Involvement of Coenzyme A Esters and Two New Enzymes, an Enoyl-CoA Hydratase and a CoA-Transferase, in the Hydration of Crotonobetaine to L-Carnitine by *Escherichia coli*†

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ABSTRACT: Two proteins (CaiB and CaiD) were found to catalyze the reversible biotransformation of crotonobetaine to L-carnitine in *Escherichia coli* in the presence of a cosubstrate (e.g., γ-butyrobetainyl-CoA or crotonobetainyl-CoA). CaiB (45 kDa) and CaiD (27 kDa) were purified in two steps to electrophoretic homogeneity from overexpression strains. CaiB was identified as crotonobetainyl-CoA: carnitine CoA-transferase by MALDI-TOF mass spectrometry and enzymatic assays. The enzyme exhibits high cosubstrate specificity to CoA derivatives of trimethylammonium compounds. In particular, the N-terminus of CaiB shows significant identity with other CoA-transferases (e.g., FldA from *Clostridium sporogenes*, Frc from *Oxalobacter formigenes*, and BbsE from *Thauera aromatica*) and CoA-hydrolases (e.g., BaiF from *Eubacterium* sp.). CaiD was shown to be a crotonobetainyl-CoA hydratase using MALDI-TOF mass spectrometry and enzymatic assays. Besides crotonobetainyl-CoA CaiD is also able to hydrate crotonyl-CoA with a significantly lower V_{max} (factor of 10^3) but not crotonobetaine. The substrate specificity of CaiD and its homology to the crotonase confirm this enzyme as a new member of the crotonase superfamily. Concluding these results, it was verified that hydration of crotonobetaine to L-carnitine proceeds at the CoA level in two steps: the CaiD catalyzed hydration of crotonobetainyl-CoA to L-carnitinyl-CoA, followed by a CoA transfer from L-carnitinyl-CoA to crotonobetaine, catalyzed by CaiB. When γ-butyrobetainyl-CoA was used as a cosubstrate (CoA donor), the first reaction is the CoA transfer. The optimal ratios of CaiB and CaiD during this hydration reaction, determined to be 4:1 when crotonobetainyl-CoA was used as cosubstrate and 5:1 when γ-butyrobetainyl-CoA was used as cosubstrate, are different from that found for *in vivo* conditions (1:3).

Different genera of Enterobacteriaceae, such as *Escherichia*, *Proteus*, and *Salmonella*, are able to convert L-carnitine (3-hydroxy-4-*N*-trimethylaminobutyrate) via crotonobetaine (4-*N*-trimethylamino-2-butenoate) into γ-butyrobetaine (4-*N*-trimethylaminobutyrate) in the presence of nitrogen and carbon sources under anaerobic conditions (*1*). γ-Butyrobetaine is accumulated in the culture medium as the final product of this reaction sequence. Recent studies have shown that the metabolism of L-carnitine by some species, e.g., *Escherichia coli* ATCC 25922, *Proteus vulgaris*, and *Proteus mirabilis*, also occurs under aerobic conditions (*2*).

In *E. coli* an inducible carrier-mediated system is responsible for the uptake of L-carnitine (*3*). Two inducible enzymes, L-carnitine dehydratase and crotonobetaine reductase, have been postulated to catalyze the conversion of L-carnitine via crotonobetaine into γ-butyrobetaine (*4*, *5*). A carnitine racemase activity able to convert D-carnitine to L-carnitine was subsequently also described (*6*). L-Carnitine dehydratase and crotonobetaine reductase have been purified and characterized (*4*, *7*). Both enzymes require for its activity crotonobetainyl-CoA and/or γ-butyrobetainyl-CoA as cosubstrate (*8*). The L-carnitine dehydratase is composed of two identical subunits with a molecular mass of 45 kDa (*4*). *CaiB* was isolated by oligonucleotide screening from a genomic library of *E. coli* O44K74 and suggested to encode the L-carnitine dehydratase (*9*). CaiB and further proteins involved in carnitine metabolism in *E. coli* are encoded by the *caiTABCDE* operon (*10*). Functions have been assigned to each putative protein of the *cai* operon on the basis of amino acid sequence similarities and enzymatic analysis. CaiD was postulated to be involved in the racemization of D-carnitine. Previous studies have shown that for the transformation of crotonobetaine to γ-butyrobetaine two proteins, CaiA and CaiB, are necessary (*7*). Besides these enzymes a cosubstrate, either crotonobetainyl-CoA and/or γ-butyrobetainyl-CoA, is essential for the reduction reaction of crotonobetaine to γ-butyrobetaine (*7*, *8*). CaiA is composed of four identical subunits with a molecular mass of 41.5 kDa (*7*). Cross-linking studies have shown that in the crotonobetaine reductase reaction one dimer of CaiB associates with one tetramer of CaiA in the presence of either crotonobetainyl-CoA and/or γ-butyrobetainyl-CoA. Recently, it was shown that the biotransformation of crotonobetaine to L-carnitine in *Proteus* sp. is also catalyzed by two enzymes

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(*11*). Both proteins involved in this hydration reaction showed amino acid sequence identities with CaiD and CaiB from *E. coli*.

In this study the mechanism of the biotransformation of crotonobetaine to L-carnitine in *E. coli* is described in detail. Furthermore, we disclose new enzymatic functions of CaiB and CaiD.

EXPERIMENTAL PROCEDURES

Chemicals. L(−)-Carnitine and crotonobetaine (inner salt) were gifts from Sigma Tau, Rome, Italy. Crotonobetaine hydrochloride and γ-butyrobetaine hydrochloride were gifts from Lonza, Basel, Switzerland. Carnitine acetyltransferase was purchased from Boehringer Mannheim, Germany. Fractogel EMD-DEAE 650 (S) was purchased from Merck, Darmstadt, Germany.

DEAE-Sepharose FF, Resource Q, and Superose 12 HR were obtained from Pharmacia, Uppsala, Sweden. Crotonyl-CoA and other CoA derivatives (with the exception of crotonobetainyl-CoA and γ-butyrobetainyl-CoA) were purchased from Sigma-Aldrich, Deisenhofen, Germany. Standard proteins for size exclusion chromatography and for sodium dodecyl sulfate (SDS)[1] gel electrophoresis were obtained from Merck, Darmstadt, Germany and Boehringer, Mannheim, Germany, respectively. All other chemicals were of analytical grade.

Strains, Plasmids, and Cultivation Conditions. E. coli O44K74 (wild type) was cultivated anaerobically at 37 °C in complex medium (17 g of pancreatic peptone, 3 g of yeast extract, 5 g of NaCl, 2 g of fumarate, and 10 mL of glycerol per liter of deionized water, pH 7.0) containing 0.13% (w/v) L-carnitine and harvested as described previously (*8*). *Enterobacter cloacae* was also cultivated under anaerobic conditions at 37 °C in complex medium containing 0.5% L-carnitine and harvested in the same manner. For overexpression of *caiB* from *E. coli* O44K74 a pT7-6 plasmid carrying the *caiB* gene was transformed into *E. coli* BL21 (DE3). For overexpression of *caiD* from *E. coli* O44K74 a pT7-5 plasmid carrying the *caiD* gene was used and also transformed into *E. coli* BL21 (DE3). Bacteria were cultivated under anaerobic conditions at 37 °C in L-broth (10 g of bacto tryptone, 5 g of yeast extract, and 5 g of NaCl per liter of deionized water, pH 7.5) containing ampicillin (100 mg L^{-1}). Cells were induced by addition of 0.4 mM IPTG[1] to the culture medium at the beginning of anaerobic cultivation.

The following operations were carried out at 0−4 °C. After 10 h incubation (OD_{600} 0.4−0.5), cells were harvested by centrifugation (5000*g*, 15 min) and washed twice with Na^+,K^+-phosphate buffer (67 mM, pH 7.5). Cells were disrupted by grinding with alcoa, and protein was extracted with K^+-phosphate buffer (10 mM, pH 7.5). Cell-free extracts were obtained by centrifugation at 15000*g* for 45 min at 4 °C.

Enzyme Assays and Definition of Enzyme Activities. L-Carnitine dehydratase was assayed according to Jung et al. (*4*).

Activity of the crotonobetaine hydrating system was assayed in a reaction mixture (200 μL) containing K^+-phosphate buffer (10 mM, pH 7.5), crotonobetaine (0.1 M), an appropriate amount of crotonobetainyl-CoA or γ-butyrobetainyl-CoA, and appropriate amounts of CaiB and CaiD. After 5 min incubation at 37 °C the reaction was stopped by addition of trichloroacetic acid [15% (w/v)]. The supernatant was subsequently neutralized with KOH. Formation of L-carnitine was determined using carnitine acetyltransferase (*12*). Enzyme activity was defined as micromoles of L-carnitine formation per minute per milligram of protein (units/mg).

The activitities of purified or partially purified CaiB and CaiD were determined from the transformation rate of crotonobetaine into L-carnitine. Each enzyme (CaiB or CaiD) is therefore saturated with the corresponding other enzyme (CaiD or CaiB) and either crotonobetainyl-CoA or γ-butyrobetainyl-CoA in the incubation mixture.

Enoyl-CoA hydratase activity of CaiD was determined spectrophotometrically by measuring the decrease in absorbance of crotonobetainyl-CoA ($\epsilon = 22.5$ l $mmol^{-1}$ cm^{-1}) at 220 nm and the decrease in absorbance of crotonyl-CoA ($\epsilon = 3.6$ l $mmol^{-1}$ cm^{-1}) at 280 nm. Specific activity was defined as micromoles of crotonobetainyl-CoA and crotonyl-CoA hydration, respectively, and decrease of absorbance at 220 and 280 nm, respectively, per minute per milligram of protein (units/mg).

Protein concentration was determined according to Bradford (*13*) using bovine serum albumin as standard. During purification, proteins were determined by measuring the absorption at 280 nm.

Synthesis and Purification of Crotonobetainyl-CoA and γ-Butyrobetainyl-CoA. Crotonobetainyl-CoA and γ-butyrobetainyl-CoA were synthesized and purified as described by Elssner et al. (*8*). *trans*-Crotonobetaine hydrochloride and γ-butyrobetaine hydrochloride were activated to the corresponding acid chlorides with phosphorus trichloride and converted to crotonobetainyl-CoA and γ-butyrobetainyl-CoA, respectively, by addition of CoA in sodium bicarbonate. Nonreacted CoA and crotonobetaine or γ-butyrobetaine, respectively, were separated from the corresponding CoA esters by chromatography on Dowex 50 WX8 (*8*).

Enrichment of L-Carnitine Dehydratase from E. coli O44K74. L-Carnitine dehydratase was enriched in a four-step procedure (EMD-TMAE, TSK-Butyl, EMD-DEAE, Superose 12 HR) as described by Preusser et al. (*7*). Ion-exchange chromatography on EMD-DEAE was carried out on an alternative chromatography system (ÄKTA purifier 100, Pharmacia, Umeå, Sweden).

Purification of CaiB from E. coli BL21 (DE3). The cell-free extract from *E. coli* BL21 (DE3), containing overexpressed *caiB*, was loaded onto a DEAE-Sepharose FF column (16 by 200 mm) previously equilibrated with K^+-phosphate buffer (10 mM, pH 7.4). The flow rate was 0.8 mL min^{-1}. The column was washed with the same buffer until no protein could be detected. CaiB was eluted using a linear gradient (6 column volumes) between 10 and 200 mM K^+-phosphate buffer (pH 7.4). Active fractions were concentrated and desalted by ultrafiltration (membrane YM 30, Amicon, Danvers, MA). Concentrated CaiB was applied to a Resource Q column (16 by 30 mm) that had been equilibrated with Na^+,K^+-phosphate buffer (80 mM, pH 7.5). After the column

[1] Abbreviations: ABTS, 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt; ELISA, enzyme-linked immunosorbent assays; HPLC, high-performance liquid chromatography; IPTG, isopropyl β-D-thiogalactoside; mabs, monoclonal antibodies; MALDI-TOF, matrix-assisted laser desorption/ionization time of flight; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate.

was washed with the same buffer, CaiB was eluted using a linear gradient (12 column volumes) between 80 and 200 mM Na^+,K^+-phosphate buffer (pH 7.5). The flow rate was 1.5 mL min^{-1}.

Purification of CaiD from E. coli BL21 (DE3). The cell-free extract from *E. coli* BL21 (DE3) containing overexpressed *caiD* was loaded onto a EMD-DEAE column (16 by 100 mm) previously equilibrated with K^+-phosphate buffer (10 mM, pH 7.5). The flow rate was 0.8 mL min^{-1}. The column was washed with K^+-phosphate buffer (50 mM, pH 7.5). CaiD was eluted using a linear gradient (12 column volumes) between 50 and 200 mM K^+-phosphate buffer (pH 7.5). Active fractions were concentrated and desalted by ultrafiltration (membrane YM 10, Amicon). Concentrated CaiD was applied to a Resource Q column (16 by 30 mm) that had been equilibrated with K^+-phosphate buffer (10 mM, pH 7.5). After the column was washed with the same buffer, CaiD was eluted using a linear gradient (42 column volumes) between 10 and 200 mM K^+-phosphate buffer (pH 7.5). The flow rate was 1 mL min^{-1}.

Purification of CaiB and CaiD from Proteus sp. The purification of these proteins (CaiB *Proteus* and CaiD *Proteus*) to electrophoretic homogeneity has been described previously (*11*).

Determination of the N-Terminal Amino Acid Sequence. The second component of L-carnitine dehydratase was separated into subunits by SDS gel electrophoresis and blotted to a poly(vinylidene difluoride) membrane (Bio-Rad, Hercules, CA). Amino-terminal sequencing was performed with a sequencer (473A, Applied Biosystems, Foster City, CA) by the Edman method. Phenylthiohydantoin amino acids were analyzed by HPLC[1] with a reversed-phase column (*14*).

Purification Control and Estimation of Molecular Mass. Gel electrophoresis was performed as described by Sambrook et al. (*15*) in 10% or 12% polyacrylamide gels containing 0.1% SDS using a discontinuous buffer system. The SDS gels were silver stained and used to determine the molecular mass of subunits as well as to screen for purity (*16*).

The apparent molecular mass of the native CaiD was estimated by size exclusion chromatography on two combined Superose 12 HR columns (10 by 300 mm) equilibrated with K^+-phosphate buffer (50 mM, pH 7.5) containing 150 mM NaCl. The flow rate was 0.3 mL min^{-1}.

Monoclonal Antibodies. Monoclonal antibodies (mabs)[1] against CaiB were obtained according to Preusser et al. (*7*). Production of mabs against CaiD was performed as described for CaiB (*7*). Female mice were used for generation of hybridomas. Hybridoma supernatants were assayed for specific mabs using enzyme-linked immunosorbent assays (ELISA)[1] according to Preusser et al. (*7*).

Determination of IgG Concentration by ELISA. The IgG concentration of mab-containing hybridoma supernatants was determined by ELISA based on the sandwich principle. ELISA plates (Greiner, Frickenhausen, Germany) were coated overnight at 4 °C with 50 μL of goat anti-mouse IgG (5 μg mL^{-1} PBS; Jackson ImmunoResearch Laboratory, West Grove, PA). After the coating solution was discarded, the wells were washed three times with washing buffer (PBS/0.1% Tween 20). Binding of IgG was performed by incubation of 50 μL of mab-containing hybridoma supernatants diluted 5-fold with PBS/0.1% Tween or 50 μL serial dilutions of mouse IgG1 standard (0.1 mg mL^{-1}, Southern Biotechnology Associates, Inc., Birmingham, Great Britain) for 1 h at 4 °C. Subsequently, the wells were washed three times. Bound mabs were detected by peroxidase-conjugated goat anti-mouse IgG (1:1000 diluted in PBS/0.1% Tween 20, 1 h, 4 °C; Dianova, Hamburg, Germany). After extensive washing, a total volume of 50 μL of 1 mM ABTS[1]/0.1 M citrate buffer, pH 4.35, containing 0.02% H_2O_2 was added to each well for determining the residual peroxidase activity as evidence of bound mabs. After incubation at room temperature for 30 min, the OD was measured at 405 nm on a microplate reader (Emax precision microplate reader, Molecular Devices Corp., Sunnyvale, CA). The IgG concentration was calculated using a standard curve of mouse IgG1.

Competitive ELISA. To determine the optimum detection range for the competitive ELISA, an indirect antigen-binding assay was performed. ELISA plates were coated with purified CaiB or CaiD (5 μg mL^{-1} in PBS, 50 μL per well) overnight at 4 °C. After three washing steps with PBS/0.1% Tween 20, the wells were incubated with mab-containing hybridoma supernatants in various concentrations (50 μL per well) at 4 °C for 2 h. Protein-recognizing mabs were detected by peroxidase-conjugated goat anti-mouse IgG as described above.

For competitive ELISA, the plates were coated with the appropriate solutions of CaiB or CaiD (5 μg mL^{-1} in PBS, 50 μL per well). Additionally, different concentrations of purified CaiB or CaiD or cell-free extracts of Enterobacteriaceae were mixed with the hybridoma supernatants in a 1:1 ratio. The mixtures were added to the coated ELISA plates and incubated at 4 °C for 4 h. Using a modification of this method to increase sensitivity, the mixtures were preincubated at 4 °C for 24 h, followed by incubation of the plate at 4 °C for 2 h. After washing, visualization was performed as described above.

MALDI-TOF[1] Mass Spectrometry. The MALDI matrix, 2-cyano-4-hydroxycinnamic acid, was prepared by dissolving 5 mg in 0.5 mL of solution containing water/3% trifluoroacetic acid/acetonitrile (4:1:5) solution. One microliter of sample and 1 μL of matrix were spotted into the wells of the MALDI plate and air-dried. Molecular mass measurements were performed using a Voyager DE-RP mass spectrometer provided by PerSeptive Biosystems (Framingham, MA). A 20 kV acceleration voltage was applied, and 30−100 shots from the nitrogen laser (337 nm) were summed for each mass spectrum. Data were calibrated with an internal/external calibration with the molecular ion of the matrix and its dimer (190.05 and 379.093 Da, respectively) using the GRAMS 386 (Galactic, Salem, NH) software.

RESULTS

Two Proteins Catalyze the Biotransformation of Crotonobetaine to L-Carnitine by E. coli. To study the mechanism of the reversible transformation of crotonobetaine into L-carnitine, homogeneous L-carnitine dehydratase was required. Purification of L-carnitine dehydratase from *E. coli* O44K74 according to Preusser et al. (*7*) using an alternative chromatography system led to the surprising observation that a second protein besides CaiB is necessary for the conversion of crotonobetaine to L-carnitine. The N-terminal amino acid sequence (Table 1) determined from the separated second protein (27 kDa) was shown to be identical to that of the *caiD* gene product (*10*).

Table 1: N-Terminal Amino Acid Sequence of the 27 kDa Protein Essential for Transformation of Crotonobetaine into L-Carnitine in Comparison with CaiD from *E. coli* (*10*)

	1				5					10
27 kDa protein	Ser	Glu	Ser	Leu	His	–	Thr	–	Asn	Gly
CaiD	Ser	Glu	Ser	Leu	His	Leu	Thr	Arg	Asn	Gly

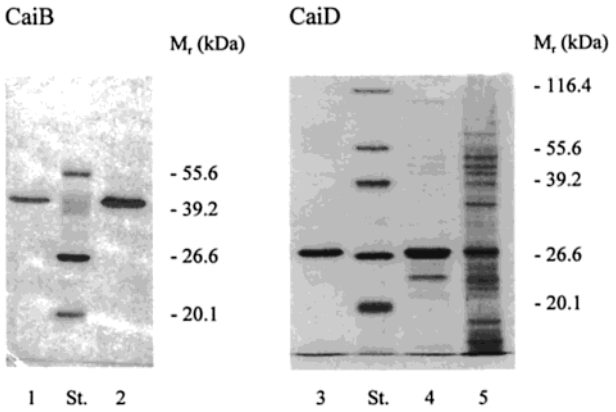


FIGURE 1: SDS gel electrophoresis of purified CaiB and CaiD from *E. coli*. Lanes: 1, CaiB (0.3 μg); 2, CaiB (0.5 μg); 3, CaiD (0.3 μg); 4, CaiD purified by chromatography on Fractogel EMD-DEAE (1 μg); 5, cell-free extract from *E. coli* BL21 (DE3) containing overexpressed CaiD (1.5 μg); St., protein standard as indicated.

Table 2: Purification of CaiB and CaiD from *E. coli* BL21 (DE3)

enzyme	purification step	total protein (mg)	total act. (units)	sp act. (units/mg)	purification (*x*-fold)	yield (%)
CaiB	cell-free extract	101.4	1688	16.6	1	100
	DEAE-Sepharose	17.5	1312	75.0	4.5	78
	Resource Q	5.7	1074	187.8	11.3	64
CaiD	cell-free extract	205.1	12364	60.3	1	100
	EMD-DEAE	12.5	5950	476.5	7.9	48
	Resource Q	4.1	2839	699.9	11.6	23

To characterize in more detail the functions of CaiB and CaiD in the transformation of crotonobetaine to L-carnitine, CaiB and CaiD were purified from overexpression strains. *E. coli* BL21 (DE3) was used, transformed with plasmids containing either *caiB* or *caiD*, because expression of the chromosomal *cai* operon is strongly reduced in this strain. CaiB was purified to electrophoretic homogeneity (Figure 1) from the overexpression strain by anion-exchange chromatography on DEAE-Sepharose FF and Resource Q. The protein was enriched about 11-fold, resulting in a specific activity of 188 units/mg and a yield of 64% (Table 2). CaiD was also purified from an overexpression strain to homogeneity (Figure 1) by anion-exchange chromatography on EMD-DEAE 650 (S) and Resource Q. CaiD was enriched about 12-fold with an overall yield of 23% of the original activity (Table 2). The native relative molecular mass of CaiD was estimated to be 87 kDa as judged by gel filtration on Superose 12 HR.

Reconstitution studies were carried out after purification of CaiB and CaiD. As shown in Table 3, L-carnitine formation from crotonobetaine was only observed in the presence of CaiB and CaiD plus the cosubstrate crotonobetainyl-CoA. CaiB or CaiD alone or in combination is unable to hydrate crotonobetaine. Crotonobetainyl-CoA is replaceable by γ-butyrobetainyl-CoA. Both compounds are almost equally effective. Crotonobetaine hydrating activity could also be reconstituted if CaiB and CaiD from *E. coli* were respectively replaced with CaiB and CaiD from *Proteus* sp.

Table 3: Formation of L-Carnitine from Crotonobetaine after Reconstitution of the Crotonobetaine Hydrating System by Combining CaiB and CaiD from *E. coli* and by Replacing CaiB and CaiD from *E. coli*, Respectively, with CaiB *Proteus* and CaiD *Proteus*

E. coli		*Proteus* sp.			
CaiB (μg)	CaiD (μg)	CaiB (μg)	CaiD (μg)	crotonobetainyl-CoA (nmol)	formation of L-carnitine (nmol)
2	2	–	–	10	101
2	–	–	–	10	0[a]
–	2	–	–	10	0
2	2	–	–	–	0
–	–	2	2	10	113
–	–	2	–	10	0
–	–	–	2	10	0
–	–	2	2	–	0
2	–	–	2	10	107
–	2	2	–	10	116

[a] The detection limit is $\geq$0.5 nmol.

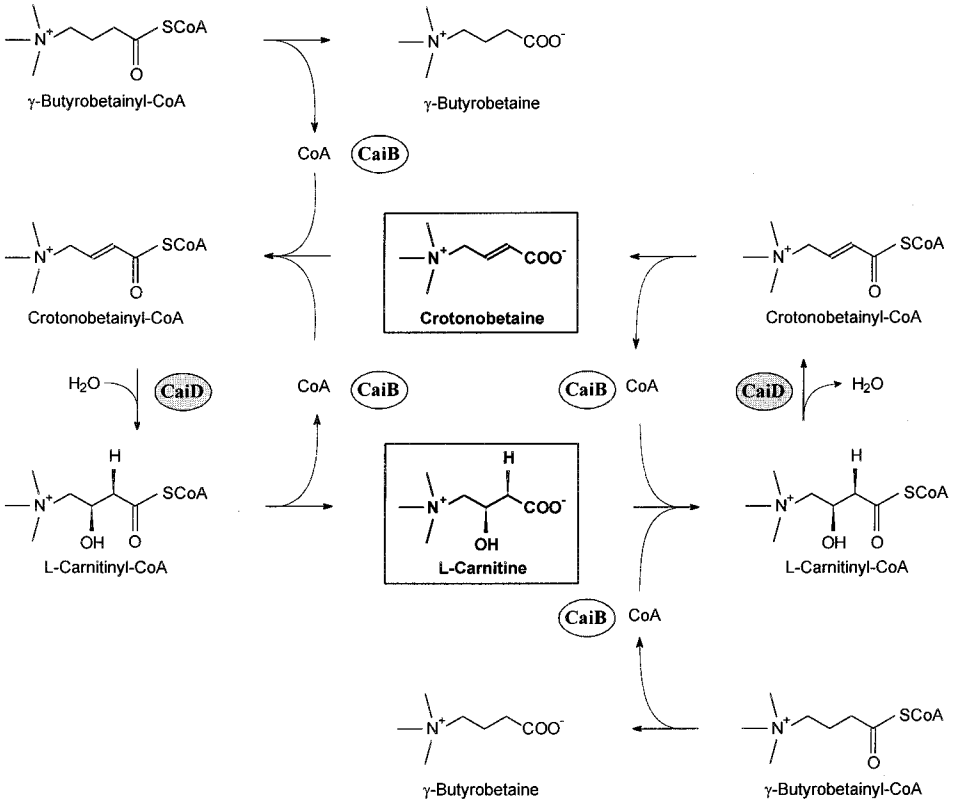
Enzymatic Function of CaiD. On the basis of the amino acid sequence homology (30.2%) observed between CaiD and enoyl-CoA hydratase from *Rattus norvegicus* (*10*), an enoyl-CoA hydratase activity for CaiD was postulated. Therefore, CaiD should hydrate crotonobetainyl-CoA to carnitinyl-CoA (Scheme 1). Crotonobetainyl-CoA has a molecular mass of 893 Da (*8*). The calculated molecular mass for carnitinyl-CoA is 911 Da. Indeed, formation of carnitinyl-CoA could be detected by means of MALDI-TOF mass spectrometry after incubation of 0.1 μg of homogeneous CaiD with 55 nmol of crotonobetainyl-CoA (Figure 2). To establish whether L-carnitinyl-CoA or D-carnitinyl-CoA was formed, carnitinyl-CoA was hydrolyzed with NaOH (end concentration 1 M) and subsequently neutralized with HCl, and the released carnitine was detected using carnitine acetyltransferase. This enzyme is specific for L-carnitine and does not accept L-carnitinyl-CoA as substrate. Until now, no sensitive method exists for detection of such small amounts of D-carnitine. Starting with 58.1 nmol of crotonobetainyl-CoA, a maximum of 37.1 nmol of L-carnitinyl-CoA could be formed (Figure 3). On condition that no D-carnitinyl-CoA is formed, the reaction equilibrium constant, [L-carnitinyl-CoA]/[crotonobetainyl-CoA], is 1.8. The conversion of crotonobetainyl-CoA into carnitinyl-CoA in the reverse reaction could not be investigated, as carnitinyl-CoA is not available in pure form.

Enzymatic activity of homogeneous CaiD can be measured directly with crotonobetainyl-CoA as the substrate by following the decrease in absorbance of crotonobetainyl-CoA at 220 nm, which should be absent in carnitinyl-CoA. The K_m value of CaiD for crotonobetainyl-CoA was determined to be 12 μM using Lineweaver–Burk and Michaelis–Menten plots. V_{max} for the enzyme is 186 μmol min^{-1} (mg of protein)$^{-1}$.

CaiD is also able to hydrate crotonyl-CoA. Formation of hydroxybutyryl-CoA could be observed by means of MALDI-TOF mass spectrometry (spectrum not shown). The K_m value of CaiD for crotonyl-CoA was determined to be 38 μM, and V_{max} for the enzyme is 0.3 μmol min^{-1} (mg of protein)$^{-1}$.

Enzymatic Function of CaiB. The ability of CaiD to hydrate crotonobetainyl-CoA to L-carnitinyl-CoA led us

Scheme 1: Mechanism for Transformation of Crotonobetaine into L-Carnitine Catalyzed by Two Enzymes, Crotonobetainyl-CoA Hydratase (CaiD) and Carnitine CoA-Transferase (CaiB)



suspect that CaiB must be a CoA-transferase. CaiB should then catalyze the transfer of the CoA moiety between γ-butyrobetainyl-CoA/crotonobetainyl-CoA/L-carnitinyl-CoA and γ-butyrobetaine/crotonobetaine/L-carnitine, respectively (Scheme 1). To verify this hypothesis, homogeneous CaiB was incubated with L-carnitine and either crotonobetainyl-CoA or γ-butyrobetainyl-CoA, respectively. By means of MALDI-TOF mass spectrometry, the formation of a compound with a molecular mass of 911 (Figure 4) was shown, which is identical to carnitinyl-CoA. Formation of carnitinyl-CoA could only be observed in the presence of all three components (CaiB, L-carnitine, and crotonobetainyl-CoA or γ-butyrobetainyl-CoA). The time course of the decrease in L-carnitine is shown in Figure 5. The decrease in L-carnitine was only linear during approximately 20 s when crotonobetainyl-CoA was the cosubstrate and 30 s with γ-butyrobetainyl-CoA as the cosubstrate. Determination of the decrease in L-carnitine after 10 and 15 s led to nonreproducible results. Hydrolysis of the incubation mixture containing CaiB, γ-butyrobetainyl-CoA, L-carnitine, and L-carnitinyl-CoA after the reaction confirmed that L-carnitinyl-CoA was indeed produced in the reaction catalyzed by CaiB. The determined amount of L-carnitine released was identical with the starting concentration. No decrease in

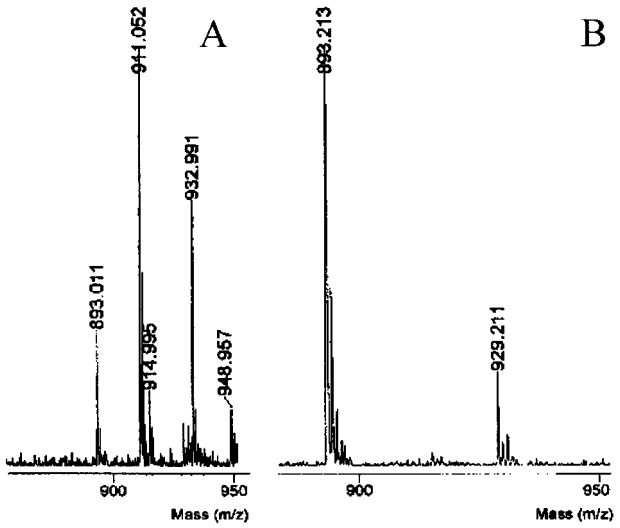


FIGURE 2: Detection of carnitinyl-CoA (*m*/*z* 911) in the hydration reaction catalyzed by CaiD from *E. coli* using MALDI-TOF mass spectrometry. (A) 55 nmol of crotonobetainyl-CoA (*m*/*z* 893) was incubated together with 0.1 μg of homogeneous CaiD at 37 °C and pH 7.5 for 5 min. (B) The reaction mixture before incubation.

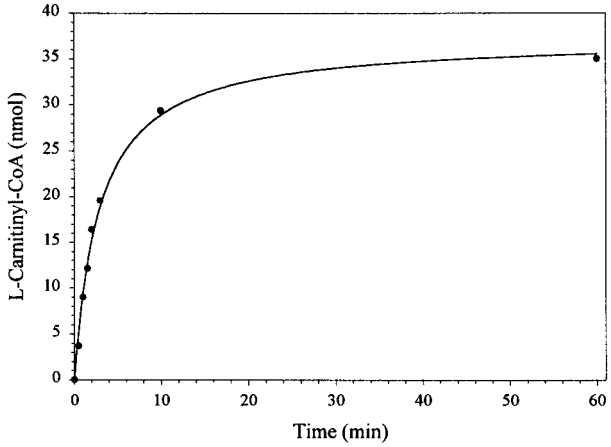


FIGURE 3: Time course of L-carnitinyl-CoA formation from crotonobetainyl-CoA in the hydration reaction catalyzed by CaiD from *E. coli*. Crotonobetainyl-CoA (58.1 nmol) was incubated together with CaiD (0.01 μg) at 37 °C and pH 7.5.

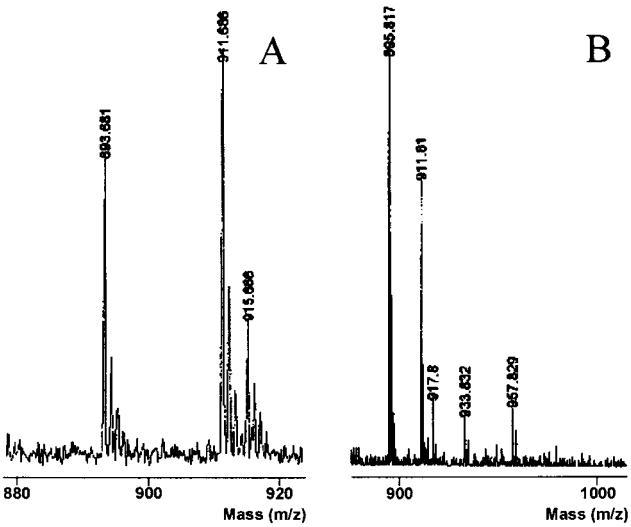


FIGURE 4: Detection of carnitinyl-CoA (*m*/*z* 911) in the transferase reaction catalyzed by CaiB from *E. coli* using MALDI-TOF mass spectrometry. (A) 60 nmol of crotonobetainyl-CoA (*m*/*z* 893) or (B) 60 nmol of γ-butyrobetainyl-CoA (*m*/*z* 895) was incubated together with 120 nmol of L-carnitine and 1 μg of homogeneous CaiB at 37 °C and pH 7.5 for 5 min.

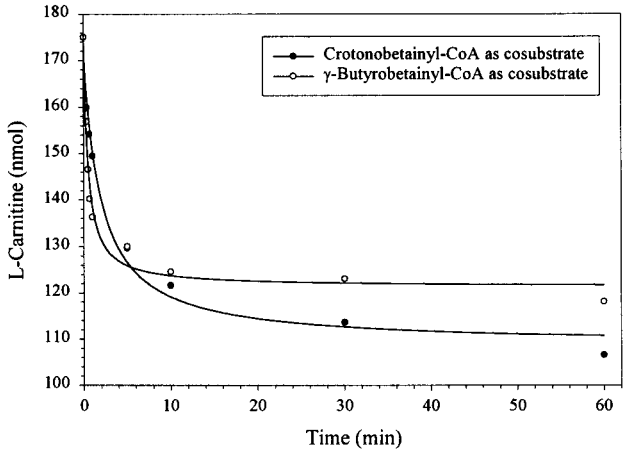


FIGURE 5: Time course of L-carnitine decrease in the transferase reaction catalyzed by CaiB from *E. coli*. Cosubstrates crotonobetainyl-CoA (91.5 nmol) or γ-butyrobetainyl-CoA (91.5 nmol) were incubated with CaiB (0.1 μg) and L-carnitine (175 nmol) at 37 °C and pH 7.5.

L-carnitine was observed in the reaction when the cosubstrate (crotonobetainyl-CoA or γ-butyrobetainyl-CoA) was substituted by CoA or other CoA derivatives (e.g., acetyl-CoA, acetoacetyl-CoA, crotonyl-CoA, DL-β-hydroxybutyryl-CoA, palmitoyl-CoA, and succinyl-CoA).

Monoclonal Antibodies against CaiB and CaiD from E. coli. One hybridoma cell line was established producing mabs against CaiD. The specificity of the mabs to CaiD used for immunization was tested by ELISA and immunoblotting experiments. Anti-CaiD-mab 11F2 was selected for immunological enzyme analysis on the basis of its pattern and intensity of reaction. As shown by ELISA (Table 4), mab 11F2 binds specifically to the corresponding enzyme antigen (CaiD). By Western blotting, mab 11F2 was found to recognize the 27 kDa subunit of purified CaiD, while no reaction against CaiB was observed. The specificity of the mab 13C7 (anti-CaiB-mab) (*7*) was tested against CaiD by immunoblotting experiments (Table 4). Anti-CaiB bound specifically to the corresponding enzyme antigen.

Table 4: Antigen Specificity of Monoclonal Antibodies Analyzed by ELISA and Immunoblotting Experiments

	antigen specificity			
	ELISA[a]		Western blotting	
mab	CaiD	CaiB	CaiD	CaiB
anti-CaiD (11F2)	1.8	−	+	−
anti-CaiB (13C7)	−	1.6	−	+

[a] Antibody reactivities are expressed as absorbance values at 405 nm.

Optimum Ratio of CaiB and CaiD from E. coli for the Hydration of Crotonobetaine to L-*Carnitine*. For determination of the optimum ratio CaiD purified to electrophoretic homogeneity was gradually saturated with purified CaiB in the presence of an excess of either crotonobetainyl-CoA or γ-butyrobetainyl-CoA. For crotonobetainyl-CoA as cosubstrate the optimum ratio between CaiB and CaiD was 4:1; for γ-butyrobetainyl-CoA the optimum was 5:1.

Ratio of CaiB and CaiD in Cell-Free Extracts. For evidence of the cellular ratios of CaiB and CaiD the concentrations of both enzymes were determined in cell-free extracts of *E. coli* using anti-CaiB-mab and anti-CaiD-mab in the competitive ELISA. Both mabs were confirmed to bind specifically to the corresponding enzyme antigen when employed in solution. To determine an optimum detection range for the mab-containing hybridoma supernatants for the competitive ELISA, an indirect antigen-binding assay was performed. For anti-CaiB-mab an optimum IgG concentration of 110 ng mL^{-1} was established and for anti-CaiD-mab 270 ng mL^{-1}. These concentrations were used in further experiments. For studies on the concentration of CaiB and CaiD in cell-free extracts, *E. coli* O44K74 was cultivated on complex medium in the presence or absence of the inducer L-carnitine. Figure 6 shows the competition of mab binding (anti-CaiB-mab and anti-CaiD-mab) to the corresponding antigen (CaiB and CaiD) adsorbed to the solid phase by cell-free extracts containing CaiB and CaiD, respectively. CaiB and CaiD purified to homogeneity were used for the standard curve. Cell-free extracts of *E. cloacae* were used as a negative control because *E. cloacae* is unable to convert L-carnitine, via crotonobetaine, to γ-butyrobetaine in the presence of carbon and nitrogen sources under aerobic and anaerobic conditions (*1*, *2*). The concentrations of CaiB and CaiD in cell-free extracts were determined from the linear part of the competition curves using the standard curves for CaiB and CaiD, respectively (panels A and B of Figure 6). As expected, CaiB and CaiD were not detectable in cell-free extracts of *E. cloacae*. Table 5 summarizes the calculated total amounts of CaiB and CaiD in cell-free extracts of *E. coli* O44K74. CaiD could only be detected in cell-free extracts of *E. coli* O44K74 grown in the presence of L-carnitine, while CaiB was also detectable in bacteria grown without L-carnitine, although only in very small amounts (3% in comparison with induced cells). The ratio between CaiB and CaiD was calculated to be 1:3.

DISCUSSION

Studies on the mechanism of the transformation of crotonobetaine to L-carnitine in *E. coli* confirmed that this reaction proceeds at the CoA level. In accordance with the

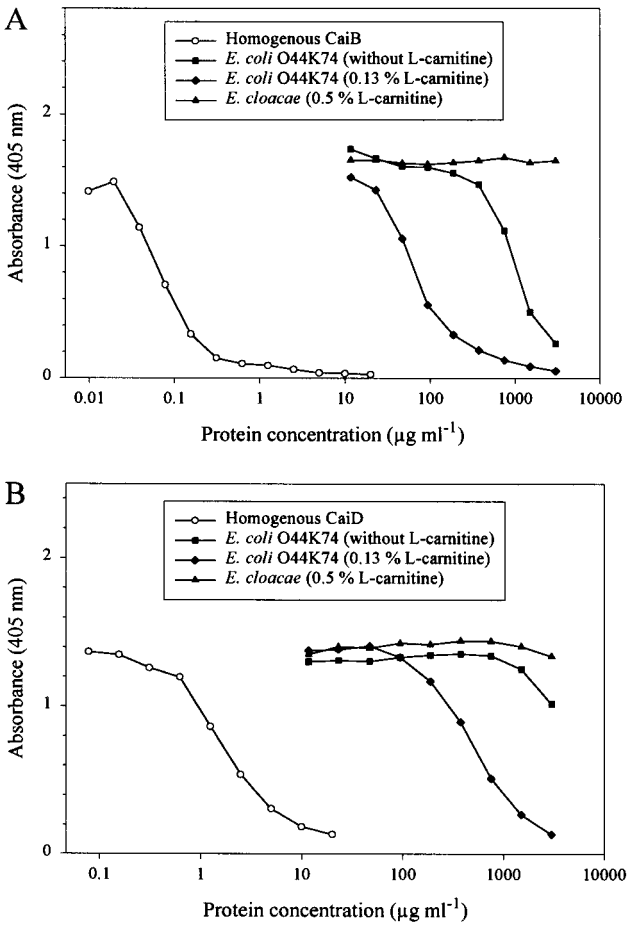


FIGURE 6: Competitive ELISA with cell-free extracts of *E. coli* O44K74 cultivated in the presence or absence of L-carnitine using anti-CaiB-mab (A) and anti-CaiD-mab (B).

Table 5: Determination of the Ratio of CaiB and CaiD in Cell-Free Extracts of *E. coli* O44K74 Grown on Complex Medium under Anaerobic Conditions in the Presence (0.13%) or Absence of L-Carnitine

inducer	total protein (mg)	total amount of CaiB (μg)	total amount of CaiD (μg)	% of total protein CaiB	% of total protein CaiD	ratio CaiB/CaiD
−	64.4	3.8	−	0.006	−	−
L-carnitine	105.6	117.4	351.2	0.11	0.33	1:3

results obtained in *Proteus* sp. (*11*), the reaction is also catalyzed by two enzymes, CaiB and CaiD in *E. coli*. Until now, CaiB was assumed to be the "L-carnitine dehydratase" (*9*). Consequently, this study has shown that the enzyme L-carnitine dehydratase does not exist. Former preparations of L-carnitine dehydratase (*4*, *7*) must also have contained very small amounts CaiD besides CaiB. Eichler et al. (*10*) observed a significant augmentation of L-carnitine dehydratase activity when *caiB* was overexpressed in *E. coli* K38, most probably because CaiD was not saturated with CaiB in the cell-free extracts. We found that the ratio of CaiB and CaiD in cell-free extracts of *E. coli* O44K74 is 1:3. However, the hydration of crotonobetaine to L-carnitine proceeds optimally when CaiB and CaiD were combined in a ratio of 4:1 together with crotonobetainyl-CoA or, 5:1 if γ-butyrobetainyl-CoA was used as the cosubstrate.

Reconstitution studies of CaiB and CaiD from *E. coli* demonstrated that both are functionally replaceable by the corresponding equivalent proteins from *Proteus* sp. These results suggest that CaiB and CaiD from *Proteus* sp. are analogues to CaiB and CaiD in *E. coli*.

Eichler et al. (*10*) reported about 30% sequence identity between CaiD and the enoyl-CoA hydratase of *R. norvegicus*. The previous identification of crotonobetainyl-CoA as a cosubstrate involved in carnitine metabolism (*8*), and the demonstrated involvement of CaiD besides CaiB in the hydration of crotonobetaine to L-carnitine have led to the conclusion that CaiD possesses enoyl-CoA hydratase activity. Although formation of carnitinyl-CoA (*m*/*z* 911) was observed by means of MALDI-TOF mass spectrometry, the hydration of crotonobetainyl-CoA to carnitinyl-CoA could not at first be detected spectrophotometrically at 280 nm. However, the hydration of crotonyl-CoA to hydroxybutyryl-CoA was detectable at 280 nm. Considering the absorption maxima of the difference spectra of crotonobetainyl-CoA and CoA (*8*), the hydration reaction was measurable at 220 nm directly. Furthermore, the carnitinyl-CoA formed was identified as the L-enantiomer after hydrolysis. Dehydration of L-carnitinyl-CoA could not be investigated because carnitinyl-CoA was not available. Removal of the trimethylammonium group from crotonobetainyl-CoA reduced V_{max} of CaiD by a factor of 10^3 in the case of crotonyl-CoA, whereas the K_m values remained significantly unchanged. Concluding these results, CaiD should be renamed crotonobetainyl-CoA hydratase or carnitinyl-CoA dehydratase.

In summary, the results obtained in this study have confirmed the postulated affiliation of CaiD to the crotonase superfamily (*17*−*20*). Members of this superfamily catalyze several reactions that require stabilization of an oxyanion intermediate. Two active site glutamate residues, Glu144 and Glu164, have been found to act as the catalytic acid and catalytic base in the catalytic mechanism of enoyl-CoA hydratase, a typical member of the crotonase superfamily (*21*, *22*). Equivalent Glu residues are located at positions 147 and 167 in the amino acid sequence of CaiD (*19*).

Elucidation of the enzymatic function of CaiD as crotonobetainyl-CoA hydratase leads to the conclusion that CaiB must be a CoA-transferase catalyzing the reversible transfer of the CoA moiety between γ-butyrobetainyl-CoA/crotonobetainyl-CoA/L-carnitinyl-CoA and the corresponding free betaines. Indeed the transfer of CoA from crotonobetainyl-CoA and γ-butyrobetainyl-CoA to L-carnitine could be verified by means of MALDI-TOF mass spectrometry. A decrease in L-carnitine could simultaneously be followed spectrophotometrically using carnitine acetyltransferase. CaiB is obviously an unusual CoA-transferase, because it accepts only betainyl-CoA derivatives (e.g., crotonobetainyl-CoA, γ-butyrobetainyl-CoA, and L-carnitinyl-CoA) as cosubtrates. This enzyme should be named crotonobetainyl-CoA:carnitine CoA-transferase. The N-terminus of CaiB shows 39% sequence identity to that of FldA from *Clostridium sporogenes* (*23*) (Figure 7) recently identified as cinnamoyl-CoA:phenyllactate CoA-transferase, which is specific for derivatives of 3-phenylpropionate and 4-phenylbutyrate. A BLAST (*24*) search using the amino acid sequence of CaiB revealed further identities with BaiF from *Eubacterium* sp., Frc from *Oxalobacter formigenes*, and BbsE from *Thauera aromatica* (Figure 7). The *frc* gene from *O. formigenes* encodes the formyl CoA-transferase, which transfers the CoA moiety

FIGURE 7: Alignment of the amino acid sequence of *E. coli* CaiB and those of functionally related enzymes: *C. sporogenes* FldA, *Eubacterium* sp. BaiF, *O. formigenes* Frc, and *T. aromatica* BbsE.

from formyl-CoA to activate oxalic acid (*25*). The *baif* gene was found to encode a bile acid-CoA hydrolase (*26*), whereas BbsE from *T. aromatica* is proposed to be a subunit of a succinyl-CoA:benzylsuccinate CoA-transferase (*27*). Additionally, CaiB also shows identities with the 2-arylpropionyl-CoA epimerase from *R. norvegicus*, a key enzyme in the ibuprofen metabolism (*28*). Interestingly, all of these proteins have a relative molecular mass between 42 and 47 kDa and, in most cases, form homodimers (CaiB, FldA, and BaiF) or heterodimers (BbsE). Most of the enzymes studied reflect high substrate specificities. Comparison of the amino acid sequences revealed highly conserved motifs, one being located near to the N-terminus (Figure 7). In summary, all of these observed similarities mainly at the molecular level lead us to propose that these enzymes form a new CoA-transferase/CoA-hydrolase family.

Summarizing the results obtained in this study, we have confirmed a new mechanism for the biotransformation of crotonobetaine into L-carnitine at the CoA level (Scheme 1), as was recently proposed (*8*). This mechanism is related to that of citrate lyase from *Klebsiella pneumoniae* (*29*) and the dehydration of phenyllactate to cinnamate by *C. sporogenes* (*23*). But this reaction sequence is different from the metabolic pathway found in strain HK4, which is taxonomically situated between *Agrobacterium* and *Rhizobium* (*30*). Kulla (*30*) postulated that this pathway is analogous to fatty acid degradation. In the first step γ-butyrobetaine is transferred by a synthetase to CoA and then oxidized to crotonobetainyl-CoA by a FAD-dependent dehydrogenase. In *E. coli* the reduction reaction of crotonobetaine to γ-butyrobetaine is irreversible and catalyzed by two enzymes, CaiB and CaiA (*7*). While CaiB was shown to be a CoA-transferase, CaiA should catalyze the irreversible reduction of crotonobetainyl-CoA to γ-butyrobetainyl-CoA. The transferase reaction catalyzed by CaiB must be reversible as well as the hydration of crotonobetainyl-CoA to L-carnitinyl-CoA catalyzed by CaiD, because the conversion of crotonobetaine to L-carnitine was shown to be reversible (*4*).

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