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Structural basis of Na⁺-independent and cooperative substrate/product antiport in CaiT

Sabrina Schulze¹, Stefan Köster¹, Ulrike Geldmacher¹, Anke C. Terwisscha van Scheltinga^{1†} & Werner Kühlbrandt¹

Transport of solutes across biological membranes is performed by specialized secondary transport proteins in the lipid bilayer¹, and is essential for life. Here we report the structures of the sodium-independent carnitine/butyrobetaine antiporter CaiT from *Proteus mirabilis* (PmCaiT) at 2.3-Å and from *Escherichia coli* (EcCaiT) at 3.5-Å resolution. CaiT belongs to the family of betaine/carnitine/choline transporters (BCCT), which are mostly Na⁺ or H⁺ dependent, whereas EcCaiT is Na⁺ and H⁺ independent². The three-dimensional architecture of CaiT resembles that of the Na⁺-dependent transporters LeuT³ and BetP⁴, but in CaiT a methionine sulphur takes the place of the Na⁺ ion to coordinate the substrate in the central transport site, accounting for Na⁺-independent transport. Both CaiT structures show the fully open, inward-facing conformation, and thus complete the set of functional states that describe the alternating access mechanism⁵. EcCaiT contains two bound butyrobetaine substrate molecules, one in the central transport site, the other in an extracellular binding pocket. In the structure of PmCaiT, a tryptophan side chain occupies the transport site, and access to the extracellular site is blocked. Binding of both substrates to CaiT reconstituted into proteoliposomes is cooperative, with Hill coefficients up to 1.7, indicating that the extracellular site is regulatory. We propose a mechanism whereby the occupied regulatory site increases the binding affinity of the transport site and initiates substrate translocation.

The three protomers of the CaiT trimer⁶ crystallized in an inward-facing, fully open conformation. The cytoplasmic side of the protomer shows a funnel approximately 25 Å deep and 15 Å wide that narrows to roughly 7 Å in the centre (Fig. 1a). Access to the central transport site from the cytoplasm is unhindered by obstructing side chains. As in the related BetP⁴, the central transport site is a tryptophan box (Fig. 1b, c), defined by Trp 142 and Trp 147 from the partly unwound fourth transmembrane helix (TM4), and Trp 323 and Trp 324 from the continuous but highly tilted TM8. Outside access is blocked by Trp 107 (TM3), Trp 316 (TM8) and Tyr 320 (TM8), which is hydrogen bonded to the carboxyl group of Glu 111 (Fig. 2a). Hydrophobic contacts between TM3, TM5, TM8 and TM12 help to seal the channel towards the outside (Fig. 2a). As expected from 87% sequence identity (Supplementary Fig. 1), the overall structures of PmCaiT and EcCaiT resemble one another closely (Supplementary Table 1).

The transport site of EcCaiT contains a bound γ -butyrobetaine substrate (Fig. 1b), carried over from the expression host, held in place by cation- π interactions characteristic of proteins that bind quaternary ammonium substrates^{7–10}. In EcCaiT, the indole ring of Trp 323 in TM8 is well placed for this interaction. The other residues in the transport site hold the substrate by van der Waals contacts. In contrast, the transport site of PmCaiT does not contain bound substrate. The side chain of Trp 323 is rotated by 60° to a position that leaves no room for

either substrate (Fig. 1c). The orientation of Trp 323 is the most striking difference between the two CaiT structures.

Unlike BetP and LeuT, CaiT is Na⁺ independent. This is explained by a comparison of the positions of side chains or ions that coordinate the substrate. LeuT contains two Na⁺ ions³. Na1 coordinates the substrate in the transport site and stabilizes the unwound region of TM6. Na2 stabilizes the unwound stretch of TM1, equivalent to TM3 in CaiT (Supplementary Table 2). The Na2 position in CaiT is occupied by Arg 262 (TM7) (Fig. 2b). The positive charge links TM3 to TM10 and stabilizes the unwound TM3, which contributes to substrate-binding in the external binding pocket through Tyr 114 (Fig. 1d).

In sodium-dependent BetP, Na1 coordinates the betaine substrate⁴. Neither CaiT structure shows a Na⁺ ion in this vicinity. Instead, there is an attractive interaction between the carboxyl group of the γ -butyrobetaine, and the sulphur of Met 331 (Fig. 1b). This methionine is conserved in CaiT and mammalian organic cation/carnitine transporter (OCTN) transporters, but not in BetP (Supplementary Fig. 1). The methionine sulphur in CaiT evidently takes over the role Na1. Interestingly, ApcT, which is H⁺ but not Na⁺ dependent, also has a methionine in this position, whereas Na2 is replaced by a lysine¹¹. Both Na⁺-independent transporters thus stabilize the unwound TM3 with a positively charged side chain, and have replaced Na1 by a methionine sulphur.

A second γ -butyrobetaine is bound in an extracellular cavity of EcCaiT near the exit of the closed substrate pathway (Fig. 1a, d and Supplementary Figs 4 and 7b). In PmCaiT this site is occupied by two ordered water molecules, linking TM12 to TM8 (Fig. 1e and Supplementary Fig. 7b). A third water molecule connects Trp 316 to the main-chain carbonyl of Tyr 114 in TM3 and is hydrogen-bonded to Arg -2 of a symmetry-related protomer (Fig. 1e and Supplementary Fig. 7b) in the linker peptide connecting the amino (N) terminus to a hexa-histidine affinity tag introduced for purification, which had been removed in EcCaiT before crystallization. The crystal contact blocks access to the second binding site, which explains why the site is empty in PmCaiT. Sequence comparison (Supplementary Fig. 1) suggests that the extracellular site is conserved in BetP. A second site has also been proposed for LeuT¹², but its predicted position is different and its functional role is unclear.

Substrate binding to CaiT is readily monitored by Trp fluorescence (Fig. 3b and Supplementary Fig. 5). The observed changes in fluorescence intensity and emission wavelength must reflect the re-orientation of Trp 323, as no other Trp side chain is subject to a comparable change of environment upon substrate binding. Assays were performed both in detergent solution (Supplementary Fig. 5) and with proteoliposomes (Fig. 3b). Titration of wild-type PmCaiT with or without NaCl indicated a dissociation constant (K_d) of 2–3 mM and a maximal substrate binding concentration $B_{\max}$ of

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Figure 1 | CaT structure and bound substrates. **a**, A cut through the protomer shows the open cytoplasmic funnel, and the two bound γ -butyrobetaine molecules (arrows). **b**, The tryptophan box of the central transport site binds the substrate to be translocated. The Met 331 sulphur coordinates the substrate (blue; $2F_o - F_c$ map at 1.0σ) by polar interaction with the carboxyl group (dotted) in EcCaT, instead of Na⁺ in the Na⁺-dependent transporters^{4,6}. **c**, In PmCaT, the Trp 323 indole group (circled) is rotated by 43° each in χ_1 and χ_2 , preventing substrate binding. In the

approximately 40 mM for either substrate (Supplementary Table 3). In Na⁺-dependent transporters, the substrate-binding affinity with Na⁺ increases about fivefold to tenfold^{13,14}. The large difference between the Michaelis-Menten constant (K_m) and K_d is consistent with more than one functionally significant substrate-binding site.

Transport measurements indicated high substrate specificity (Fig. 3a). Kinetic analysis of CaT proteoliposomes preloaded with γ -butyrobetaine gave a value of K_m for L-carnitine of 40–80 μ M, and a maximal transport velocity v_{max} of about 4,800 nmol substrate per minute and mg of protein for both wild-type transporters, with or without NaCl (Supplementary Table 3). Assuming that every CaT is active, the calculated uptake rate is roughly 270 molecules per minute (20 °C), similar to BetP¹³.

extracellular second binding site of EcCaT (**d**), a bound γ -butyrobetaine (contoured as above) is coordinated by cation– π interaction with Tyr 114 and Trp 316, plus an H bond of the γ -butyrobetaine carboxyl to the Gly 315 backbone nitrogen. In the substrate-free second binding site of PmCaT (**e**), two water molecules connect TM8 and TM12. A third water molecule links TM3 to the indole nitrogen of Trp 316, and forms a hydrogen bond to Arg –2 (grey) between the N terminus and His₆ tag of a symmetry-related protomer, blocking the second site.

Mutations of Met 331 mimicking Na⁺ in the transport site, or of Trp 316 in the second binding site, both affect CaT function. Replacing Trp 316 by alanine reduces K_d , K_m and v_{max} by factors of approximately 5, 3.5 and 2.5, respectively (Supplementary Table 3), indicating that substrate in this site has a profound effect on activity. Mutating Met 331 to valine reduces K_d for substrate binding by a factor of around 4, but v_{max} by a factor of 10 (Supplementary Table 3), which highlights the pivotal role of this residue for transport. The mutation does not make CaT Na⁺ dependent. A simple side-chain substitution is not sufficient to recreate a Na⁺-binding site in this position.

Substrate-binding curves for CaT reconstituted into proteoliposomes are sigmoidal (Fig. 3b), indicating positive cooperativity¹⁵.

Figure 2 | Hydrogen bond networks in PmCaT.

a, Hydrogen bonds (dotted) around Glu 111, connecting TM3 and TM4 to TM8. These helices must move apart to release the substrate in the outside-open conformation. **b**, Arg 262, which replaces Na2 in the Na⁺-dependent transporters, is located between TM3 and TM10, stabilizing the unwound stretch of TM3 in the membrane by hydrogen bonds.

Figure 3 | Substrate transport and binding in CaiT. **a**, Substrate uptake in wild-type EcCaiT (pink), wild-type PmCaiT (black) and PmCaiT mutants M331V (blue), W316A (green) and E111A (red) reconstituted into proteoliposomes in sodium-free buffer (left) or with 100 mM NaCl. **b**, Binding of γ -butyrobetaine or L-carnitine to PmCaiT reconstituted into proteoliposomes in sodium-free buffer. Error bars, s.e.m. of triplicate experiments.

Transport is thus allosterically regulated, such that a substrate bound to one site increases the binding affinity of the other. It is reasonable to assume that the external substrate-binding site is the regulatory site. This assumption is strongly supported by our finding that the PmCaiT structure does not contain bound substrate, whereas that of EcCaiT does. In PmCaiT, access to the second site is fortuitously blocked by a crystal contact (Fig. 1e), which cannot form when substrate is bound. An empty regulatory site would reduce the affinity of the central transport site, which indeed is also empty in PmCaiT.

The affinity of CaiT for γ -butyrobetaine is significantly lower than for L-carnitine (Supplementary Table 3), and this must reflect the binding properties of the regulatory site. With its extra hydroxyl group, an L-carnitine molecule in this site can establish another hydrogen bond to the ϵ -amino group of Lys 470 (Fig. 1d and Supplementary Fig. 7b), whereas there are no such polar interactions in the central transport site. Our finding that the Trp 316 mutant is strongly impaired in transport, but only moderately so in substrate binding (Supplementary Table 3) supports our conclusion that the external binding site is regulatory, as the structure of the transport site, and therefore its affinity for either substrate, will be largely unaffected by the W316A mutation. If the external site were not the regulatory site, no strong effect on transport would be expected, contrary to what we observe (Fig. 3a).

The Hill coefficients derived from the binding curves (Fig. 3b) range from 1.4 to 1.7 (Supplementary Table 3), indicative of pronounced cooperativity. Many allosteric proteins are oligomers, although they mostly have only one binding site per protomer, whereas CaiT has two. It is therefore interesting to ask whether the cooperativity applies to the two binding sites in the protomer, or to all six in the CaiT trimer⁶. Substrate binding is accompanied by structural changes both in the protomer and in the trimer, where the substrate-bound protomer tilts by 3° tilt towards the threefold axis (Supplementary Fig. 7a).

The overall root mean squared deviations (r.m.s.d.) of 0.7 Å between protomers and of 0.9 Å between trimers with and without bound substrate (Supplementary Table 2) indicate that these changes are significant, given that the r.m.s.d. between spinach and pea LHC-II, two similarly homologous, trimeric membrane proteins that do not undergo a conformational change, is 0.35 Å (ref. 16). The Hill coefficient greater than 1 signifies an interaction of at least two binding sites, which are likely to be those we observe in the protomer, as cooperativity between all six sites in the trimer should result in a higher Hill coefficient. However, the role of the CaiT trimer⁶ in allosteric regulation remains to be determined.

How might the regulatory site communicate its substrate-bound state to the transport site? In the occupied regulatory site, bound substrate displaces two water molecules (Fig. 1d, e and Supplementary Fig. 7b). This causes small shifts of Tyr 114 (TM3), Trp 316 (TM8), Lys 470 and Gln 473 (TM12) (Supplementary Fig. 7b), which propagate to Tyr 320 (TM8) and Glu 111 (TM3). The buried Glu 111 that coordinates the network of hydrogen bonds (Fig. 2a) connecting the two binding sites is conserved in CaiT, BetP and mammalian OCTN (Supplementary Fig. 1). Replacing this residue by alanine renders the transporter inactive (Fig. 3a) and destabilizes the trimer (Supplementary Fig. 6), highlighting its crucial role for CaiT assembly and function.

These differences add up to small but significant helix movements in the region between the regulatory site and the transport site. TM3 of EcCaiT tilts out of the substrate pathway by roughly 1 Å, and TM8 of the other repeat moves in the opposite direction by a similar distance (Supplementary Fig. 7b). As a result, Trp 142 in the tryptophan box and Trp 363 each move by approximately 1 Å. These movements most likely facilitate the replacement of solvent molecules in the transport site, and the rotation of Trp 323. The latter moves TM12 out of the substrate pathway by roughly 1 Å. A similar movement of TM5 makes the EcCaiT transport site more accessible. The r.m.s.d. values (Supplementary Table 1) and extreme sequence conservation of 95% in the TM regions suggest that these differences are functionally relevant.

We propose a mechanism of allosterically regulated substrate uptake by CaiT that encompasses all our findings (Fig. 4). It should be regarded as speculative, however, until structures of all intermediate stages have been determined. First, L-carnitine binds to the regulatory extracellular site. This triggers the re-orientation of Trp 323 in the central transport site, increasing its substrate-binding affinity. The open cytoplasmic funnel ensures free access from the cell interior, so that substrate can bind as soon as Trp 323 re-orientates. The substrate binding from the inside would be γ -butyrobetaine, the metabolic product of L-carnitine.

Transition of CaiT from the fully inside-open to the occluded state of BetP involves an iris-like movement of TM helices surrounding the transport site (Supplementary Fig. 8), along with the re-orientation of bulky hydrophobic side chains blocking the pathway. A superposition of TM helices describes the associated helix movements, which have been discussed in detail⁴. When modelled on the outside-open conformation of LeuT^{3,4,14,17–20}, the extracellular ends of helices TM3 and TM4 of CaiT move by 3.2 and 5 Å, whereas TM8 and TM12 move by 6.2 and 5 Å out of the extracellular substrate pathway. Although sequence similarity is low (Supplementary Table 1), the overall structures of the protomers are conserved. In this transition, the hydrogen bonds between Glu 111, Tyr 320, Tyr 137 and Thr 317 (Fig. 2a) must break for the outside funnel to open. Several of the hydrogen bonds around Glu 111 are longer by up to 1.0 Å in EcCaiT than in PmCaiT, suggesting that substrate binding to the external site weakens the central hydrogen-bonding network.

In the outside-open state, γ -butyrobetaine would diffuse out of the transport site, L-carnitine would bind instead and the process would go into reverse. The extracellular pathway would close and the cytoplasmic funnel would open, releasing L-carnitine to the cell interior. The constant consumption of L-carnitine in the cell would maintain a

Figure 4 | Proposed mechanism of cooperative transport. The empty transporter, represented by the PmCaiT structure, is activated by L-carnitine (yellow diamond) binding to the regulatory site. This triggers the re-orientation of Trp 323 (blue disk), and γ -butyrobetaine (red diamond) binds to the central transport site from the cytoplasm. The state in which both binding sites are occupied is represented by the EcCaiT structure. In the occluded state, represented by the BetP structure⁴, the substrate in the transport site is inaccessible. In the outside-open state, represented by the LeuT structure⁶, γ -butyrobetaine in the central transport site is replaced by L-carnitine. CaiT then changes conformation through the occluded state back to the inside-open state, releasing L-carnitine to the cytoplasm, where it is metabolized. The next γ -butyrobetaine product binds, and the transport cycle repeats. When the outside L-carnitine concentration drops below a critical level, the substrate diffuses out of the regulatory site, and the transporter switches off.

concentration gradient that drives uptake. In the CaiT structure modelled on the outward-open state of LeuT, the regulatory site remains intact (Supplementary Fig. 9), suggesting that substrate remains bound throughout the transport cycle (Fig. 4). When the external L-carnitine concentration drops below a critical level, the substrate would diffuse out of the regulatory site, and the transporter would switch off.

While this paper was under review, an X-ray structure of CaiT from *E. coli* at 3.15-Å resolution was reported²¹. It is largely identical to our structure of the EcCaiT trimer, except that the protomer binds four molecules of L-carnitine, apparently because of an excess of this substrate added for crystallization. Two of the binding sites confirm those we find to contain γ -butyrobetaine, whereas the other two most likely contain unspecifically bound substrate. Conclusions about the structural basis of Na^+ -independent transport have not been drawn, nor are there any measurements to show cooperative substrate binding.

METHODS SUMMARY

Genes encoding CaiT from *E. coli* and *P. mirabilis* were cloned into pET-15b (Novagen) and expressed in *E. coli* strain BL21(DE3) pLysS. Point mutations were inserted using site-directed mutagenesis. The *E. coli* membrane fraction was solubilized and CaiT was purified by Ni-NTA affinity chromatography. Crystals were grown in hanging drops. Data sets of native CaiT were collected on the X10SA beamline at the Swiss Light Source. The structure was determined by molecular replacement with BetP (Protein Data Bank accession number 2WIT). The structure of PmCaiT was refined to final working (R_{work}) and free (R_{free} , calculated for 5% of the data excluded from refinement) reliability factors of 0.209 and 0.238, respectively. The EcCaiT model was refined to an R_{work} value of 0.237 and an R_{free} value of 0.271, with strict non-crystallography symmetry restraints enforced during refinement. For transport measurements, CaiT was reconstituted into liposomes and uptake of [¹⁴C]L-carnitine was recorded. Substrate binding was assayed by tryptophan fluorescence. Binding curves were evaluated using the program Origin 7.5.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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Author Contributions Experiments were performed by S.S. and U.G.; cloning and mutagenesis was performed by S.K. Crystals were grown by S.S., diffraction data were collected and processed by S.S. and A.C.T.v.S., and the structures were analysed by S.S., A.C.T.v.S. and W.K. S.S. and W.K. wrote the manuscript.

Author Information Coordinates and structure factors for *P. mirabilis* CaiT and *E. coli* CaiT with bound substrate are deposited in the Protein Data Bank under accession numbers 2WSW and 2WSX, respectively. 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 this article at www.nature.com/nature. Correspondence and requests for materials should be addressed to W.K. (werner.kuehlbrandt@biophys.mpg.de).

METHODS

Cloning, expression and protein purification. *E. coli* BL21(DE3) pLysS cells were transformed with plasmids containing the EcCaIT or PmCaIT genes cloned into the pET-15b (Novagen) vector, using the restriction enzymes NdeI and XhoI. Recombinant protein was overexpressed at 30 °C in 2×YT media supplemented with 50 µg ml⁻¹ ampicillin and 50 µg ml⁻¹ chloramphenicol. Overexpression of recombinant protein was induced with 0.5 mM isopropyl-β-D-thiogalactopyranoside at an absorbance ($A_{600\text{ nm}}$) 0.6–0.9. Cells were harvested, homogenized in buffer containing 25 mM HEPES (pH 7.5), 100 mM NaCl and 0.1 mM Pefabloc (Biomol) and disrupted using a microfluidizer (Model M-110L, Microfluidics) with three passes at 60,000 pounds per square inch. Cell debris was removed by centrifugation (10,000g, 4 °C, 30 min), after which membranes were collected by ultracentrifugation (125,000g, 4 °C, 90 min). The membrane pellet was re-suspended in buffer containing 25 mM HEPES (pH 7.5), 100 mM NaCl, 2 mM Tris[2-carboxyethyl]-phosphine (TCEP) and 20% glycerol. Aliquots were frozen in liquid nitrogen and kept at -80 °C. Membranes were solubilized by incubation overnight at 4 °C with 2% 5-cyclohexyl-1-pentyl-β-D-maltoside (Cymal-5, Anatrace) and unsolubilized material was removed by ultracentrifugation (125,000g, 4 °C, 40 min). The supernatant was loaded onto Ni²⁺-chelating sepharose (Amersham) equilibrated with a buffer containing 25 mM HEPES (pH 7.5), 100 mM NaCl, 1–2 mM TCEP, 0.15–0.2% Cymal-5 and 20% glycerol. The protein was washed with 50 mM imidazole and eluted with 200 mM imidazole in the same buffer containing 10% glycerol, and then with imidazole-free buffer on a PD-10 Sephadex G-25M (GE Healthcare) column. For tryptophan fluorescence and transport assays, the buffer was replaced by 50 mM Tris (pH 7.5) and 50 mM KCl. The His₆-tag of EcCaIT was removed using the Thrombin-Cleavage Kit (Sigma). The purified protein was concentrated to 5 mg ml⁻¹ (Vivaspin 100kDa, Sartorius). Site-directed mutagenesis of Met 331 Val and Trp 316 Ala of PmCaIT was performed with the QuikChange Site Directed Mutagenesis Kit (Stratagene) according to the manufacturer's instructions. All constructs were verified by nucleotide sequencing.

Crystallization. The protein solution was clarified by centrifugation (100,000g, 4 °C, 30 min) before crystallization. Crystals were grown at 4 °C by vapour diffusion in hanging drops of 2 µl protein solution and 1 µl reservoir (50 mM Mg²⁺-acetate or Ca²⁺-acetate (pH 4.5–5.5), 0–200 mM NaCl, 19–30% PEG400). Crystals appeared after 3–10 days. They were frozen directly in liquid nitrogen using PEG400 as cryoprotectant. The quality of the crystals varied and pre-screening of a large number was essential. PmCaIT crystals diffracted isotropically to a resolution of 2.3–2.7 Å. The best EcCaIT crystals diffracted isotropically to a resolution of 3.5 Å.

Data collection and phasing. Data were collected on beamline X10SA at the Swiss Light Source (Villigen, Switzerland). Data were initially processed in P1 using XDS²². The correct space group was determined using POINTLESS²³ and data were scaled with SCALA²⁴. PmCaIT crystallized in the space group *H*3 with three molecules in the asymmetric unit arranged around a crystallographic threefold axis. EcCaIT crystallized in *P*3₂ with three molecules in the asymmetric unit. The structure of PmCaIT was solved by molecular replacement²⁵ with a poly-alanine model of BetP (Protein Data Bank accession number 2WIT) using PHASER²⁶, and the structure of EcCaIT by MR with the PmCaIT structure. Model bias was minimized by using the prime-and-switch program²⁷. The primary density was improved by density modification in RESOLVE²⁸.

Model building and refinement. The structure was refined with iterative rounds of manual rebuilding in COOT²⁹ and O³⁰, and simulated annealing followed by maximum-likelihood-based energy minimization and isotropic B-factor refinement in PHENIX³¹. Figures were generated with PovScript+³², Povray (<http://www.povray.org>) and PyMOL³³. Superpositions were performed with the SSM³⁴ superposition routine of COOT²⁹. Superpositions to compute r.m.s.d. values were performed with the DaliLite server^{35,36} (<http://www.ebi.ac.uk/DaliLite/>).

Transport assays. Low-sodium buffer for transport and substrate-binding studies was prepared with sodium-free water (Fluka) and, as far as possible, sodium-free chemicals. The estimated residual Na⁺ concentration from the Tris buffer (Trizma base, BioUltra, Fluka) was 2.5 µM. Purified PmCaIT was reconstituted into liposomes of *E. coli* polar lipids (Avanti). Substrate exchange was measured

by recording the uptake of L-[N-methyl-¹⁴C]carnitine hydrochloride (¹⁴C-L-carnitine, Perkin Elmer) into CaIT-containing liposomes preloaded with a saturating concentration of non-radioactive substrate. Usually 10 mM of potential substrate such as glycine (Sigma), glycine betaine (Sigma), γ-butyrobetaine (Lonza) or L-carnitine (Sigma) was used. Proteoliposomes were loaded by two rounds of freezing and thawing in the presence of substrate. After the second freeze-thaw cycle the proteoliposomes were extruded first through a 200-nm membrane filter (Avestin) followed by extrusion through a 400-nm membrane filter (Avestin). The extraliposomal substrate was removed by ultracentrifugation (100,000g, 20 °C, 30 min), and proteoliposomes used for transport measurements were washed with buffer (50 mM KP_i (pH 7.5), 1–2 mM TCEP). Substrate uptake was initiated by 2-µl aliquots of the proteoliposome suspension diluted into 400 µl buffer (50 mM KP_i (pH 7.5), 1–2 mM TCEP) with concentration of ¹⁴C-L-carnitine (2.5 µCi ml⁻¹) ranging from 6 to 620 µM. At time points of 5–30 s, ¹⁴C-L-carnitine uptake was determined with a TRI-CARB 1500 scintillation counter (Canberra-Packard). All transport assays were repeated at least three times.

Binding assays. Binding assays were performed with 1.5 µM purified CaIT protein in 0.15–0.2% Cymal-5 detergent, or with the transporter reconstituted into proteoliposomes as above. Concentrations of potential substrates such as glycine betaine, γ-butyrobetaine, L-carnitine or choline ranged from 0.25 to 40 mM. Tryptophan fluorescence emission between 305 and 390 nm was recorded on a Hitachi F-4500 fluorescence spectrophotometer and averaged over three to five readings, with the excitation wavelength set to 295 nm and a slit width of 2.5 or 5 nm for excitation or emission, respectively, at different NaCl concentrations (1–50 mM) or without added NaCl. The mean value and standard deviation at the 342-nm emission maximum was plotted for each substrate concentration. Data were fitted to the Michaelis–Menten equation² and apparent *K_m* values were calculated using the program Origin 7.5. Hill coefficients were determined by fitting sigmoidal binding curves using the program Origin.

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