Isolation, Identification, and Synthesis of γ-Butyrobetainyl-CoA and Crotonobetainyl-CoA, Compounds Involved in Carnitine Metabolism of *E. coli*†

Thomas Elssner,‡ Lothar Hennig,§ Holm Frauendorf,‡,|| Dieter Haferburg,‡ and Hans-Peter Kleber*,‡

Institut für Biochemie, Fakultät für Biowissenschaften, Pharmazie, und Psychologie, Universität Leipzig, Talstrasse 33, D-04103 Leipzig, Germany, Institut für Organische Chemie, Fakultät für Chemie und Mineralogie, Universität Leipzig, Johannisallee 29, D-04103 Leipzig, Germany, and Institut für Analytische Chemie, Fakultät für Chemie und Mineralogie, Universität Leipzig, Linnéstrasse 3, D-04103 Leipzig, Germany

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ABSTRACT: A still unknown low-molecular-mass cofactor essential for the activity of carnitine-metabolizing enzymes (e.g., L-carnitine dehydratase, crotonobetaine reductase) from *E. coli* has been purified to homogeneity from a cell-free extract of *E. coli* O44K74. The purity of the cofactor was confirmed by HPLC analysis. Biosynthesis of the unknown compound was only observed when bacteria were cultivated anaerobically in the presence of L-carnitine or crotonobetaine. The determined properties, together with results obtained from UV−visible, ^{1}H NMR, and mass spectrometry, indicate that the compound in question is a new CoA derivative. The esterified compound was suggested to be γ-butyrobetaine—a metabolite of carnitine metabolism of *E. coli*. Proof of structure was performed by chemical synthesis. Besides γ-butyrobetainyl-CoA, a second new CoA derivative, crotonobetainyl-CoA, was also chemically synthesized. Both CoA derivatives were purified and their structures confirmed using NMR and mass spectrometry. Comparisons of structural data and of the chemical properties of γ-butyrobetainyl-CoA, crotonobetainyl-CoA, and the isolated cofactor verified that the unknown compound is γ-butyrobetainyl-CoA. The physical and chemical properties of γ-butyrobetainyl-CoA and crotonobetainyl-CoA are similar to known CoA derivatives.

Different genera of Enterobacteriaceae, such as *Escherichia*, *Proteus*, and *Salmonella*, are able to convert L-carnitine into γ-butyrobetaine, via crotonobetaine (Scheme 1), in the presence of carbon and nitrogen sources under anaerobic conditions (*1*). Previous studies have shown that *E. coli* ATCC 25922, *P. vulgaris*, and *P. mirabilis* also metabolize L-carnitine in the same manner under aerobic conditions (*2*). In both cases, γ-butyrobetaine is accumulated in the culture medium as the final product of the reaction sequence.

In *E. coli*, two enzymes—L-carnitine dehydratase (EC 4.2.1.89) and crotonobetaine reductase—catalyze this reaction sequence (*3*, *4*). L-Carnitine was shown to serve as an osmoprotectant in *E. coli* (*5*) and other microorganisms (*6*−*10*). A role as an external electron acceptor, like nitrate or fumarate (*11*), was postulated for crotonobetaine (*12*). The stimulation of anaerobic growth by crotonobetaine supports this hypothesis. L-Carnitine dehydratase, which catalyzes the reversible dehydration of L-carnitine into crotonobetaine, has been purified and characterized (*3*). A low-molecular-mass cofactor (<1000) essential for enzyme activity was separated during the purification of L-carnitine dehydratase. The addition of the cofactor to the inactive L-carnitine dehydratase caused the reactivation of the apoenzyme. It was not possible to replace this compound by other known cofactors and coenzymes of enzyme reactions involved in dehydration (hydration) reactions (e.g., pyridoxal phosphate) (*3*). Other well-known coenzymes [e.g., coenzyme A (CoA)[1]] also show no effect. This still unknown low-molecular-mass cofactor described for L-carnitine dehydratase is also necessary for the crotonobetaine reductase activity (*13*). Crotonobetaine reductase from *E. coli* is composed of two proteins (CaiA and CaiB). *CaiA* and *caiB* belong to the *caiTABCDE* operon, which encodes the structural components of the carnitine pathway in *E. coli* (14). The *caiB* gene was found to encode the L-carnitine dehydratase of *E. coli* O44K74 (*15*). CaiA was postulated to be an oxidoreductase from comparison of amino acid sequence similarities. CaiA was purified previously from an overexpression strain to electrophoretic homogeneity (*13*). It was shown by cross-linking studies that in the crotonobetaine reductase reaction, one dimer of CaiB associates with one tetramer of CaiA in the presence of the unknown cofactor. These results emphasize the importance of the unknown low-molecular-mass cofactor for carnitine-metabolizing enzymes. In this study, we describe the purification, identification, and chemical synthesis of this compound.

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* To whom correspondence should be addressed. Fax: +49(341)-97-36998; E-mail: Kleber@rz.uni-leipzig.de.

‡ Institut für Biochemie, Universität Leipzig.

§ Institut für Organische Chemie, Universität Leipzig.

|| Current address: Fraunhofer Institute for Process Engineering and Packaging, Department of Product Safety and Analysis, Giggenhauser Strasse 35, D-85354 Freising, Germany.

[1] Abbreviations: CFU, cofactor unit; CoA, coenzyme A; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); ESI, electrospray ionization; FPLC, fast protein liquid chromatography; HPLC, high-performance liquid chromatography; MALDI-TOF, matrix-assisted laser desorption/ionization-time-of-flight; NMR, nuclear magnetic resonance.

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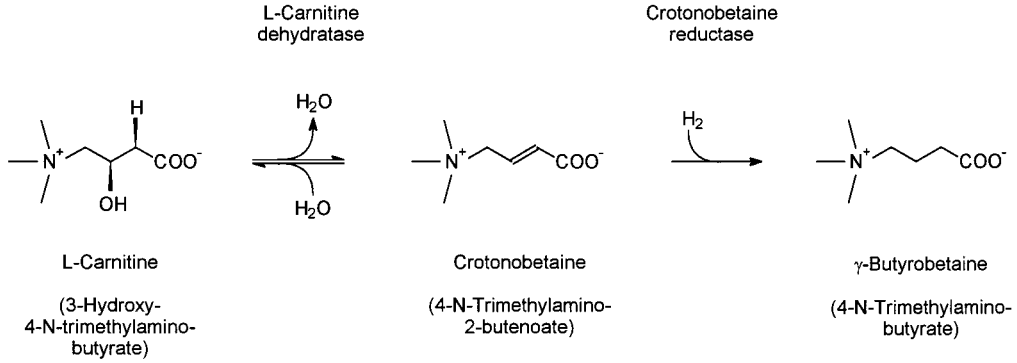
Scheme 1



EXPERIMENTAL PROCEDURES

Chemicals. L-Carnitine, D-carnitine, and crotonobetaine (inner salt) were gifts from Sigma Tau, Rome, Italy. *Trans*-crotonobetaine hydrochloride, γ-butyrobetaine (inner salt), and γ-butyrobetaine hydrochloride were gifts from Lonza, Basel, Switzerland. Carnitine acetyltransferase was purchased from Boehringer Mannheim, Germany. DEAE-Sepharose CL-6B and Sephadex G-15 and G-10 were obtained from Pharmacia, Uppsala, Sweden. Dowex 50 WX8 (200−400 mesh) was from Serva, Heidelberg, Germany. Phosphorus trichloride was purchased from Merck-Schuchardt, Hohenbrunn, Germany. CoA, trilithium salt, was obtained from Boehringer Mannheim, Germany. All other chemicals were of analytical grade.

Cultivation Conditions and Cell Disruption. E. coli O44K74 was cultivated under anaerobic conditions at 37 °C in complex medium (17 g of pancreatic peptone, 3 g of yeast extract, and 5 g of NaCl per liter of deionized water, pH 7.0) containing 8 mM L-carnitine, 137 mM glycerol, and 17 mM fumarate as described previously (*3*). The cultivation was carried out in 1000 mL flasks filled to the neck with medium and stoppered airtight. At the end of the exponential growth phase (8−10 h incubation, OD_{600} 1.2−1.4), cells were harvested by centrifugation (5000*g*, 15 min) and washed twice with 67 mM phosphate buffer (pH 7.5). The cells were disrupted by being passed twice through a French pressure cell (SLM Instruments, Urbana, IL) operating at 20 000 lb/in^2. Unbroken cells and debris were removed by centrifugation at 15000g for 45 min at 4 °C.

Enzyme Assays and Definition of Cofactor Unit (CFU).[1] L-Carnitine dehydratase assay was carried out according to Jung et al. (*3*). For determination of cofactor activity, an excess of partially purified L-carnitine dehydratase was used. Partially purified L-carnitine dehydratase does not transform crotonobetaine into L-carnitine without addition of the unknown cofactor. The cofactor activity was defined as CFU. One CFU is equivalent to an initial rate of substrate turnover of 1 μmol min^{-1} by the reactivated L-carnitine dehydratase.

Preparation of Partially Purified L-Carnitine Dehydratase. E. coli O44K74 was cultivated as described above. The cell-free extract was ultrafiltered (membrane YM 01, Amicon, USA) and the supernatant loaded onto a DEAE-Sepharose CL-6B column (15 by 150 mm) previously equilibrated with potassium phosphate buffer (10 mM, pH 7.5). Cofactor and L-carnitine dehydratase bound to the column. The cofactor was eluted using 150 mM potassium phosphate buffer (pH 7.5). Subsequently, the cofactor-free L-carnitine dehydratase was eluted using 200 mM potassium phosphate buffer (pH 7.5) at a flow rate of 0.8 mL min^{-1}. The purification procedure was performed at 4 °C on a chromatography system (Econo System, Bio-Rad, USA). When necessary, fractions containing L-carnitine dehydratase were concentrated with an ultrafiltration membrane (YM 30, Amicon).

Purification of Cofactor from E. coli O44K74. All purification methods were carried out with the chromatography system mentioned above. The cofactor was separated from the L-carnitine dehydratase by ultrafiltration as described, lyophilized (Alpha 1-2, Christ, Germany), and dissolved in 4 mL of distilled water, and the solution was loaded onto a DEAE-Sepharose CL-6B column equilibrated with distilled water. Cofactor was eluted with a stepwise gradient (0.1−0.15 M NaCl) using a flow rate of 0.8 mL min^{-1}. Active fractions (150 mM NaCl) were pooled and concentrated by lyophilization. The lyophilized cofactor was redissolved in distilled water and loaded onto a Sephadex G-15 column (16 by 400 mm) equilibrated with distilled water. The cofactor was eluted with distilled water and collected in 2 mL fractions. The flow rate was 0.5 mL min^{-1}. Active fractions were pooled and concentrated by lyophilization. The same procedure was repeated using a Sephadex G-10 column (16 by 400 mm).

Synthesis of γ-Butyrobetainyl-CoA and Crotonobetainyl-CoA. γ-Butyrobetaine hydrochloride and *trans*-crotonobetaine hydrochloride were dried over phosphorus pentoxide under vacuum for at least 12 h. The dried betains (10 mmol) were mixed with phosphorus trichloride (12 mmol) at 20 °C for 5−7 days. The reaction flasks were stoppered airtight. The reaction was complete when a colorless viscous oil was formed. After removal of the excess phosphorus trichloride, the residual oil remained stable undistilled for several weeks. γ-Butyrobetainyl chloride and crotonobetainyl chloride (approximately 22 μmol) were converted to γ-butyrobetainyl-CoA and crotonobetainyl-CoA, respectively, by addition of CoA (13 μmol) in 0.5 M sodium bicarbonate (pH 8.5). The reaction was monitored for free thiol groups with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB[1]) (*16*). When no free thiol was observed (approximately 15 min), the reaction mixtures were titrated to pH 5 if necessary, with HCl.

Purification of γ-Butyrobetainyl- and Crotonobetainyl-CoA. The purification of γ-butyrobetainyl- and crotonobetainyl-CoA was performed at room temperature on an LKB II FPLC[1] system (Pharmacia, Uppsala, Sweden). The reaction mixture was loaded onto a Dowex 50 WX8 column (15 by 300 mm) previously equilibrated with distilled water. The

CoA derivatives were eluted using distilled water at a flow rate of 1 mL min^{-1}. Fractions containing the CoA derivative were pooled and lyophilized.

HPLC[1] Analysis of Isolated Cofactor from E. coli O44K74, γ-Butyrobetainyl-CoA, and Crotonobetainyl-CoA. The cofactor and the CoA-esters were separated isocratically on a reversed phase column (RP-18, 250 × 4 mm, 5 μm particle size; Spherisorb ODS II, Bischoff Chromatography, Leonberg, Germany) in methanol/water [60:40 (v/v)] at a flow rate of 0.8 mL min^{-1}. The compounds were detected at 260 nm.

UV−Visible Spectral Measurements. The absorption spectra were recorded between 190 and 820 nm on a diode array spectrophotometer (8452 A, Hewlett-Packard, USA). Determination of ε was performed on a UV-160A spectrophotometer (Shimadzu, Kyoto, Japan).

NMR[1] Spectroscopy. ^{1}H NMR spectra were measured in D_2O (4.65 ppm) using Varian Gemini 2000 (200.041 MHz) and Varian Unity (399.952 MHz) spectrometers (Palo Alto, CA) at 26 °C, partially with water suppression by decoupler presaturation. ^{13}C NMR spectra were recorded using Varian Gemini 2000 (50.300 MHz) and a Bruker DRX (100.623 MHz) machine (Karlsruhe, Germany). Chemical shifts are given in ppm.

MALDI-TOF[1] Mass Spectrometry. The MALDI matrix, 2-cyano-4-hydroxycinnamic acid, was prepared by dissolving 5 mg in 0.5 mL of a water/3% trifluoroacetic acid/acetonitrile (4:1:5) solution. Then 1 μL 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.

ESI[1] Mass Spectrometry. Electrospray investigations were carried out by means of a VG PLATFORM mass spectrometer (Micromass, Manchester, U.K.) equipped with an electrospray ion source. The liquid eluant water/acetonitrile (1:1, v/v) was delivered by an HPLC instrument (Jasco, Gross-Umstadt, Germany) at a flow rate of 10 μL min^{-1}. Samples were dissolved in formic acid (0.2%) or in water. Sample volumes of 10 μL were applied by loop injection. Electrospray mass spectrometry was performed in the positive-ion and negative-ion modes. Nitrogen was used as nebulizing and drying gas. Mass spectra were acquired in scan ranges from *m*/*z* 100 to *m*/*z* 2000.

RESULTS

Biosynthesis and Purification of the Cofactor. The cofactor requires a carnitine-metabolizing enzyme for determination of its activity. Therefore, cofactor activity in cell-free extracts can only be measured indirectly (e.g., via formation of L-carnitine from crotonobetaine using cofactor-free, partially purified L-carnitine dehydratase). Cofactor activity could be detected only in cell-free extracts from *E. coli* O44K74 grown anaerobically in the presence of L-carnitine (31 mM) or crotonobetaine (35 mM). Other quaternary ammonium compounds (e.g., D-carnitine, γ-butyrobetaine, choline, glycinbetaine) did not show any inducing effect on the biosynthesis of the cofactor. The specific activity of the L-carnitine dehydratase in cell-free extracts was approximately 160 milliunits/mg. Addition of cofactor isolated by ultrafiltration (30 mCFU) to the cell-free extract increased the formation of L-carnitine from crotonobetaine by about 70%. This indicated that L-carnitine dehydratase was not saturated with the cofactor.

Table 1: Purification of Cofactor of Carnitine-Metabolizing Enzymes from *E. coli* O44K74

purification step	cofactor activity (CFU)	yield (%)
ultrafiltration on Amicon YM 01	129	100
DEAE-Sepharose CL-6B	108	84
Sephadex G-15	59	46
Sephadex G-10	50	39

For purification of the cofactor, 20 L of complex medium was used in the cultivation. The first purification step—ultrafiltration on Amicon YM 01—served to separate the cofactor from the carnitine-metabolizing enzymes with minimal loss of activity. The achieved yield was assessed to be 100%. Further purification of the cofactor was carried out by ion exchange chromatography on DEAE-Sepharose CL6-B (cofactor was eluted between 100 and 150 mM NaCl) and two size exclusion chromatographies on Sephadex G-15 and G-10, respectively. Fractions containing the cofactor were identified by measuring the transformation of crotonobetaine into L-carnitine after addition of a cofactor-free solution containing L-carnitine dehydratase prepared from *E. coli*. It must be emphasized that the prepared cofactor-free solution containing L-carnitine dehydratase from *E. coli* was unable to transform crotonobetaine into L-carnitine without the cofactor. The purified cofactor gave a single peak when analyzed by HPLC. The overall yield was 39% of the original activity (Table 1).

Properties of the Cofactor. Treatment with proteases and nucleases (*3*) or 5′-nucleotidase did not influence the stability of the cofactor, whereas acid and alkaline phosphatases and 3′-nucleotidase inactivate the cofactor completely.

The purified cofactor is very hygroscopic, and it was not possible to store the cofactor lyophilized. In water it lost about 40% of its activity when stored at −20 °C for 120 h. It was stable in neutral and acid solutions (down to pH 2) for about 1 h. In alkaline solutions (pH 10), the cofactor exhibited about 30% loss of activity after 1 h. The binding properties of the cofactor to the anion exchanger (DEAE-Sepharose) indicated an anionic character of the unknown compound. The ultraviolet spectrum of purified cofactor dissolved in H_2O showed absorption maxima at 208 and 260 nm.

Structure of the Cofactor. The achieved yield of purified cofactor was sufficient for recording of the ^{1}H NMR spectrum but not for the ^{13}C NMR spectrum. It was therefore not possible to assign an unambiguous structure for the cofactor based on the results obtained from the ^{1}H NMR spectrum (Table 2). In comparison, the ^{1}H NMR spectrum of the purified cofactor showed similarities to ^{1}H NMR spectra of CoA and CoA derivatives, respectively (*17, 18*). Sharp resonance signals resulting from the two methyl groups of CoA were observed at 0.67 and 0.79 ppm. Two signals due to the hydrogens at carbon atoms 2 and 8 of the adenine

Table 2: Chemical Shifts of ^{1}H NMR Spectra of Crotonobetainyl- and γ-Butyrobetainyl-CoA and the Isolated Cofactor from *E. coli* O44K74

position	crotonobetainyl-CoA	γ-butyrobetainyl-CoA	cofactor
a	8.51s	8.54s	8.47s
b	8.28s	8.31s	8.21s
c	6.05d	6.09d	6.09d
d	4.69	4.73	4.75
e	3.10	3.21	ni[a]
f	4.45	4.47	4.50
g	3.46/3.73	3.48/3.73	ni
h	4.12s	4.14s	4.13s
i	0.67s, 0.79s	0.69s, 0.80s	0.67s, 0.79s
k	3.89s	3.88s	3.89s
l, m	3.31t, 2.29t	3.32t, 2.30t	ni, 2.35t
n, o	3.26t, 2.95t	3.20t, 2.89t	3.24t, 2.90t
I: CH_2CO	−	2.63t	2.63
II: CH_b	6.53d (J = 15.4 Hz)	−	−
I: CH_2	−	1.98m	2.00m
II: CH_a	6.73m (J = 15.4 Hz)	−	−
CH_2N^+	3.98d	3.19t	ni
$N^+(CH_3)_3$	3.00s	2.98s	3.02s

[a] Not clearly identified.

moiety were also identified at 8.21 and 8.47 ppm. Different signals resulting from methylene groups or hydrogens of the sugar were observed between 1.2 and 6.5 ppm. The singlet at 3.0 ppm was an exception. This resonance signal was probably due to a $(CH_3)_3N^+$group, because a singlet was observed at the same position in the ^{1}H NMR spectrum of crotonobetaine (not shown). These results suggest that the cofactor is a CoA derivative, because CoA alone or CoA and ATP together did not reactivate partially purified L-carnitine dehydratase. Because of the singlet at 3.0 ppm, the esterified compound could be a metabolite of carnitine metabolism in *E. coli* (e.g., carnitine, crotonobetaine, or γ-butyrobetaine).

The molecular mass of the purified component is smaller than 1000 Da, because it passed through membranes with a molecular mass cutoff of 1000 Da. For determination of molecular mass, two different ionization methods—ESI and MALDI—were used. The positive-ion ESI mass spectrum of purified cofactor is dominated by singly and doubly charged molecular ions (Figure 1). The molecular ion (pH < 7) should have a positive charge due to the $(CH_3)_3N^+$ group if it is a CoA derivative of a quaternary ammonium compound. The singly charged molecular ion $(M)^+$ and its adducts were observed between *m/z* 895.0 and 971.0 and the doubly charged $(M+H)^{2+}$ between *m/z* 447.7 and 505.5. The signal at *m/z* 895.0 was identified as the peak corresponding to the molecular mass of the cofactor. Further peaks are due to adducts, e.g., at *m/z* 917.0 $(M+Na)^+$, *m/z* 932.8 $(M+K)^+$, *m/z* 955.6 $(M+Na+K)^+$, and *m/z* 971.0 $(M+K+K)^+$. The doubly charged molecular ion peak was observed at *m/z* 447.7. The negative ion spectrum (not shown) also reveals singly and doubly charged peaks at *m/z* 446 and 893. The calculated molecular mass of crotonobetainyl-CoA is 893.2 Da, γ-butyrobetainyl-CoA 895.2 Da, and carnitinyl-CoA 911.2 Da. The molecular mass of the cofactor resulting from these spectra assumes crotonobetainyl-CoA or γ-butyrobetainyl-CoA as isolated cofactor. This attribution was finally assessed by MALDI-TOF analysis in the positive ion mode. The data were almost in agreement with the molecular mass established by ESI-MS. The molecular ion $(M)^+$ was observed at 895.1 (Figure 2 A). Concluding all these results, it is most probable that γ-butyrobetainyl-CoA is the purified cofactor from *E. coli*.

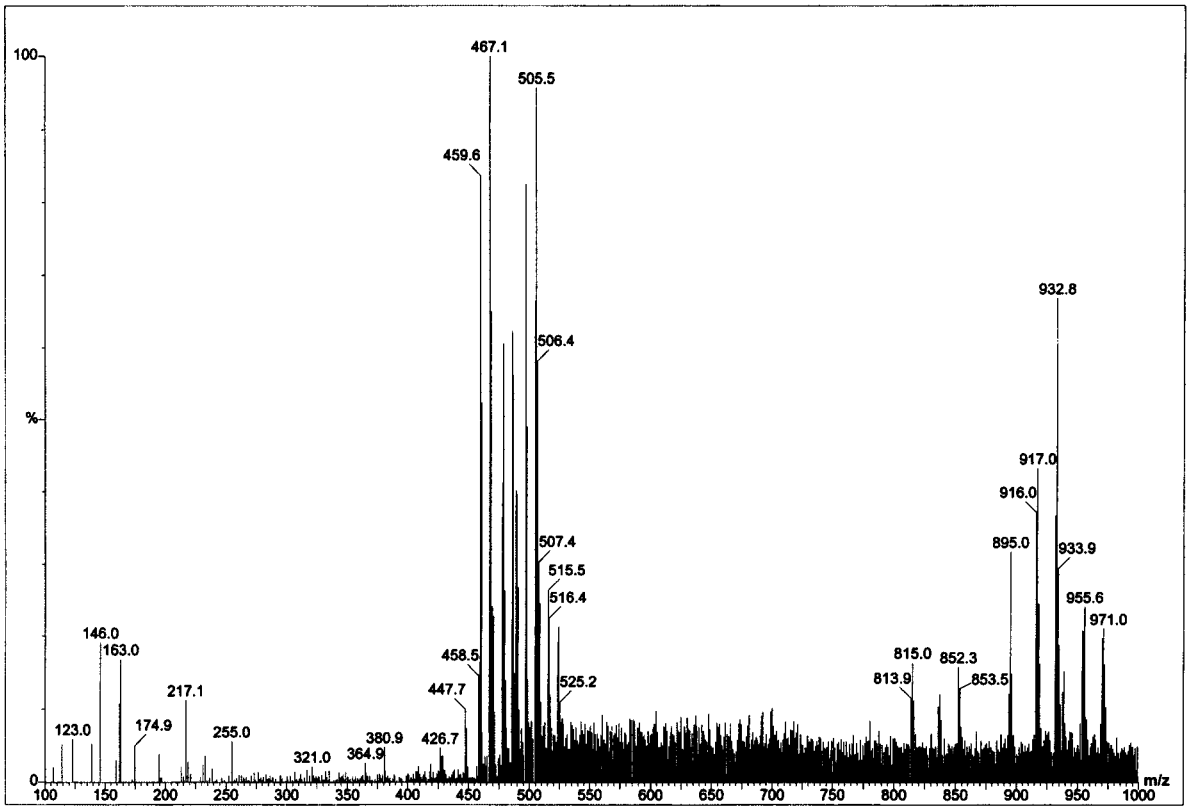


FIGURE 1: Positive ion ESI mass spectrum of the isolated and purified cofactor of carnitine-metabolizing enzymes from *E. coli* O44K74. The molecular ion peak of the unknown cofactor was identified within the singly (*m/z* 895.0) and the doubly (*m/z* 447.7) charged ions.

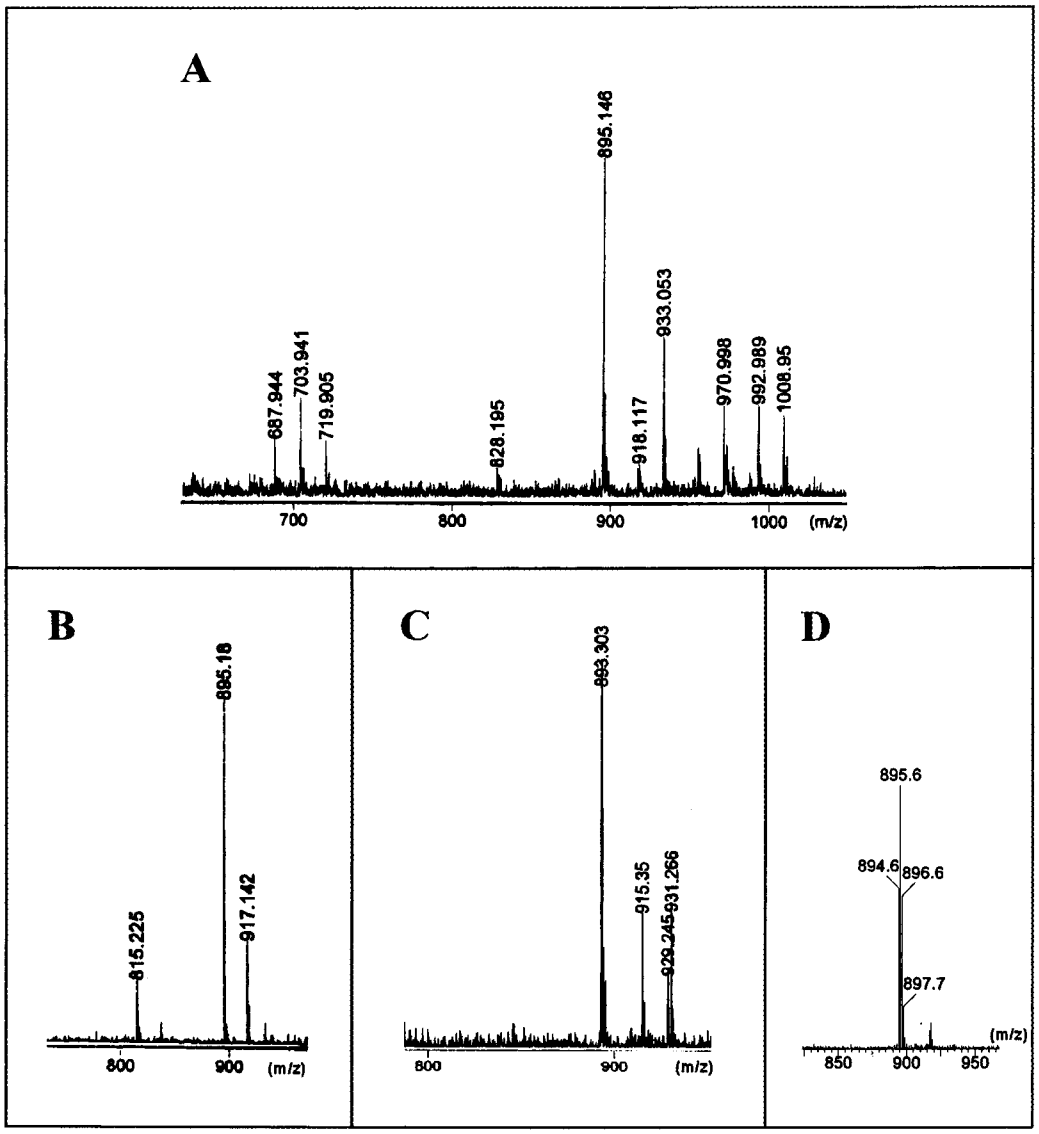


FIGURE 2: (A) Analysis of purified cofactor of carnitine-metabolizing enzymes from *E. coli* O44K74 by MALDI-TOF mass spectrometry. (B−D) Analysis of γ-butyrobetainyl-CoA and crotonobetainyl-CoA by MALDI-TOF- and ESI-MS. (B) MALDI-TOF mass spectrum of γ-butyrobetainyl-CoA. (C) MALDI-TOF mass spectrum of crotonobetainyl-CoA. (D) ESI mass spectrum of γ-butyrobetainyl-CoA.

Chemical Synthesis and Purification of γ-Butyrobetainyl-CoA and Crotonobetainyl-CoA. To verify the identity of the purified compound, it was necessary to synthesize the postulated CoA derivative chemically. γ-Butyrobetainyl-CoA and crotonobetainyl-CoA were synthesized starting from γ-butyrobetaine and *trans*-crotonobetaine hydrochloride, which were converted to the respective acyl chloride with phosphorus trichloride. Formation of a colorless compound with a viscous consistency was observed after approximately 1 week at room temperature. The formation of γ-butyrobetainyl chloride and crotonobetainyl chloride proceeded completely as no γ-butyrobetaine or crotonobetaine remained. Transesterification of γ-butyrobetainyl chloride/crotonobetainyl chloride with CoASH at pH 8 (*19*) produced γ-butyrobetainyl-CoA and crotonobetainyl-CoA, respectively. Unreacted CoA, γ-butyrobetaine/crotonobetaine, and γ-butyrobetainyl-CoA/crotonobetainyl-CoA were separated by chromatography on Dowex 50 WX8. Both CoA derivatives gave a single peak when analyzed by HPLC. γ-Butyrobetainyl-CoA eluted at 2.4 min and crotonobetainyl-CoA at 2.5 min.

Structural Comparison of Crotonobetainyl-CoA, γ-Butyrobetainyl-CoA, and the Isolated Cofactor. The structures of crotonobetainyl-CoA and γ-butyrobetainyl-CoA (Figure 3) were verified using ^{1}H NMR (Table 2) and ^{13}C NMR spectra (not shown). Resonance signals due to the CoA moiety are in agreement with data of known CoA derivatives (*18*). The synthesized crotonobetainyl-CoA had a *trans*-configuration as revealed by the coupling constants (15.4 Hz). Almost all signals (except positions e, g, and l) detected in ^{1}H NMR spectrum of γ-butyrobetainyl-CoA could be recognized in the ^{1}H NMR spectrum of the cofactor. Signals resulting from the crotonobetaine moiety (e.g., at 6.53 and 6.73 ppm) in the crotonobetainyl-CoA spectrum were not observed in the cofactor spectrum. However, two of three resonance signals due to the γ-butyrobetaine moiety (at 1.98 and 2.63 ppm) were identified in the cofactor spectrum. Significantly, the singlet resulting from $N^+(CH_3)_3$ was identified in all spectra. Comparison of data obtained from ^{1}H NMR spectra of the isolated cofactor and both synthesized CoA derivatives suggests γ-butyrobetainyl-CoA as the isolated compound.

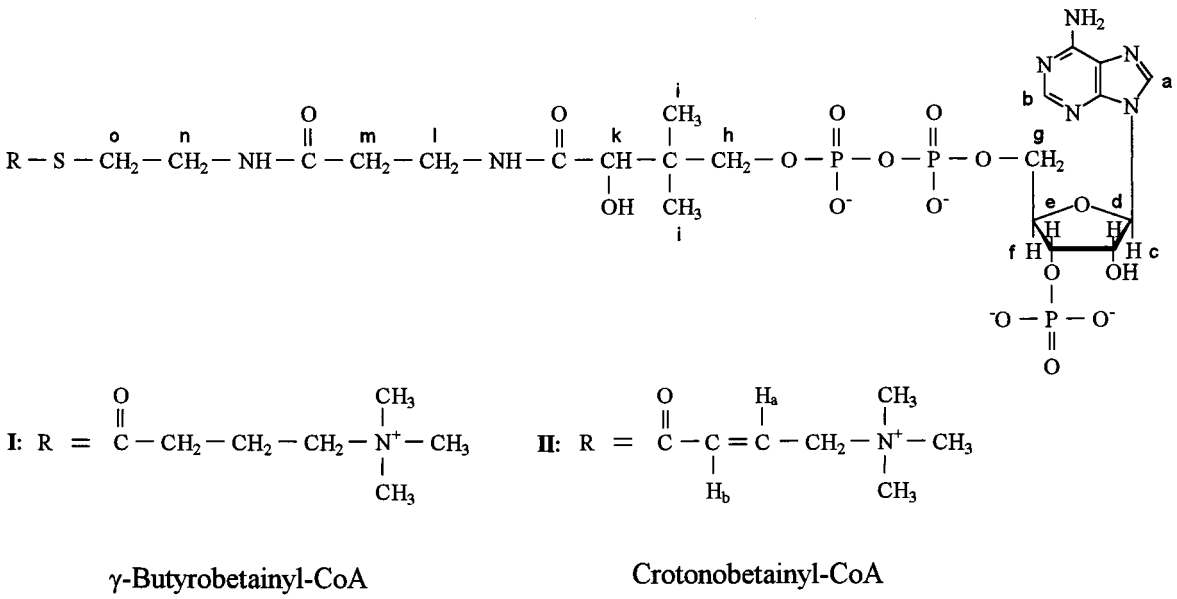


FIGURE 3: Structures of γ-butyrobetainyl-CoA (I) and crotonobetainyl-CoA (II).

Table 3: Properties of γ-Butyrobetainyl-CoA, Crotonobetainyl-CoA, and the Isolated Cofactor from *E. coli* O44K74

properties	γ-butyrobetainyl-CoA	crotonobetainyl-CoA	isolated cofactor
molecular mass	895.2	893.3	895.1
absorption maxima	258 nm	208 nm, 260 nm	208 nm, 260 nm
ϵ_{260}	16.1 L mmol^{-1} cm^{-1}	20.2 L mmol^{-1} cm^{-1}	nd[a]
temperature stability (1 h)	100%: −20 °C >90%: 0−90 °C	100%: −20−0 °C 75%: 0−60 °C	100%: −20−60 °C
pH stability (1 h)	3−10	2−10	2−8
solubility	H_2O	H_2O	H_2O
general remarks	white powder, hygroscopic	white powder, hygroscopic	white powder, hygroscopic

[a] Not determined.

The structure proposed for the isolated cofactor from *E. coli* has a molecular mass of 895 Da. A MALDI-TOF mass spectrum of γ-butyrobetainyl-CoA lends strong support for this structure (Figure 2B). The molecular ion was observable at *m*/*z* 895.2. The MALDI-TOF mass spectrum of crotonobetainyl-CoA showed a main peak at *m*/*z* 893.3 (Figure 2C). Both masses agree with the calculated formulas $C_{28}H_{50}N_8O_{17}P_3S_1$ and $C_{28}H_{48}N_8O_{17}P_3S_1$ for γ-butyrobetainyl-CoA and crotonobetainyl-CoA, respectively. A second mass spectrum of γ-butyrobetainyl-CoA obtained using electrospray ionization verified the molecular mass (Figure 2D). Data for γ-butyrobetainyl-CoA obtained with MALDI and ESI are in agreement with data for the isolated cofactor.

Functional Importance of γ-Butyrobetainyl-CoA and Crotonobetainyl-CoA. Addition of γ-butyrobetainyl-CoA (2 nmol) or crotonobetainyl-CoA (2 nmol) to partially purified L-carnitine dehydratase (10 μg) allowed conversion of crotonobetaine to L-carnitine. However, no transformation of crotonobetaine into L-carnitine was observed when γ-butyrobetainyl-CoA or crotonobetainyl-CoA was substituted by other CoA derivatives (e.g., acetyl-CoA, acetoacetyl-CoA, crotonyl-CoA, DL-β-hydroxybutyryl-CoA, palmitoyl-CoA). The apparent K_m values for γ-butyrobetainyl-CoA and crotonobetainyl-CoA were 40 and 29 μM, respectively, in the presence of an excess of crotonobetaine during the incubation. V_{max} was determined as 30.5 units mg^{-1} for γ-butyrobetainyl-CoA and 37 units mg^{-1} for crotonobetainyl-CoA under identical conditions. It is also worth noting that the cofactor described for the crotonobetaine reductase activity (*13*) can be substituted by γ-butyrobetainyl-CoA or crotonobetainyl-CoA.

Physicochemical Properties of γ-Butyrobetainyl-CoA, Crotonobetainyl-CoA, and the Isolated Cofactor from E. coli O44K74. In Table 3 some properties of γ-butyrobetainyl-CoA, crotonobetainyl-CoA, and the cofactor are compared. Because of the small amount of purified cofactor, it was not possible to determine the extinction coefficient, ε, at 260 nm. For determination of stability, γ-butyrobetainyl-CoA and crotonobetainyl-CoA were incubated for 1 h in acid and alkaline solutions and subsequently neutralized, and the resulting decomposition was measured via transformation of crotonobetaine into L-carnitine catalyzed by the L-carnitine dehydratase. However, the most noticeable difference between the two CoA derivatives are the absorption maxima. Contrary to γ-butyrobetainyl-CoA, crotonobetainyl-CoA and the isolated cofactor showed two absorption maxima at 208 and 260 nm. All other determined parameters of the isolated cofactor are in agreement with properties of γ-butyrobetainyl-CoA.

DISCUSSION

The unknown compound essential for the activity of carnitine-metabolizing enzymes in *E. coli* O44K74 was unequivocally identified as γ-butyrobetainyl-CoA. The biosynthesis of γ-butyrobetainyl-CoA is induced in the presence of either L-carnitine or crotonobetaine in the growth medium under anaerobic conditions. L-Carnitine dehydratase and

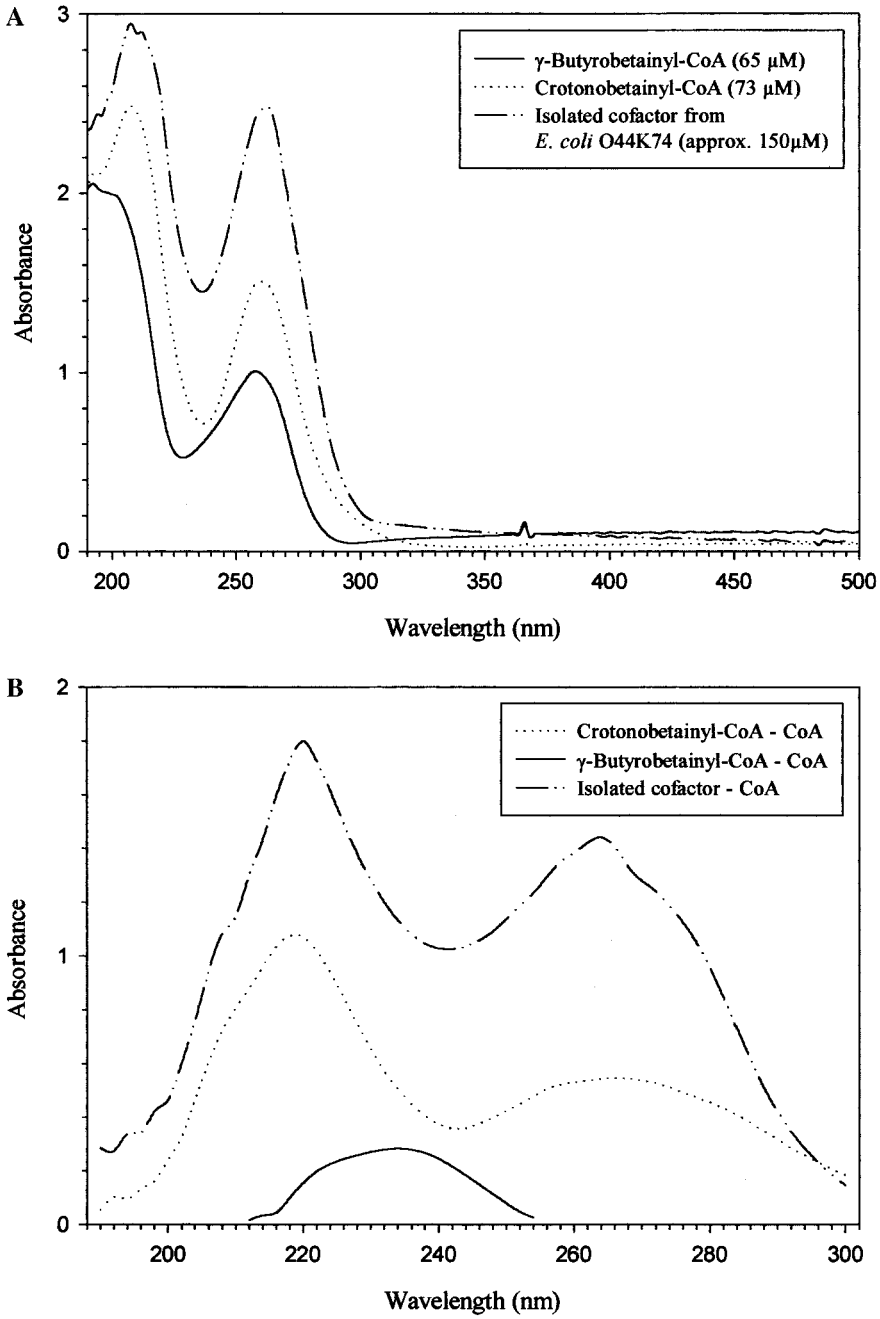
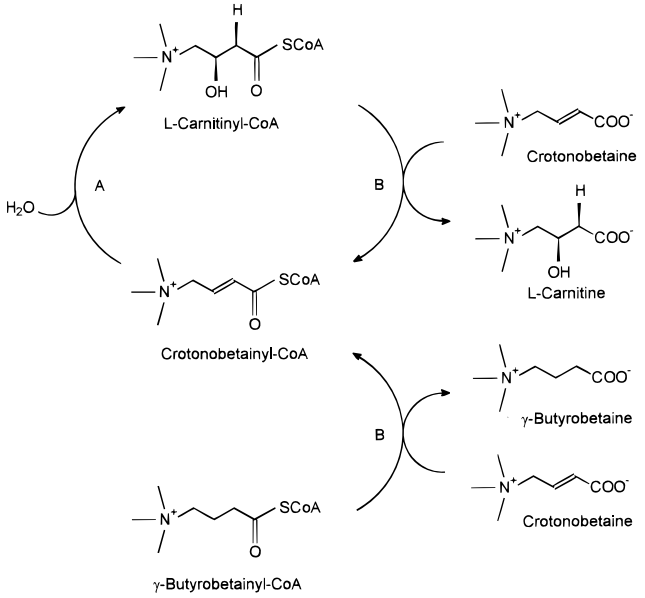


FIGURE 4: (A) UV−visible spectra of γ-butyrobetainyl-CoA, crotonobetainyl-CoA, and the isolated cofactor from *E. coli* O44K74. All spectra were obtained in H_2O at 25 °C. (B) Difference spectra of γ-butyrobetainyl-CoA, crotonobetainyl-CoA, the isolated cofactor, and CoA.

crotonobetaine reductase are inducible enzymes detectable in cells grown anaerobically in the presence of L-carnitine or crotonobetaine (*20*). Furthermore, biosynthesis of γ-butyrobetainyl-CoA and the expression of crotonobetaine reductase in *E. coli* O44K74 seem to be repressed by oxygen (*2*). These studies suggest the same epigenetical control for the biosynthesis of γ-butyrobetainyl-CoA and the induction of carnitine-metabolizing enzymes. For the formation of γ-butyrobetainyl-CoA, a synthetase could be responsible, because the gene product of *caiC* of the *cai* operon has a high similarity to eukaryotic and prokaryotic proteins with ligase activity (*14*).

Comparison of NMR spectra of the isolated compound from *E. coli* O44K74 and the chemically synthesized γ-butyrobetainyl-CoA showed that only a few signals of the ^{1}H NMR spectrum of the cofactor could not be assigned to γ-butyrobetainyl-CoA. These are most likely due to contaminants not detected by HPLC in the purified cofactor that produced resonance signals which masked γ-butyrobetainyl-CoA signals in the cofactor NMR spectrum. The comparison of properties of γ-butyrobetainyl-CoA and the isolated compound revealed only few differences between the absorption spectra (Figure 4 A). The main difference between the UV spectra of synthesized γ-butyrobetainyl-CoA and the isolated cofactor is the shift of the absorption minimum of the former from 238 to 226 nm. The UV spectrum of crotonobetainyl-CoA shows an absorption minimum at 238 nm. Concentrations for determination of absorption maxima of γ-butyrobetainyl-CoA and crotonobetainyl-CoA were 65 and 73 μM in H_2O, but it was not possible to determine the

Scheme 2: Proposed Mechanism for the Transformation of Crotonobetaine into L-Carnitine Catalyzed by either L-Carnitine Dehydratase or an Enzyme System Consisting of an Enoyl-CoA Hydratase (A) and a CoA Transferase (B)



concentration of the isolated compound. The concentration of the isolated cofactor was only estimated to be 150 μM using the ϵ_{260} of γ-butyrobetainyl-CoA and the absorption spectrum of the purified compound. The maximum of crotonobetainyl-CoA at 260 nm is composed of the absorption of adenine ($\epsilon = 16$ l mmol^{-1} cm^{-1}) and the unsaturated thiol ester ($\epsilon = 4.2$ l mmol^{-1} cm^{-1}), whereas the spectrum of γ-butyrobetainyl-CoA is composed of the absorption of the adenine at 260 nm and that of an unconjugated thiol ester at 232 nm. In general, the thiol ester band of CoA derivatives is overlapped by the strong absorption of the adenine (*21*). The thiol ester of α,β-unsaturated acids is correlated with the formation of two absorption maxima at 225 and 263 nm. Difference spectra of crotonobetainyl-CoA (crotonobetainyl-CoA − CoA) revealed also two maxima at 220 and 264 nm (Figure 4B). These spectra are very similar to difference spectra of the isolated cofactor. In contrast, the difference spectra of γ-butyrobetainyl-CoA showed only one maximum due to the unconjugated thiol ester at 232 nm. Therefore it cannot be excluded that the preparation of cofactor used for UV spectroscopy was a mixture of crotonobetainyl-CoA and γ-butyrobetainyl-CoA. This seems possible because crotonobetainyl-CoA could be metabolized/transformed into γ-butyrobetainyl-CoA (Scheme 1). Whether the isolated cofactor solution contains crotonobetainyl-CoA, γ-butyrobetainyl-CoA, or even both depends most likely on the time of cultivation.

All other determined properties of γ-butyrobetainyl-CoA and crotonobetainyl-CoA resemble those of known CoA derivatives. The absorption spectra of acetyl-CoA, malonyl-CoA, and succinyl-CoA also show maxima at 260 nm (*22*). For acetyl-CoA, the ϵ is 16.4 mmol^{-1} cm^{-1} at 260 nm and for α,β-unsaturated CoA derivatives 22.6 l mmol^{-1} cm^{-1}. As with most known CoA derivatives, γ-butyrobetainyl-CoA and crotonobetainyl-CoA are stable in neutral and moderately acid solutions and are hydrolyzed in alkaline solutions.

At first sight, the involvement of γ-butyrobetainyl-CoA and/or crotonobetainyl-CoA in the transformation of crotonobetaine to L-carnitine seems remarkable. Jung et al. (*3*) reported that L-carnitine dehydratase catalyzes the reversible dehydration of crotonobetaine to L-carnitine (Scheme 1). In general, CoA derivatives are responsible for acyl group transfer in different reactions, e.g., β-oxidation, fatty acid synthesis, or pyruvate oxidation. Therefore, it is likely that γ-butyrobetainyl-CoA and crotonobetainyl-CoA are both substrates or cosubtrates in the conversion of L-carnitine to crotonobetaine and the reaction proceeds at the CoA level. For this to occur, a CoA transferase activity and an enoyl-CoA hydratase activity are necessary (Scheme 2). A CoA transferase activity would transfer the CoA moiety between γ-butyrobetainyl-CoA/crotonobetainyl-CoA/postulated L-carnitinyl-CoA and γ-butyrobetaine/crotonobetaine/L-carnitine, respectively. An enoyl-CoA hydratase should then catalyze the reversible hydration of crotonobetainyl-CoA to L-carnitinyl-CoA. These two activities could be incorporated in a single enzyme, L-carnitine dehydratase, based on the observations of Eichler et al. (*15*) and Jung et al. (*3*), or, alternatively they may be found in separate enzymes: a CoA transferase and an enoyl-CoA hydratase.

Recently, single-crystal neutron diffraction studies have shown that the addition of D_2O across the C═C double bond of *trans*-crotonobetaine proceeded by a stereospecific *syn* pathway (*23*). In all enzymatic dehydration reactions so far examined in which there is a *syn* elimination of water, the proton abstracted was α to a carbonyl group of a thioester or a ketone (*24*). An enzyme catalyzing the hydration of *trans*-crotonobetaine to L-carnitine via *trans*-crotonobetainyl-CoA and L-carnitinyl-CoA would be compatible with the proposed duality in classification of hydratase−dehydratase enzymes.

In further studies, we intend to investigate the mechanism of the transformation of crotonobetaine into L-carnitine catalyzed by the L-carnitine dehydratase in more detail. To verify the proposed bifunctionality of the L-carnitine dehydratase, we also want to clarify the role of the enzyme in the reduction of crotonobetaine to γ-butyrobetaine.

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