5 DISCUSSION

5.1.1 SapC

In vivo, lysosomal cleavage of ceramide by ASAH requires activator proteins saposins, which make the amide bond of the ceramide more accessible to the active side of the enzyme. The malfunction of saposins is associated with several severe inborn errors of metabolism, known as lysosomal storage disorders. In addition, saposins are essential cofactors of lipid-triggered immune response and facilitate the extraction of antigenic lipids from intralysosomal membranes for loading to CD1 molecules, which present them to T cells. Although the general fold of proteins belonging to the saposin family is known, our understanding of the structural basis for their varying specificities and modes of interaction with biological membranes is still very limited. The crystal structure of the human SapC in the open dimeric configutation provided new insights into flexibility of the SapC. The obtained SapC crystal structure revealed that tetragonal SapC forms a homodimer with domain swapped boomerang-shaped monomers. Composite omit maps generated by CNS unambiguously approved the novel course of the chains.

Oligomerization seems to be a common feature of saposins. Analytical ultracentrifugation showed that SapD (Rossmann et al, 2008), SapA and SapB form dimers in solution (Ahn et al., 2006), and SapC was shown to form even trimers in the presence of detergents (Ahn et al., 2006). By contrast, SAXS data presented here indicated that SapC can form also larger oligomers in solution. It was not possible to fit the theoretical scattering curves calculated with program CRYSOL (Svergun D., 1995) from the SapC monomers or dimers found in crystal structures to the experimental one. Total forward scattered intensity, excluded volume and pair distribution function indicated the presence of particles in solution with a max. size of 350 Å and molecular weight of about 120 kDa. These findings are consistent with atomic force microscopy (AFM) experiments showing that SapC molecules form patches on the membrane surface (Alattia et al., 2006). Additionally, the CD spectra presented here also indicated the presence of SapC aggregates solution which can be solubilized by organic solvents like TFE and isopropanol. The association of SapC

to oligomer structures seems not to be dependent on the presence of lipid membranes or vesicles and occurs already in lipid-free solutions at low pH. The biological role of the oligomerization remains unclear.

The mechanism by which the saposin-like activator proteins work is still a matter of conjecture. Only three polar residues that are not cysteines are conserved between the four saposins: Asn22, Thr24, and Tyr54/Phe54 (Fig. 19c), and the conservation of the first two residues is only due to the presence of glycosylation sites N-X-T in prosaposin. As aromatic Tyr and Phe side chains were found to be involved in glycolipid binding in diverse proteins (Mahfoud et al., 2002; Malinina et al., 2004; Wright et al., 2005), they consequently are also candidates for glycolipid recognition centres in saposins. In fact, the solvent accessible and conserved Tyr54 and Phe4 are adjoined and seal the buried hydrophobic inside of SapC drawn in red in Fig. 20a. Conformational flexibility at the opening to a hydrophobic cavity has been suspected to be important for the recognition and extraction of lipids by saposins and other lipid-transport proteins (Ahn et al., 2003; Niere et al., 2001; Wright et al., 2005), and the kinking of helix a3 at the conserved residue Tyr54 was proposed to be a key for the plasticity of saposins (Ahn et al., 2006). The conservation of this kink in the novel extended open conformation of SapC (Table 7) emphasizes its importance.

Two modes of interactions of saposins are currently mostly discussed: a ‘solubilizer’ (Ahn et al., 2003) and a ‘liftase’ mode (Sandhoff and Kolter, 1996) (Fig. 55a). A ‘solubilizer’ is a protein that can break down phospholipids-containing membranes at critical phospholipid concentrations (Ciaffoni et al., 2001). In contrast, a protein acting as a “liftase” recognizes a lipid within the membrane plane, complexes it, and lifts it out of the lipid bilayer, thereby exposing its carbohydrate head group to the metabolizing enzyme (Sandhoff and Kolter, 1996). Based on the results presented here and in previous works, the following model for the functioning of SapC can be proposed (Fig. 55 a, b):

Prior to interaction with lipid in a membrane, saposins rest in the closed compact conformation (Fig. 55b). Interaction of the positively charged patch of lysines at the “top” (helices 1 and 2) of saposin aligns the molecule on the surface of intralysosomal

membranes, which are rich in negatively charged lipids, characteristically BMP (Möbius et al., 2003) (Fig. 55a, step 1).

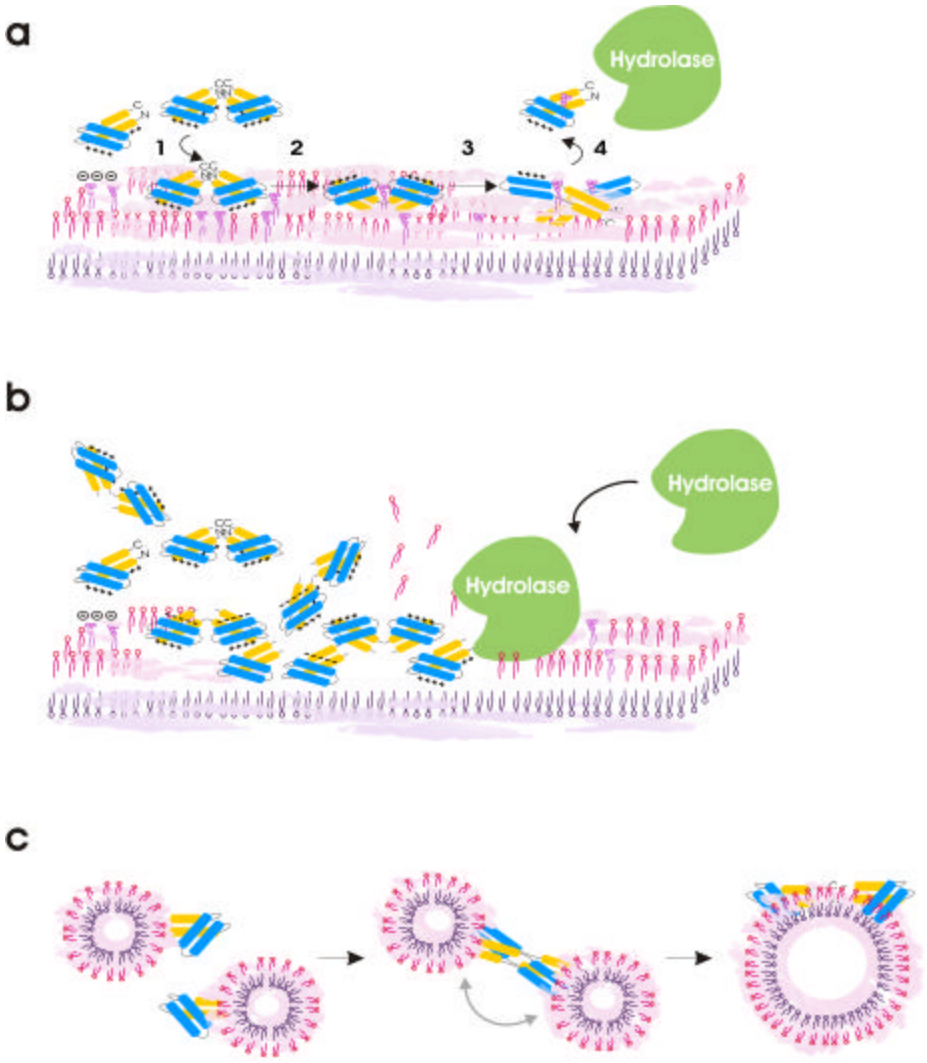

Figure 55. Schematic models for saposin stimulated lipid activation. (a) **Liftase model**. Step 1, water-soluble Sap monomers and dimers bind to the negatively charged membrane surface. Step 2, Sap rotates such that the hydrophobic "top" of the dimer faces the membrane surface. Step 3, Sap changes configuration to the boomerang-shape found for SapC, Fig. 3a, and amphipathic a-helices stretch parallel to the lipid bilayer, exposing polar residues to the solvent. The hydrophobic surface dips into the membrane and perturbs its structure. Step 4, Sap changes configuration to the closed form, lifts a lipid out of the membrane, and may leave the membrane with bound lipid. (b) **Perturbation model.** Sap accumulates on the membrane surface forming patches and replacing the "top" lipid monolayer. This leads to perturbation of the membrane structure and makes sphingolipids more solvent accessible. Hydrolases are probably acquired by Sap containing patches, bind to the membrane surface and hydrolyse perturbed sphingolipids. (*c*) **Schematic model for saposin stimulated vesicle fusion.** A "Clip-on" model for SapC-induced

vesicle fusion was proposed by Wang *et al.* (Wang et al., 2003). Two SapC molecules anchored to phospholipid bilayers of vesicles (Step 1) clip to each other through domain swapping (Step 2) and bring vesicles close enough for fusion (Step 3). The size of the vesicle and saposins are not on the same scale.

The bottom of the SapC is rich in apolar residues favoring a hydrophobic surrounding, which may promote a rolling movement on the surface of membrane that buries hydrophobic residues in the bilayer and exposes positively charged residues to the solvent (Fig. 55a, step 2). The interaction of carbohydrate and alkyl moieties with the gatekeeper amino acids Tyr4 and Tyr54, which seal the hydrophobic interior of the SapC, results in a hinge-bending opening of the saposin. The hydrophobic surfaces of the a-helices dip into the membrane (Abu-Baker et al., 2005), thereby perturbating the membrane the structure (Fig. 55a, step 3). Supported by thermal motion of the membrane bilayer, the orientation process could be accompanied by opening and closing cycles of the saposin monomer at hinges between helices a1/a2 and a3/a4. This could lock the lipid molecules within the hydrophobic cavity of SapC, lift them out of the membrane and make them available to the hydrolytic enzymes (Sandhoff and Kolter, 1996), although the perturbation of the lipid bilayer structure alone could already be sufficient for lipid activation.

It is conceivable that a saposin-lipid complex could leave the membrane surface (Fig. 25a, step 4), although no crystals of SapC in complex with lipids were obtained till now. SapC was rather shown to participate in fusion and destabilization of acidic vesicles (Vaccaro et al., 1994; Wang et al., 2003). With respect to the destabilization process, Alattia et al. were able to visualize the events occurring at the membrane interface using AFM (Alattia et al., 2006; Alattia et al., 2007). They showed that SapC induced a membrane remodeling that was characterized by a reduced membrane thickness. SapC was located in the remodeled areas replacing the top lipid leaflet, whereas its amphipathic nature allowed it to shield the hydrophobic tails of the acyl chains in the bottom leaflet (Fig. 55b). The organization of lipids at the interface between intact bilayers and transformed areas likely exposed lipid molecules to hydrolases and probably enhanced their binding to the membrane. The question

remains, how the lipid fractions removed by SapC are loaded onto CD1 molecules (Winau et al., 2004).

Bilayer fusion is a fast process completed within less than a millisecond. In the living world, fusion intermediates are sometimes stabilized by proteins, whereas phospholipid vesicles often seem to rely on fusogenic proteins (Cevc and Richardsen, 1999).

Fusogenic proteins induce prepare the fusion by:

- facilitating vesicle coalescence
- generating membrane defects
- increasing surface tension
- exchanging material between the bilayers
- changing the morphology of the vesicle
- increasing the bilayer permeability
- decreasing interfacial dielectric constant

Thus, fusogenic proteins require very special structural properties enabling the protein to induce the effects named above. SapC is the only saposin shown to have a fusogenic activity, but its mechanism could not be derived from the structure of the closed SapC monomer. Wang et al. postulated the hypothetical ‘clip-on’ model for SapC mediated vesicle fusion (Qi and Chu, 2004; Wang et al., 2003), in which two SapC molecules clip to each other by interactions between helix pairs a2/a3 and are anchored by helix pairs a1/a4 on opposing liposomal vesicles, thereby bringing the vesicles close enough for fusion (Fig. 55c). The ‘clip-on’ model was supported by FRET experiments that showed helices a1 and a4 to be embedded in negatively charged membrane surfaces, whereas helices a2 and a3 were found to be solvent exposed (Abu-Baker et al., 2005; Wang et al., 2003).

The postulated model presumes the presence of the open SapC dimer configuration. The only reported crystal structure of a SAPLIP in the open conformation is SapB, where two monomers of SapB form a dimeric V-shaped shell containing a population of *E. coli* lipids. Using NMR, John et al. have shown that a similar structure may also

exist for SapC (John et al., 2006; Wang et al., 2003). In their NMR experiments with SapC, they observed unknown conformational changes of SapC after incubation of SapC monomers at 37°C (John et al., 2006). In absence of detergents the obtained spectra could only be explained by formation of hydrophobic intermolecular contacts between SapC monomers, and this interaction is only possible in the open dimeric configuration which is now also supported by the here presented crystallographic data. The preparation of the crystallization drops with pre-warmed reservoir solutions at 37° C obviously induced the conformational transition in SapC.

Based on the conserved disulfide bridges and conserved amphipathic helices, the question arises as to whether other saposins could form similar dimers or oligomers through domain swapping and whether heterodimers formed between different saposins could exist as well *in vitro* and *in vivo*.

5.1.2 Acid ceramidase

SapC is required for the degradation of plasma membrane-derived ceramides by acid ceramidase (ASAH). Since malfunction of ASAH is associated with Farber disease, structural information on ASAH might help to understand the molecular basis of this disease. Additionally, ASAH was proposed to be a suitable target for anti-tumor therapeutics because inhibition of ASAH causes apoptosis in cancer cells (Saad et al., 2007), and consequently structural information could guide the design of specific ASAH inhibitors. ASAH was recombinantly expressed in Sf21 cell using the baculovirus expression system (AG Sandhoff, university Bonn). Unfortunately, infection of Sf21 cells with a recombinant baculovirus encoding acid ceramidase precursor led to a mixture of ASAH precursor and mature enzyme secreted into the medium. Since the initial purification strategy did not allow to separate these two forms (Schulze et al., 2007), there was not enough pure and homogeneous protein available for crystallization. Although X-ray diffracting crystals of ASAH were obtained, it was not possible to improve them, and the crystal structure of ASAH could not be determined.

The following finding could not be pursued further but might lead to pure homogeneous protein. When the culture supernatant of the insect cells was acidified and kept at 37°C for 5 days, no precursor was detected. In addition, an exchange of

Cys122 by Ala122 inactivated ASAH, and in this case only the precursor of ASAH was present in cell culture supernatant (personal commumication of Dr. Schulze, Bonn). Thus, the obtained homogeneous protein samples might improve the crystal quality.

To gain structural insights for ASAH, a homology model of the β-subunit of human acid ceramidase was built on the basis of the crystal structure of CBAH determined in this work. According to the obtained model, ASAH belongs to the Ntn-hydrolase family and uses N-terminal Cys122 (generated by autoprocessing of the ASAH precursor) as a nucleophile. All five known mutations in the β-subunit of ASAH that were proposed on the basis of the structure of CBAH did not affect the catalytic site residues. This observation supported the correctness of the homology model because a complete inactivation of ASAH is lethal, in agreement with results obtained earlier from knock-out experiments in mice (Li et al., 2002). Thus, the known mutations seem to be only responsible for partial ASAH deactivation caused by lower substrate affinity to this protein. Mutation-induced changes in solubility, stability or oligomerization state of ASAH are also possible taking into account the numerous proteases present in lysosomes.

5.1.3 Conjugated bile salt hydrolase

Bacterial bile salt hydrolases catalyze the degradation of conjugated bile acids in the mammalian gut. The crystal structures of conjugated bile acid hydrolase (CBAH) from *Clostridium perfringens* as apoenzyme and in complex with deoxycholyl taurine, cholylglycine, and cholyl sarcosine that were hydrolyzed to the reaction products are described in the present work. The crystal structures revealed close relationship between CBAH and penicillin V acylase (PVA) from *Bacillus sphaericus*. This similarity together with the N-terminal cysteine classify CBAH as a member of the N-terminal nucleophile (Ntn) hydrolase superfamily.

The structure analysis of *C. perfringens* CBAH identified critical residues in catalysis, substrate recognition, and tetramer formation. The catalytically active residues of CBAH were inferred from biochemical experiments and from analogy to the active sites of other Ntn-hydrolases. Besides Cys2, residues Asp21, Asn82, Asn175, and

Arg228 have been identified as catalytically important in CBAH. PVA uses the same catalytic residues except for Asn82 which is replaced by Tyr82. This, however, does not alter the nature of the active site since only the peptide NH atoms of these two residues are important in catalysis by providing hydrogen bond donors in the oxyanion hole together with Asn175. *In silico* simulation of substrate binding by CBAH and PVA revealed a possible involvement of Asn82/Tyr82 in the recognition of the leaving group.

It has long been proposed that cysteine is involved in the catalytic function of *C. perfringens* CBAH. This work has shown that CBAH was inactivated by the single substitution of Cys2 to Ala2 or to Ser2. The expression rates of these CBAH variants were very low, and the variants remained mostly insoluble and unprocessed, suggesting that protein processing occurs autocatalytically. Notably, the exchange Arg18Leu also inhibited the auto-cleavage of Met1 from the N-terminus and led to low amount of soluble protein in which the N-terminal methionine seems to be removed enzymatically by methionine amino-peptidase of the expression host.

The here presented structures of CBAH suggest that Arg18 could play a triple role. This residue may be involved in the processing of the N-terminal methionine, in decreasing the pK_a value of the nucleophilic SH-group, and in substrate or product binding, because in all the here described CBAH-product complexes, Arg18 was found hydrogen bonded to the carbonyl oxygen of cholate or deoxycholate. As mentioned above, exchange of Arg18 by Leu18 inhibited the processing of Met1 and resulted in insoluble CBAH. In contrast, the processing of the CBAH variant R18H was five times more effective than the R18L variant (see point 3.11), indicating that His18 can partially replace Arg18. The crystal structure of the CBAH variant R18L has shown that this exchange does not influence the geometry of the catalytic center, suggesting that conservation of Arg18 is due to its involvement in catalysis rather than in conferring substrate specificity.

Thus, two catalytic centers in CBAH which are based on Cys2 can be proposed. The first catalytic center is responsible for the processing of Met1 and contains the catalytic triad Cys2-Arg18-Asp69. The processing of Met1 liberates the N-terminal

amino group of Cys2 that becomes a part of the second catalytic center (dyad) consisting of Cys2 and Asp21.

Semi-synthetic cephalosporins are primarily synthesized from 7-aminocephalosporanic acid (7-ACA), which is usually obtained by chemical deacylation of cephalosporin C (CPC). Since the chemical production of 7-ACA includes several expensive steps and requires thorough treatment of chemical wastes, an enzymatic conversion of CPC to 7-ACA is of great interest. So far, none of the numerous attempts to modify Ntn-hydrolases from penicillin acylase family for this purpose have been successful. CBAH, which features the same catalytic mechanism, but belongs to another subfamily, could serve to achieve this goal. The structures of apoCBAH and of CBAH in complexes with reaction products revealed that the substrate specificity of this enzyme is associated with recognition of the cholate moiety and does not well recognize the leaving group (amino acid), because only the side chain of Asn82 is involved in its binding. CBAH being a homologue to PVA was shown to have low catalytic activity towards penicillin V (Kumar et al., 2006), raising the hope that CBAH specificity to penicillin V could be further improved. Because CBAH does not recognize the leaving group well (β-lactam ring in case of penicillin V as a substrate) and has a larger binding pocket for cholate compared to PVA, it can be more easily modified to accept the side chain (adipyl moiety) of cephalosporin C. Using structural information from PVA with respect to binding of the ß-lactam leaving group and among others, for example, the exchange of Asn82 by Tyr82 could improve the specificity of CBAH to different β-lactam rings including that of cephalosporin C. Alternatively, using structural information on CBAH, it might be possible to modify the substrate binding pocket of PVA with the aim to extend its specificity to the adipyl moiety.

Finally, Ntn-hydrolases use a catalytical mechanism very similar to that of classical serine proteases. In analogy to well-characterized inhibitors for serine/cysteine proteases, an irreversible inhibitor for CBAH was proposed (Fig. 47). The same inhibition principle could probably also be used for the design of inhibitors of human acid ceramidase, an Ntn-hydrolase belonging to the same subfamily of hydrolases as CBAH.