Structural basis of the alternating-access mechanism in a bile acid transporter

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Bile acids are synthesized from cholesterol in hepatocytes and secreted through the biliary tract into the small intestine, where they aid in absorption of lipids and fat-soluble vitamins. Through a process known as enterohepatic recirculation, more than 90% of secreted bile acids are then retrieved from the intestine and returned to the liver for resecretion1. In humans, there are two Na+-dependent bile acid transporters involved in enterohepatic recirculation, the Na+-taurocholate co-transporting polypeptide (NTCP; also known as SLC10A1) expressed in hepatocytes, and the apical sodium-dependent bile acid transporter (ASBT; also known as SLC10A2) expressed on enterocytes in the terminal ileum2. In recent years, ASBT has attracted much interest as a potential drug target for treatment of hypercholesterolaemia, because inhibition of ASBT reduces reabsorption of bile acids, thus increasing bile acid synthesis and consequently cholesterol consumption3,4. However, a lack of three-dimensional structures of bile acid transporters hampers our ability to understand the molecular mechanisms of substrate selectivity and transport, and to interpret the wealth of existing functional data5-8. The crystal structure of an ASBT homologue from Neisseria meningitidis (ASBTNM) in detergent was reported recently9, showing the protein in an inward-open conformation bound to two Na+ and a taurocholic acid. However, the structural changes that bring bile acid and Na+ across the membrane are difficult to infer from a single structure. To understand the structural changes associated with the coupled transport of Na+ and bile acids, here we solved two structures of an ASBT homologue from Yersinia frederiksenii (ASBTYf) in a lipid environment, which reveal that a large rigid-body rotation of a substrate-binding domain gives the conserved 'crossover' region, where two discontinuous helices cross each other, alternating accessibility from either side of the cell membrane. This result has implications for the location and orientation of the bile acid during transport, as well as for the translocation pathway for Na+.

Purified ASBTYf, when reconstituted into liposomes, mediates Na+-dependent transport of the conjugated bile acid taurocholic acid (TCA) with an apparent Michaelis constant (Km) of 46.8 ± 7.4 μM (Fig. 1a, b and Extended Data Fig. 1). ASBTYf was crystallized in lipidic cubic phase (LCP), and the structure solved to 1.95 Å (Extended Data Table 1). ASBTYf has ten transmembrane segments (TM1–10) divided into two domains: a panel domain, formed by TM1, 2, 6 and 7; and a core domain, formed by TM3–5 and 8–10. The first and last five transmembrane helices are structurally homologous, and due to their respective domain, formed by TM3–5 and 8–10. The first and last five transmembrane helices are structurally homologous, and due to their respective domain, formed by TM3–5 and 8–10. The first and last five transmembrane helices are structurally homologous, and due to their respective domain, formed by TM3–5 and 8–10.

The first and last five transmembrane helices: a panel domain, formed by TM1, 2, 6 and 7; and a core domain, formed by TM4 and 9. TM4 and 9 are highly conserved between ASBTNM and ASBTYf (Extended Data Fig. 4), but there is no obvious electron density at Na1 in the ASBTYf structure that could be attributed to Na+, and a very weak density at Na2 (Extended Data Fig. 3c, d). A closer examination of the residues forming the putative Na+-binding sites in ASBTYf showed that they are not in a position to coordinate Na+ optimally, probably due to a conformational change of TM4b. Whereas other transmembrane helices in the core domain of ASBTYf align closely with those of ASBTNM, TM4b tilts ~11° away from the cytoplasm, and its first helix turn unwinds (Figs 1e). These changes bring Asn 109 and Ser 108 out of range for coordination of Na+ in Na1, and may also affect the orientation of backbone carbonyls that form part of Na2 (Fig. 1d and Extended Data Fig. 5a, b). To test whether ASBTYf contains two Na+-binding sites, like ASBTNM, we measured 22Na binding by purified ASBTYf (Fig. 1f). Wild-type ASBTYf bound 22Na with an apparent half-maximum effective concentration (EC50) of 5.37 ± 0.01 mM and a Hill coefficient of 1.56 ± 0.06, suggesting cooperative binding between more than one Na+-binding site. Consistent with the notion that Na1 and Na2 are two Na+-binding sites in ASBTYf replacing Glu 254 in Na1 or Gln 258 in Na2 with Ala reduced binding of 22Na to 49% and 68% when compared with wild-type ASBTYf, respectively, and reduced the Hill coefficients to 1.06 ± 0.02 and 0.5 ± 0.1. The structure of ASBTYf thus represents an inward-facing unliganded state lacking Na+ and bile acid. Interestingly, the rotation of TM4b also renders Na1 accessible to the solvent from the intracellular side (Extended Data Fig. 5c, d), presenting a potential pathway for release of Na+ into the cytosol.

To obtain ASBTYf in an alternative conformation, we perturbed Na1 by mutating the highly conserved Glu 254 to Ala. Although ASBTYf(E254A) is still capable of mediating Na+-dependent transport (Extended Data Fig. 1c–e), the rate of TCA uptake is substantially reduced. Like wild-type ASBTYf, the E254A mutant was crystallized in LCP, and a complete data set was collected to 2.5 Å resolution. Interestingly, molecular replacement using the full structure of wild-type ASBTYf as a search model did not yield a valid solution. However, when the panel and core domain were used as two independent rigid bodies, a single solution was obtained (Fig. 2a and Extended Data Table 1). The core and panel domains from ASBTYf(E254A) individually align well with those of the wild type, with n-carbon root mean squared deviation (r.m.s.d.)

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As the amphipathic helices in the panel domain probably remain at the membrane–solvent interfaces after aligning both domains. This indicates that there is relative motion between the core and panel domains. As the discontinuous helices TM4 and TM9 relative to the intracellular cavity. Locations of the Na$^+$-binding sites in the previously reported ASBT$_{NM}$ structure are marked with dotted circles. Inset on the right shows a magnified view of residues that coordinate Na$^+$ in the ASBT$_{NM}$ structure.

To address the question of how this rigid-body motion of the core domain can translocate bile acid across the membrane, we compared the solvent-accessible surfaces of the inward- and outward-facing cavities in the two structures. This analysis reveals a narrow area running across the centre of the core and panel domains that is accessible to the solvent in both the inward-open and outward-open conformations (Fig. 2c). The dual-accessibility region includes the crossover region, and contains residues that are highly conserved among ASBT homologues. To test whether the crossover region is indeed accessible from the periplasm, as predicted by the E254A structure, and that the out-

Figure 1 | Function and crystal structure of ASBT$_{Yf}$. a, Time course of uptake of $^3$H-TCA into empty or ASBT$_{Yf}$-containing proteoliposomes in the presence of 100 mM external NaCl or choline chloride. b, Uptake of $^3$H-TCA 30 s after addition to ASBT$_{Yf}$-containing proteoliposomes in the presence of 100 mM external NaCl or choline chloride, as a function of the external $^3$H-TCA concentration. c, Cartoon representation of the ASBT$_{Yf}$ structure shown from two perpendicular directions in the plane of the membrane with the periplasm on top (left and middle), and from the extracellular side (right). The transmembrane helices are coloured in pseudosymmetry-related pairs. d, A cutaway surface representation of ASBT$_{Yf}$ showing the locations of the discontinuous helices TM4 and TM9 relative to the intracellular cavity. Locations of the Na$^+$-binding sites in the previously reported ASBT$_{NM}$ structure are marked with dotted circles. Inset on the right shows a magnified view of residues that coordinate Na$^+$ in the ASBT$_{NM}$ structure. e, Alignment of the discontinuous helices in the ASBT$_{Yf}$ (light blue) and ASBT$_{NM}$ (dark blue) structures. The partly unwound region of TM4b is marked with a black arrow. f, Na$^+$-binding kinetics of ASBT$_{Yf}$ and the Na$^+$-site mutants. Equilibrium binding of 0.95 nM [${}^{22}$Na]Cl (5.92 Ci mmol$^{-1}$) to 250 ng of wild-type (WT), E254A or Q258A ASBT$_{Yf}$ was measured with the SPA in the presence of increasing NaCl concentrations ranging from 0–100 mM. Isotopic replacement of $^{22}$Na$^+$ was plotted as a function of the concentration of non-labelled NaCl. The means of triplicate measurements ± standard error of the mean (s.e.m.) were subjected to nonlinear regression fitting in Prism 5 (GraphPad) for panels a, b and f.
for this apparent conflict is that the transporter possesses another as yet unobserved binding site for TCA, which, unlike the binding site shown in ASBT\textsubscript{NM}, has alternating access to both the periplasm and cytoplasm. For example, the bile acid could bind in a lateral orientation to the dual-access region described earlier (Extended Data Fig. 7c), with its mostly hydrophobic \( \beta \)-face oriented towards the hydrophobic panel domain, and its hydrophilic \( \alpha \)-face close to polar residues on the core domain (Extended Data Fig. 7d). In this configuration, the rigid-body motion of the core domain revealed by the two ASBT\textsubscript{Yf} structures would be sufficient to translocate the bile acid across the membrane. Mutation of polar residues capable of forming hydrogen bonds to the three hydroxyls on the steroid nucleus of TCA in a speculative horizontal orientation reduces TCA binding relative to wild-type ASBT\textsubscript{Yf} (Extended Data Fig. 7e, f). However, introducing mutations to the protein can affect substrate binding in detergent indirectly through a variety of mechanisms not involving direct contact with the substrate, and further experimental validation will be required to demonstrate the existence of a horizontal binding site in ASBT.

Experimental observation of distinct conformations of secondary transporters during the transport cycle is a major challenge in understanding the transport-associated dynamics of these molecular machines. We have presented two alternative conformations of a transporter, produced by a point mutation in a Na\textsuperscript{1} site. It cannot be fully excluded that mutating Na1 resulted in perturbations from the native structure, but the two structures appear to correspond to ligand-free inward- and outward-open states. To investigate further the states of the ASBT transport cycle, we measured the interdependency of Na\textsuperscript{+} and TCA binding with the scintillation proximity assay (SPA). Binding of TCA to ASBT\textsubscript{Yf} is strongly dependent on the concentration of Na\textsuperscript{+} (Fig. 3a), whereas TCA has a minimal effect on Na\textsuperscript{1} binding (Fig. 3b). This suggests that the Na\textsuperscript{1} sites are occupied before TCA can bind to the transporter. From these results, we can begin to enumerate and order conformational states in a preliminary model of the ASBT transport cycle (Fig. 3c).

ASBT\textsubscript{Yf} and ASBT\textsubscript{NM} share their fold with NhaA (a member of the Na\textsuperscript{+}/H\textsuperscript{+} antiporter family). NhaA also possesses inverted pseudosymmetry repeats that form a substrate-binding core domain and a panel domain, although the panel domain typically contains two or more additional helices that form a homodimer interface\textsuperscript{21,22, which correspond to inward-open and outward-open states. Despite the substantial difference in the size of the substrates involved, the rigid-body movement of the core domain that converts between the two states in the Na\textsuperscript{+}/H\textsuperscript{+} antiporters is remarkably similar to that observed for ASBT\textsubscript{Yf} (Extended Data Fig. 8b, c). As in ASBT\textsubscript{Yf}, the conformational change provides alternating access to the crossover region, where Na\textsuperscript{+} and protons are predicted to bind to a cluster of conserved acidic residues. However, this site is not equivalent to either Na1 or Na2 in the bile acid transporter. Examination of the site on NapA corresponding to Na2 shows that two of the polar residues coordinating Na\textsuperscript{+} in ASBT\textsubscript{Yf} (Gln 258 and His 71; Extended Data Fig. 8f) are in fact replaced with two positively charged side chains (Arg 331 and Lys 305; Extended Data Fig. 8g), which form hydrogen bonds with the C-terminal ends of helices TM4a and TM11a (TM4a and TM9a in ASBT\textsubscript{Yf}). In the structurally unrelated antiporter CaiT, which is Na\textsuperscript{+} independent, an arginine residue has recently been shown to mimic binding of Na\textsuperscript{+} to a site found in Na\textsuperscript{1} dependent symporters of the same fold\textsuperscript{23. It might therefore be
Distinct conformations captured by crystallography are indicated with the transporter17,24 (Extended Data Fig. 8d). Drives release of Na in the absence of 100 μM TCA. Error bars are the s.e.m. of triplicate measurements. Key conformational states of ASBT during the translocation of substrates. Feasible to speculate that these residues in NapA can have a role analogous to bound Na+ in ASBTYf and ASBTNM. Further comparison of these two families of transporters may provide insight into how the bilayer. Although they belong to an unrelated fold, the structures of ASBTYf and ASBTNM show how the energy from Na+ or H+ and the cognate substrate could trigger a conversion between the two alternate conformations. Further studies are necessary to understand how the energy from Na+ or H+ binding triggers and drives the conformational changes required for binding and translocation of the cognate substrate.

In humans, ASBT inhibitors have received considerable attention as potential therapeutics for the treatment of hypercholesterolemia23 and type 2 diabetes26. Another possible medical application of compounds targeting bile acid transporters involves conjugating bile acids to drugs with poor oral bioavailability, so that they are recognized as substrates by ASBT and NTCP and absorbed in the intestine and liver27,28. Both approaches would greatly benefit from an improved understanding of bile acid transporter structure and mechanism of action. ASBTYf shares 22% sequence identity and 59% similarity with human ASBT. Additionally, the residues forming the two Na+–binding sites are highly conserved (Extended Data Fig. 4). This suggests that the overall fold and transport mechanism are similar between the two proteins, and that ASBTYf may serve as a useful model system for understanding mechanisms of transport and inhibition in the mammalian ASBT homologues.

**METHODS SUMMARY**

The gene encoding ASBTYf (RefSeq accession ZP_04633709.1) was obtained by PCR from the genomic DNA of Y. frederiksenii, ligated into a modified PET vector, and expressed in E. coli. The ASBTYf protein was extracted from the cell membrane, purified by a metal affinity column, and further purified by size exclusion chromatography. Crystals were grown in LCP with 30% (v/v) PEG-400, 0.1 M NaCl and 3% (w/v) D-trehalose for the wild-type protein and 2,000 c.p.m., counts per minute. WT ASBTYf exposure to lower Na+ concentrations in the cytoplasm drives release of Na+, possibly by the pathway opened by the rotation of T4Mb in the ASBTYf structure (VI), which in turn triggers release of TCA.

**Online Content** Any additional Methods, Extended Data display items and Source Data are available in the online version of the paper; references unique to these sections appear only in the online paper.

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Supplementary Information is available in the online version of the paper.

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Author Information Atomic coordinates and structure factors have been deposited at the Protein Data Bank under accessions 4N7W and 4N7X. Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to M.Z. (mzhou@bcm.edu or mingzhou@mail.kiz.ac.cn) or M.Q. (mq2102@columbia.edu).
METHODS
SPA-based binding assay. Binding of radiolabelled Na\(^+\) and TCA to His-tagged ASBT\(_Yf\) was measured with the SPA, using Cu\(^{2+}\)-coated polyvinyl tosylate (PVT) or yttrium silicate (YSi) SPA beads were diluted to 2.5 mg ml\(^{-1}\) in assay buffer. For the Na\(^+\) binding experiment the assay buffer was composed of 200 mM HEPES-Tris, pH 7.5, 20% glycerol, 1 mM tris(2-carboxyethyl)phosphine (TCEP; Sigma), and 0.19% (w/v) DM (Anatrace), whereas for the TCA-binding experiments 200 mM HEPES-Tris was equimolarly replaced with 50 mM HEPES-Tris, pH 7.5 and 150 mM NaCl. Two-hundred and fifty nanograms of the indicated purified recombinant (His-tagged) ASBT\(_Yf\) variants were added to 100 µl of SPA beads and binding of 0.5 µM TCA or 100 nM Na\(^+\) was measured in the presence of 0–5 mM TCA, and binding of 0.95 µM [\(^{22}\)Na]Cl (5.92 Ci mmol\(^{-1}\)) (Perkin Elmer) was assayed in the presence of 0–100 mM non-labelled NaCl in individual wells of clear-bottom/white-wall 96-well plates. Equilibrium binding was performed in the dark for 16 h at 4 °C with vigorous shaking on a vibrating platform and the counts per minute (c.p.m.) were recorded. Data were normalized with regard to the activity of wild-type ASBT\(_Yf\) in the absence of non-labelled ligand and set as 100%. All experiments were performed at least in duplicate with repeats of 3–5 and data are expressed as mean ± s.e.m. Data fits of kinetic analyses were performed using nonlinear regression algorithms in Prism 5 (GraphPad) and errors represent the s.e.m. of the fit. Data in Fig. 1c were fit to a dose-response curve in the form of

\[
Y = \frac{\text{max} - \min}{1 + \left(\frac{[\text{Na}^+]_{EC_{50}}}{[\text{Na}^+]_{EC_{50}}}ight)^n}\]

where n is the Hill coefficient.

Uptake of TCA in proteoliposomes. Before reconstitution, the N-terminal His tag on cobalt-affinity-purified ASBT\(_Yf\) was removed by digestion with \(
\text{z}\)-chymotrypsin followed by gel filtration as described below for protein used for crystallization. The chymotrypsin-treated wild-type ASBT\(_Yf\) variants and variants were reconstituted at a 100:1 (w/w) ratio in preformed Triton X-100 (0.12% (w/w)) destabilized liposomes that were prepared of 100 mM potassium phosphate, pH 7.5, 2 mM \(\text{C}_{18}\)/\(\text{C}_{19}\)-mercaptoethanol and 1 mM phenylmethanesulfonyl fluoride (PMSF). Cells were lysed by sonication and 10 mM dodecyl-\(\beta\)-d-maltopyranoside (DDM). The purified ASBT\(_Yf\) protein was concentrated to ~50 mg ml\(^{-1}\) as approximated by ultraviolet absorbance and mixed with 1-oleoyl-rac-glycerol (monolein; Sigma-Aldrich) at a 2:3 (w/w) protein-to-lipid ratio using the twin-syringe mixing method\(^{32}\). The protein/lipid mixture was dispensed manually in ~50 nl drops onto 96-well glass sandwich plates and overlaid with 1.5 µl precipitant solution per drop. The wild-type ASBT\(_Yf\) crystals were grown in 30% (v/v) PEG-400, 0.1 M Na-citrate pH 5.5, 0.1 M NaCl and 3% (w/v) \(\text{z}\)-trehalose, and the ASBT\(_Yf\) (E254A) crystals were grown in 35% (v/v) PEG-400, 0.1 M Tris-HCl pH 8.5, 0.1 M KC1 and 10 mM MgCl\(_2\). The crystals reached full size within 5–7 days at 20°C. The E254A crystals were soaked in 10 mM taurocholic acid for 2 h before harvest, and both crystals were flash frozen in liquid nitrogen without additional cryoprotectant.

Data collection and structure solution. Diffraction data were collected on beamline 8.2.2 at the Advanced Light Source, on beamline X29 at the National Synchrotron Light Source and on beamlines 24ID-E and 17ID-B at the Advanced Photon Source. The data were indexed, integrated and scaled using the HKL2000 software suite. The wild-type ASBT\(_Yf\) structure was solved by molecular replacement with Phaser\(^{33}\) using the ASBT\(_{NaM}\) structure as a search model. The asymmetric unit contained two ASBT\(_Yf\) monomers, and strong NCS restraints were used during early model building and refinement. Manual model building and refinement were carried out using Coot\(^{34}\) and Phenix\(^{35}\), respectively. Molprobity\(^{36}\) was used to monitor and improve protein geometry. The final model contains residues 1–301 in chain A and residues 3–306 in chain B, 147 water molecules, 2 monoolein molecules and 2 molecules of citrate, which was included in the crystallization solution as a buffer, and appears to bind directly to the crossover region (Extended Data Fig. 9a, c). To rule out the concern that the bound citrate molecules were perturbing the Na\(^+\)-binding sites, 22Na\(^+\) binding in the presence/absence of 5 mM potassium citrate was measured by SPA, which showed very little effect (Extended Data Fig. 9b). The E254A structure was solved by molecular replacement using sequential searches with the separate core and panel domains of wild-type ASBT\(_Yf\). Model building and refinement followed the same methodology as for the wild-type structure. The final asymmetric unit contained residues 1–301 of ASBT\(_Yf\) (E254A) and 14 water molecules. Although the crystal was soaked in 10 mM TCA, no density consistent with TCA could be identified in the structure. Five residues from the TEV cleavage site on the N terminus are also resolved in the structure, and form a continuation of TMI. Solvent accessibility in Fig. 2c and Extended Data Fig. 7c was visualized by selecting residues within 4 A˚ of spheres placed by the program HOLLOW\(^{37}\), using a probe radius of 1.4 A˚. VMD\(^{38}\) was used to calculate r.m.s.d. values, transformation matrices, and miscellaneous other molecular properties. Intermediate states for the morphing animation in Supplementary Video 1 were calculated using LSQMANN\(^{39}\). All Coot\(^{34}\) and Phenix\(^{35}\) structures were prepared for PyMOL (Schrodinger PyMOL Molecular Graphics System) for molecular visualization.

Pegylation assay. Site-directed mutagenesis was performed using the QuickChange Kit (Agilent). For pegylation experiments, the native cysteines of ASBT\(_Yf\) (C196 and C248) were mutated to serines before the introduction of single cysteine binding sites, methoxypolyethylene glycol maleimide 5,000 (mPEG-Mal-5K; Sigma-Aldrich). Briefly, E. coli cells expressing ASBT\(_Yf\) carrying specific single cysteine mutations were harvested and washed twice with pegylation buffer containing 20 mM HEPES pH 7.5, 150 mM NaCl and 10% (v/v) glycerol. The cells were resuspended in the pegylation buffer (per ml culture) and split into four aliquots. The first is a control that did not contain any pegylation reagents; the second one was treated with 10 mM mPEG-Mal-5K at room temperature (21–23 °C) for 1 h with shaking; the third sample was sonicated first to break the cells in the presence of 10 mM mPEG-Mal-5K and then incubated at room temperature for 1 h; and the fourth sample was first treated with 20 mM NEM (2 h at room temperature) to block free cysteines and then incubated at a concentration of 30 mM. The mixtures were then shaken at 20°C for 2 h to extract membrane proteins, and cleared with centrifugation (40,000 g, 45 min). The supernatant was then loaded onto columns packed with 0.5 ml of cobalt beads, washed with 30 bed volumes of pegylation buffer containing 20 mM imidazole pH 8, and eluted with 5 bed volumes of pegylation buffer containing 300 mM
imidazole pH 8. The protein eluates were concentrated using Amicon Ultra centrifugal filter units (50 kDa nominal molecular weight limit), and analysed by SDS–PAGE and Coomassie staining.

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Extended Data Figure 1 | Purification and functional characterization of wild-type and Na\(^+\)-site-mutant ASBT\(_{Yf}\). a, The elution profiles of wild-type (WT), E254A, Q258A and E254A/Q258A ASBT\(_{Yf}\) from a size-exclusion column. Inset shows SDS–PAGE gel of fast-performance liquid chromatography (FPLC)-purified wild-type ASBT\(_{Yf}\) before (lane 2) and after (lane 3) cleavage of the affinity tag with TEV protease.

b, Chemical structures of bile acids. The primary bile acid cholic acid (top) contains a steroid nucleus, with a five-carbon side chain terminating in a carboxylic acid attached to carbon 17. Further modification of cholic acid by attachment of the amino acid taurine to the side chain results in the conjugated bile acid TCA (bottom). Both structures are oriented with the β-face towards the viewer and the α-face away from the viewer.

c–e, Time courses of 1 μM \(^{3}\)H-TCA (20 Ci mmol\(^{-1}\)) uptake into proteoliposomes reconstituted with wild-type (red) or E254A (blue) ASBT\(_{Yf}\) or control liposomes (black) without protein, in the presence of 100 mM external NaCl. Uptake was measured under three conditions: in intact liposomes with an inwardly directed Na\(^+\) gradient (c); in the presence of 25 μg ml\(^{-1}\) of the Na\(^+\)-selective ionophore gramicidin, collapsing the Na\(^+\) gradient (d); and in the presence of 25 μg ml\(^{-1}\) gramicidin and 0.05% of the detergent n-dodecyl-β-D-maltopyranoside (e). Under the latter condition, the liposomes are permeabilized, and only \(^{3}\)H-TCA bound to the lipids and protein is measured.
Extended Data Figure 2 | Topology diagram of the bile acid transporter fold. A schematic of the membrane topology of ASBT<sub>Ye</sub>, oriented with the periplasm on top. The helices are grouped by domain, and the blue and yellow trapezoids denote transmembrane helices in the first and second inverted repeats, respectively. Pseudosymmetry-equivalent transmembrane helices are coloured identically.
Extended Data Figure 3 | The wild-type ASBTYf structure is in a Na\(^+\)-free state. a–d, Stereo images of the residues forming Na1 (a, c) and Na2 (b, d) in the ASBT\(_\text{NM}\) (a, b) and ASBT\(_\text{Yf}\) (c, d) structures, shown with the 2\(F_o\) – \(F_c\) electron density maps in blue and the \(F_o\) – \(F_c\) density maps in green. Contour levels are set at 1.5 and 3.0\(\sigma\), respectively, and the sodium ions were omitted from the \(F_o\) – \(F_c\) map calculation for the ASBT\(_\text{NM}\) structure. The purple spheres in all four images correspond to the positions of Na\(^+\) in the ASBT\(_\text{NM}\) structure.
Extended Data Figure 4 | Sequence conservation of the bile acid transporter family. Sequence alignments of human (h) NTCP, ASBT and bacterial homologues from *N. meningitidis* and *Y. frederiksenii* were calculated with CLUSTALW. The coloured bars mark the locations of transmembrane helices in ASBTYf. Residues forming Na1 and Na2 are highlighted with orange and pink, respectively. Residues in ASBTYf mutated to cysteine for the accessibility experiments are coloured green; native cysteines that were mutated to serine to make the cysteine-free background are coloured cyan.
Extended Data Figure 5 | \( \text{Na}^+ \)-induced conformational changes in the \( \text{Na}^+ \)-binding sites and crossover region. a, b, Stereoimages of the \( \text{Na}^+ \)-binding sites Na1 (a) and Na2 (b) are shown in the superposed ASBT\textsubscript{Yf} (light blue) and ASBT\textsubscript{NM} (black) structures. Purple spheres correspond to the sodium ions in the ASBT\textsubscript{NM} structure. c, The ASBT\textsubscript{Yf} structure coloured by domain, with the locations of the \( \text{Na}^+ \)-binding sites from ASBT\textsubscript{NM} marked with circles. Green dots mark a solvent-accessible invagination in the surface of the core domain. TM1 is hidden for clarity. d, Closer view of Na1, formed by residues from helices TM4, TM5 and TM9 of the core domain, shown in the overlaid ASBT\textsubscript{Yf} (dark blue) and ASBT\textsubscript{NM} (black) structures, as viewed from periplasmic side. The purple sphere corresponds to the \( \text{Na}^+ \) position in the ASBT\textsubscript{NM} structure. Green dots mark a solvent-accessible invagination in the surface of the core domain leading to the central cavity in ASBT\textsubscript{Yf} which is blocked by the residue equivalent to N109 in the \( \text{Na}^+ \)-bound ASBT\textsubscript{NM} structure.
Extended Data Figure 6 | The core domain of ASBT₂f moves relative to the membrane to form the outward-open state. **a**, If the inward-open and outward-open ASBT₂f structures are aligned on the core domain only (grey), a rigid motion of the panel domain (blue) moves the amphipathic helices (red) out of the inferred bilayer–periplasm and bilayer–cytoplasm interfaces. **b**, If the inward-open and outward-open ASBT₂f structures are aligned on the panel domain only (grey), a rigid-body motion of the core domain (blue) leaves the amphipathic helices largely unaffected.
Extended Data Figure 7 | Accessibility of residues in the crossover region and potential substrate-binding sites. a, Empty liposomes or proteoliposomes reconstituted with 1:100 (mg:mg) wild-type, C196S/C248S/T106C, C196S/C248S/V123C or C196S/C248S/I269C ASBTYf were assayed for uptake of 1 μM ³H-TCA (10 Ci mmol⁻¹) in the presence of 100 mM NaCl for the indicated time periods. b, Accessibility of the T106C, V123C and I269C residues to modification by mPEG-Mal-5K, assessed by a shift in mobility on a Coomassie-blue-stained SDS–PAGE gel (same as in Fig. 2d, shown here uncropped). Each cysteine mutant was overexpressed in E. coli and subjected to four different conditions before purification: no addition of mPEG-Mal-5K; addition of mPEG-Mal-5K to the outside of whole cells; addition of mPEG-Mal-5K after sonication to rupture the cell membranes; and addition of mPEG-Mal-5K to whole cells after block of cysteines with NEM. c, The core domain of ASBTYf, viewed from the central-cavity-facing side, with the inward accessible, outward accessible, and dual accessible surface areas coloured as in Fig. 2c. A molecule of TCA is shown modelled into two potential binding sites: left, the binding site observed in the ASBTNM structure; and right, a laterally oriented binding site based on the location of residues accessible to solution in both the inward-open and outward-open ASBTYf crystal structures. d, Surface representations of the core and panel domains of ASBTYf, both oriented with the cavity-facing sides in front, coloured by element. Carbon atoms are shown as blue-grey, oxygen atoms as red, nitrogens as dark blue, and sulphurs as yellow. e, Locations of polar residues near the crossover region. TCA is shown based on the ASBTNM structure (left) and accessibility in the ASBTYf structures (right). f, Binding of 1 μM ³H-TCA in the presence of 150 mM NaCl by wild-type and mutant ASBTYf measured with the SPA. Mutations that reduce binding by more than 20% relative to the wild-type protein are labelled in red. Error bars are ±s.e.m. of triplicate measurements.
Extended Data Figure 8 | Comparison of ASBTYf to the NhaA/NapA, XylE and GlpTε transporters. a, Cartoon representation of the NapA structure (PDB accession 4BWZ) shown from two perpendicular directions. The transmembrane helices are coloured in pseudosymmetry-related pairs according to the same scheme used for the ASBT fold in Extended Data Fig. 2. Helices in the interface domain with no equivalent in the ASBT fold are coloured grey. ASBTYf is shown in the two rightmost panels for comparison. b, Outward-open (PDB accession 4BWZ, left) and inward-open (PDB accession 1ZCD, right) structures of Na⁺/H⁺ antiporters with the mobile core domain coloured dark blue and the immobile interface domain coloured red. c, Outward-open (left) and inward-open (right) structures of ASBTYf with the mobile core domain coloured dark blue and the immobile panel domain coloured red. d, Outward-open (PDB accession 1XFH, left), intermediate (PDB accession 3V8G (ref. 40), middle), and inward-open (PDB accession 3KBC, right) structures of GlpTε with the mobile substrate-binding domain coloured dark blue and the immobile interface domain coloured red. e, Outward-open (PDB accession 4GBY, left), partially inward-open (PDB accession 4JA3, middle), and inward-open (PDB accession 4JA4, right) structures of E. coli XylE with the mobile C-terminal domain coloured dark blue and the immobile N-terminal domain coloured red. f, g, The core domains of ASBTYf (f) and NapA (g) are shown viewed from the side facing the panel domain, with magnified views of the crossover regions. Polar and charged residues stabilizing the exposed backbone atoms in the unwound regions are shown as sticks in both structures. The grey circles correspond to the Na⁺-binding sites in ASBTNε or to the approximate location of the putative Na⁺ binding site in NapA.
Extended Data Figure 9 | Citrate in the crossover region of the wild-type ASBT<sub>Wt</sub> structure.  

**a**, Location of the bound citrate molecule in the wild-type ASBT<sub>Wt</sub> structure. The green surface corresponds to the F<sub><i>o</i></sub> - F<sub><i>c</i></sub> omit density for the citrate, contoured at 3.0σ. Helix TM1 is hidden for clarity.  

**b**, Specific binding of 0.48 μM [<sup>22</sup>Na]<sup>3+</sup>Cl (5.92 Ci mmol<sup>-1</sup>) to wild-type ASBT<sub>Wt</sub> measured by SPA in the presence and absence of 5 mM potassium citrate. Data are from a representative experiment performed in parallel, and data points represent the mean ± s.e.m. of triplicate measurements.  

**c**, A close-up stereo-view of the area marked with a black rectangle in panel **a**. Likelihood-weighted 2F<sub><i>o</i></sub> - F<sub><i>c</i></sub> (1.5σ) and F<sub><i>o</i></sub> - F<sub><i>c</i></sub> (3.0σ) electron density is shown as blue and green mesh, respectively. The citrate molecule was omitted from the F<sub><i>o</i></sub> - F<sub><i>c</i></sub> map calculation. Potential hydrogen bonds to the protein and ordered solvent molecules are marked with dotted lines.
### Extended Data Table 1 | Data collection, phasing and refinement statistics

|                    | WT ASBT<sub>wt</sub> | E254A ASBT<sub>wt</sub> |
|--------------------|----------------------|-------------------------|
| **Data collection**|                      |                         |
| Space group        | C2                   | P2<sub>1</sub>2<sub>1</sub>2<sub>1</sub> |
| **Cell Dimensions**|                      |                         |
| a, b, c (Å)        | 189.41, 46.22, 70.36 | 54.16, 73.86, 85.53     |
| α, β, γ (°)        | 90.0, 100.7, 90.0    | 90.0, 90.0, 90.0        |
| Resolution (Å)     | 1.95 (1.98-1.95)*    | 2.5 (2.54-2.50)         |
| R<sub>merge</sub>  | 0.076 (0.586)        | 0.085 (0.416)           |
| Compl. (%)         | 99.6 (100.0)         | 98.0 (80.5)             |
| **Refinement**     |                      |                         |
| Resolution (Å)     | 1.95 (1.99-1.95)     | 2.5 (2.75-2.50)         |
| No. reflections    | 43718 (2471)         | 12142 (2792)            |
| Compl. (%)         | 99.4 (96.0)          | 98.0 (91.9)             |
| R<sub>merge</sub>, R<sub>free</sub> (%) | 18.6/22.3       | 20.9/24.9               |
| No. atoms          |                      |                         |
| Protein            | 4580                 | 2291                    |
| Ligand/Ion         | 76                   | 0                       |
| Solvent            | 147                  | 14                      |
| **B-factors**      |                      |                         |
| Protein            | 33.2                 | 42.4                    |
| Ligand/Ion         | 55.8                 | -                       |
| Solvent            | 40.1                 | 39.5                    |
| R.m.s. deviations  |                      |                         |
| Bond lengths (Å)   | 0.007                | 0.003                   |
| Bond angles (°)    | 1.090                | 0.790                   |

Statistics from X-ray data collection and from refinement of atomic models for the wild-type (WT) and E254A ASBT<sub>wt</sub> crystals. Values in parentheses correspond to the highest resolution shell.