3 MATERIALS AND METHODS

3.1 SapC

3.1.1 Cloning and expression of SapC

The DNA sequence for human saposin C was amplified by PCR from the vector pBHE0 (Henseler et al., 1996) using primers designed to introduce a C-terminal $(His)_6$-tag and appropriate restriction sites. The PCR product was digested with *SnaB*I and *Mfe*I and cloned into pPIC9K digested with *SnaB*I and *EcoR*I generating plasmid pPIC9K-SapC. The resulting SapC sequence contained additional tyrosine and valine at the N-terminus and an additional arginine residue before the C-terminal $(His)_6$-tag. *Pichia pastoris* strain GS115 (Invitrogen) was transformed by electroporation with linearized (*Sal*I restriction) pPIC9K-SapC, and cells were grown on regeneration dextrose plates (RD) plates (1M sorbitol, 1.34% YNB, 4×10^{-5}% biotin, 2% glucose). Transformants were screened for multi-copy insertion by growth on YPD-Agar (1% yeast extract, 2% peptone, 2% agar) containing antibiotic G418 (Invitrogen) at concentrations of 500 µg/ml. The phenotype of the clone growing at highest G418 concentrations was determined to be $Mut^{(+)}$ by growth on methanol as the sole carbon source.

P. pastoris clones were inoculated into minimal glycerol medium (MGY) (1.34% YNB, 4×10^{-5}% biotin, 2% glycerol) and grown at 30° C until OD_{600} reached 4. The cells were pelleted (10 min at 4000 g), resuspended 1 : 4 in minimal methanol medium (MMM) (1.34% YNB, 4×10^{-5}% biotin, 1% methanol), and protein expression was induced at 30°C for 96 h. Methanol was supplied to 1% every 24 hours. Following induction, 1 ml aliquots of the culture were cleared by centrifugation (10 min at 4000 g), and proteins were precipitated by chloroform/methanol or applied directly to 20% SDS-PAGE. The expression level of recombinant saposins was estimated by silver staining.

3.1.2 Protein precipitation by chloroform/methanol

1 ml of yeast cell suspension was pelleted (10 min at 4000 g), and 900 µl of the supernatant were mixed with 600 µl chloroform and 500 µl methanol. The mixture was

vortexed for 1 min and centrifuged for 3 min at 13000 g. The upper and lower phases were removed, the protein precipitated at the interface of the two phases, was vacuum-dried and resuspended in 50 µl of SDS sample buffer without DTT.

3.1.3 High-density fermentation of SapC

For high cell density fermentation, 3.5 L fermentation basal salts medium containing per liter: 26.7 ml phosphoric acid, 1 g calcium chloride·$2H_2O$, 18.2 g potassium sulfate, 14.9 g magnesium sulfate·$7H_2O$, 4.13 g potassium hydroxide, and 40 g glycerol was supplemented with sterile filtered 2 ml/l trace salts solution containing per liter: $CuSO_4 \cdot 5H_2O$ 6.0 g, NaI 0.08 g, $MnSO_4 \cdot H_2O$ 3.0 g, $Na_2MoO_4 \cdot 2H_2O$ 0.2 g, H_3BO_3 0.02 g, $CoCl_2$ 0.5g, $ZnCl_2$ 20.0 g, $FeSO_4 \cdot 7H_2O$ 65.0 g, biotin 0.3 g, 5 ml concentrated H_2SO_4. This medium was inoculated with 150 ml of a 24 hour culture in MGY medium (see above) in a Bioflo3000 bioreactor (New Brunswick scientific). The temperature was set to 30° C, the pH 5.0 was adjusted by addition of 20% NH_4OH. Oxygen was added to the air flow to maintain the level of dissolved oxygen above 30%. When the glycerol from the MGY medium was metabolized, a 4 hour feed-batch phase with a feed of 15 ml/l*h of 50% aqueous glycerol was started. During the fermentation the pH was controlled using 1 M NaOH and 1M HCl. The methanol feeding was started at the fifth hour. Cells were adapted to methanol by slowly increasing the feed of methanol supplemented with 2 ml/l trace salts solution until a feed rate of 12 ml/l*h was reached. Induction with methanol was then continued for 96 hours until the medium was harvested by centrifugation for 15 min at 1000 g followed by a second centrifugation for 30 min at 15000 g.

3.1.4 Purification of SapC

SapC was captured from the medium adjusted to pH 4.0 with 50 mM sodium phosphate buffer by cation-exchange chromatography on Poros HS20 (Applied Biosystems) using 50 mM sodium phosphate pH 4.0 for washing and a linear gradient of the same buffer supplemented with 1 M NaCl for elution. The pH of the eluted protein fractions was adjusted to pH 7.5 with 0.1 M NaOH, and the protein was subsequently loaded onto a Ni^{2+}-NTA-Superflow column (Qiagen). The column was washed with 50 mM sodium phosphate buffer pH 6.0, and the protein was eluted with

300 mM NaCl in 50 mM sodium phosphate buffer pH 4.0. The buffer was exchanged by 25 mM sodium citrate pH 4.0, 100 mM NaCl by dialysis, and the protein was concentrated by Centricon-5 centrifugal filter devices (Millipore) to 25 mg/ml as determined by BCA assay (Pierce).

3.1.5 Crystallization of SapC

Crystallization of SapC was achieved by vapor diffusion using either hanging or sitting drop methods as indicated. Initial crystallization conditions were established from various sparse matrix and grid screens using 96-well Greiner plates designed for three sitting drops per reservoir. In the setups, the reservoir was 120µl, and each drop consisted of 1 µl protein solution (25 mg/ml) mixed with 1 µl reservoir solution. Fine tuning of the crystallization conditions was performed in 24-well format trays with 750µl volume reservoir solutions. An orthorhombic crystal form grew from a reservoir solution containing 50 mM sodium acetate pH 4.0, 30% (v/v) pentaerythritol ethoxylate 15/4 (15/4 EO/OH) and 100 mM ammonium sulfate, mixing 2 µl pre-warmed reservoir solution (1h at 37° C) with 2 µl protein solution. In order to improve crystal growth, the "streak-seeding" technique was used with the following optimized conditions: 42% (v/v) 15/4 EO/OH, 50 mM sodium acetate pH 4.0, 300 mM magnesium sulfate, and this condition resulted in a tetragonal crystal form.

3.1.6 Collection and processing of SapC X-ray datasets

X-ray data collection of the orthorhombic crystal form was done at room temperature in a glass capillary using an in-house Enraf-Nonius FR571 rotating anode generator equipped with an Osmic MaxFlux mirror system and a MAR345 imaging plate detector. X-ray diffraction data of the tetragonal crystal was measured at beamline ID14-2 at ESRF, Grenoble, France, on a CCD area detector Quantum 4 (ADSC, Area Detector System Corporation) at 100 K. The tetragonal crystals were incubated in reservoir solution supplemented with 22% glycerol as cryoprotectant prior to freezing in liquid nitrogen. The diffraction data were processed and scaled using the DENZO and SCALEPACK (Otwinowski and Minor, 1997).

3.1.7 Circular dichroism spectroscopy

The protein concentration was determined photometrically. The specific absorption at coefficient at 280 nm was estimated from the protein sequence using PROTPARAM tools available at http://expasy.org/tools/protparam.html. Protein solutions filtered through a 0.2 μm membrane and diluted with tridestillated water were used for CD spectroscopy. The CD spectra were recorded employing a J-600 spectropolarimeter and a quartz cuvette with 0.1 or 0.2 cm path length. Spectra were recordered in the range from 250 to 190 nm. Each CD spectrum was accumulated from 5 scans at 50 nm/min with a 1 nm slit width and 1 s response time. All spectra were corrected for buffer contributions and converted to mean residue weight ellipticity. Protein secondary structure analysis was performed with CDSSTR (Sreerama and Woody, 2000) using DICHROWEB (Whitmore and Wallace, 2004) online server accessible at http://www.cryst.bbk.ac.uk/cdweb.

3.1.8 Small-angle X-ray scattering

Small-angle-scattering (SAXS) measurements were performed on the X33-D1/2 beamline at EMBL outstation Hamburg (DESY) using multiwire proportional chambers with delay line readout. The scattering patterns were recorded at a sample-detector distance of 2.7 m with a range of momentum transfer of 0.006 $Å^{-1} < s < 0.48$ $Å^{-1}$ corresponding to a d-spacing range of $13 < d < 1046$ Å. The lower value (13 Å) presents the approximate, qualitative nature of the resolution value for the obtained measurements. The protein was diluted in 10 mM sodium acetate pH 4.5, 150 mM sodium chloride to concentrations of 5 and 10 mg/ml. Two 2min. exposures at 25°C were performed on each protein concentration with no X-ray damage as no changes in spectra were observed. A buffer blank measurement was made before and after each protein sample. The program PRIMUS (Konarev et al., 2003) was used to average the frames and subtract the buffer background. Models of the putative dimers and their fit to the experimental data were evaluated with the program CRYSOL (Svergun et al., 1995). The forward scattering intensity I (0), the longest length of the particle (D_{max}), the excluded volume V_p, and the particle distance distribution function $P(r)$ were obtained from the experimental SAXS data using programs PRIMUS and GNOM (Svergun, 1992).

3.1.9 Cloning of CBAH and CBAH mutants

Subcloning of the *cbah* gene from *C. perfringens* into the pKK233-2 plasmid was described earlier (Coleman and Hudson, 1995). This construct was used as a template to prepare CBAH variants C2S, R18H, and R18L by PCR using appropriate forward primers:

CBAH C2S: 5' - AAT GAG GAG TGA GTG TTT ATG GCT ACA GGA TTA GCC TTA GAA AC - 3'

CBAH R18H: 5' - CAA AAG ATG GAT TAC ATT TGT TTG GCC ATA ATA TGG ATA TTG AAT ATT C - 3'

CBAH R18L: 5' - CAA AAG ATG GAT TAC ATT TGT TTG GTT TAA ATA TGG ATA TTG AAT ATT C - 3'

and **TOPO-reverse** primer 5' - CTA ATT TAC ATG ATT AAT ACT TAA AGT TTT G - 3'. All obtained PCR products except **CBAH C2S** were reamplified with **TOPO-forward** primer 5' - CAC CAT GTG TAC AGG ATT AGC CTT AGA AAC G - 3' and **TOPO-reverse** primer and cloned into the T7 promotor-based directional TOPO® expression plasmid Champion™ pET 101 (Invitrogen) following the supplier's protocol. The PCR product generated by the primer pair **CBAH C2S** and **TOPO-reverse** was cloned directly into the plasmid pET 101.

The **CBAH C2A** mutant was prepared using the QuikChange® site-directed mutagenesis kit (Stratagene) with primer pair: 5'- AAT GAG GAG TGA GTG TTT ATG GCT ACA GGA TTA GCC TTA GAA AC - 3' and 5' - GT TTC TAA GGC TAA TCC TGT AGC CAT AAA CAC TCA CTC CTC ATT - 3' and pKK233-2 plasmid as a template according to the supplier's protocol. All obtained plasmids were validated by sequencing.

3.2 Conjugated Bile Salt Hydrolase (CBAH)

3.2.1 Expression and purification of CBAH and CBAH variants

The plasmids containing wild-type CBAH and mutated CBAH genes were transformed into *E. coli* BL21 (DE3) (Invitrogen), and cells were grown in Luria-Bertani medium (LB) containing 100 µg/ml ampicillin at 37° C throughout. After expression was induced at OD_{600} = 0.8 with 200 µm/l IPTG, cells were allowed to grow for another four hours before harvesting and resuspending in 25 mM HEPES (pH 7.5), 10 mM DTT, 25 mM NaCl, and 5 mM EDTA. The cells were harvested and

disrupted by two passages through a French press, and after centrifugation the protein in the supernatant was purified as described (Coleman and Hudson, 1995) with following modifications. The HEPES buffer was exchanged overnight by dialysis against 20 mM sodium acetate buffer pH 4.5 and subsequently centrifuged. The supernatant was used for ammonium sulfate precipitation (45%). The pellet was discarded and the supernatant adjusted to the bading buffer containing 100 mM phosphate pH 6.0, 1.8 M ammonium sulfate, 10 mM DTT, and 5 mM EDTA and subjected to hydrophobic interaction chromatography on a Poros 20PE (Applied Biosystems) column equilibrated with the loading buffer. CBAH was eluted by a linear gradient from 1.8 to 0 M ammonium sulfate in loading buffer. The enzymatic activity in individual chromatography fractions was monitored by a precipitation assay. Assay were performed as described (Coleman and Hudson, 1995). Briefly, aliquots (20 µl) of fractions were added to the mix containing 50 mM sodium acetate pH 6.0, 10 mM 2 mercaptoethanol, 1 mM EDTA, 10 mM sodium deoxycholoyltaurine. Samples containing CBAH activity generated free deoxycholate insoluble at the reaction pH.

Fractions containing CBAH were collected, concentrated by ultrafiltration through an Amicon YM-30 membrane, and applied to a Superdex 200 column (Amersham Bioscience) preequilibrated with buffer containing 10 mM sodium acetate pH 5.5, 400 mM NaCl, 1 mM DTT, 1 mM EDTA, 10% (v/v) glycerol with a flow rate of 1 ml/min. Fractions (1 ml) were collected and assayed for BSH activity. Prior to crystallization the protein solutions were supplemented with 10 mM DTT and concentrated to 25 mg/ml and the protein concentration was determined by BCA assay (Pierce) using BSA as a standard.

The expression of selenomethionine-substituted CBAH was performed by growing the transformed methionine-autotrophic *E.coli* strain B834 (DE3) in Luria-Bertani medium containing 100 µg/ml ampicillin at 37° C. The culture was grown to an OD_{600} of about 0.6, cells were harvested and resuspended to the same OD_{600} in minimal medium (Budisa et al., 1995) containing 0.1 mg/ml DL-selenomethionine (Acros). After the cells were grown at 37° C until OD_{600} reached about 0.8, protein expression was induced by 200 µm/l IPTG for 4 hours. After the expression, Se-CBAH was purified as described above for wild-type CBAH.

3.2.2 Immunoblot analysis

Whole-cell protein extracts and soluble fraction of CBAH were fractionated using 12.5 % SDS/PAGE. Using a dry blot device (BioRad) with blotting buffer (5.82 g Tris, 2.93 g glycine, 3.75 ml 10% SDS solution, 200 ml methanol, pH 8.5) proteins were transferred to a nitrocellulose membrane (Millipore). The membrane was blocked for 1 h in 3% milk-powder solution in TBS buffer (20 mM Tris, pH 7.5; 150 mM NaCl) and incubated for 1 hour with the rabbit polyclonal antibody against CBAH (Coleman and Hudson, 1995) diluted 1:5000 in TBS buffer to detect CBAH. The membrane was washed 3×15 min with TBS-Tween buffer (20 mM Tris, 500 mM NaCl, 0.05% Tween 20) and incubated for 1 hour with goat anti-rabbit IgG (1:10000 in TBS) conjugated to alkaline phosphatase. After the wash step (3×15 min) with TBS-Tween buffer the detection was performed using BCIP/NBT substrate system (66 µl NBT solution (5% NBT in 70% dimethylformamide) and 33 µl BCIP solution (5% BCIP in 100% dimethylformamide) in 10 ml development buffer (100 mm Tris, pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$).

3.2.3 Conjugated bile salt hydrolase assay

CBAH activity was assayed colorimetrically by measuring the amount of glycine liberated after a fixed period at 37° C. The standard assay was performed in 2ml microcentrifuge tubes. The assay mixture contained 5 µl protein solution, 10 mM of sodium acetate pH 5.5, 1 mM DTT, and 10 mM sodium choloylglycine (Fluka). The blind sample contained the same amount of protein and no substrate.

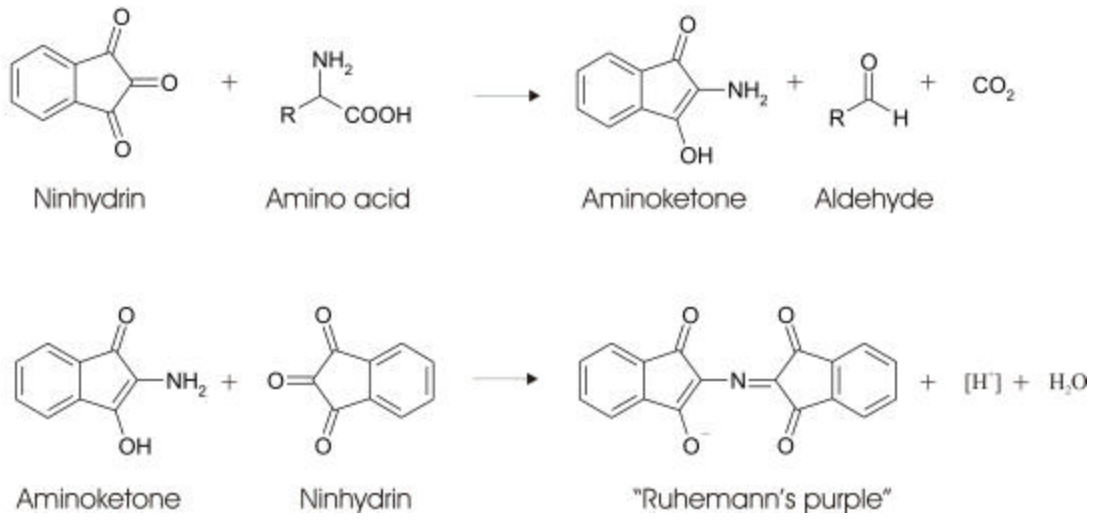

Figure 12. Detection of primary amines with ninhydrin. Primary amines from the hydrolyzed amino acid react with ninhydrin to turn to a deep blue or purple colour known as Ruhemann's purple that is quantified photometrically at 570 nm.

After 3 h incubation at 37°C the reaction was stopped by addition of 100 µl of 15% (wt/vol) trichloroacetic acid. After centrifugation for 5 min at 13 000 rpm to remove precipitated protein, the mixture was assayed for free amino groups by the ninhydrin reaction (Fig. 12): 50 µl of the reaction solution supernatant was mixed with 950 µl of ninhydrin solution containing 1 mM sodium cholate (Fluka), 200 mM HEPES pH 8 and 4 % ninhydrin (wt/vol) (Acros) in 40% glycerol. The mixtures were incubated for 15 min at 95° C and the absorbance was measured at 570 nm against the blind sample. The amount of product formed was estimated from a calibration curve obtained for glycine solutions with concentrations between 5 and 60 µg/ml. One unit of the activity was defined as that amount of protein which catalyzes the liberation of 1 µmol of glycine in 1 min. To determine the effect of basic pH on the enzymatic activity, 10 mM sodium acetate buffer pH 5.5 in the assay mixture was replaced by 10 mM HEPES buffer pH 8.

3.2.4 Crystallization of CBAH and CBAH variants

Crystallization of CBAH was achieved by vapor diffusion at 18° C. Initial crystallization conditions were established from various sparse matrix and grid screens using 96-

well Greiner plates containing 100 µl of reservoir solution. The apoCBAH crystals were grown in sitting or in hanging drops by mixing 1.5 µl protein and 1 µl reservoir solution or 3 µl protein solution and 2 µl reservoir solution, respectively. The reservoir solution contained 20-26% PEG 4000, 200 mM ammonium sulfate, 100 mM Bis-Tris pH 5.5 or 2.4-2.8 M ammonium sulfate, 100 mM sodium citrate pH 6.0.

The CBAH-product complex with deoxycholoyltaurine (dctCBAH) crystallized in hanging drops by mixing 3 µl protein solution and 2 µl reservoir solution. The reservoir solution contained 2.4-2.8 M ammonium sulfate, 100 mM sodium citrate pH 6.0, and 1 mM deoxycholoyltaurine.

Crystals of the CBAH variant C2A were grown in sitting drops in 96-well MRC™ crystallization plates by mixing 1.5 µL protein solution and 1.5 µL reservoir solution and equilibrating the droplet against 90 µl reservoir solution containing 22-25% PEG 3350, 200 mM ammonium sulfate, 100 mM Bis-Tris pH 5.5.

The selenomethionine labelled CBAH was used to obtain crystals of CBAH-product complex with sodium choloylglycine (Acros) (cgCBAH) and choloylsarcosine (Prodotti Chimici e Alimentari, Italia) (csCBAH). The crystals grew under in the conditions established for the CBAH variant C2A, but containing 5 mM sodium choloylglycine.

Crystals of the CBAH variant R18L were obtained in hanging drops by mixing of 2 µL protein and 2 µL reservoir solution. The reservoir solution contained 2.4-2.8 M ammonium sulfate, 100 mM HEPES, pH 7.5.

3.2.5 Collection and processing of CBAH datasets

All X-ray diffraction data were collected at 100 K at the Protein Structure Factory beamline BL14.2 of Freie Universität Berlin at BESSY (Berlin, Germany). Prior to cryocooling, the mother liquor of apoCBAH was replaced by reservoir solution containing 25% ethylene glycol, and the mother liquor of the CBAH complexed with reaction products and the both variants was replaced by reservoir solution containing 20% (v/v) glycerol. The datasets were processed using programs DENZO, SCALEPACK, MOSFLM (Powell, 1999), and XDS.

3.2.6 Docking of choloylglycine into the active site of CBAH

An *in silico* docking between the apoCBAH structure and choloylglycine was performed using the automated docking tools AUTODOCK (Goodsell et al., 1996). The ligand coordinate and topology files were generated using PRODRG server (Schuttelkopf and van Aalten, 2004). Prior to docking, the ligand was placed manually close to the active site, but in a position different from the position of the cholate molecule found in the crystal structure. A rigid molecular docking procedure was used to predict the initial binding mode of the substrate to CBAH. The search was done using the Lamarckian genetic algorithm with 50 runs, 150 populations per run and a maximum of 5 Mio. energy evaluations. 50 configurations were obtained and clustered with an r.m.s.d. cut-off of 2 Å for all atoms of each docked solution. The optimally docked configuration belonging to the lowest energy of the most populated cluster was selected and the results were examined visually using programs ADT (http://autodock.scripps.edu/resources/tools) and PYMOL.

The docking between the PVA structure and its ligands was calculated in the same manner and with the same settings as for the CBAH-product complex. The ligand coordinate and topology files were generated using the PRODRG server.

3.2.7 Tunnel identification using CAVER

To search for pathways leading from the active site of the Ntn-enzymes CBAH and PVA to the protein surface using program CAVER (Petrek et al., 2006) as a plug-in in PYMOL (DeLano, W.L. The PyMOL Molecular Graphics System (2002) DeLano Scientific, Palo Alto, CA, USA) was used. As starting point the empty space close to the Cys2 residue was chosen and a protein grid cell resolution of 0.7 Å was employed for calculations. The identified pathways were visualized by PYMOL.

3.3 Human acid ceramidase (ASAH)

3.3.1 Expression and purification of ASAH

Purified human acid ceramidase (ASAH) was obtained from the group of Prof. Sandhoff (Universität Bonn). ASAH was expressed and purified as described (Schulze et al., 2007). Briefly, ASAH was expressed in Sf9 cells using the baculovirus expression system. Protein was secreted into the growth medium and after

centrifugation (10 min, 3000 × g, 4° C) the supernatant was applied to an affinity column (Concanavalin A, Sigma) equilibrated with loading buffer (10 mM sodium phosphate, 0.1 mM NaCl, pH 7). After a wash step (with 10 mM sodium phosphate, 1.5 M NaCl, pH 7), the bound protein was eluted using the same buffer, but containing 0.75 M a-methyl-D-mannopyranoside, pH 7.0. ASAH containing fractions were pooled, concentrated, and further purified by gel filtration chromatography on a HiLoad Superdex 75 prep grade column (GE Healthcare). The running buffer was 10 mM NaAcetate, pH 4.2, 150 mM NaCl, 0.1% β-octylglycoside.

3.3.2 Crystallization of ASAH

Crystallization of ASAH was achieved by vapor diffusion using sitting drop methods. Initial crystallization conditions were established from various sparse matrix and grid screens using 96-well Greiner plates designed for three sitting drops per reservoir. In the setups, the reservoir was 120 µl, and each drop consisted of 1 µl protein solution (7.5 mg/ml) mixed with 1 µl reservoir solution. Fine tuning of the crystallization conditions was performed in 24-well format trays with 750 µl volume reservoir solutions. ASAH crystals grew from a reservoir solution containing 100 mM sodium acetate pH 4.0, 100 mM ammonium sulphate, 5% PEG 400 and 7.5% PEG 8000, mixing 2 µl reservoir solution with 2 µl protein solution.

3.3.3 Collecting of ASAH X-ray diffraction data

X-ray diffraction data of an ASAH crystal was measured at beamline ID14-2 at ESRF, Grenoble, France, on a CCD area detector Quantum 4 (ADSC, Area Detector System Corporation) at 100 K. The tetragonal crystals were incubated in reservoir solution supplemented with 20% glycerol as cryoprotectant prior to freezing in liquid nitrogen. The diffraction data were processed and scaled using the DENZO (Otwinowski and Minor, 1997).

3.3.4 Homology modelling of the b-subunit of ASAH

The server Phyre-3D-PSSM (Position–Specific Scoring Matrix) server (Kelley et al., 2000) available at the address http://www.sbg.bio.ic.ac.uk/3dpssm/index2.html was used to search for proteins with a homology to the primary and predicted secondary structure of the acid ceramidase. The proteins with the best score

served as starting basis for modelling with the program MODELLER 9.1 (Marti-Renom et al., 2000) using the model-default options. Program PROSA II (Wiederstein and Sippl, 2007) (https://prosa.services.came.sbg.ac.at/prosa.php) selected the best models provided by MODELLER. A *Z-score* tested the coherency and validity of the model structures. The *Z-score* is correlated to differences in the potential energy that are calculated using mean field potentials between the input structure and other randomly assigned folds with the same amino-acid sequence. A lower *Z-score* corresponds to a more favourable energy.

3.3.5 Structure analysis and generation of figures

The stereochemistry of the models was analyzed with programs PROCHECK and WHATCHECK (Hooft et al., 1996). All atomic coordinate superimpositions were carried out with program LSQKAP (Kabsch et al., 1976). Structural alignments were done using the DALI server (Holm and Sander, 1993; Holm and Sander, 1998). Amino-acid sequence alignments were calculated with program BIOEDIT developed by T.A. Hall. Programs CONTACT and AREAIMOL within the CCP4 program suite (Bailey, 1994) were used to calculate crystal contacts and buried surface areas, respectively.

The topography diagram was created with the aid of program TOPS (Flores et al., 1994). The schematic drawing of the deoxycholate binding site was generated with the aid of the program LIGPLOT (Wallace et al., 1995). Figures were drawn using the program PYMOL or using programs MOLSCRIPT (Kraulis, 1991) and RASTER3D (Merritt and Murphy, 1994) with the help of the graphical user interface MOLDRAW developed by Prof. Dr. N. Sträter. The chemical formulas were drawn using program ISIS DRAW (http://www.mdli.com/).