CaiT of Escherichia coli, a New Transporter Catalyzing L-Carnitine/γ-Butyrobetaine Exchange*

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1-L-Carnitine is essential for β-oxidation of fatty acids in mitochondria. Bacterial metabolic pathways are used for the production of this medically important compound. Here, we report the first detailed functional characterization of the caiT gene product, a putative transport protein whose function is required for L-carnitine conversion in Escherichia coli. The caiT gene was overexpressed in E. coli, and the gene product was purified by affinity chromatography and reconstituted into proteoliposomes. Functional analyses with intact cells and proteoliposomes demonstrated that CaiT is able to catalyze the exchange of L-carnitine for γ-butyrobetaine, the excreted end product of L-carnitine conversion in E. coli, and related betaines. Electrochemical ion gradients did not significantly stimulate L-carnitine uptake. Analysis of L-carnitine counterflow yielded an apparent external K_m of 105 μM and a turnover number of 5.5 s⁻¹. Contrary to related proteins, CaiT activity was not modulated by osmotic stress. L-Carnitine binding to CaiT increased the protein fluorescence and caused a red shift in the emission maximum, an observation explained by ligand-induced conformational alterations. The fluorescence effect was specific for betaine structures, for which the distance between trimethylammonium and carboxyl groups proved to be crucial for affinity. Taken together, the results suggest that CaiT functions as an exchanger (antiporter) for L-carnitine and γ-butyrobetaine according to the substrate/product antiprinciple.

L-Carnitine (R-(−)-3-hydroxy-4-trimethylaminobutyrate) is essential for transport of activated fatty acids across the inner mitochondrial membrane via the carnitine/acylcarnitine exchange protein (1, 2). Bacteria use L-carnitine in different ways (3). For example, Escherichia coli and other enterobacteria accumulate the betaine as a compatible solute under osmotic stress (4, 5). In addition, these bacteria take up L-carnitine under anaerobic growth conditions if no other electron acceptors or glucose is present and convert it into γ-butyrobetaine (4-Trimethylaminobutyrate), the excreted end product of the pathway (6). Crotonobetaine (4-Trimethylaminocrotonate) is formed as an intermediate and probably serves as an electron acceptor under these growth conditions (7, 8) (cf. also Fig. 8). This pathway is particularly interesting, as it can be reverted and used for the enantioselective production of L-carnitine (9, 10), as a prerequisite of electrochemical ion gradients, substrate and product must be transported across the cell membrane. To date, only very little is known about the mechanism underlying L-carnitine uptake and γ-butyrobetaine excretion. Early studies using a wild-type E. coli strain (O44K74) showed that the cells possess an active, carrier-mediated uptake system specific for L-carnitine (11). Transport was not assigned to a specific gene product. Therefore, we focus here on the elucidation of the transport mechanism for L-carnitine and related betaines in E. coli.

The E. coli genes responsible for carnitine conversion have been cloned and sequenced (12–14). The genes are organized in two divergent operons, one of which is the caiTABCDE operon. The products of the caiB and caiA genes, carboxydeshydrogenase and crotonobetaine reductase, have been characterized in previous studies (15–18). Furthermore, the product of the caiT gene was proposed to function as a transporter based on sequence comparison and hydrophathy profile analysis (13). The caiT gene encodes a highly hydrophobic protein of 504 amino acids, which probably form 12 membrane-spanning domains (Fig. 1). CaiT displays similarity to the glycine betaine uptake system OpdU from Bacillus subtilis, the choline transporter BetT from E. coli, BetP from Corynebacterium glutamicum, and a BetT-like protein from Haemophilus influenzae (19, 20). They form a small family of transporters, the betaine/carnitine/choline transporter (BCCT) family (Transport Protein Database TC 2.A.15).² Proteins of this family share the common functional feature of transporting molecules with a quaternary ammonium group (R-N⁺(CH₃)₃). Some of these transporters have been shown to utilize electrochemical ion gradients (H⁺, Na⁺) for substrate accumulation. In addition, some members of the family are osmotically regulated and share hydrophilic extensions at the N and/or C termini (19, 20, 22, 23).

This study represents the first detailed analysis of CaiT function. For this purpose, CaiT was purified and reconstituted into proteoliposomes. Functional analyses with intact cells and proteoliposomes demonstrated that CaiT is able to catalyze the exchange of L-carnitine for γ-butyrobetaine and related betaines, whereas electrochemical ion gradients do not significantly stimulate L-carnitine uptake. Analysis of CaiT fluorescence revealed ligand-induced conformational alterations. The fluorescence approach was used to obtain information on the substrate specificity of the transporter.

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¹ The abbreviations used are: BCCT, betaine/carnitine/choline transporter; Mes, 2-(N-morpholino)ethanesulfonic acid.
² Available at tcdb.ucsd.edu/tcdb/tcfamilybrowse.php?tsname=2.A.15.
EXPERIMENTAL PROCEDURES

**Materials**—[(S)-1-methyl-3-[14C]Carnitine (53 μCi/μmol) and horseradish peroxidase-conjugated sheep anti-mouse IgG were purchased from Amersham Biosciences (Fernwald, Germany). Ni²⁺-nitrilotriacetic acid resin was from Qiagen GmbH (Hilden, Germany). *E. coli* phospholipid extract was from Avanti Polar Lipids, Inc. (Alabaster, AL).

**Bacterial Strains and Plasmids**—*E. coli* JM109 (endA1 recA1 gyrA96 thi-1 hsdR17 supE44 Δlac-proAB F′ lacIq ΔlacZΔM15) (24) served as carrier for the plasmids described. *E. coli* BL21(DE3) pLysS (F thi hsdR17 supE44 Δlac-proAB F′ lacIq ΔlacZΔM15) (24) served as carrier for the plasmids described. *E. coli* BL21(DE3) pLysS (F thi hsdR17 supE44 Δlac-proAB F′ lacIq ΔlacZΔM15) (24) served as carrier for the plasmids described.

**Plasmid Construction**—Plasmid pTcaiT (or its derivatives) were grown aerobically in LB medium containing 100 μg/ml ampicillin and 34 μg/ml chloramphenicol. In the middle of the exponential growth phase, gene expression was induced by addition of 0.3 mM isopropyl-β-D-thiogalactopyranoside, and incubation was continued for 3 h. Cells were harvested and washed as described above. Cells were frozen and kept at −80°C until used.

**Immunological Analysis**—The amount of CaiT in *E. coli* WG389 and BL21(DE3) pLysS membranes was estimated by immunoblotting with mouse anti-FLAG IgG raised against theFLAG epitope at the carboxyl terminus of the protein, followed by incubation with horseradish peroxidase-conjugated sheep anti-mouse IgG as described (29).

**Purification of CaiTFH**—Inverted membrane vesicles of *E. coli* BL21(DE3) pLysS containing CaiTFH (CaiT with the FLAG epitope and a His₆ tag attached to the C terminus) were prepared by passage of the cell suspension through a Sorvall RF1 Ribi refrigerated cell fractionator, followed by low speed centrifugation at 12,000 × g for 30 min at 4°C to remove unbroken cells. Membranes were collected by centrifugation at 230,000 × g for 90 min at 4°C, washed with 50 mM potassium phosphate buffer (pH 7.5) and 2 mM β-mercaptoethanol, and resuspended in the same buffer, and stored at −80°C. For solubilization of CaiTFH, inverted membrane vesicles were diluted into 50 mM potassium phosphate buffer (pH 8.0) and 2 mM β-mercaptoethanol, and 10% glycerol. Dodecyl β-maltoside was added step-wise to yield a final concentration of 1.5% (w/v) while stirring on ice. After additional stirring for 30 min, the sample was centrifuged at 230,000 × g for 20 min. The supernatant was supplemented with 2 mM β-mercaptoethanol and 10% glycerol. Dodecyl β-maltoside was added step-wise to yield a final concentration of 1.5% (w/v) while stirring on ice.

**Cell Growth and Expression of caiT**—*E. coli* WG389 cells harboring plasmid pTcaiT (or its derivatives) were grown aerobically in LB medium (28) containing 200 mM l-proline and 100 μg/ml ampicillin at 37°C, and expression was initiated by addition of 0.5 mM isopropyl-β-D-thiogalactopyranoside in the middle of the exponential growth phase.

After further growth for 2 h, cells were harvested by centrifugation, washed with 100 mM potassium phosphate buffer (pH 7.5) and 2 mM β-mercaptoethanol, and immediately used for transport assays. *E. coli* BL21(DE3) pLysS cells containing the corresponding plasmids were grown as described for strain WG389, except that the LB medium contained 100 μg/ml ampicillin and 34 μg/ml chloramphenicol. In the middle of the exponential growth phase, gene expression was induced by addition of 0.3 mM isopropyl-β-D-thiogalactopyranoside, and incubation was continued for 3 h. Cells were harvested and washed as described above. Cells were frozen and kept at −80°C until used.

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To analyze the effect of N$	extsubscript{10}$ mML -carnitine), this yielded a final external L-carnitine concentration of 54.7 muM. 

Transport Measurements with Intact Cells—Fresly grown E. coli WG389 cells transformed with either pTcaiT or pT7-5 were resuspended in 100 mM potassium phosphate buffer (pH 7.5), 2 mM beta-mercaptoethanol, and 5 mM MgSO$	extsubscript{4}$ (buffer D). In the case of counterflow experiments (cells preloaded with 10 mM L-carnitine), this yielded a final external L-carnitine concentration of 54.7 muM. Incubation was carried out at 25 °C, and transport was terminated at different time points by addition of 100 mM potassium phosphate buffer (pH 6.0) and 100 mM LiCl (stop buffer) and rapid filtration as described (29).

Transport Measurements with Proteoliposomes—Proteoliposomes containing CaiTFH (1-2 mg of protein/ml) were subjected to three cycles of freeze/thaw-sonication prior to transport measurements as described (31). L-[methyl-14C]Carnitine uptake was measured with proteoliposomes in 100 mM potassium phosphate buffer (pH 7.5), 2 mM beta-mercaptoethanol, and 5 mM MgSO$	extsubscript{4}$ (buffer D). Ion-dependent transport was started by 200-fold dilution of aliquots of the proteoliposome suspension (cells preloaded with 10 mM L-carnitine), this yielded a final external L-carnitine concentration of 54.7 muM. Incubation was carried out at 25 °C, and transport was terminated at different time points by addition of 100 mM potassium phosphate buffer (pH 6.0) and 100 mM LiCl (stop buffer) and rapid filtration as described (29).

RESULTS

CaiT Activity in Intact Cells—The caiT gene of E. coli was cloned into plasmid pT7-5 as described under “Experimental Procedures,” yielding plasmid pTcaiT. To facilitate immunological detection and purification of the gene product, a nucleotide sequence encoding the FLAG epitope and a His$	extsubscript{6}$ tag was attached to the 3'-end of caiT, resulting in plasmid pTcaITFH. Plasmid DNA was transformed into E. coli WG389. This strain lacks the Na+/proline transporter PutP as well as the transport systems ProP and ProU, which catalyze the uptake of proline and betaines in response to osmotic stress. All trials to delete the caiT gene from the genome of E. coli WG389 did not produce viable cells. Transformed cells were grown under aerobic conditions, which prevent expression of the caiT gene from the genome (8, 38). Plasmid-derived caiT was expressed via the lac promoter, and Western blot analyses with monoclonal antibodies directed against the FLAG epitope or the His$	extsubscript{6}$ tag demonstrated the CaiT location in the membrane fraction of isopropyl-beta-D-thiogalactopyranoside-induced cells (data not shown).

To investigate CaiT function, uptake of L-[methyl-14C]carnitine into intact cells of E. coli WG389 transformed with either plasmid pTcaiT or pT7-5 was analyzed by a rapid filtration assay. Cells containing plasmid pT7-5 without caiT showed no or only little uptake of L-[methyl-14C]carnitine depending on the L-carnitine concentration used (data not shown). Expression of plasmid-encoded CaiT did not stimulate this uptake process independent of the presence or absence of an electrochemical H$^+$ or Na$^+$ gradient (data not shown). However, CaiT increased L-[methyl-14C]carnitine transport into cells preloaded with unlabeled L-carnitine, an activity not observed with cells lacking CaiT (Fig. 2). This L-carnitine/L-carnitine exchange (counterflow) was inhibited by reaction of CaiT with the sulphydryl reagent N-ethylmaleimide (80% inhibition of the initial rate), whereas the ionophore carbonyl cyanide m-chlorophenylhydrazone did not affect counterflow. Besides preloading with L-carnitine, also preloading with gamma-butyrobetaine stimulated uptake of external L-[methyl-14C]carnitine into cells containing CaiT (Fig. 2). In contrast, CaiT-containing cells preloaded with glycine betaine, choline, proline, or DL-gamma-amino-beta-hydroxybutyrate did not accumulate L-[methyl-14C]carnitine (data not shown). Finally, cells transformed with plasmid pTcaITFH showed the same counterflow activity as cells expressing caiT from plasmid pTcaIT, indicating that the attachment of the FLAG epitope and a His$	extsubscript{6}$ tag to the C terminus does not significantly influence CaiT function (data not shown).
Solubilization, Purification, and Reconstitution of CaiTFH—To test whether CaiT is solely responsible for the observed transport activity, the corresponding gene was overexpressed via the T7 promoter of plasmid pTcaiT (+caiT cells). Cells containing plasmid pT7-5 (−caiT cells) served as negative control. For exchange experiments, cells were resuspended in resuspension buffer (7 mg of total cell protein/ml) and equilibrated with the indicated trimethylammonium compound (10 mM). Aliquots (1 µl) of the cell suspension were diluted into 200 µl of resuspension buffer containing 4.7 µM l-[methyl-14C]carnitine (53 Ci/mmol). l-[methyl-14C]Carnitine uptake was measured in unloaded +caiT cells (○), unloaded −caiT cells (□), +caiT cells preloaded with either l-carnitine (●) or γ-butyrobetaine (▴), and −caiT cells preloaded with l-carnitine (●). In the l-carnitine counterflow experiment, 106 cpm/mg corresponded to 16.7 nmol of l-carnitine/mg of total cell protein.

Subsequent to solubilization, CaiTFH was purified by Ni2+-chelate affinity chromatography as described under “Experimental Procedures.” SDS-PAGE analysis of the resulting peak fractions revealed a broad band with an apparent molecular mass of ~40 kDa (Fig. 3A). The protein of this band reacted with monoclonal antibodies directed against the His6 tag or the FLAG epitope (Fig. 3B). A relatively broad band was also observed for other transport proteins (e.g. LacY (31) and could reflect different stages of protein unfolding in SDS. N-terminal sequencing indicated a single protein species in the band with an amino acid sequence (MKNEKRKTGIEP) that was 100% identical to the sequence deduced from the caiT gene. The lower than predicted (56.6 kDa) apparent molecular mass of the protein was explained by the highly hydrophobic nature of CaiTFH. A minor band appearing at ~80 kDa corresponded to the CaiTFH dimer. As judged from silver-stained SDS gels, the obtained protein (monomer plus dimer) was a minimum of 95% pure (Fig. 3). Overall, starting from 90 mg of membrane protein, ~4 mg of purified CaiTFH were obtained.

For reconstitution, CaiTFH was incubated with preformed, detergent-denatured liposomes made of E. coli phospholipids, followed by stepwise addition of Bio-Beads SM-2 to remove detergent. Using a lipid/protein ratio of 100:1 (w/w), ~60% of solubilized CaiTFH was reconstituted into proteoliposomes (as judged from protein determinations).

Functional Properties of CaiTFH in Proteoliposomes—The function of isolated and reconstituted CaiTFH was probed by measuring l-[methyl-14C]carnitine uptake into proteoliposomes. A membrane potential (ΔΨ, interior negative) and desired ion gradients were imposed across the proteoliposome membrane by creating an outwardly directed K+ diffusion gradient in the presence of valinomycin and changing the ionic composition and/or pH of the dilution buffer. However, inwardly directed electrochemical H+ and Na+ gradients did not cause significant accumulation of l-[methyl-14C]carnitine in proteoliposomes (data not shown). In contrast, uptake of l-[methyl-14C]carnitine was observed upon preloading the proteoliposomes with unlabeled l-carnitine (l-carnitine counterflow) (Fig. 4). Labeling of CaiTFH with the sulphydryl reagent N-ethylnmaleimide inhibited this transport process as observed for intact cells. In addition, proteoliposomes preloaded with γ-carnitine, crotonobetaine, or γ-butyrobetaine were able to accumulate external l-[methyl-14C]carnitine, with initial rates similar to those observed with proteoliposomes preloaded with γ-carnitine. Glycine betaine, choline, and proline inside the proteoliposomes did not have a significant stimulatory effect on transport (Fig. 4).

In further studies, the temperature dependence of l-carnitine counterflow was analyzed. Plotting the data according to Arrhenius gave a straight line in the range between 13.5 and 38 °C (data not shown). The activation energy was determined as 51 kJ/mol, a value that is somewhat lower than the corresponding value of the mitochondrial carnitine/acylcarnitine carrier (133 kJ/mol) (39).

Kinetic Analysis of l-Carnitine Counterflow—For determination of kinetic parameters, proteoliposomes preloaded with 10 mM unlabeled l-carnitine were diluted into buffer containing various concentrations of l-[methyl-14C]carnitine. The filter assay was performed at a relatively low temperature (18 °C) to facilitate determination of initial rates of l-carnitine uptake particularly at l-carnitine concentrations approaching saturation. Plotting the initial rates of counterflow against the external concentration of l-[methyl-14C]carnitine resulted in a saturation curve (Fig. 5). Replotting according to Eadie-Hofstee yielded an apparent Km for l-carnitine of 105 ± 20 µM and a Vmax of 6058 ± 888 nmol/min/mg (Fig. 5, inset). Assuming that all the transporter molecules were reconstituted functionally, this reflected a turnover number of 5.5 s−1 at 18 °C.

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FIG. 4. L-Carnitine uptake into proteoliposomes preloaded with different trimethylammonium compounds. Proteoliposomes containing CaiTFH were resuspended in buffer B (1.22 mg of protein/ml) and equilibrated with the indicated trimethylammonium compound (10 mM) as described under “Experimental Procedures.” Aliquots (2 µl) of the proteoliposome suspension were diluted into 400 µl of resuspension buffer containing 4.7 µM [1-14C]carnitine (53 Ci/mol). L-[1-methyl-14C]Carnitine uptake was measured in unloaded proteoliposomes and in proteoliposomes preloaded with glycine betaine, choline, or proline (the results obtained with these unloaded and preloaded proteoliposomes are represented by a single curve (●)); in proteoliposomes preloaded with L-carnitine (counterflow) (○), D-carnitine (▲), crotonobetaine (□), or γ-butyrobetaine (◇); and in proteoliposomes preincubated with 2 mM N-ethylmaleimide for 10 min and subsequently preloaded with L-carnitine (◇). In the L-carnitine counterflow experiment, 10^6 cpm/mg corresponded to 83.5 nmol of L-carnitine/mg of protein.

FIG. 5. Kinetics of L-carnitine counterflow in proteoliposomes. CaiTFH-containing proteoliposomes were preloaded with 10 mM unla-beled L-carnitine by the freeze/thaw-extrusion method. Aliquots of the proteoliposome suspension (1–2 µg of protein) were diluted 200- or 400-fold into buffer B containing 1-[1-methyl-14C]carnitine at the indicated concentrations. After incubation at 18 °C for 5, 10, or 20 s, the transport assay was stopped and rapidly filtered. The data were fitted to the Michaelis-Menten equation and replotted according to Eadie-Hofstee ( inset).

CaiT shows similarities to transport proteins involved in osmoadaptation, the effect of medium osmolality on the function of CaiT was investigated. For this purpose, L-[1-methyl-14C]carnitine uptake into intact cells or proteoliposomes containing CaiT (or CaiTFH) was analyzed under various conditions in the presence and absence of 0.4 M NaCl or 0.6 M sucrose. Elevated salt or sucrose concentrations did not stimulate L-[1-methyl-14C]carnitine transport in the presence of an imposed electrochemical ion gradient (H⁺/Na⁺). Furthermore, increased medium osmolality only slightly stimulated L-carnitine counterflow (2.5-fold maximum stimulation) in intact cells as well as in proteoliposomes (data not shown).

Influence of L-carnitine on CaiTFH Fluorescence—To analyze L-carnitine binding to CaiTFH, a nonradioactive binding assay was developed. The assay utilized the effect of ligand on the tryptophan fluorescence of CaiTFH. Fig. 6 shows the emission spectrum (290-nm excitation wavelength) obtained with CaiTFH-containing proteoliposomes and reveals the spectral changes observed upon addition of L-carnitine. In the absence of substrate, the fluorescence spectrum was characterized by a maximum emission wavelength of 334 nm. Addition of 50 mM L-carnitine caused a substantial increase in the fluorescence measured between 310 and 370 nm. This increase was accompanied by a shift in the emission maximum by 4 nm toward longer wavelengths (Fig. 6). Recording the fluorescence intensity (F) at 338 nm in the presence and absence of 50 mM L-carnitine yielded changes in signal intensity (expressed as ΔF/F) of 25–30% depending on the preparation. Titrating the L-carnitine effect on CaiTFH fluorescence and plotting ΔF/F against the substrate concentration yielded a typical saturation curve (Fig. 7). The concentration of L-carnitine causing half-maximum stimulation of the fluorescence signal (K_0.50) at 338 nm was determined as 1.92 ± 0.44 mM. In addition to the analysis of CaiTFH in proteoliposomes, the fluorescence experiments were performed with protein solubilized with dodecyl β-d-maltoside. Addition of L-carnitine caused a change in the fluorescence emission spectrum similar to that observed for the reconstituted protein (data not shown). Titration of the L-carnitine effect yielded a K_0.50 of 3.82 ± 0.03 mM.
The results demonstrate that solubilized CaiTFH is able to bind substrate and that its apparent substrate affinity is slightly lower than the corresponding value for the reconstituted protein.

**Substrate Specificity of CaiTFH**—The binding assay described for l-carnitine was used to analyze the substrate specificity of CaiTFH. For this purpose, the effect of various compounds structurally related to l-carnitine on the fluorescence of reconstituted CaiTFH was measured. The experiments showed that besides l-carnitine, also the d-enantiomer as well as acetyl-l-carnitine, γ-butyrobetaine, and glycine betaine induced changes in CaiTFH fluorescence (increase in the fluorescence intensity and red shift in the emission maximum), thereby demonstrating that these compounds are able to interact with the protein. The apparent affinities of CaiTFH for d-carnitine and γ-butyrobetaine were ~3-fold lower than the corresponding value for l-carnitine, whereas acetyl-l-carnitine and glycine betaine showed only very weak binding to the protein (Table I). Choline, γ-amino-β-hydroxybutyrate, γ-aminobutyrate, and trimethylamine did not have a significant influence on CaiTFH fluorescence, suggesting that these compounds do not interact with the protein. A precise analysis of crotonobetaine binding could not be performed by the fluorescence assay because light absorption by the double bond of the potential ligand interfered with the measurements. Finally, proline caused a small increase in the fluorescence intensity (−9% at 338 nm), which was not accompanied by a red shift in the emission maximum. d-Carnitine and γ-butyrobetaine affected the fluorescence also of solubilized CaiTFH, for which the \( K_{0.5} \) values were 3–4-fold higher than for the reconstituted protein. In conclusion, the fluorescence studies suggest that a trimethylammonium group and a carboxylate are required for ligand binding to CaiTFH, for which the distance between the two charged groups is crucial for the affinity. Also proline appears to interact with the transporter, but the mechanism of interaction obviously differs from that of the betaines.

**DISCUSSION**

Based on hydropathy profile analyses and sequence comparisons, CaiT of *E. coli* has been suggested to function as a transport protein specific for l-carnitine (12). This study tests this assumption and provides the first experimental evidence on the function of the putative transporter. For this purpose, caiT was cloned and overexpressed in *E. coli*, and the protein was purified and reconstituted into proteoliposomes. Based on the strategy developed by Rigaud et al. for other membrane proteins (40, 41), the highest CaiT activities were obtained upon reconstitution of the protein into preformed liposomes titrated with Triton X-100 until the onset of liposome solubilization.

CaiT function was probed in intact cells and in the proteoliposome system. Although electrochemical H⁺ or Na⁺ gradients proved to be inefficient to drive l-carnitine uptake, cells or proteoliposomes preloaded with l-carnitine, d-carnitine, crotonobetaine, or γ-butyrobetaine showed high levels of exchange activity, which were not affected by ionophores. The apparent \( K_m \) and turnover number (105 μμm and 5.5 s⁻¹ of protein, respectively) for l-carnitine counterflow are in the same order of magnitude as the corresponding values for the non-homologous carnitine/acylcarnitine exchanger of rat liver mitochondria (39). These observations suggest that CaiT functions as a betaine exchanger (antiporter) rather than as an ion-coupled betaine uptake system. Taking into account that γ-butyrobetaine is the stable excreted end product of l-carnitine conversion in *E. coli*, it appears likely that CaiT works as a l-carnitine/γ-butyrobetaine antiporter under physiological conditions. This proposal is in line with increasing evidence showing that microorganisms that metabolize their substrates (precursors) only partially often link substrate uptake and product excretion in a single antiport mechanism (Fig. 8) (42). Crotonobetaine is not available as a substrate of CaiT because the intermediate occurs probably only in an activated form (coenzyme A ester) within the cell (15). In addition, the studies with purified CaiT demonstrate that the product of the caiT gene is sufficient to catalyze the observed transport processes.

The conclusion that CaiT functions as a betaine antiporter partly contradicts earlier studies suggesting that l-carnitine transport in *E. coli* is driven by an electrochemical ion (H⁺) gradient (11). However, it has to be noted that those studies were performed with a wild-type *E. coli* strain grown in the presence of l-carnitine to induce expression of the cai operon. As a consequence, the cells used for uptake measurements may have contained d-l-carnitine or its degradation products, thereby stimulating l-carnitine exchange without extra preloading of the cells. Furthermore, other transport systems capable of taking up l-carnitine (for example, ProP and ProU) were probably present and led to overlapping transport activities.

In contrast to the proposed function of CaiT, all other known members of the BCCT family are proposed to catalyze H⁺/(Na⁺)/solute symport processes (19, 20). However, as more and more primary structures of transporters become available, it appears impossible to infer information on the mechanism of energy coupling from sequence comparisons. For example, the galactoside-pentose-hexuronide family (Transport Protein Database TC 2.A.2)³ is a mixed family containing proteins capable of catalyzing symport and/or antiport processes (43).

Increasing medium osmolality did not stimulate ion-coupled l-carnitine uptake and had only a very little effect on CaiT-dependent exchange processes in intact cells as well as in proteoliposomes. In this way, CaiT clearly differs from other betaine

³Available at tcdb.ucsd.edu/tcdb/tcfamilybrowse.php?tcname=2.A.2.
A significant role in osmoregulation is consistent with the inability of the protein to mediate changes in substrate/product antiport according to the substrate/product antiport principle (Fig. 8). Such a mechanism minimizes the energy costs for transport, which is particularly important as the cells grow under anaerobic conditions without a functional respiratory chain.

The intrinsic fluorescent properties of CaiT were significantly altered upon addition of l-carnitine. The observed increase in the fluorescence intensity and the red shift in the fluorescence maximum arose most likely from conformational alterations induced by binding of the substrate to the transporter, as described, for example, for sugar-dependent changes in the tryptophan fluorescence of melibiose permease of *E. coli* (46–48). Furthermore, changes in protein fluorescence may be caused by direct interaction of the trimethylammonium group with the substrate/product antiport principle.

The fluorescence effect yielded a saturation curve with a half-maximum effect at an L-carnitine concentration \((K_{0.5})\) of 1.9 ± 0.44 mM for the proteoliposomes system. The latter value is only slightly lower than the value determined for solubilized CaiT, suggesting that solubilization has only a very little effect on CaiT structure. However, the \(K_{0.5}\) values for both compounds are only slightly increased compared with the l-carnitine value. Also acetylation of the hydroxyl group did not prevent binding. These results suggest that the hydroxyl group of L-carnitine does not play a pivotal role in the binding process and that CaiT cannot discriminate between the L- and D-enantiomers. This conclusion agrees with the observation that preloading of proteoliposomes with L-carnitine increased uptake rates without a functional respiratory chain. The distance between the trimethylammonium group and the carboxylate appears to be crucial for binding because glycine betaine has only a very low apparent affinity for the transporter.

Taken together, the results of this study suggest that CaiT functions as a transporter specific for betaine derivatives of 4-trimethylaminobutyrate. The observed high L-carnitine/L-carnitine, L-carnitine/D-carnitine, and L-carnitine/y-butyrobetaine exchange rates support the idea that CaiT functions as an L-carnitine/y-butyrobetaine exchanger (antiporter) according to the substrate/product antiport principle (Fig. 8). Such a mechanism minimizes the energy costs for transport, which is particularly important as the cells grow under anaerobic conditions without a functional respiratory chain.

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**TABLE I**

**Substrate specificity of CaiTFH**

Fluorescence was measured, and \(K_{0.5}\) values were determined as described in the legends to Figs. 6 and 7.

| Ligand                  | CaiTFH reconstituted into proteoliposomes | Solubilized CaiTFH |
|-------------------------|-------------------------------------------|---------------------|
| 1-Carnitine             | 1.92 ± 0.44                               | 3.85 ± 0.05         |
| D-Carnitine             | 5.80 ± 0.51                               | 27.6 ± 4.95         |
| y-Butyrobetaine        | 5.83 ± 0.62                               | 23.1 ± 3.65         |
| Acetyl-l-carnitine      | 30 ± 3                                    | ND                  |
| Glycine betaine        | >60                                       | ND                  |
| L-Proline              | (2.88 ± 0.24)\(^a\)                       | ND                  |
| Choline                | No change in fluorescence                  | ND                  |
| y-Amino-β-hydroxybutyrate | No change in fluorescence              | ND                  |
| y-Aminobutyrate        | No change in fluorescence                  | ND                  |
| Trimethylamine         | No change in fluorescence                  | ND                  |

\(^{a}\) Not determined.

\(^{b}\) Determination of \(K_{0.5}\) (L-Pro) was based on an L-proline-induced increase in the fluorescence intensity at 338 nm, which was not associated with a shift in the fluorescence maximum.

**Fig. 8.** Model of CaiT function in the L-carnitine metabolism of *E. coli*. Under anaerobic conditions, l-carnitine is reduced to y-butyrobetaine in a two-step pathway with crotonobetaine as intermediate (6, 7, 15–18). Recent studies have demonstrated that carnitine and its derivatives are activated during this process by reaction with coenzyme A (15). CaiT is proposed to function as an L-carnitine/y-butyrobetaine exchanger (antiporter) according to the substrate/product antiport principle.
