Carboxymethylproline Synthase (CarB), an Unusual Carbon-Carbon Bond-forming Enzyme of the Crotonase Superfamily Involved in Carbapenem Biosynthesis*

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Carboxymethylproline synthase (CarB) catalyzes the committed step in the biosynthesis of (S)-1-carbapen-2-em-3-carboxylate, the simplest member of the carbapenem family of β-lactam antibiotics, some of which are used clinically. CarB displays sequence homology with members of the crotonase family including enoyl-CoA hydratase (crotonase) and methylmalonyl-CoA deacetylase. The CarB reaction has been proposed to comprise condensation of acetyl coenzyme A (AcCoA) and glutamate semi-aldehyde to give (2S,5S)-carboxymethylproline (2S,5S)-CMP. (2S,5S)-CMP is then cyclized in an ATP-driven reaction catalyzed by CarA to give a carbapenam, which is subsequently epimerized and desaturated to give a carbapenem in a CarC-mediated reaction. Here we report the purification of recombinant CarB and that it exists predominantly in a trimeric form as do other members of the crotonase family. AcCoA was not found to be a substrate for CarB. Instead malonyl-CoA was found to be a substrate, efficiently producing (2S,5S)-CMP in the presence of glutamate semi-aldehyde. In the absence of glutamate semi-aldehyde, mass spectrometric analysis indicated that CarB catalyzed the decarboxylation of malonyl-CoA to AcCoA. The reactions of CarB, CarA, and CarC were coupled in vitro demonstrating the viability of malonyl-CoA as a carbapenem precursor. CarB was also shown to accept methylmalonyl CoA as a substrate to form 6-methyl-(2S,5S)-CMP, which in turn is a substrate for CarA. The implications of the results for the biosynthesis of both carbapenem-3-carboxylate and C-2/C-6-substituted carbapenems, such as thienamycin, are discussed.

Members of the carbapenem family of β-lactam antibiotics are medicinally useful as they possess a broad range of activity and display resistance to hydrolysis by serine β-lactamases (1, 2). Although some carbapenems are natural products, the medicinally used antibiotics are produced by chemical synthesis, as it has not yet been possible to develop fermentation based procedures. Therefore there is interest in defining the biosynthetic pathway to the carbapenems with a view to its manipulation to enable more efficient production. Although at least 11 enzymes have been implicated in the biosynthesis of the C-2- and C-6-substituted carbapenem thienamycin (3), only three enzymes are required for the biosynthesis of the simplest naturally occurring carbapenem (R)-1-carbapen-2-em-3-carboxylate ((S)-carbapenem), which is produced by Pectobacterium carotovora, Serratia marcescens, and Photorhabdus luminescens (4–8) (Scheme 1). The latter two enzymes in this pathway, CarC (carbapenem synthase) and CarA (carbapenem synthetase) have been isolated and their crystal structures reported (9–11). CarA and CarC are closely related to β-lactam synthetase and clavamic acid synthase, which also catalyze β-lactam formation and oxidative reactions, respectively, in the biosynthesis of the β-lactamase inhibitor clavulanic acid and other clavams (12–15). However CarB, which catalyzes the interesting first step in the pathway to (5R)-carbapenem, has no homologue in clavam biosynthesis, where the committed step is catalyzed by a thiamine pyrophosphate-dependent enzyme, carboxethyl-arginine synthase (16), the crystal structure of which has now been solved (17). CarB catalysis is proposed to involve condensation of acetyl coenzyme A (AcCoA) with glutamate semi-aldehyde (GSA) (9). It has been reported to display sequence homology with enoyl-CoA hydrolases and napthoate synthetases (5). Both of these subfamilies of enzymes belong to the crotonase superfamily, which encompasses a diverse range of enzymes catalyzing reactions involving enolates derived from CoA ester substrates. Structural studies imply that all members of the family feature a similar scaffold, which enables binding of the CoA substructure and provides an oxyanion hole to stabilize an enolate anion. The rest of the active sites appear to be tailored to the individual reaction being catalyzed (18–20) (Fig. 1).

After some uncertainty regarding the stereochemical assignment of the naturally occurring carbapenems, the substrate and product of CarA have been assigned as (2S,5S)-carboxymethylproline (2S,5S)-CMP and (3S,5S)-carbapenem, respectively (9, 21, 22). CarC not only catalyzes desaturation but also the highly unusual epimerization of the carbapenam at the C-5 position and, like CarA, has been shown to accept several diastereomers of its natural substrate (21). Thus the “stereochemical course” of the pathway to the (5R)-carbapenem is determined, at least in part, by the selectivity of CarB. Here we report the purification of recombinant CarB and the discovery

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1 The abbreviations used are: (5R)-carbapenem, (R)-1-carbapen-2-em-3-carboxylate; GSA, glutamate semi-aldehyde; LC/MS, liquid chromatographic/mass spectrometry; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); DPPGSE, double pulsed field gradient spin echo; TOCSY, total correlation spectroscopy; CMP, carboxymethylproline; P5C, pyroline-5-carboxylate.

2 M. C. Sleeman, P. Smith, B. Kellam, S. R. Chhabra, B. W. Bycroft, and C. J. Schofield, submitted for publication.
that its substrate is most likely malonyl-CoA rather than AcCoA as previously proposed (5, 9).

EXPERIMENTAL PROCEDURES

Materials—All chemicals were obtained from Sigma. GSA was prepared from DL-α-pyruvate-5-carboxylic acid, 2,4-dinitrophenylhydrazine hydrochloride double salt (Sigma) by the method of Farrant et al. (24) SS-(carboxymethyl)-S-proline was prepared as described (7, 9).

CarB Cloning, Expression, and Purification—A PCR-amplified DNA product corresponding to the P. carotovora carB gene was engineered as a NdeI-BamHI fragment into the pET24a expression vector (Novagen) and transformed into Escherichia coli BL21(DE3) supercompetent cells. Cells were grown in shake flasks at 37 °C using 2TY medium containing 50 μg/ml kanamycin. After 0.7 h, cells were induced with 1 mM isopropyl-β-D-galactopyranoside, and growth was allowed to continue for 4 h; CarB expression was ~30% of the total soluble protein. Cultured cells were resuspended in Tris-HCl (15 ml, 50 mM, pH 7.5) and sonicated (HeatSystems), and the lysate centrifuged (35,000 × g) for 20 min. The resultant supernatant was filtered (0.22 μm, Millipore) and then applied directly to a Q-Sepharose FF column (12 ml) pre-equilibrated with Tris-HCl (50 mM, pH 7.5). Protein was eluted with a 0.2 M NaCl gradient in Tris-HCl (50 mM, pH 7.5). The purest samples as judged by SDS-PAGE analysis were pooled, glycerol was added to a final concentration of 10%, and then the mixture was concentrated to a volume of 3 ml. The resultant supernatant was applied directly to a DEAE-Sepharose column (10 ml) pre-equilibrated with Tris-HCl (50 mM, pH 7.5) and 10% glycerol. The purest samples were again judged by SDS-PAGE analysis, pooled, and applied to a Superdex-S75 column (320 ml) pre-equilibrated with Tris-HCl (100 mM, pH 7.5). The purest fractions (~95% pure by SDS-PAGE) were stored at ~80 °C until further use. (Molecular mass by negative ion electrospray MS: 27575 Da, cf. calcd. 27575.2 Da.)

CarA Cloning, Expression, and Purification—A PCR-amplified DNA product corresponding to the P. carotovora carA gene was engineered as a NdeI-BamHI fragment into the pET24a expression vector (Novagen) and transformed into E. coli BL21(DE3) supercompetent cells. Cells were grown in shake flasks at 37 °C using 2TY medium containing 50 μg/ml kanamycin. At A600 0.7, cells were induced with 1 mM isopropyl-β-D-galactopyranoside, and growth was allowed to continue for 4 h; CarA production was ~25% of the total soluble protein. Cultured cells were resuspended in Tris-HCl (15 ml, 50 mM, pH 8), sonicated (HeatSystems), and the lysate centrifuged (35,000 × g) for 20 min. The resultant supernatant was applied directly to a DEAE-Sepharose column (10 ml) pre-equilibrated with Tris-HCl (50 mM, pH 8). Protein was eluted with a 0.0–0.5 M gradient of NaCl in Tris-HCl (50 mM, pH 8). The purest samples as judged by SDS-PAGE analysis were pooled, and an equal volume of Tris-HCl (25 mM, pH 7.5) was added dropwise with stirring. This solution was applied through a 0.45 μm filter to a Mono-Q column (Amersham Biosciences), concentrated to 32 mg/ml (calculated mass without N-termi-
matography and by native PAGE analyses. Calibration for size exclusion chromatography (Superdex 200 HR) was carried out using cytochrome c (12 kDa), chymotrypsin (25 kDa), ovalbumin (43 kDa), bovine serum albumin (67 kDa), aldolase (158 kDa) and blue dextran (2000 kDa) (Gel Filtration Calibration Kit, Amersham Biosciences). An elution volume parameter ($K_{av}$) was calculated for each of the calibration proteins and a calibration curve constructed. By calculation $K_{av}$ for CarB the native molecular weight was established. Native PAGE employed 15% Tris-glycine polyacrylamide gels lacking SDS and run at 20 mV for 10 h.

DTNB Assay—Production of free CoA (CoASH) was assayed by reaction with 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) to produce thionitrobenzoate, which has a maximum absorbance at $\lambda_{max} = 412$ nm (25). Assay mixtures (50 $\mu$L) were diluted 4-fold and derivatized with DTNB reagent (50 $\mu$L). Absorbance was measured at 405 nm (Shimadzu UV-1601). Calibration was carried out using a range of CoASH concentrations. A typical assay mixture consisted of Tris-HCl, pH 7.5 (20 mM), malonyl CoA (0.8 mM), GSA (1.6 mM), CarB (0.32 mg ml$^{-1}$). The assay mixture was incubated at 37 °C for 10 min prior to derivatization.

LC/MS—LC/MS was performed using a Waters high performance liquid chromatography system connected to a Micro-Mass ZMD mass spectrometer in the negative ion mode. Assay mixtures as for the DTNB assay were precipitated with acetone (equal volume) and centrifuged (17,800 g) for 5 min before analysis. Controls were carried out under identical conditions but with CarB omitted. The assay mixture (20 $\mu$L) was injected onto a Waters Bondpak NH2 (270 mm x 4.6 mm) column (Phenomenex) equilibrated in 5% MeOH at 1 ml min$^{-1}$. After 5 min, a gradient to 5% MeOH, 0.05% formic acid was run over 10 min. These conditions were maintained for 10 min before returning to 5% MeOH over 5 min and re-equilibration for 15 min.

Preparative LC/MS used an 0.75-ml injection onto a C 18/NH2 5-µm Spherisorb mixed mode column (250 x 9.4 mm) equilibrated in 5% MeOH at 4 ml min$^{-1}$. A gradient to 5% MeOH, 0.1% formic acid was run over 5 min. These conditions were maintained for 20 min before returning to 5% MeOH over 5 min and re-equilibration for 25 min. Coupled CarB/CarA/CarC assays contained Tris-HCl, pH 9 (10 mM), 2-oxoglutarate (1 mM), CarC (1.6 mg ml$^{-1}$), FeSO$_4$ (1 mM), MgCl$_2$ (2 mM), ATP (3 mM), CarA (2.5 mg ml$^{-1}$), malonyl- or methylmalonyl-CoA (3 mM), GSA (7.5 mM), and CarB (1.2 mg ml$^{-1}$). Coupled CarB/CarA assays were as stated above with the omission of CarC, 2-oxoglutarate, and FeSO$_4$. The assay mixture was incubated at 37 °C for 30 min. For the bioassays, assay mixtures were transferred into holes (11-mm diameter) bioassay plates (E. coli X580) and incubated at 28 °C overnight. For LC/MS analysis, assay mixtures were mixed with MeCN (equal volume), chilled on ice for 5 min, and then centrifuged (17,800 x g) for 5 min before analysis. The assay mixture (100 $\mu$L) was injected onto a Synergi Polar-RP (250 mm x 4.6 mm) column (Phenomenex) equilibrated in 5% MeCN at 1 ml min$^{-1}$. After 15 min, a gradient to 90% MeCN was run over 5 min. These conditions were maintained for 5 min before returning to 5% MeCN over 5 min and re-equilibration for 10 min.

CD spectra were obtained in H$_2$O at 20 °C with a Jasco J-720 spectropolarimeter using a 1 mm path length. $^1$H NMR spectra were obtained in D$_2$O at 500 MHz on a Bruker DRX-500 spectrometer. DPPGSE-TOCSY $^1$H NMR was carried out as described by Sharman.
Native mass spectrometry was carried out on a Micromass Platform quadrupole mass spectrometer in 3 mM ammonium acetate with the enzyme concentration at 8 pmol/µl and a range of cone voltages.

RESULTS

Recombinant CarB and CarA were produced in E. coli and highly purified (>95% by SDS-PAGE analysis). For CarB, a three-column procedure employing anion exchange (Q-Sepharose), hydrophobic interaction (Phenyl-Resource), and gel filtration (Superdex-S75) steps (see “Experimental Procedures” and Fig. 2) was used. For CarA, anion exchange (DEAE-Sepharose) was followed by a second anion exchange step (Mono-Q) and gel filtration (Superdex-S75).
CarB, an Unusual Carbon-Carbon Bond-forming Enzyme

Quantitative gel filtration and native PAGE analyses indicated that purified CarB existed primarily as a trimer, consistent with crystallographic analyses of other members of the crotonase family (27–30).

Initially the activity of purified CarB was assayed by spectrophotometric determination of CoASH employing DTNB as a derivatization reagent (25). Assays were carried out with AcCoA and synthetic racemic GSA. However, no production of CoASH above background levels was observed, and no CMP was observed by LC/MS analyses. Because enzymes from the crotonase family have been reported to use a variety of acyl-CoA derivatives as substrates (18–20), we examined various acyl-CoA derivatives as potential CarB substrates. Stearoyl-CoA and acetoycetyl-CoA were not substrates, but in the presence of malonyl-CoA and GSA the DTNB derivatization assay revealed that CarB clearly catalyzed the conversion of malonyl-CoA to CoASH (specific activity of 4.0 μmol·min⁻¹·mg protein⁻¹).

The spectrophotometric observations that malonyl-CoA acts as a substrate for CarB were supported by nanospray mass spectrometry analyses. Under native conditions CarB was observed predominantly as a trimer by mass spectrometry, supporting the native PAGE and quantitative gel filtration analyses. When incubated with malonyl-CoA in the absence of GSA, CarB catalyzed its decarboxylation to AcCoA, observed by mass spectrometry both free in solution and as an enzyme-bound complex (Fig. 3). In contrast, in the presence of both malonyl-CoA and GSA, production of both an enzyme-bound complex with CoASH and free CoASH in solution was observed. CarB-mediated catalytic conversion of 2-methylmalonyl-CoA to CoASH was also observed by using the DTNB assay.

Production of CMP and 6-methyl-CMP (probably as a mixture of C-6 epimers) was confirmed using LC/MS and, in the case of the former, NMR analyses (500 MHz) of both crude reaction mixtures (using DGGFSE-TOCSY 1H NMR (26)) and high pressure liquid chromatography-purified product (Fig. 4).

A comparison of the NMR data for CMP produced via CarB catalysis with synthetic standards of (2S,5S)-CMP and (2S,5R)-CMP implied the production of only (>95%) the “trans” (5S)-CMP diastereomer (or its enantiomer). There was no evidence for the formation of the “cis” (5R)-CMP diastereomer (or its enantiomer) within the limits of detection. Assignment of the stereochemistry of the predominant CMP isomer produced via CarB catalysis as (2S,5S)-CMP was performed by comparison of the CD spectra of the enzymatically produced CMP with the chemically synthesized CMP.

LC/MS assays of the coupled CarB and CarA reactions demonstrated production of CMP or 6-methyl-CMP from malonyl-CoA or methylmalonyl-CoA, respectively. Bioassays conducted on CarA, CarB, and CarC resulted in the production of a zone of antibiosis, indicating the production of an active antibiotic when malonyl-CoA was used as a starting material. However, with methylmalonyl-CoA under analogous conditions, no antibiosis was observed, indicating either that CarC is unable to epimerize/desaturate the 6-methyl-carbapenem or, less likely, because 6-ethyl-carbapenem antibiotics have been reported, that the 6-methyl-carbapenem produced is not an active antibiotic.

DISCUSSION

Although malonyl-CoA is commonly used as a two-carbon source in polyketide biosynthesis (see e.g. Refs. 31 and 32), with the exception of methylmalonyl-CoA decarboxylase, it is not used by known members of the crotonase family, making its use by CarB unusual. Further, although preceded (β-hydroxysibutryl-CoA lyase and feruloyl-CoA hydratase/lyase), it is also unusual for crotonases to catalyze CoA ester hydrolysis. Intermolecular C–C bond formation involving heterocyclic substrates is unprecedented in the family. The sequence alignment of CarB with other members of the crotonase family highlights a high degree of similarity in the β-sheet core of the protein, which is very well conserved structurally in the other members of the family (18–20). Residues with a functionality similar to those responsible for binding the CoA portion of the substrate are also predicted to be present in a similar location in CarB (Fig. 1).

The observed decarboxylation of malonyl-CoA by CarB in the absence of GSA is consistent with a mechanism involving an initial decarboxylation to form an enzyme-bound enolate, analogous to the mechanism proposed for methylmalonyl-CoA decarboxylase (28). However, in the reaction catalyzed by CarB, instead of protonation the enolate formed can react with GSA/pyrroline-5-carboxylate (P5C) to produce CMP (Scheme 2). The enolate may react with the protonated imine form of P5C to give CMP directly. Alternatively, it may react with GSA (in an
aldol reaction) to give, after loss of water, an alkene, which undergoes an intramolecular Michael reaction in a kinetically favored 5-exo-trig reaction (33). The distinct lack of activity with AcCoA under standard assay conditions suggests the absence of an appropriately positioned, or strong enough, base to form an enolate for this potential substrate. The use of malonyl-CoA rather than AcCoA, may reflect a requirement to physically separate any base involved in enolate formation from the electrophilic P5C/GSA, thus avoiding unwanted reactions of the latter with the enzyme-bound base/nucleophile.

Because in vivo malonyl-CoA can be produced from AcCoA in a reaction catalyzed by AcCoA carboxylase, the observation of activity of CarB with malonyl-CoA agrees with previous observations of the incorporation of carbons derived from an intact acetate unit into positions 6 and 7 of the β-lactam ring (34). Given the apparent lack of absolute stereoselectivity of CarA (10), which minimally accepts three stereoisomers of CMP (albeit with different efficiencies), the production (at least predominantly) of a single enantiomer of CMP by CarB rationalizes the observation of only the (3S,5S)-carbapenam as the sole in vivo product of CarA. It is likely that CarD and CarE, which display sequence similarity with proline oxidase and 2Fe2S ferrodoxin, are responsible for increasing the level of the GSA/P5C via its biosynthesis from L-proline (5).

Aside from its synthetic implications, the observation that methylmalonyl-CoA is a substrate for CarB has possible implications for the biosynthesis of substituted carbapenems such as thienamycin (3) (Scheme 3). Most naturally occurring carba-
peptidoglycan} including antibiotics with the medicinally useful hydroxyethyl side chain, are substituted at the C-6 position, (35, 36). Recently, the sequence for the biosynthetic gene cluster for thienamycin biosynthesis in Streptomyces castellato has been reported and a pathway proposed (3) (Scheme 3). In this proposal a CarB homologue, ThnE, is proposed to catalyze the formation of a CoA derivative of dehydro-CMP from γ-glutamyl phosphate and AcCoA (following from earlier suggestions by Houck et al. (37) and Williamson (36)). In the next step it is proposed that a methyl transfer involving S-adenosylmethionine occurs to give a C-6 methyl dehydro-CMP CoA derivative, cyclization of which is mediated by ThnM (a homologue of CarA (29% identity, 53% similarity)) to give a 6-methyl-carbapen-2-one-carbon pool (37). The known biosynthetic routes to methylmalonyl-CoA all proceed via propanoyl-CoA, which can be biosynthesized by a number of different routes. However, these routes do not (to our knowledge) involve methylation of malonyl-CoA (or a derivative) is a possibility.

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