Carboxymethylproline Synthase from *Pectobacterium carotorova*: A Multifaceted Member of the Crotonase Superfamily†

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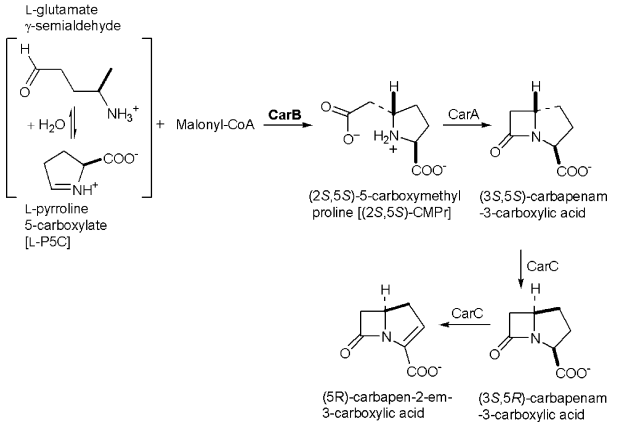
ABSTRACT: The simplest carbapenem antibiotic, (5*R*)-carbapen-2-em-3-carboxylic acid, is biosynthesized from primary metabolites in *Pectobacterium carotorova* by the action of three enzymes, carboxymethylproline synthase (hereafter named CarB), carbapenam synthetase, and carbapenem synthase. CarB, a member of the crotonase superfamily, catalyzes the formation of (2*S*,5*S*)-5-carboxymethylproline from malonyl-CoA and L-pyrroline-5-carboxylate. In this study we show that, in addition, CarB catalyzes the independent decarboxylation of malonyl-CoA and methylmalonyl-CoA and the hydrolysis of CoA esters such as acetyl-CoA and propionyl-CoA. The steady-state rate constants for these reactions are reported. We have identified the intermediates in the CarB reactions with L-pyrroline-5-carboxylate and malonyl-CoA or methylmalonyl-CoA as the CoA esters of (2*S*,5*S*)-5-carboxymethylproline and (2*S*,5*S*)-6-methyl-5-carboxymethylproline, respectively. The data provided indicate that these intermediates partition between completing turnover and dissociating from the enzyme. On the basis of the steady-state rate constants measured for the CarB-catalyzed hydrolysis of synthetic (2*S*,5*S*)-5-carboxymethylprolyl-CoA and for the CarB reaction with malonyl-CoA and L-pyrroline-5-carboxylate, we have calculated the rate constants for each step of these reactions. The results identify CarB as a particularly interesting member of the crotonase superfamily that combines in one net reaction three activities of this superfamily, decarboxylation, C−C bond formation, and CoA ester hydrolysis.

As the number of sequenced natural product gene clusters has increased, the often difficult task of decrypting the biosynthetic roles of their encoded proteins has revealed examples of remarkable evolutionary divergence from their antecedents recognizable in primary metabolism to carry out new, specialized functions (*1*−*3*). Impressive synthetic efficiencies can be seen in these enzymes where more than one mechanistically related reaction is frequently catalyzed as part of an overall biosynthetic pathway. The formation of the simplest of the carbapenem β-lactam antibiotics, (5*R*)-carbapen-2-em-3-carboxylic acid, by only three enzymes from simple primary metabolites is a telling illustration of these processes in nature and underscores the potential for redesign of these proteins for other synthetic purposes, for example, the preparation of structural variants of this important class of antibiotics to overcome resistance.

(5*R*)-Carbapen-2-em-3-carboxylic acid is formed in a poorly understood transformation whereby (3*S*,5*S*)-carbapenam-3-carboxylic acid, having the opposite bridgehead stereochemistry, is inverted by CarC,[1] an α-ketoglutarate-dependent non-heme iron oxygenase, to the epimeric (3*S*,5*R*)-carbapenam-3-carboxylic acid, which is desaturated by the same enzyme to (5*R*)-carbapen-2-em-3-carboxylic acid (Scheme 1) (*4*−*6*). CarA catalyzes β-lactam ring formation in the synthesis of (5*R*)-carbapen-2-em-3-carboxylic acid by coupling the cyclization step to ATP hydrolysis (*7*) in a fashion similar to that of β-lactam synthetase, an enzyme involved in clavulanic acid biosynthesis (*8*−*10*). The kinetic

Scheme 1: Biosynthesis of (5*R*)-Carbapen-2-em-3-carboxylic Acid



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[1] Abbreviations: CarB, carboxymethylproline synthase; CarA, carbapenam synthetase; CarC, carbapenem synthase; IPTG, isopropyl β-D-thiogalactopyranoside; PMSF, phenylmethanesulfonyl fluoride; (2*S*,5*S*)-CMPr, (2*S*,5*S*)-5-carboxymethylproline; 6-methyl-(2*S*,5*S*)-CMPr, (2*S*,5*S*)-6-methyl-5-carboxymethylproline; (2*S*,5*R*)-CMPr, (2*S*,5*R*)-5-carboxymethylproline; (2*R*,5*R*)-CMPr, (2*R*,5*R*)-5-carboxymethylproline; MeCMPr-CoA, (2*S*,5*S*)-6-methyl-5-carboxymethylproline CoA ester; CMPr-CoA, (2*S*,5*S*)-5-carboxymethylproline CoA ester; ESI-MS, electrospray ionization mass spectrometry; LC/ESI-MS, high-pressure liquid chromatography coupled to electrospray ionization mass spectrometry; P5C, pyrroline-5-carboxylate; 4PDS, 4,4′-dipyridyl disulfide.

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mechanism and the structure of CarA have been characterized (*7*, *11*). CarA, with its remarkably broad substrate specificity, was shown to be an ideal candidate for the synthesis of novel β-lactam antibiotics (*7*). The natural substrate of CarA, (2*S*,5*S*)-CMPr, is the product of the CarB-catalyzed reaction, the first essential step in the biosynthesis of (5*R*)-carbapen-2-em-3-carboxylic acid (*4*).

A preliminary characterization of the reactions performed by CarB, recently reported (*12*), identifies malonyl-CoA together with L-glutamate semialdehyde (or its closed, dehydrated form at equilibrium, pyrroline-5-carboxylate) (*5*) as the substrates. On the basis of amino acid sequence analysis, CarB is proposed to belong to the crotonase superfamily (*13*). Sequence homology and structural comparisons among members of this family are poor, but two consensus sequences reveal a common mechanistic feature, stabilization of the enolate anion intermediate (or transition state) derived from a thioester substrate by an "oxyanion hole" (*3*). By means of this conserved structural motif, members of the crotonase superfamily catalyze a wide range of reactions such as dehalogenation, hydration/dehydration, decarboxylation, formation and cleavage of carbon−carbon bonds, and the hydrolysis of thioesters (*3*).

In this work, we present HPLC, ESI-MS, and ^{1}H NMR data aimed at investigating the different reactions catalyzed by CarB and characterizing their products and intermediates. We have identified the intermediates of the CarB reaction with L-P5C and malonyl-CoA or methylmalonyl-CoA as CMPr-CoA and MeCMPr-CoA, respectively. We have shown that these intermediates partition between dissociating from the enzyme and completing turnover to CoASH and CMPr or MeCMPr. Using the measured steady-state kinetic constants for the CarB reaction with malonyl-CoA and L-P5C and for the CarB-catalyzed hydrolysis of CMPr-CoA, we have calculated the rate constants for each step of this reaction. By HPLC analysis we describe the decarboxylation of malonyl-CoA and methylmalonyl-CoA and the hydrolysis of propionyl-CoA and acetyl-CoA catalyzed by CarB. We also report the steady-state rate constants for these reactions. Finally, we describe a new expedient synthesis of L-P5C that can be easily adapted for the synthesis of D-P5C or isotopically labeled P5C.

EXPERIMENTAL PROCEDURES

Materials. Plasmid pET24a/*carB* was a generous gift from Dr. Rongfeng Li of this laboratory (*4*). Butyl-Sepharose 4FF was obtained from Amersham Biosciences (Piscataway, NJ) while all of the other resins, buffers, coupling enzymes, and CoASH esters were purchased from Sigma (St. Louis, MO). All of the reagents used in the chemical syntheses and in the colorimetric assays were from Aldrich (Milwaukee, WI) with the exception of DL-hydroxylysine and L-hydroxylysine, which were obtained from Sigma (St. Louis, MO) and from Degussa (Curbevoie, France), respectively.

Methods. The CarB extinction coefficient was obtained by performing quantitative amino acid analysis (AAA Laboratory, Merce Island, WA) on triplicate samples with measured A_{280} values. NMR spectra were obtained on a Varian Unity^{Plus} 400 spectrometer. NMR spectra recorded in D_2O are reported in parts per million (δ) relative to D_2O 4.80 ppm (^{1}H). (2*S*,5*S*)- and (2*S*,5*R*)-CMPr were synthesized as previously described (*7*). DL- and L-P5C were synthesized by the method of Williams and Frank (*14*) and quantified by the colorimetric assay with *o*-aminobenzaldehyde (ϵ_{440} = 2.58 mM^{-1} cm^{-1}) as described by Mezl and Knox (*15*). CoA ester concentrations were determined using ϵ_{260} = 15.4 mM^{-1} cm^{-1} (*16*). HPLC analysis was carried out on a Hewlett-Packard 1050 HPLC with a variable wavelength detector or an Agilent (Foster City, CA) 1100 HPLC with a diode array detector. ESI-MS spectra were acquired in the negative ion mode on a Finnigan LCQ Deca ion trap mass spectrometer with an ESI source. The capillary temperature was maintained at 225 °C. The samples, unless otherwise noted, were prepared in 1:1 dd_2O/methanol with 0.1% NH_4-OH prior to direct injection into the ESI chamber using a syringe pump at a flow rate of 25 μL/min. Typically 30 scans were averaged for each spectrum.

Overexpression and Purification of CarB. BL21(DE3) cells were transformed with pET24a/*carB* and grown at 37 °C in 2 × YT medium supplemented with 50 μg/mL kanamycin A. The cells were induced (OD_{600} = 0.65) with 1 mM IPTG at 28 °C, harvested by centrifugation 3 h postinduction, and frozen as a pellet in liquid nitrogen. Usually 14 g of cells was obtained from 3 L of medium. All purification procedures were carried out on ice or at 4 °C. The frozen cells (7 g) were resuspended to 0.3 g/mL in ice-cold lysis buffer (100 mM Tris-HCl, pH 8.0, 1.8 mM EDTA, 1 mM PMSF, 1 mM DTT) and broken by French press at 12000 psi. After removal of the cell debris by centrifugation, streptomycin sulfate was added to a final concentration of 3%. The supernatant obtained by centrifugation was subjected to ammonium sulfate fractionation at 35% and 65% saturation. The pellet from the 65% cut was resuspended in 25 mL of dialysis buffer (50 mM Tris-HCl, pH 7.5, 10 μM EDTA, 1 mM benzamidine, 1 mM DTT, 1 mM PMSF) and was dialyzed against the same buffer. The dialyzed suspension was loaded at 3 mL/min on a Q-Sepharose FF column (2.5 × 33 cm) preequilibrated with 50 mM Tris-HCl, pH 7.5, 1 mM DTT, and 100 mM NaCl. CarB was eluted with a 100−350 mM linear gradient of NaCl in 50 mM Tris-HCl, pH 7.5, and 1 mM DTT, and fractions were pooled on the basis of SDS−PAGE analysis. Ammonium sulfate was then added to a final concentration of 0.8 M. The ammonium sulfate solution was loaded on a butyl-Sepharose 4FF column (2.5 × 15 cm) preequilibrated with 50 mM Tris-HCl, pH 7.5, 1 mM DTT, and 0.8 M ammonium sulfate. CarB was eluted with a 0.8−0 M linear gradient of ammonium sulfate in 50 mM Tris-HCl, pH 7.5, and 1 mM DTT, and the fractions pooled on the basis of SDS−PAGE analysis were dialyzed against 50 mM Tris-HCl, pH 7.5. The enzyme was concentrated by ultrafiltration in an Amicon stirred cell over a YM10 membrane to 10 mg/mL and delivered dropwise into liquid nitrogen and stored at −80 °C. An average yield of the CarB purification is 8.6 mg of pure protein/g of wet cell paste (based on SDS−PAGE analysis; Figure S1). The protein concentration was measured spectrophotometrically at 280 nm using the extinction coefficient determined for CarB [ϵ_{280} = 0.96 (±0.03) mL mg^{-1} cm^{-1} (pH 7.0, 25 °C)].

Synthesis of L-*Pyrroline-5-carboxylate*. L-P5C was obtained by deprotection of (2*S*)-*tert*-butyl-*N*-Boc-5-hydroxy-L-prolinate (*7*) (0.1 g, 0.322 mmol) for 5 h in 1 N HCl with vigorous stirring. L-P5C was stored in 20 mM aliquots at 4 °C. A control reaction was run in 1 N DCl to confirm

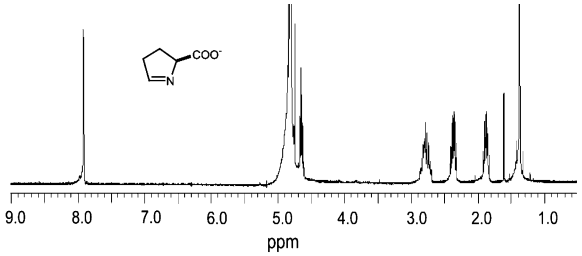


FIGURE 1: 400 MHz ^{1}H NMR spectrum in D_2O, pH 7.7, of L-P5C synthesized from (2*S*)-*tert*-butyl-*N*-Boc-5-hydroxy-L-prolinate.

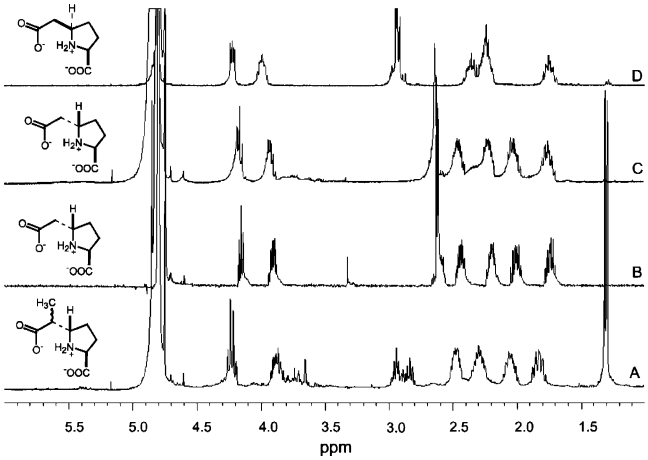


FIGURE 2: ^{1}H NMR spectra in D_2O of 6-methyl-(2*S*,5*S*)-CMPr (A) isolated from the CarB-catalyzed reaction with 1.3 mM methylmalonyl-CoA and 1.5 mM L-P5C, of chemically synthesized (2*S*,5*S*)-CMPr (B), of (2*S*,5*S*)-CMPr (C) isolated from the CarB-catalyzed reaction with 1.0 mM malonyl-CoA and 1.7 mM DL-P5C, and of chemically synthesized (2*S*,5*R*)-CMPr (D).

completion by ^{1}H NMR analysis (Figure 1) in D_2O, pH 7.7 with NaOD: δ 7.93 (1 H), 4.64 (1H), 2.77 (2H), 2.35 (1H), and 1.87 (1H).

D-P5C vs L-P5C as Substrate of CarB Reaction. Reactions were run at 22 °C in 100 mM KP_i, pH 7.8, with 40 μg/mL CarB for 1 h. When DL-P5C or L-P5C was limiting, the concentrations of DL-P5C or L-P5C and malonyl-CoA were 0.15 and 0.3 mM, respectively. Controls with malonyl-CoA limiting were run with concentrations of DL-P5C or L-P5C and malonyl-CoA of 0.25 and 0.1 mM, respectively. CoASH formation was determined by the colorimetric assay with the thiol reagent 4PDS (*17*).

Isolation and Characterization of (2S,5S)-CMPr and 6-Methyl-(2S,5S)-CMPr, Products of the CarB Reactions with Malonyl-CoA and with Methylmalonyl-CoA. Reactions were run at 22 °C in 100 mM KP_i, pH 7.8, and 1 mg/mL CarB. The reactions with 1.0 mM malonyl-CoA and with 1.3 mM methylmalonyl-CoA contained 1.7 mM DL-P5C and 1.5 mM L-P5C, respectively. After removal of CarB by ultrafiltration in an Amicon stirred cell over a YM10 membrane, the solution, titrated to pH 3−4 with acetic acid, was loaded onto a Dowex 50W-X8 column in the proton form preequilibrated with 0.2 N CH_3COOH. The column was then washed with 0.2 N CH_3COOH, and subsequently with D_2O. (2*S*,5*S*)-CMPr and 6-methyl-(2*S*,5*S*)-CMPr eluted with 0.3 N pyridine. After removal of the pyridine by rotary evaporation, the products were characterized by ^{1}H NMR spectroscopy (Figure 2) and ESI-MS. (2*S*,5*S*)-CMPr ^{1}H NMR and ESI-MS: δ 4.17 (t, 1H), 3.93 (m, 1H), 2.62 (m, 2H), 2.46 (m, 1H), 2.23 (m, 1H), 2.04 (m, 1H), 1.77 (m, 1H); *m*/*z* 172.5 [M − H] [MS/MS 128.1 ($-CO_2$)] for (2*S*,5*S*)-CMPr (Figure S2). 6-Methyl-(2*S*,5*S*)-CMPr ^{1}H NMR and ESI-MS: δ 4.22 (q, 1H), 3.89 (m, 1H), 2.94 and 2.84 (two m, 1H total), 2.47 (m, 1H), 2.30 (m, 1H), 2.05 (m, 1H), 1.83 (m, 1H), 1.30 (d, 3H); *m*/*z* 186.4 [M − H] [MS/MS 142.2 ($-CO_2$)] (Figure S3).

Investigations of the Reactions Catalyzed by CarB by HPLC. Reactions were run at 22 °C in 100 mM KP_i, pH 7.8, with 0.3 mM CoA esters and, if added, 0.5 mM L-P5C synthesized from L-hydroxylysine. The concentrations of the CoA ester substrates were 0.3 mM. CarB concentration varied from 1.28 to 1280 μg/mL depending on the substrates. Control reactions without enzyme were performed in parallel. Aliquots of the reaction were removed at different times and quenched in ice to a final concentration of 0.1 N HCl followed by the addition of CCl_4 with vigorous mixing and centrifugation. The aqueous layer was frozen in liquid nitrogen and stored at −20 °C prior to the analysis on an analytical Phenomenex Prodigy ODS3 column (4.6 × 250 mm). The column was preequilibrated in 60% solvent A (0.1 M $NH_4H_2PO_4$, 0.075 M $CH_3COO^-NH_4^+$, pH 4.65, with CH_3COOH) and 40% solvent B (70% solvent A, 30% CH_3OH) at a flow rate of 1 mL/min. The various compounds were eluted with a linear gradient (40−85% solvent B in 28 min, jump to 100% solvent B in 1 min, and hold for 10 min) and detected at 260 nm. This HPLC method is a modification of that previously reported (*18*). Unless otherwise noted, each peak was identified by co-injection with standards purchased from Sigma (St. Louis, MO). Retention times vary slightly due to the volatile nature of the solvents used.

Investigations of the Reactions Catalyzed by CarB by ESI-MS. The reactions were run and quenched as described above but with 1.5 mM L-P5C and 1.1 mM malonyl- or methylmalonyl-CoA. The products of the CarB reaction with methylmalonyl-CoA and L-P5C were purified by the HPLC method described above. The two unknown peaks were collected from multiple runs and desalted with a Phenomenex STRATA X column preequilibrated with 0.1% TFA. After the column was washed with 0.1% TFA, the CoA esters were eluted with a solution of 80% methanol and 20% of 0.1% TFA, and the lyophilized eluant was analyzed by ESI-MS as described in the Methods section: *m*/*z* 935.2 [M − H] for the first peak and *m*/*z* 935.3 [M − H] for the second peak (Figure S4). The products of the CarB reaction with malonyl-CoA and L-P5C were isolated and characterized by LC/ESI-MS on a ThermoFinnigan HPLC connected to a Finnigan LCQ Deca ion trap mass spectrometer with an ESI source. The reaction mixture was injected on an analytical Phenomenex Prodigy ODS3 column (4.6 × 250 mm) preequilibrated in 92% solvent C (20 mM $CH_3COO^-NH_4^+$, pH 6.3) and 8% solvent D (20 mM $CH_3COO^-NH_4^+$, pH 6.3, 80% methanol) at a flow rate of 0.5 mL/min. A linear gradient (8−80% solvent D for 20 min and hold at 80% solvent D for 10 min) was applied to the column, and the various compounds were detected at 260 nm and by ESI-MS in the negative ion mode. Malonyl-CoA, CMPr-CoA, and CoASH elute at 14.3 min (*m*/*z* 852.3 [M − H]), 15.6 min (*m*/*z* 921.4 [M − H]), and 16.5 min (*m*/*z* 766.5 [M − H]), respectively.

Enzymatic Synthesis and Purification of CMPr-CoA. CMPr-CoA was synthesized in a 1.0 mL CarB-catalyzed reaction, pH 8.0, consisting of 1 mM malonyl-CoA and 1

mM L-P5C. The 40−85% solvent B HPLC gradient method was used to purify CMPr-CoA (Figure S5). The CMPr-CoA peak was collected from multiple runs and desalted on a Phenomenex STRATA X column as described above (Figure S5): 0.288 μmol, 28.8% yield. ESI-MS: *m*/*z* 921.2 [M − H].

Determination of the Steady-State Kinetic Parameters for the CarB-Catalyzed Reactions. The reactions were run at 22 °C in a buffer system of piperazine/HEPES (*19*), pH 8.0, and were quenched in HCl/CCl_4 as described above at four different time points (usually 4, 8, 12, and 16 min) in addition to a zero time point. Each time point was analyzed by HPLC with the solvent B gradient method described above, and the amount of product formed was determined from a standard curve of known concentrations of CoASH obtained under identical conditions. All of the reactions were run in duplicate, and controls were performed in the absence of CarB. The initial velocities in the CarB-catalyzed reaction of L-P5C and malonyl-CoA were obtained from a linear fit of the sum of CoASH and CMPr-CoA produced at different time points. L-P5C was synthesized from L-glutamic acid as described above. Malonyl-CoA and L-P5C concentrations were respectively 50 and 500 μM when held constant. The concentrations for malonyl-CoA and L-P5C when varied were 1−25 and 2−100 μM, respectively. Initial velocity studies for the CarB-catalyzed hydrolysis of CMPr-CoA were performed under the same conditions, varying the CMPr-CoA concentrations from 1 to 50 μM. The CarB reactions with propionyl-CoA (concentrations varying from 0.05 to 6 mM) were monitored on an analytical Phenomenex Prodigy ODS3 column (4.6 × 250 mm) at 1 mL/min with the 65% solvent B isocratic method. Propionyl-CoA and CoASH elute at 21 and 5 min under this condition. The initial velocities of formation of propionyl-CoA from methylmalonyl-CoA (concentrations varying from 0.2 to 4 mM) were measured by analysis of the time point aliquots with the 70% solvent B isocratic method. Methylmalonyl-CoA and propionyl-CoA elute at 4 and 17 min. The 60% solvent B isocratic method was used in the initial velocity studies for the hydrolysis of acetyl-CoA (concentrations varying from 0.5 to 20 mM) to CoASH and for the decarboxylation of malonyl-CoA (concentrations varying from 0.1 to 4 mM) to acetyl-CoA. Malonyl-CoA, acetyl-CoA, and CoASH elute at 4.5, 11.5, and 6 min, respectively. To ensure the absence of competing hydrolysis of either propionyl-CoA or acetyl-CoA, respectively, in the decarboxylation reactions of methylmalonyl-CoA and malonyl-CoA, the longest time point aliquots were also analyzed with the 40−85% solvent B gradient method described above. All data were fitted using the FORTRAN programs of Cleland (*20*). All initial velocities were fitted to eq 1, except the data for the decarboxylation of malonyl-CoA which were fitted to eq 2.

$$v/[E_0] = k_{cat}A/(K_m + A) \quad (1)$$

$$\log(v/[E_0]) = \log(k_{cat}A/(K_m + A + A^2/K_i)) \quad (2)$$

RESULTS

Characterization of the CarB Reactions with Malonyl-CoA and with Methylmalonyl-CoA. CarB catalyzes the reaction of malonyl-CoA and DL-P5C to CoASH and CMPr (Figures 2 and S2) (*12*). NMR analysis of the purified product of this reaction was carried out by us (Figure 2) and others (*12*) to narrow the assignments of the stereochemistry of the products by excluding (2*R*,5*S*)- and (2*S*,5*R*)-CMPr. Although the NMR spectra of the isolated product of the CarB reaction and of chemically synthesized (2*S*,5*S*)-CMPr are identical, this comparison does not distinguish between the two enantiomers, (2*R*,5*R*)- and (2*S*,5*S*)-CMPr, that can be formed from DL-P5C. Colorimetric quantification of the CoASH product in the CarB reaction with either DL- or L-P5C with excess malonyl-CoA was used to determine the stereochemical requirement of the reaction. When the reaction was run to completion, the moles of CoASH with DL-P5C as a substrate were half of those obtained in the reaction with L-P5C, thus establishing that the latter is the only substrate of CarB, and the product of this reaction is (2*S*,5*S*)-CMPr. When methylmalonyl-CoA is taken as one of the substrates, CarB catalyzes the formation of 6-methyl-CMPr as shown by ESI-MS and ^{1}H NMR (Figures 2 and S3). Although this activity was previously reported, no experimental details were provided (*12*). The ^{1}H NMR spectrum of the purified product showed that it is a mixture of epimers at C-6 (δ 2.94 and 2.84, two multiplets that integrate for 1 H total) with the *S* configuration at C-2 and C-5 (Figure 2).

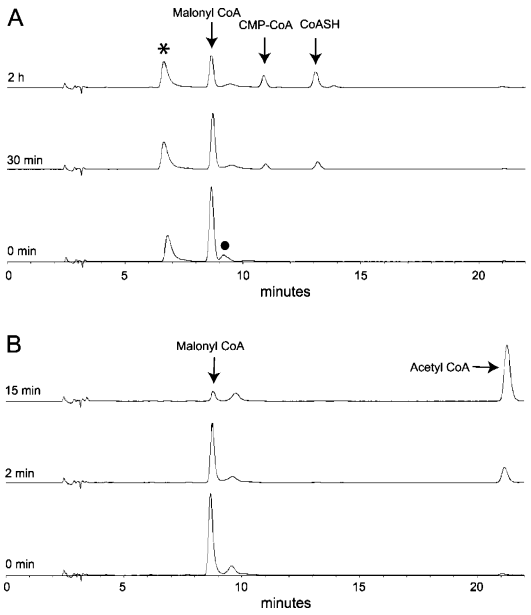


FIGURE 3: Reverse-phase HPLC analysis using the 40−85% solvent B gradient method of the reaction catalyzed by CarB in 100 mM KP_i, pH 7.8; detection at 260 nm. (A) HPLC time analysis of the reaction of 0.3 mM malonyl-CoA and 0.5 mM L-P5C catalyzed by 1.28 μg/mL CarB. (B) HPLC time analysis of the decarboxylation of 0.3 mM malonyl-CoA catalyzed by 25.6 μg/mL CarB. (*) Impurity from L-P5C obtained from L-hydroxylysine. (•) Less polar impurity present in all commercially obtained CoA compounds.

Identification of an Intermediate in the CarB Reaction with Malonyl-CoA and Methylmalonyl-CoA. A time course HPLC analysis of the CarB reaction with malonyl-CoA and L-P5C showed, concomitant with the decrease of the malonyl-CoA peak, the appearance and then increase of a peak coeluting with CoASH and of a second unknown peak (Figure 3A). When the reaction was run in the presence of increased concentrations of CarB, the unknown compound appeared after 2 min and eventually was all converted to CoASH after 2 h (Figure S6). To further investigate the identity of the

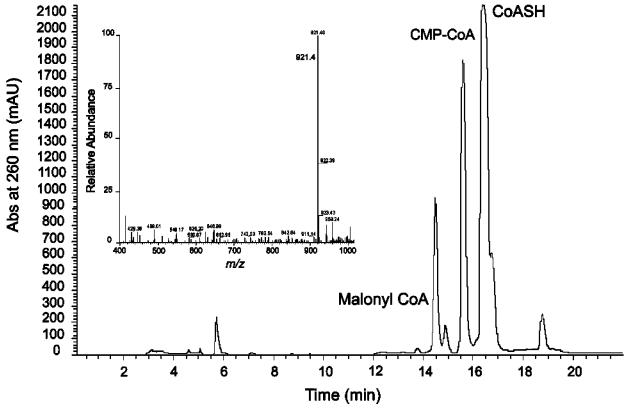


FIGURE 4: Identification of CMPr-CoA (15.6 min, *m*/*z* 921.4) in the CarB-catalyzed reaction with 1.1 mM malonyl-CoA and 1.5 mM L-P5C, pH 7.8, by LC/ESI-MS analysis, performed as described in Experimental Procedures. The HPLC trace was detected at 260 nm. Inset: ESI spectrum of the 15.6 min peak obtained as described in Experimental Procedures.

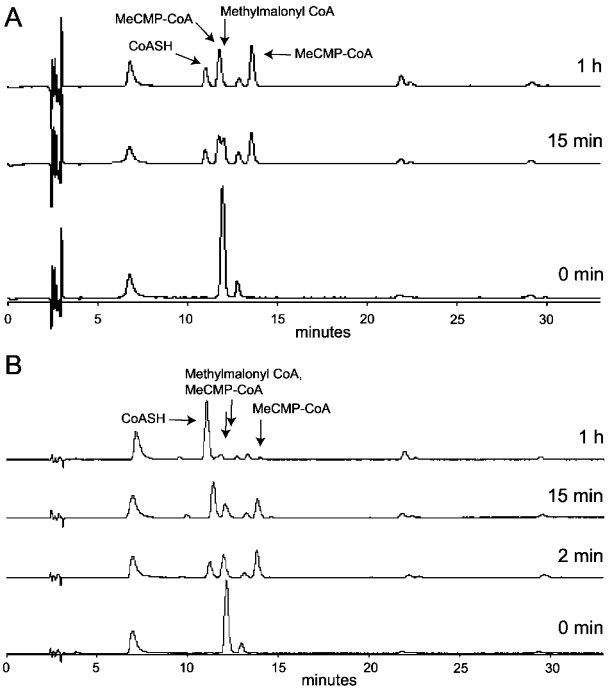


FIGURE 5: Reverse-phase HPLC analysis with the 40−85% solvent B gradient method of the reaction catalyzed by CarB in 100 mM KP_i, pH 7.8, with 0.3 mM methylmalonyl-CoA and 0.5 mM L-P5C; detection at 260 nm. (A) HPLC time analysis of the reaction catalyzed by 25.6 μg/mL CarB. (B) HPLC time analysis of the reaction catalyzed by 512 μg/mL CarB.

unknown compound, a new HPLC gradient was devised to separate the CoASH, malonyl-CoA, and the unknown peak with a solvent system compatible with the ESI-MS (low concentration of a volatile buffer). LC/ESI-MS of a time aliquot of the CarB reaction revealed that the unknown peak had a *m*/*z* of 921.4 [M − H] corresponding to CMPr-CoA (Figure 4). Moreover, the absorbance spectrum of CMPr-CoA showed the typical absorbance shoulder at 232 nm of CoA esters (*21*) (data not shown). When the CarB reaction with methylmalonyl-CoA was monitored by HPLC, not one but two peaks appeared and increased with the CoASH peak simultaneous with the disappearance of the methylmalonyl-CoA peak (Figure 5A). The ratio of these two peak heights remained constant during the reaction, and they eventually

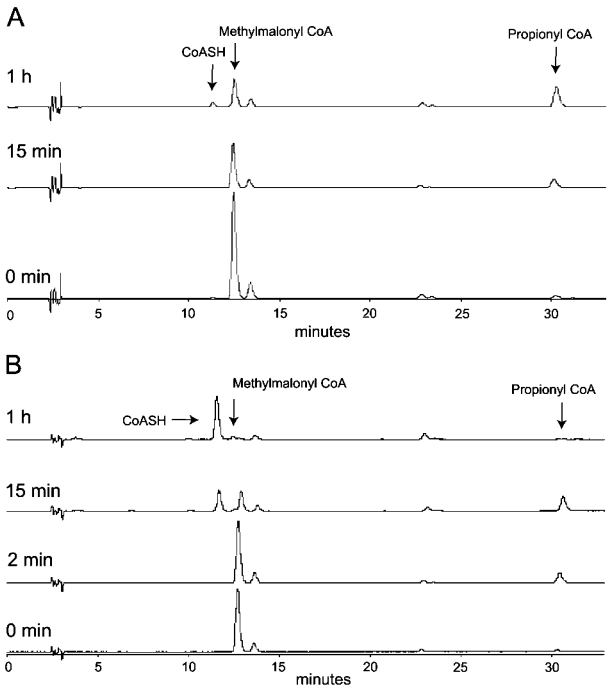


FIGURE 6: Reverse-phase HPLC analysis with the 40−85% solvent B gradient method of the decarboxylation of 0.3 mM methylmalonyl-CoA in 100 mM KP_i, pH 7.8; detection at 260 nm. (A) HPLC time analysis of the reaction catalyzed by 51.2 μg/mL CarB. (B) HPLC time analysis of the reaction catalyzed by 512 μg/mL CarB.

were converted to CoASH (Figure 5). As for CMPr-CoA, the absorbance spectra of these compounds showed the typical shoulder at 232 nm for CoA esters (*21*). Unfortunately, the HPLC method applied in the LC/ESI-MS analysis did not separate these two peaks from the methylmalonyl-CoA and the CoASH peaks. Purification and desalting steps were, therefore, necessary to obtain a solution of each unknown compound for ESI-MS analysis. The *m*/*z* 935.2 [M − H] (Figure S4) measured for both compounds corresponds to the two C-6 epimers of MeCMPr-CoA. CarB-catalyzed hydrolysis of these compounds yields the two MeCMP epimers observed by ^{1}H NMR (Figure 2).

CarB-Catalyzed Decarboxylation and Hydrolysis Reactions. Time course HPLC analyses of the CarB reactions with malonyl-CoA or with methylmalonyl-CoA in the absence of L-P5C showed the ability of CarB to catalyze the decarboxylation of these CoA esters by the appearance of peaks coeluting with acetyl-CoA and propionyl-CoA, respectively (Figures 3B and 6). An additional activity of CarB was observed in the study of the decarboxylation reaction of methylmalonyl-CoA. In Figure 6A the 1 h HPLC analysis showed, besides the increase in the propionyl-CoA peak, the appearance of a CoASH peak. This result was further confirmed by running the reaction with 10 times more CarB (Figure 6B). As shown after 2 min, only the decarboxylation product can be observed, but after 15 min a similar amount of CoASH and propionyl-CoA can be detected. After 1 h all of the methylmalonyl-CoA and the propionyl-CoA was transformed to CoASH (Figure 6B). The ability of CarB to catalyze the hydrolysis reaction of propionyl-CoA and acetyl-CoA to CoASH was verified by a time course HPLC analysis as shown in Figure 7. In the absence of L-P5C, CarB produces CoASH when acetyl-CoA is the substrate (Figure 7B). CarB was also able to catalyze the hydrolysis of CMPr-

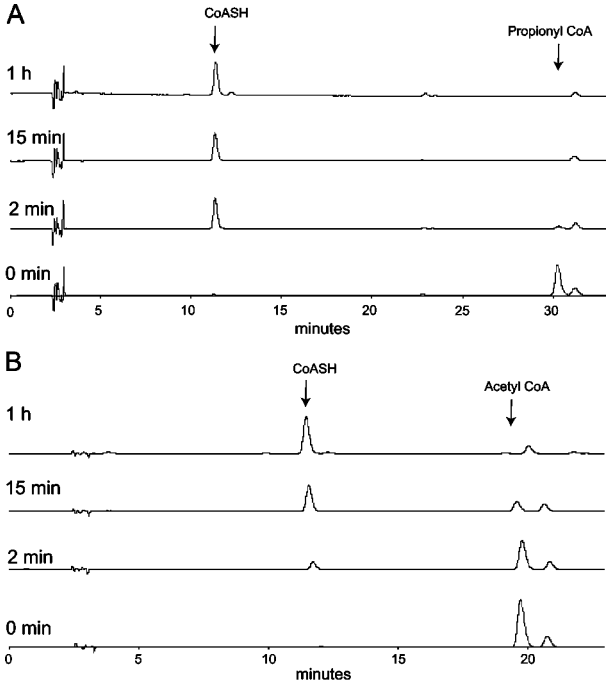


FIGURE 7: Reverse-phase HPLC analysis with the 40−85% solvent B gradient method of the hydrolysis of 0.3 mM propionyl-CoA (A) and 0.3 mM acetyl-CoA (B) catalyzed by 1280 μg/mL CarB in 100 mM KP_i, pH 7.8; detection at 260 nm.

CoA as observed in Figure S6 and confirmed in the initial velocity studies described below.

A New Synthesis of L*-P5C*. Published procedures for the synthesis DL-P5C entail periodate oxidation of DL-*allo*-δ-hydroxylysine and regeneration from DL-pyrroline-5-carboxylic acid 2,4-dinitrophenylhydrazine hydrochloride double salt (both starting materials can be purchased from Sigma) (*15*, *22*). Synthesis of L- or D-P5C is hampered by long and low-yielding synthetic protocols for L- and D-*allo*-δ-hydroxylysine (*23*). The high cost and the presence of high percentages of salt and impurities (see peak at 6.7 min in the HPLC traces of Figures 3A and 5) in the commercially available L-hydroxylysine compelled us to develop a new procedure for the synthesis of L-P5C. We have previously reported (*7*) the four-step synthesis of (2*S*)-*tert*-butyl-*N*-Boc-5-hydroxy-L-prolinate from L-pyroglutamate with an overall yield of 69%. The stability of this protected form of L-P5C allows for storage over a long time period. L-P5C can then be easily obtained, when needed, following an acidic deprotection step and is stable for 4 weeks when stored at 4 °C in 20 mM aliquots, as previously reported (*22*). P5C has to be stored in aqueous solution since polymerization will occur following any concentration step such as rotary evaporation or lyophilization (*15*, *22*). Because of these limitations, complete removal of *tert*-butyl alcohol, a product of the deprotection, is difficult, and its presence is shown by the peak at 1.37 ppm in the NMR spectrum of L-P5C (Figure 1). The chemical shifts of the ^{1}H NMR of L-P5C are consistent with previously reported NMR analysis (*22*). The main advantage of this procedure is that it can be easily and cheaply modified to synthesize D-P5C and radiolabeled or stable isotope labeled P5C using commercially available D- and labeled glutamic acids, respectively. L-P5C obtained with this procedure was used in the determination of the steady-state rate constants for the reactions catalyzed by CarB.

Steady-State Kinetic Parameters for the CarB-Catalyzed Reactions. The steady-state kinetic constants measured at 22 °C and at pH 8.0 for all of the CarB-catalyzed reactions are summarized in Table 1. Since CMPr-CoA is released as well as CoASH in the CarB-catalyzed reaction with L-P5C and malonyl-CoA, the initial velocities were measured by HPLC and not by a colorimetric assay with a thiol reagent such as 4PDS or 5,5′-dithiobis(2-nitrobenzoic acid). Unfortunately, the HPLC assay devised for the reaction with malonyl-CoA could not be applied to measure the initial velocities in the CarB-catalyzed reaction with L-P5C and methylmalonyl-CoA due to the absence of baseline separation between the methylmalonyl-CoA peak and one of the MeCMPr-CoA diastereomers (Figure 5A). An estimate of the rate of the reaction with methylmalonyl-CoA can be obtained by comparing the sum of the areas of the CMPr-CoA and CoASH peaks after 1 h (data not shown) of the reaction with 1.28 μg/mL CarB to the sum of the areas of the MeCMPr-CoA and CoASH peaks of the reaction at the same time point with 25.6 μg/mL CarB (Figure 5A). After correction for the amount of enzyme present, the reaction with methylmalonyl-CoA is approximately 10-fold slower than the reaction with malonyl-CoA.

As shown in the time course HPLC analyses, CMPr-CoA and MeCMPr-CoA are formed and released from the enzyme during the course of the reaction. The intermediacy of these compounds was first supported by their hydrolysis to CoASH after 1 or 2 h of the CarB reaction (Figures 5B and S6). To further support the intermediate nature of CMPr-CoA and to kinetically characterize its hydrolysis to CoASH, the CarB-catalyzed hydrolysis of isolated CMPr-CoA was monitored by HPLC, and its steady-state kinetic parameters were measured (Table 1). Likewise, CarB also catalyzes the hydrolysis of propionyl-CoA and acetyl-CoA to CoASH (Figure 7). Removal of the proline moiety in CMPr-CoA, as shown in the reaction with acetyl-CoA, causes a 600-fold increase in K_m. By addition of a methyl group approaching the CMPr-CoA structure, as in the reaction with propionyl-CoA, the K_m decreases by 3-fold compared to the acetyl-CoA, and it is 190-fold higher than the K_m for the CMPr-CoA hydrolysis (Table 1).

The steady-state rate constants of the previously reported decarboxylation reactions of malonyl-CoA and methylmalonyl-CoA in the absence of L-P5C were measured by HPLC assay. CarB displayed a marked substrate inhibition with malonyl-CoA but an unusual steady-state kinetic plot probably due to the different inhibitory effects of the two enantiomers of methylmalonyl-CoA. Therefore, the standard substrate inhibition equation (eq 2) was used to fit the kinetic data for malonyl-CoA but not for methylmalonyl-CoA. To obtain approximate values for the steady-state rate constants for the decarboxylation of methylmalonyl-CoA, the data were tentatively fitted to the Michaelis−Menten equation (eq 1).

DISCUSSION

CarB is a member of the crotonase superfamily. Of all the members of this superfamily, extensive mechanistic and structural studies have been reported only for crotonase itself and 4-chlorobenzoyl-CoA dehalogenase (*24*−*26*). Sequence

Table 1: Steady-State Kinetic Parameters for CarB Measured at 22 °C and pH 8.0[a]

substrate	reaction	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m ($mM^{-1}\ s^{-1}$)
L-P5C	(2*S*,5*S*)-CMPr formation	1.52 ± 0.04	0.0015 ± 0.0004	984
malonyl-CoA	(2*S*,5*S*)-CMPr formation	1.7 ± 0.2	0.0027 ± 0.0009	630
malonyl-CoA[b]	decarboxylation	1.5 ± 0.3	1.2 ± 0.3 (K_i = 0.9 ± 0.2)	1.23
methylmalonyl-CoA[c]	decarboxylation	0.07 ± 0.01	0.6 ± 0.3	0.10
CMPr-CoA	hydrolysis	0.53 ± 0.05	0.009 ± 0.002	58
propionyl-CoA	hydrolysis	0.190 ± 0.009	1.7 ± 0.2	0.110
acetyl-CoA	hydrolysis	0.043 ± 0.002	5.4 ± 0.7	0.0081

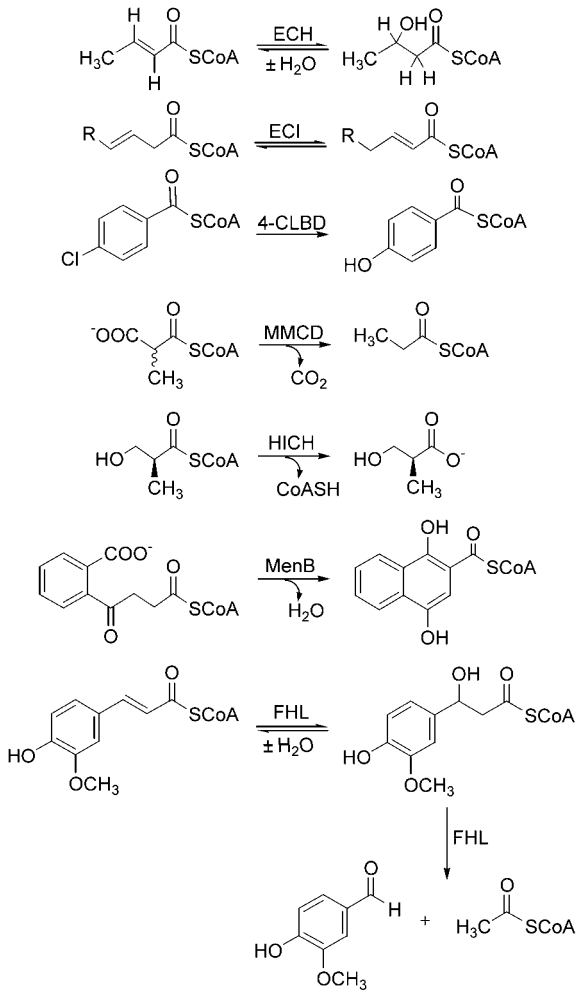
[a] The kinetic parameters were determined by fitting the data to eq 1 unless otherwise noted. Reactions were performed as described under Experimental Procedures. [b] These kinetic parameters were determined by fitting the data to eq 2. [c] See Results.

homology among these enzymes is low (often less than 20%), and no common denominator between function and active site residues has been found (*27*). Two perpendicular layers of β-sheet surrounded by α-helices are the structural elements of the common fold characteristic of members of this superfamily (*3*). Within this fold, located in the active site, are two structurally conserved peptidic NH groups that form an oxyanion hole, the key to the apparent mechanistic diversity among members of the crotonase superfamily (*3*). By stabilizing an anionic intermediate, either a thioester enolate or a thioester tetrahedral intermediate in the oxyanion hole, the enzymes of this superfamily are able to catalyze a wide variety of reactions from hydration (crotonase, ECH), isomerization (3,2-*trans*-enoyl-CoA isomerase, ECI), and dehalogenation (4-chlorobenzoyl-CoA dehalogenase, 4-CLBD) to decarboxylation (methylmalonyl-CoA decarboxylase, MMCD), hydrolysis of CoA esters (3-hydroxyisobutyryl-CoA hydrolase, HICH), C−C bond formation (1,4-dihydroxynaphthoyl-CoA synthase, MenB), and C−C bond cleavage (feruloyl-CoA hydratase/lyase, FHL) (Scheme 2) (*3*).

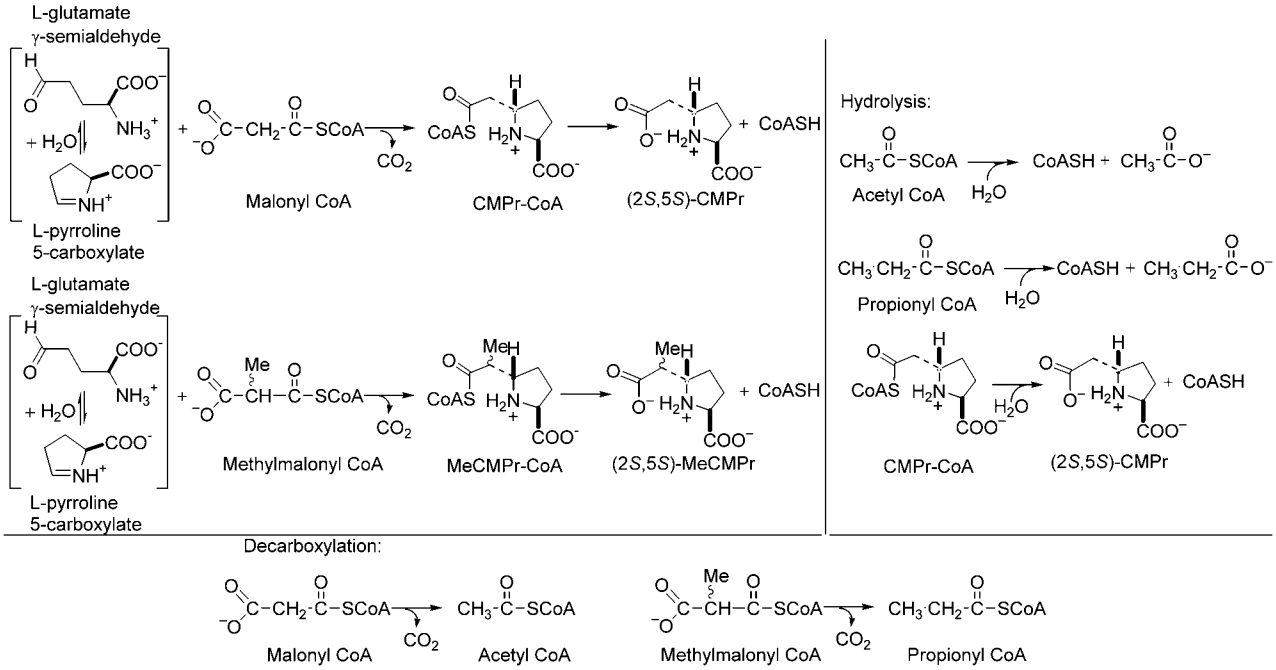
Among these homologues, CarB stands out as the first enzyme to combine in one overall reaction three activities of the crotonase superfamily. By HPLC and ESI-MS analysis we have shown that the reaction catalyzed by CarB with malonyl-CoA and L-P5C encompasses decarboxylation, C−C bond formation, and thioester bond hydrolysis steps (Scheme 3). Only one other member of the crotonase superfamily catalyzes a C−C bond forming reaction, 1,4-dihydroxynaphthoyl-CoA synthase (24% sequence identity to CarB) (*28*), and the CarB reaction is the first case with an amino acid as a cosubstrate. The decarboxylation of malonyl-CoA and methylmalonyl-CoA catalyzed by CarB is reminiscent of the decarboxylation of methylmalonyl-CoA by methylmalonyl-CoA decarboxylase (22% sequence identity to CarB) (*29*). None of the catalytic residues assigned on the basis of the crystal structures (*29*, *30*) for either enzyme is conserved in CarB. We have also described the hydrolase activity of CarB with acetyl-CoA, propionyl-CoA, and CMPr-CoA that is reminiscent of the hydrolysis of 3-hydroxyisobutyryl-CoA to CoASH catalyzed by 3-hydroxyisobutyryl-CoA hydrolase (14.4% sequence identity to CarB) (*31*).

The formation and characterization of CMPr-CoA, upon reaction with malonyl-CoA and L-P5C, and the ability of CarB to independently hydrolyze synthetic CMPr-CoA to CoASH are proof of the intermediacy of CMPr-CoA in the CarB-catalyzed reaction (Scheme 3, Table 1). Curiously, in the reaction catalyzed by CarC, the last enzyme in the carbapenem biosynthetic pathway, the intermediate, (3*S*,5*R*)-carbapenem-3-carboxylic acid, has been detected in solution and, when added to a CarC solution with the necessary cofactor, was transformed into (5*R*)-carbapenem-3-carboxylic acid (*6*). "Sloppy" wild-type enzymes that release intermediates into solution have been previously reported in the literature, especially for secondary metabolic enzymes (*32*−*35*). Among these, of particular interest is feruloyl-CoA hydratase/lyase, the only other member of the crotonase superfamily that combines two activities in one net reaction (*36*). This enzyme catalyzes the hydration and cleavage of feruloyl-CoA to acetyl-CoA and vanillin (Scheme 2). The intermediate of this reaction, 4-hydroxy-3-methoxyphenyl-

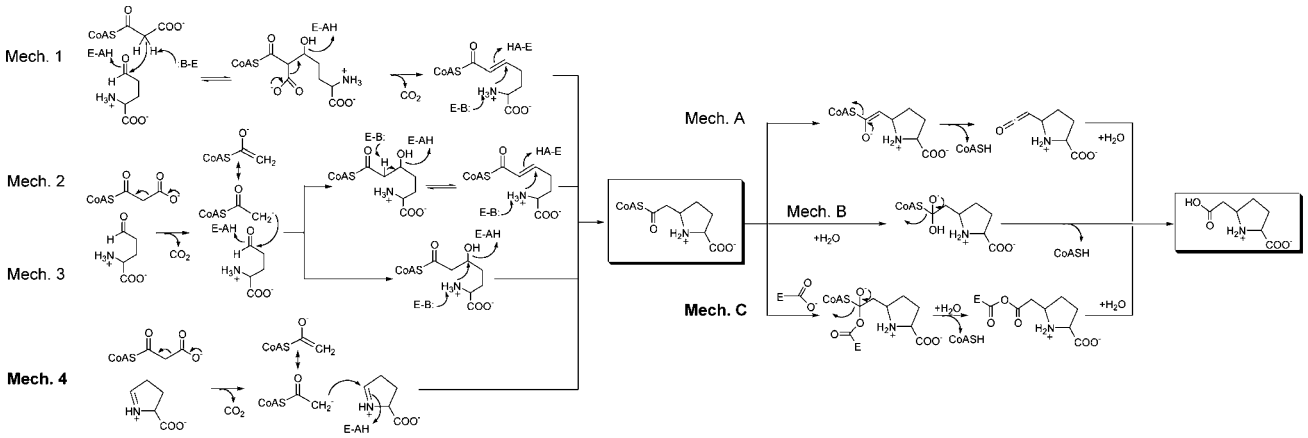
Scheme 2: Reactions Catalyzed by the Crotonase Superfamily



Scheme 3: Three Reactions Catalyzed by CarB



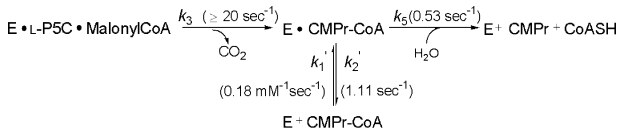
Scheme 4: Possible Mechanisms for the CarB-Catalyzed Reaction



hydroxypropionyl-CoA, was found to be a substrate for the cleavage reaction and also for the reverse hydration reaction (*36*). The peculiar characteristic of releasing the intermediates of CarB and feruloyl-CoA hydratase/lyase is a consequence of evolutionary divergence to less than catalytic perfection (*3*). Nature seems to preserve a specific structural feature involved in a mechanistic strategy (such as the conserved oxyanion hole responsible for stabilizing an anionic thioester intermediate in the crotonase superfamily) and modifies other aspects of the reaction to give rise to a new enzymatic activity based on a conserved mechanistic theme and new substrate specificity (*3*). Therefore, the common fold present in the CarB active site, and responsible for stabilizing the enolate intermediate formed from the decarboxylation of malonyl-CoA, is now employed in the thioester hydrolysis step. What would have been the product of this reaction, CMPr-CoA, now becomes the intermediate for the last step of the reaction reflecting changes in the active site to accommodate an additional catalytic event employing the same mechanistic strategy.

Unfortunately, the assignment of CMPr-CoA as an intermediate in the CarB-catalyzed reaction with malonyl-CoA and L-P5C does not distinguish among the four mechanisms that can be predicted for this reaction (Scheme 4). These mechanisms are differentiated by two main features: first, the order of the decarboxylation step with respect to the C−C bond formation step; second, the chemical structure of L-P5C bound in the active site of the enzyme. In mechanism 1, decarboxylation succeeds the Perkin-like condensation of glutamate semialdehyde, whereas decarboxylation of malonyl-CoA occurs prior to the addition step in the other mechanisms. The characterized independent decarboxylation reactions of methylmalonyl-CoA and malonyl-CoA exclude mechanism 1. It is more difficult to distinguish among the remaining three mechanisms. Mechanism 4 stands out because it assumes that the closed form of the substrate binds to the active site, whereas the active form of the substrate for the remaining mechanisms is glutamate semialdehyde. The pH-dependent equilibrium between the open and closed forms of P5C previously reported (*37*) shows that at pD 6.2 the ratio of closed to open form, where the open form is the hydrated glutamate semialdehyde (*37*), is unity. However, this ratio becomes 26 times more favorable to the imine at pD 7.4 (*37*). The extremely low K_m for L-P5C measured at

Scheme 5: Minimal Kinetic Scheme of the CarB-Catalyzed Reactions



pH 8.0 (Table 1), where the equilibrium is shifted fully toward the closed form (*37*), points to mechanism 4 as the most likely scenario. We propose that the enolate formed after decarboxylation of malonyl-CoA reacts with the iminium form of the substrate to yield the identified intermediate CMPr-CoA (Scheme 4). Nucleophilic attack of the enolate occurs stereospecifically on the *re* face of the iminium ion as shown by the formation of only (2*S*,5*S*)-CMPr as the product of reaction with L-P5C. In the CarB-catalyzed reaction with methylmalonyl-CoA the two epimers of MeCMPr-CoA are formed in a 7:8 ratio. This result is in agreement with both enantiomers acting as substrates of CarB, given that the unreacted methylmalonyl-CoA racemizes rapidly in solution and that the reaction is likely to proceed through inversion of configuration as shown in 2-ketocyclohexanecarboxyl-CoA hydrolase (*38*).

Thioester hydrolysis in the crotonase superfamily was recently proposed to proceed through three different mechanisms via a ketene intermediate (Scheme 4, mechanism A), an anionic tetrahedral intermediate (Scheme 4, mechanism B), and an anhydride intermediate (Scheme 4, mechanism C) (*39*). In 3-hydroxyisobutyryl-CoA hydrolase, the only other member of the crotonase superfamily known to hydrolyze CoA esters, the reaction proceeds through an anhydride enzyme-bound intermediate (*39*). Mutagenesis studies on this enzyme allowed the assignment of Glu143 as the nucleophile in the hydrolysis reaction (*39*). This residue is conserved in CarB and its orthologues. Similarly, the CarB-catalyzed hydrolysis of CMPr-CoA could involve an anhydride intermediate with Glu131 acting as the nucleophile (Scheme 4, mechanism C). The implication that the common fold of the crotonase superfamily stabilizes the CarB reaction, both at the enolate intermediate of the decarboxylation step and at the anionic tetrahedral intermediate/transition state of the hydrolysis step, is one added piece of evidence of the adaptability of this structural motif to decrease the overall activation energy for different reactions.

The ability of CarB to accept either malonyl-CoA and L-P5C or CMPr-CoA as substrates allows the calculation of the rate constants for both the conversion of malonyl-CoA and L-P5C to CMPr-CoA and of CMPr-CoA to (2*S*,5*S*)-CMPr and CoASH (Scheme 5). In Scheme 5 a minimal kinetic scheme of these reactions is shown. The product ratio, *R*, of [CMPr-CoA]/[CoASH] was found on average to equal 2.1 in the CarB reaction with malonyl-CoA and L-P5C. By solving the equations for the product ratio [$=k_2'/k_5$] and for the K_m for CMPr-CoA [$=(k_2'+k_5)/k_1'$] and knowing that the k_{cat} value obtained when CMPr-CoA is the substrate is equal to k_5, the values for k_2' and k_1' were calculated and are reported in Scheme 5. The k_{cat} value measured for the reaction with L-P5C and malonyl-CoA is a function of k_3, k_5, and k_2' [$k_{cat} = k_3(k_5 + k_2')/(k_3 + k_5 + k_2')$, eq 3]. Once k_2' and k_5 were determined, eq 3 could be written as a function of $k_3 = 1.64k_{cat}/(1.64 - k_{cat})$ (eq 4). A value of 20 s^{-1} for k_3 was obtained from the turnover number measured with saturating malonyl-CoA ($k_{cat} = 1.52 \pm 0.04$ s^{-1}). Since the k_{cat} values measured with saturating L-P5C or malonyl-CoA are so close to 1.64, it is not possible to get a precise solution other than that k_3 is large and is equal to or greater than 20 s^{-1}.[2] These calculated rate constants suggest that either the hydrolysis of CMPr-CoA and/or the (2*S*,5*S*)-CMPr and CoASH dissociation is partially rate limiting while the decarboxylation and the C−C bond formation steps are faster. The k_2' of CMPr-CoA is roughly 6 and 2 times faster than k_1' and k_5, respectively, allowing detection of the intermediate in solution. Considering that no active site catalytic residues are conserved in the crotonase superfamily and the paucity of mechanistic studies on these enzymes, this analysis in combination with the L-P5C-independent decarboxylation and hydrolysis reactions will be extremely useful in future mutagenesis studies aimed at the identification of the residues involved in each catalytic step of the CarB-catalyzed reaction.

Even though the active site of CarB is specific for L-P5C and catalyzes addition to the *re* face of this probable intermediate, it has some degree of flexibility for substitution at the methylene position of malonyl-CoA as shown by the CarB-catalyzed reactions with racemic methylmalonyl-CoA. These results, in combination with previous studies on the broad substrate specificity of CarA (*7*), highlight the feasible application of CarB and CarA in the preparation of new carbapenems. In addition, this work illustrates the importance of CarB as a new paradigm among members of the crotonase superfamily and whose understanding could shed light on the evolutionary process of the crotonase fold. The reported kinetic analysis will facilitate the assignment of catalytic residues in future mutagenesis studies on the three reported activities of CarB, decarboxylation, hydrolysis, and C−C bond formation. Conserved residues predisposing specific activities could eventually be identified in the crotonase superfamily by comparing the catalytic residues of CarB with those, for example, of methylmalonyl-CoA decarboxylase, 1,4-dihydroxynaphthoyl-CoA synthase, and 3-hydroxyisobutyryl-CoA hydrolase. If such an assignment is possible, the link between structure and conserved residues with function might allow activity predictions for various members of the crotonase superfamily.

ACKNOWLEDGMENT

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SUPPORTING INFORMATION AVAILABLE

SDS−PAGE analysis of CarB purification, ESI-MS and MS-MS spectra of (2*S*,5*S*)-CMPr and 6-methyl-(2*S*,5*S*)-CMPr, and ESI-MS spectra of MeCMPr-CoA, plus HPLC analysis of the enzymatic synthesis and of the isolated CMPr-CoA and time course HPLC analysis of the CarB reaction with malonyl-CoA and L-P5C. This material is available free of charge via the Internet at http://pubs.acs.org.

[2] Considering that k_2' and k_5 are very similar and contribute to the overall rate limitation of the reaction, calculation of k_3 using the turnover number measured with saturating L-P5C ($k_{cat} = 1.7 \pm 0.2$ s^{-1}) is hampered by the comparatively large experimental error on this k_{cat} value.

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