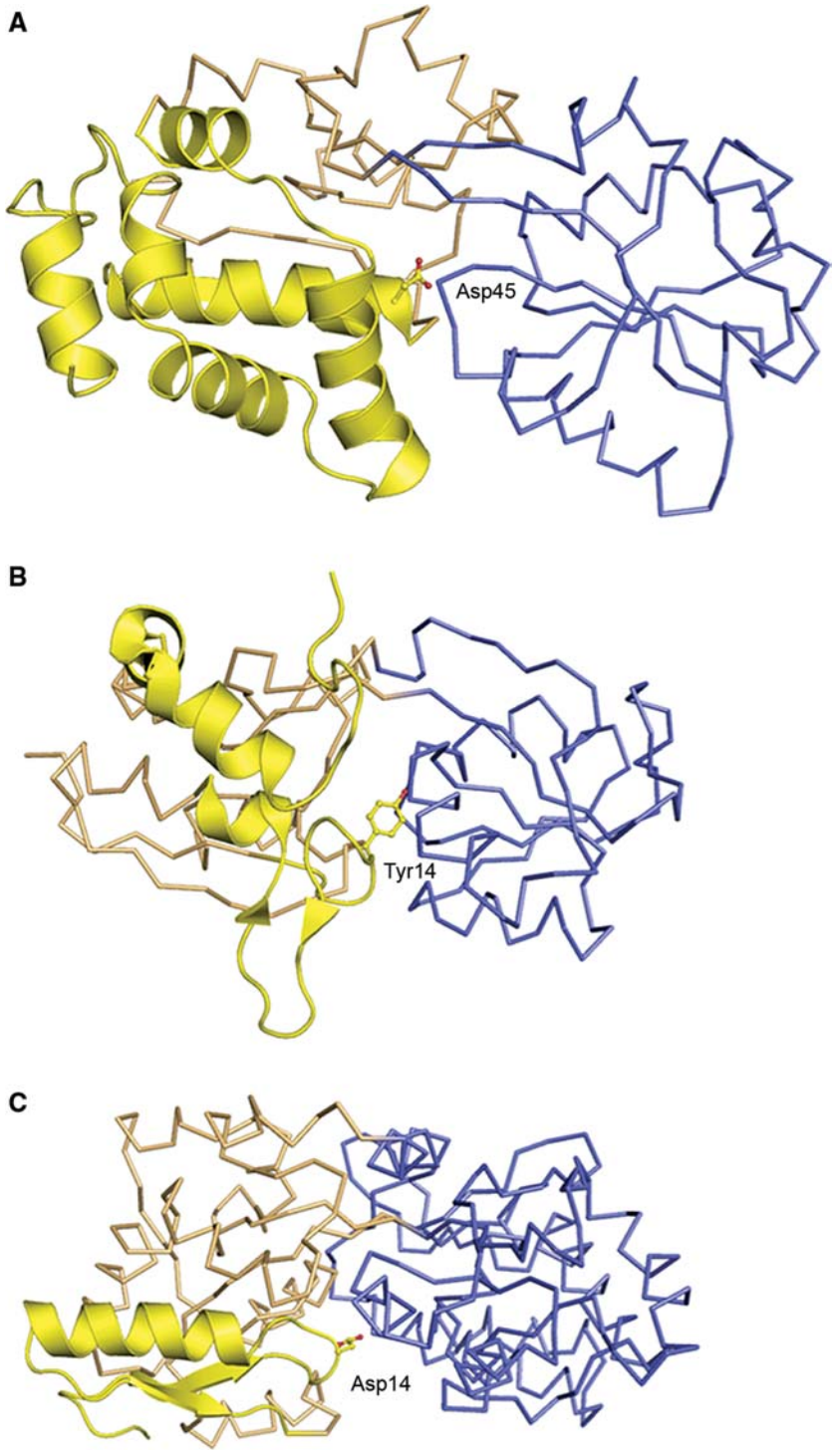

Figure 4 Putative locations of subdomain Ia in ChoX (A), HisJ (B) and MBP (C).
Subdomain Ia is shown in yellow sketch representation, the remaining part of domain I in ribbon representation, whereas domain II is represented as blue ribbons. Amino acids participating in ligand binding and located in the putative subdomain Ia are shown in yellow ball-and-stick representation.

MBP has recently been defined by single-molecule force spectroscopy as one of the four 'unfoldons' present in MPB and independently also by paramagnetic relaxation enhancement NMR studies (Tang et al., 2007; Bertz and Rief, 2008). This subdomain comprises Asp14 which participates directly in ligand binding; its mutational change to a Tyr residue does not affect the binding affinity of MPB towards maltose, but drastically reduces substrate uptake via the maltose ABC transport system (Hor and Shuman, 1993).

Possible function of subdomain I in various binding proteins

Substrate binding proteins exhibit equilibrium between the open and the closed state (Sack et al., 1989; Oh et al., 1993), and upon ligand binding this equilibrium is shifted towards the liganded-closed state (Mao et al., 1982; Quiocho and Ledvina, 1996). In ChoX, an intermediate step in this cycle is observed that is mediated by subdomain I. Our inspections of the appropriate crys-

tal structures of MPB and HisJ detected such subdomains in these SBPs as well. Biochemical studies have demonstrated that the ATPase activity of *in vitro* reconstituted ABC transporters is stimulated by the SBP even in the absence of substrate (Ames and Joshi, 1990; Davidson et al., 1992; Horn et al., 2005). This stimulation of ATPase activity occurs, however, with lower efficiency when compared with the stimulation of ATPase activity in the presence of SBP and substrate (Davidson et al., 1992; Horn et al., 2005). Furthermore, in the histidine transporter from *S. typhimurium*, the SBP displayed identical affinities towards the cognate TMD of the ABC transporter independent of the presence or absence of ligand (Ames et al., 1996). Thus, the unchanged SBP affinity towards the ABC transporters with no substrate present combined with a change in ATPase stimulation suggests that a distinction or substrate sensing is indeed prevailing in SBPs. One might thus speculate that sensing the substrate and thereby altering ATP hydrolysis rates of the complete ABC transporter could be the function of the outward rotation of subdomain I as detected in ChoX.

The recently published AFM and NMR studies of MBP (Tang et al., 2007; Bertz and Rief, 2008) as well as the data presented here clearly demonstrate that the lobes of SBPs are not rigid entities as anticipated before. Rather, at least one subdomain seems to be present in one of the two lobes of SBP that is capable to undergo individual motions that are uncoupled from the motions conducted by the rest of this lobe. The formation of such subdomains within the SBPs might be important for the communication of the SBP with the other components of a given ABC importer.

Materials and methods

Protein expression and purification

The *S. meliloti* ChoX protein was overproduced in the *E. coli* strain BL21(DE3)(pLysS) resulting in the synthesis of ChoX as a C-terminal His_6-tagged fusion protein (Dupont et al., 2004). Purification of ChoX was performed as described previously (Dupont et al., 2004). The purified ChoX-His_6 protein was concentrated to 10 mg/ml in 10 mM Tris-HCl (pH 7.0) for crystallization experiments.

Crystallization

To obtain crystals of ChoX in the ligand-free state, streak-seeding, utilizing ChoX/choline microcrystals, was used (Oswald et al., 2008b). ChoX crystals were obtained using the hanging drop method at 293 K, against a reservoir solution of 100 mM Na-acetate pH 4.8–5.0 and 15–25% polyethylene glycol 3350. Crystals grew in 1 d and were flash frozen in liquid nitrogen. The mother liquor, supplemented with 20–25% ethylenglycol was used as cryoprotectant.

Data collection, refinement, and structure analysis

The 2.9 Å dataset of the ChoX protein in its semi-closed form was processed with XDS (Kabsch, 1993). The ChoX unliganded closed model (PDB code: 2RF1) (Oswald et al., 2008a) was used as a template to obtain initial phases, using MOLREP (Vagin and Teplyakov, 2000). The algorithm of Padilla and Yeates (Padilla and Yeates, 2003) revealed a perfect twin (twinning factor $\alpha > 0.479$) and therefore the structure was further refined using REFMAC (Murshudov et al., 1997) with (h,-k,-l) as twinning operator. Dataset and refinement statistics are listed in Table 1. As analyzed with Procheck (Laskowski et al., 1993), the Ramachandran plot of semi-closed ChoX shows 1.0% of the residues in the disallowed regions. However, these residues are taking part in crystal contacts, explaining this rather high percentage. To predict possible rigid parts of the protein, the HingeProt-Server (http://bioinfo3d.cs.tau.ac.il/HingeProt/hingeprot.html) was used (Emekli et al., 2008). Figures of protein molecules were created using PyMol (www.pymol.org).

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