Determination of the Chemical Pathway for 4-Chlorobenzoate:Coenzyme A Ligase Catalysis†

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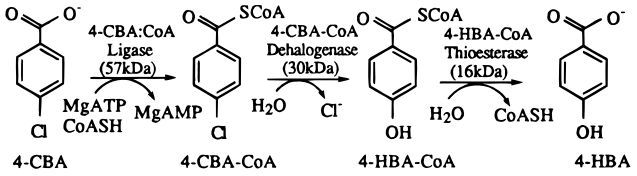
Received May 31, 1996; Revised Manuscript Received July 30, 1996⊗

ABSTRACT: 4-Chlorobenzoate:coenzyme A ligase (4-CBA:CoA ligase) catalyzes the first step of the 4-CBA degradation pathway of *Pseudomonas* sp. strain CBS3. In this reaction, 4-CBA-CoA thioester synthesis is coupled to ATP cleavage. The studies described in this paper examine the intermediacy of 4-chlorobenzoyl−adenosine 5′-phosphate diester (4-CBA-AMP) in the ligase reaction. The 4-CBA-AMP adduct was isolated from the ligase reaction mixture generated from magnesium adenosine 5-triphosphate (MgATP) and 4-CBA in the absence of CoA. The structure of the 4-CBA-AMP was verified by ^{1}H-, ^{13}C-, and ^{31}P-nuclear magnetic resonance analysis. Single-turnover reactions carried out with ^{14}C-labeled 4-CBA in a rapid quench apparatus demonstrated formation of the enzyme•4-CBA-AMP•$MgPP_i$ complex from the enzyme•4-CBA•MgATP complex at a rate of 135 s^{-1}. The rate of ligand release from the enzyme•4-CBA-AMP•$MgPP_i$ complex was measured at 0.013 s^{-1}. Single-turnover reactions of [^{14}C]-4-CBA, MgATP, and CoA catalyzed by the ligase revealed that the 4-CBA-AMP intermediate formed reaches a maximum level of 25% of the starting 4-CBA within 10 ms and then declines with the formation of the 4-CBA-CoA. The rates of the adenylation and thioesterification partial reactions, determined by kinetic simulation of the rate data, are nearly equal (135 and 100 s^{-1}). Substitution of CoA with the slow substrate pantetheine did not significantly alter the rate of the adenylation step but did reduce the rate of the thioesterification step to 2 s^{-1}. The maximum level of 4-CBA-AMP reached during the single-turnover reaction of 4-CBA, MgATP, and pantetheine corresponded to one-half of the starting 4-CBA.

The 4-chlorobenzoate (4-CBA)[1] degrading pathway has been discovered in certain strains of soil-dwelling bacteria exposed to 4-CBA or its progenitor, 4-chlorobiphenyl [for recent reviews see Dunaway-Mariano and Babbitt (1994) and Janssen et al. (1994)]. The pathway, which converts 4-CBA to the metabolite 4-hydroxybenzoate (4-HBA), allows these bacteria to utilize 4-CBA as an alternate carbon source. The 4-CBA pathway, shown in Scheme 1, consists of three chemical steps catalyzed by 4-CBA:CoA ligase, 4-CBA-CoA dehalogenase, and 4-HBA-CoA thioesterase. This paper examines catalysis by the first enzyme of the pathway, 4-CBA:CoA ligase of the bacterium *Pseudomonas* sp. strain CBS3 (Chang et al., 1992; Löffler et al., 1992).

The 4-CBA:CoA ligase catalyzes the reaction of 4-CBA, MgATP, and CoA to 4-CBA-CoA, AMP, and $MgPP_i$ at a turnover rate of 40 s^{-1} (pH 7.5, 25 °C) (Chang et al., 1992). Sequence analysis of the ligase allowed us to connect it with a family of structurally and functionally related enzymes (Babbitt et al., 1992; Dunaway-Mariano & Babbitt, 1994). The members of this group share an ATP/AMP binding motif (Scholten et al., 1991; Bairoch, 1992; Chang, 1994) and sufficient global sequence identity (25−38%) to suggest common ancestry (Babbitt et al., 1992; Turgay et al., 1992). Each enzyme within this group catalyzes the reaction of ATP, a carboxylic acid, and an acyl acceptor to form an oxygen ester, a thioester, or an amide. Two subfamilies are distinguished on the basis of catalysis of acyl-CoA thioester formation or thiol (pantetheine) template-directed synthesis of macromolecules (Babbitt et al., 1992). The 4-CBA:CoA ligase belongs to the carboxylic acid:CoA ligase subfamily, which also includes acetyl-CoA synthetase, long-chain fatty acid acyl-CoA synthetase, 4-coumarate:CoA ligase, *O*-succinyl:CoA ligase, carnitine:CoA ligase, bile acid:CoA ligase, and acyl-CoA synthetase (Dunaway-Mariano & Babbitt, 1994).

Scheme 1: Chemical Steps of the 4-CBA to 4-HBA Pathway Catalyzed by (a) 4-CBA:CoA Ligase, (b) 4-CBA-CoA Dehalogenase, and (c) 4-HBA-CoA Thioesterase (Scholten et al., 1991)



Implicit in the reactions catalyzed by this large family of enzymes is the intermediacy of an acyl-adenylate formed by the reaction of the carboxylic acid substrate and MgATP. Formation of an acyl-adenylate adduct in fatty acyl-synthetase (Londesborough & Webster, 1974), gramicidin S and tyrocidine synthetase (Lee & Lipman, 1975; Kleinkauf & van Döhren, 1987, 1990), and enterobactin synthase (Rusnak et al., 1989, 1991) catalysis has been probed by measuring catalyzed isotope exchange between ATP and [^{32}P]PP_i (and

† This work was supported by N.I.H. Grant GM 36260.

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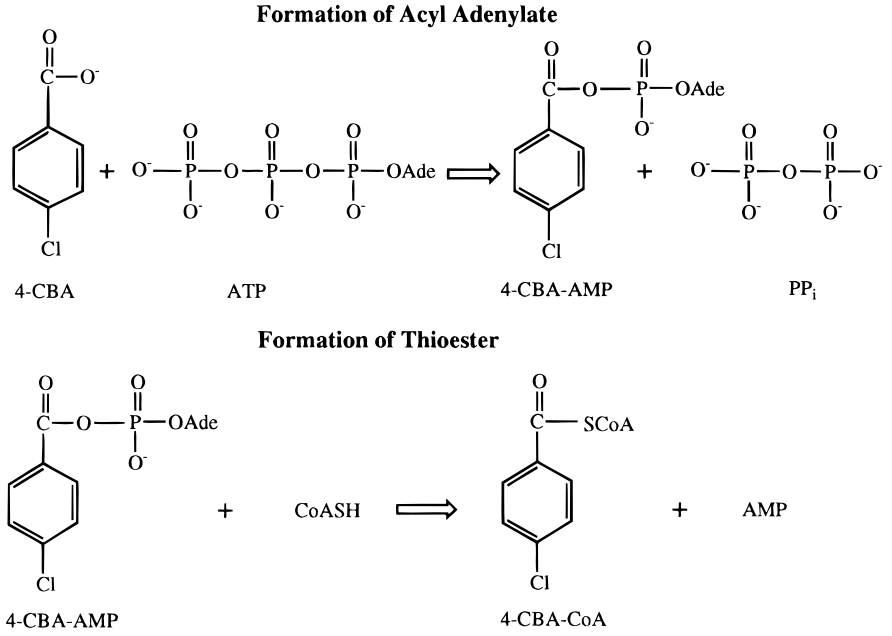
⊗ Abstract published in *Advance ACS Abstracts,* September 15, 1996.

[1] Abbreviations: 4-CBA, 4-chlorobenzoate; 4-CBA-CoA, 4-chlorobenzoyl−coenzyme A; 4-HBA, 4-hydroxybenzoate; 4-HBA-CoA, 4-hydroxybenzoyl−coenzyme A; 4-CBA-AMP, 4-chlorobenzoyl−adenosine 5′-phosphate diester; 4-CBA-Pan, 4-chlorobenzoyl−pantetheine; K^+Hepes, (*N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid]) potassium salt; ATP, adenosine 5′-triphosphate; AMP, adenosine 5′-phosphate; NADP, micotinamide adenine dinucleotide phosphate; HPLC, high-performance liquid chromatography; NMR, nuclear magnetic resonance.

S0006-2960(96)01284-6 CCC: $12.00

Scheme 2: Adenylation and Thioesterification Partial Reactions Proposed for 4-CBA:CoA Ligase Catalysis



between [^{18}O]acid and AMP) and by identifying tightly bound ligand formed in the enzyme-catalyzed half-reaction of radiolabeled carboxylic acid and ATP. Although the kinetics of acyl-adenylate formation and reaction catalyzed by these enzymes have not been determined, such studies have been carried out with an analogous enzyme system, tyrosyl-tRNA synthetase (Fersht et al., 1975, 1988; Fersht & Jakes, 1975; Fersht & Kaethner, 1976; Fersht, 1987; Wells et al., 1991), that belongs to the family of aminoacyl-tRNA synthetases (Soll & Schimmel, 1974; Fersht et al., 1975; Schimmel, 1987). Transient kinetic studies evidenced a two-step reaction involving rapid formation of the enzyme·tyrosyl-AMP complex followed by a comparatively slow reaction with tRNA to form the tyrosyl-tRNA product.

In analogy to the chemical pathway of the tyrosyl-tRNA ligase catalyzed reaction, we propose that the 4-CBA:CoA ligase-catalyzed reaction consists of an adenylation step followed by thioesterification with CoA (Scheme 2). This paper describes the isolation and structural characterization of the 4-CBA-AMP adduct formed by the first partial reaction. In addition, we describe transient kinetic studies which define the rates of the two partial reactions and demonstrate the intermediacy of the 4-CBA-AMP adduct in the overall reaction.

MATERIALS AND METHODS

Enzymes and Chemicals. 4-CBA:CoA ligase was isolated using the procedure described in Chang et al. (1992). Pantetheine was prepared according to the method of Butler et al. (1976). [7-^{14}C]-4-CBA (21 mCi/mmol) was purchased from California Bionuclear Corporation. [U-^{14}C]ATP (587 mCi/mmol, tetrasodium salt) was obtained from NEN. Buffers and reagents were obtained from either USB or Sigma. Enzymes used in coupling assays were purchased from Sigma. Bio-Gel P2 was purchased from Bio-Rad. Liquid scintillation cocktail (Bio-Safe II) was purchased from Research Products International Corporation.

Equipment. Enzymatic assays were performed on a Gilford spectrophotometer model 250. ^{1}H- and ^{13}C-NMR spectra were recorded on a Bruker AMX500 multinuclear spectrometer operating at a frequency of 500 and 125 MHz, respectively. ^{31}P-NMR spectra were recorded on a Bruker WP-200 multinuclear spectrometer operating at 84 MHz. UV absorption spectra were recorded using a Milton Roy Spectronic 3000 spectrophotometer. The rapid quench experiments were performed on a KinTek quench flow apparatus (three-syringe model equipped with a thermostatically controlled circulator). HPLC analysis was performed on a Beckman model 110A HPLC system equipped with a Hitachi model 100-10 spectrophotometer. Liquid scintillation counting was performed on a Beckman model LS 5801 counter.

HPLC Analysis of Product Formation in Multiple-Turnover Reactions Catalyzed by 4-CBA:CoA Ligase. Three reaction mixtures (1 mL, 25 °C) were prepared for product analysis, and each contained 2 mM 4-CBA, 3.5 mM ATP, 5 mM $MgCl_2$, 5 units of 4-CBA:CoA ligase (7 μM), and 10 units of inorganic pyrophosphatase (EC 3.6.1.1) in 50 mM K^+Hepes (pH 7.5). One reaction contained 0.1 μCi of [^{14}C]-4-CBA, and another contained 0.1 μCi of [^{14}C]ATP. Each reaction mixture was incubated for 6 h and quenched by adding 500 μL of CCl_4 with vigorous vortexing. The precipitated protein was pelleted by centrifugation at 14 000 rpm for 2 min. An aliquot (100 μL) of the supernatant was injected onto an HPLC column for analysis (see below). A fourth reaction mixture (1 mL) containing 2 mM [^{14}C]-4-CBA (0.1 μCi), 3.5 mM ATP, 5 mM $MgCl_2$, 5 units of 4-CBA:CoA ligase (7 μM), and 10 units of inorganic pyrophosphatase in 50 mM K^+Hepes (pH 7.5) was incubated for 6 h, after which CoA was added to the reaction mixture to a final concentration of 2 mM. The mixture was incubated for an additional 2 min. The reaction was quenched, and the supernatant was prepared in the same way as described above. An aliquot (100 μL) of the supernatant was injected into an HPLC column. Two control reactions were performed: one contained everything except the inorganic pyrophosphatase, and the other contained everything except the 4-CBA:CoA ligase.

The HPLC separation of the quenched ligase reactions was performed on a C-18 reversed-phase column (Beckman

Ultrasphere; 4.6 mm × 25 cm) at a flow rate of 1 mL/min. A linear gradient was employed in which solvent A was 5% methanol, 2.5% triethylamine (TEA), and 25 mM K^+P_i (adjusted to pH 6.5 with H_3PO_4) and solvent B was 50% methanol in the same buffer. The linear gradient program was as follows: 100% A for 2 min, 0−100% B in 45 min, and hold at 100% B for 5 min. The column eluant was monitored at 260 nm. The retention times of AMP, ADP, ATP, CoA, 4-CBA, and 4-CBA-CoA were determined by injecting standards to be 7, 9, 12, 25, 35, and 50 min, respectively. When radiolabeled substrates were used, the effluent (1−2 mL) from the HPLC column corresponding to the peak of interest was collected and mixed with 5 mL of liquid scintillation cocktail. Radioactivity was then measured by scintillation counting.

Isolation and Structure Determination of 4-CBA-AMP Formed in Multiple-Turnover Reactions of 4-CBA + MgATP Catalyzed by 4-CBA:CoA Ligase. A 2 mL reaction mixture containing 10 mM 4-CBA, 8 mM ATP, 10 mM $MgCl_2$, 25 units of 4-CBA:CoA ligase (17 μM), and 30 units of inorganic pyrophosphatase in 50 mM K^+Hepes (pH 7.5) was incubated at room temperature overnight and then quenched by adding 200 μL of 0.1 N HCl and 1 mL of CCl_4. Following vigorous vortexing, the precipitated protein was removed by centrifugation at 14 000 rpm for 2 min. The supernatant was injected onto an HPLC column for separation. The HPLC separation was performed in the same manner as described previously except that the elution buffers and the gradient program employed were different. A linear gradient was used in which solvent A was 25 mM sodium acetate buffer (pH 5) and solvent B was acetonitrile. The linear gradient program was as follows: 100% A for 2 min, 0−100% B in 23 min, and hold at 100% B for 5 min. The retention times of ATP, 4-CBA-AMP, and 4-CBA were 3, 14, and 16 min, respectively. The peak eluting at 14 min was collected and concentrated to 1 mL by rotary evaporation. The concentrate was loaded to a Bio-Gel P2 column (1.5 cm × 85 cm) which had been equilibrated with deionized distilled H_2O (ddH_2O). The column was eluted with ddH_2O at a flow rate of 8 mL/h, and the eluant was monitored at 260 nm. The 4-CBA-AMP-containing fractions were pooled and then lyophilized. The residue was dissolved in D_2O (pH 6.0) for ^{1}H-, ^{13}C-, and ^{31}P-NMR analysis and for wavelength scanning (200−700 nm). Chemical shifts (δ) for ^{1}H-NMR are reported in Table 1 in parts per million (ppm) relative to HDO (δ 4.77, 25 °C). Chemical shifts (δ) for ^{13}C-NMR are reported in parts per million (ppm) relative to $CDCl_3$ (δ 77.00, 25 °C). Assignment of the ^{13}C-NMR resonances was aided by use of the DEPT technique to determine the numbers of attached hydrogens. Chemical shifts for ^{31}P-NMR were referenced to 85% H_3PO_4 (δ 0.00, 25 °C).

Steady-State Kinetic Analysis of 4-CBA-AMP Formation from the Reaction of 4-CBA + MgATP Catalyzed by 4-CBA:CoA Ligase. The steady-state rate of ligase-catalyzed 4-CBA-AMP formation from reaction of ATP and 4-CBA in the absence of CoA, was determined by detecting the rate at which PP_i is released into the solution using an inorganic pyrophosphatase-based coupled assay (Knight et al., 1981). A 1 mL amount assay solution contained 2 mM 4-CBA, 3.5 mM ATP, 5 mM $MgCl_2$, 1 mM NADP, 4% (w/v) glycogen, 1 unit of 4-CBA:CoA ligase (1.4 μM), 5 units of inorganic pyrophosphatase (EC 3.6.1.1), 5 units of phosphorylase a (EC 2.4.1.1), 3 units of phosphoglucomutase (EC 5.4.2.2), and 5 units of glucose-6-phosphate dehydrogenase (EC 1.1.1.49) in 50 mM K^+Hepes (pH 7.5) at 25 °C. The increase in absorbance at 340 nm due to the reduction of NADP ($\Delta\epsilon$ = 6.2 mM^{-1} cm^{-1}) was monitored.

Table 1: Summary of the ^{1}H-, ^{13}C-, and ^{31}P-NMR Spectral Data Measured for the 4-CBA-AMP Intermediate Formed in the Ligase Reaction

δ (ppm)	description	assignment
	^{1}H-NMR [D_2O, pH 6.00, 500 MHz, relative to HDO (δ 4.77, 25 °C)][a]	
4.64	m, 3H	ribose H-4 and H-5
5.93	d, 1H, J = 5.9 Hz	ribose H-1
7.14	d, 2H, J = 8.5 Hz	phenyl H-3 and H-5
7.49	d, 2H, J = 8.6 Hz	phenyl H-2 and H-6
8.13	s, 1H	adenyl H-3
8.22	s, 1H	adenyl H-8
	^{13}C-NMR [D_2O, pH 6.0, 125 MHz, relative to $CDCl_3$ (δ 77.00, 25 °C)]	
66.57	CH_2	ribose C-5
70.25	CH	ribose C-3
73.08	CH	ribose C-2
83.70	CH	ribose C-4
86.44	CH	ribose C-1
118.18 and 126.86 and 139.73	quaternary	adenyl
128.37 and 130.74	CH	phenyl
139.36 and 152.59	CH	adenyl
148.75 and 155.08	quaternary	phenyl
163.16	C═O	C═O
	^{31}P-NMR [D_2O, pH 6.0, 84 MHz, referenced to 85% H_3PO_4 (δ 0.00, 25 °C)]	
−5.53	m	

[a] The signal of the ribose H-2 and H-3 were overlapped with that of H_2O.

4-CBA-AMP Formation in Single-Turnover Reactions of [^{14}C]-4-CBA + ATP Measured as a Function of 4-CBA:CoA Ligase Concentration. Each reaction was initiated by mixing 43 μL of the enzyme solution (at different concentrations: 26, 40, 50, 60 and 100 μM) in 50 mM K^+Hepes (pH 7.5, 25 °C) with 43 μL of the solution containing 16 μM [^{14}C]-4-CBA (0.01 μCi), 7 mM ATP, and 10 mM $MgCl_2$ in 50 mM K^+Hepes (pH 7.5, 25 °C). The final concentrations after mixing (in a total volume of 86 μL) were 8 μM [^{14}C]-4-CBA (0.01 μCi), 3.5 mM ATP, 5 mM $MgCl_2$, and 13, 20, 25, 30, and 50 μM 4-CBA:CoA ligase. After incubation for 5 s, the reaction mixture was quenched with 164 μL of 0.1 N HCl and 100 μL of CCl_4. Following vigorous vortexing, the precipitated protein was pelleted by centrifugation for 2 min at 14 000 rpm in a microfuge. The supernatant (250 μL) was stored at −80 °C (for ∼24 h) until it was examined by HPLC analysis. The 4-CBA and 4-CBA-AMP of the reaction mixture were separated by HPLC. Unlabeled 4-CBA and 4-CBA-AMP were added into the quench solutions to a final individual concentration of 20 μM before injection onto the HPLC in order to locate the peak positions. The HPLC separation of the reaction mixture employed a linear gradient in which solvent A was 25% methanol, 2.5% triethylamine, and 25 mM K^+P_i (adjusted to pH 6.5 with H_3PO_4) and solvent B was 50% methanol in the same buffer. The linear gradient program was as follows: 100% A for 2 min, 0−100% B in 23 min, and hold at 100% B for 5 min. The AMP and ATP eluted at the solvent front (at 3−4 min), and the retention times of 4-CBA and 4-CBA-AMP were 19 and 22 min, respectively. The

peak eluant was collected and assayed for ^{14}C content by liquid scintillation counting. A control experiment in which the reaction was carried out in the absence of the ligase was conducted in order to ensure that the observed radioactivity coeluting with 4-CBA-AMP was derived solely from the enzyme catalyzed reaction. Another control reaction in which the enzyme was added to a premixed solution of acid and substrates was carried out to ensure that the acid was adequately quenching the enzyme reaction.

Measurement of the Single-Turnover Time Course for the 4-CBA:CoA Ligase-Catalyzed Reaction of [^{14}C]-4-CBA and MgATP. All reactions were performed at 25 °C. Each reaction was initiated by mixing (in the rapid quench instrument) 43 μL of 60 μM (or 120 μM) of the enzyme solution (~3.5 mg of protein/mL) in 50 mM K^+Hepes (pH 7.5) with 43 μL of the solution containing 16 μM [^{14}C]-4-CBA (0.01 μCi), 7 mM ATP, and 10 mM $MgCl_2$ in 50 mM K^+Hepes (pH 7.5). The final concentrations after mixing (in a total volume of 86 μL) were 30 μM (or 60 μM) enzyme, 8 μM [^{14}C]-4-CBA (0.01 μCi), 3.5 mM ATP, and 5 mM $MgCl_2$. After incubation for a specified period of time (ms), the reaction mixture was quenched with 164 μL of 0.1 N HCl. The quench solution was collected in a 1-mL syringe and then transferred to a 1.5-mL Eppendorf tube containing 100 μL of CCl_4. Following vigorous vortexing the precipitated protein was pelleted by centrifugation for 2 min at 14 000 rpm in a microfuge. The supernatant (250 μL) was stored at −80 °C (for ~24 h) until it was subjected to HPLC separation and scintillation counting as described in the previous section.

Measurement of the Single-Turnover Time Course for the 4-CBA:CoA Ligase-Catalyzed Reaction of 4-CBA, MgATP, and CoA or Pantetheine. All reactions were performed at 25 °C. Each reaction was initiated by mixing (in the rapid quench instrument) 43 μL of 60 μM ligase in 50 mM K^+-Hepes (pH 7.5) with 43 μL of the solution containing 16 μM [^{14}C]-4-CBA (0.01 μCi), 7 mM ATP, 10 mM $MgCl_2$, and 2 mM CoA or 20 mM pantetheine in 50 mM K^+Hepes (pH 7.5). The final concentrations after mixing (in a total volume of 86 μL) were 30 μM ligase, 8 μM [^{14}C]-4-CBA (0.01 μCi), 3.5 mM ATP, 5 mM $MgCl_2$, and 1 mM CoA or 10 mM pantetheine. After incubation for a specified period of time (ms), the reaction was quenched with acid and treated in the same way as described previously. The protein-free solution (250 μL) was kept at −80 °C (for ~24 h) until HPLC analysis was performed. The CoA reaction mixture was separated as described in the previous section. The retention times of 4-CBA, 4-CBA-AMP, and 4-CBA-CoA were determined to be 19, 22, and 33 min, respectively. For separation of the pantetheine reaction mixture a linear gradient was employed in which solvent A was 25 mM acetate buffer (pH 5) and solvent B was acetonitrile. The linear gradient program was as follows: 100% A for 2 min, 0−100% B in 23 min, and hold at 100% B for 5 min. The retention times of ATP, 4-CBA-AMP, 4-CBA, and 4-CBA-pantetheine were 3, 14, 16, and 20 min, respectively. The peak eluant was collected and assayed for radioactivity by scintillation counting.

Single-Turnover Kinetic Data Analysis. The rate constants observed for the single-turnover reactions were determined by computer fitting the data (input reaction time versus concentration as *x*,*y* pairs) obtained from the rapid quench experiments to the first-order rate equations shown below using the Kaleidagraph program.

$$[S]_t = [S]_{max} - ([P]_{max}(1 - e^{-kt}))$$

$$[P]_t = [P]_{max}(1 - e^{-kt})$$

where k represents the first-order rate constant, and $[S]_t$ and $[P]_t$ stand for the substrate and product concentrations at a certain time, respectively. The rate constants calculated in this manner are denoted k_{obs}. With experimentally obtained rate constants and a kinetic model, the computational program KINSIM (Barshop et al., 1983) was then used to simulate kinetic progress curves to fit (by an iterative process) single-turnover time course data. Adjustments were made in the values of rate constants until the simulated curves closely matched (superimposed) the experimentally obtained time courses. The rate constants k_1, k_{-1}, k_2, and k_{-2} thus obtained were cross-checked with those measured from single-turnover reaction profiles.

RESULTS AND DISCUSSION

Detection of 4-CBA-AMP Formation in the 4-CBA:CoA Ligase-Catalyzed ATP + 4-CBA Half-Reaction by HPLC Analysis. The first approach taken to observe the formation of the intermediate of the ligase reaction, 4-CBA-AMP, was to carry out the multiple-turnover reaction of ATP and 4-CBA in the absence of CoA. Inorganic pyrophosphatase was included in the reaction mixture for the purpose of driving the reaction forward via the catalyzed hydrolysis of the PP_i product. The reaction mixture was analyzed by separating its components on a reversed-phase HPLC column, monitoring the absorbance of the eluate at 260 nm. The HPLC chromatogram of the initial reaction mixture (without enzyme) consisted of peaks from ATP (12 min) and 4-CBA (35 min) (see Figure 1A). The HPLC chromatogram measured following 6 h of incubation with the ligase revealed unconsumed ATP and 4-CBA and a new peak, which was not observed with the control reactions, eluting at 39 min. Excess CoA was added to the 6 h incubation mixture, and, following a 2 min incubation period, the resulting mixture was separated on the HPLC column. AMP (7 min), CoA (25 min), and 4-CBA-CoA (50 min) peaks were observed in place of the 4-CBA, ATP, and the unidentified "39 min" peaks. In order to test whether or not the material eluting at 39 min is 4-CBA-AMP, the same reaction was carried out using [^{14}C]-4-CBA or [^{14}C]ATP (Figure 1A,B). In both instances the ^{14}C radiolabel was found to be associated with the 39 min peak in the amount predicted on the basis of the specific activity of the labeled reactant and the percent conversion derived from the relative areas of the A^{260} peaks. The material eluting at 39 min was collected, concentrated, and reinjected onto the HPLC column before and after reacting it with excess CoA in the presence of the ligase. The first chromatogram revealed a single peak at 39 min, thus demonstrating the chemical stability of the putative 4-CBA-AMP adduct (Figure 1C). The second chromatogram revealed AMP, CoA, and 4-CBA-CoA, thus demonstrating the competence of the adduct as a reactant in the CoA half-reaction.

Spectral Characterization of the 4-CBA-AMP Reaction Intermediate. Purification of the 4-CBA-AMP adduct from the ligase-catalyzed reaction of ATP and 4-CBA was carried

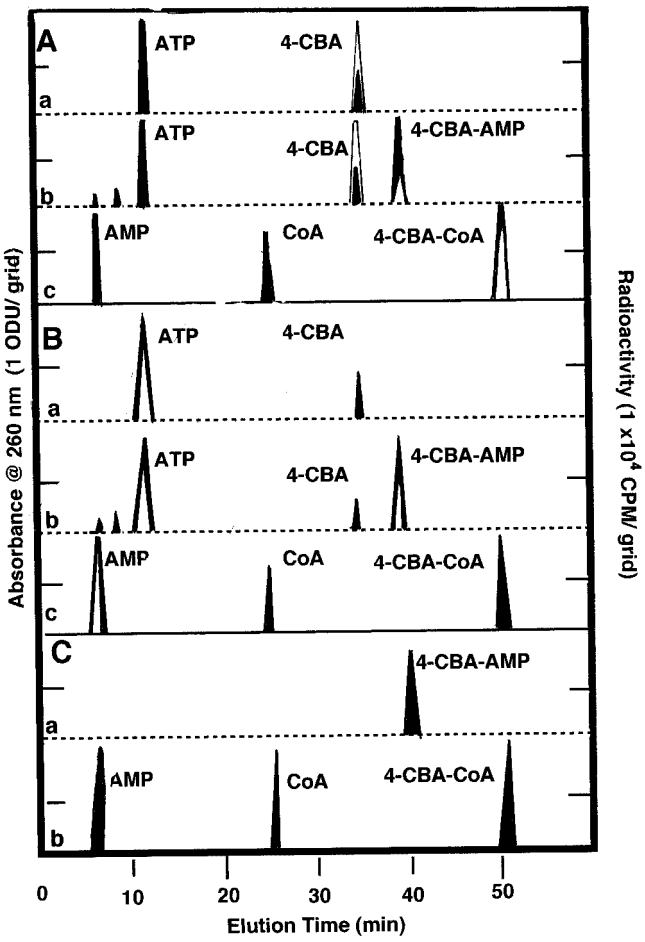


FIGURE 1: Illustration of the reversed-phase HPLC traces of the chromatographies of (A) the reaction of 2 mM [^{14}C]-4-CBA (0.1 μCi), 3.5 mM ATP, 5 mM $MgCl_2$, 10 units of inorganic pyrophosphatase, and 5 units of 4-CBA:CoA ligase (7 μM) in 50 mM K^+-Hepes (pH 7.5) and (B) the reaction of 2 mM 4-CBA, 3.5 mM [^{14}C]ATP (0.1 μCi), 5 mM $MgCl_2$, 10 units of inorganic pyrophosphatase, and 5 units of 4-CBA:CoA ligase (7 μM) in 50 mM K^+-Hepes (pH 7.5). One-tenth (100 μL) of the total volume of the reaction mixture was injected into the HPLC column. The reaction (a) at 0 min, (b) after 6 h of incubation at room temperature, and (c) after 6 h of incubation followed by 2 min of incubation with 2 mM CoA. The shaded area under the peak represents 260 nm absorbance, and the unshaded area of the peak corresponds to the ^{14}C radioactivity (CPM) content of the fraction. (C) HPLC analysis of the purified 4-CBA-AMP intermediate before (a) and after (b) reaction with 1 mM CoA and 0.01 unit of the ligase in 50 mM K^+Hepes (pH 7.5) for 2 min at room temperature (see Methods for details).

out using reversed-phase HPLC and gel filtration column chromatographic techniques. The ^{1}H-, ^{13}C-, and ^{31}P-NMR spectral data measured for the purified compound are presented in Table 1 along with the spectral assignments (spectra are shown in Figures 1−3 of the supporting information). The UV absorption spectrum revealed a λ_{max} at 203 nm ($\epsilon = 20$ mM^{-1} cm^{-1}) and 249 nm ($\epsilon = 15$ mM^{-1} cm^{-1}) (H_2O, pH 6.0, 25 °C) (spectrum provided in Figure 4 of the supporting information).

Kinetics of 4-CBA-AMP Formation and Reaction. The steady state rate of 4-CBA-AMP formation from multiple turnovers of ATP and 4-CBA catalyzed by the ligase was measured at saturating levels of substrate and Mg(II) using the inorganic pyrophosphatase/glycogen phosphorylase/glucose isomerase/glucose dehydrogenase-coupled assay to monitor PP_i formation (Knight et al., 1981). The rate measured at pH 7.5, 25 °C, is 0.013 s^{-1}, which is considerably slower than the turnover rate of the enzyme catalyzing the full reaction (ATP + 4-CBA + CoA to 4-CBA-CoA + AMP + PP_i), which is 40 s^{-1} (Chang et al., 1992). *A priori*, the slow rate of 4-CBA-AMP formation under steady state conditions could be attributed to a requirement for CoA to be bound for efficient catalysis and/or to slow release of 4-CBA-AMP and/or PP_i from the enzyme complex.

The rate of 4-CBA-AMP formation in the presence and absence of CoA was measured for a single-turnover on the enzyme using transient kinetic techniques. [^{14}C]-4-CBA (K_m = 10 μM; Chang et al., 1992) was chosen to serve as the limiting substrate in the reaction because it binds to the enzyme significantly more tightly than does ATP (K_m = 300 μM; Chang et al., 1992). A study of the amount of 4-CBA-AMP formed by 5 s hand-quenched reactions of 8 μM [^{14}C]-4-CBA and 3.5 mM ATP with 13−50 μM ligase (in 50 mM K^+Hepes, pH 7.5, 5 mM $MgCl_2$) showed that 80% conversion of the 4-CBA to 4-CBA-AMP could be expected from a single turnover of 30 μM ligase compared to 90% conversion determined by extrapolation to an infinite concentration of ligase. The time courses for the single-turnover reaction of 4-CBA and ATP, with and without CoA, were measured using 30 μM ligase, 8 μM [^{14}C]-4-CBA and saturating cosubstrate/Mg(II). These conditions met the technical limits set by the ligase supply/solubility and [^{14}C]-4-CBA specific activity while, at the same time, approximating single-turnover conditions (in which, ideally, all of the limiting reactant is enzyme bound).

The single-turnover reaction catalyzed by the ligase was analyzed at varying conversion by mixing the reactants/Mg(II) with buffered enzyme in a rapid quench apparatus and then quenching the reaction at various time intervals with acid. The [^{14}C]-4-CBA-AMP and/or [^{14}C]-4-CBA-CoA were separated from unconsumed [^{14}C]-4-CBA by HPLC and quantitated by liquid scintillation counting. The time course measured for the reaction carried out in the absence of CoA (Figure 2A) shows formation of 4-CBA-AMP in parallel with 4-CBA consumption. A maximum level of conversion is reached within 50 ms. The rate data were fit to a first-order equation to give a k_{obs} for 4-CBA consumption of 140 ± 10 s^{-1} and a k_{obs} for 4-CBA-AMP formation of 130 ± 20 s^{-1}. In order to demonstrate that the values of k_{obs} reflect the rate of catalysis and not substrate binding, the reaction was repeated at double the concentration of ligase (60 μM). In this case, the observed rate constants were determined to be 150 ± 10 and 120 ± 10 s^{-1}, respectively. Since the rate constants were not doubled by doubling the enzyme concentration, we assume that they are a measure of the rate of catalysis and are not limited by the rate at which substrate is bound. The rate of formation of 4-CBA-AMP (120 ± 10 s^{-1}) in the single-turnover experiment exceeds the steady state rate (0.013 s^{-1}) by several orders of magnitude. This rate difference is ascribed to the slow release of 4-CBA-AMP and/or PP_i from the enzyme, i.e., at 0.013 s^{-1}. The rate of formation of 4-CBA-AMP in the single-turnover reaction also exceeds the steady state rate of product formation (k_{cat} = 40 s^{-1}), thus demonstrating the kinetic competence of 4-CBA-AMP as an intermediate of the overall reaction.

The experimentally determined rate constants (k_{obs}) were used in conjunction with the kinetic model for the partial

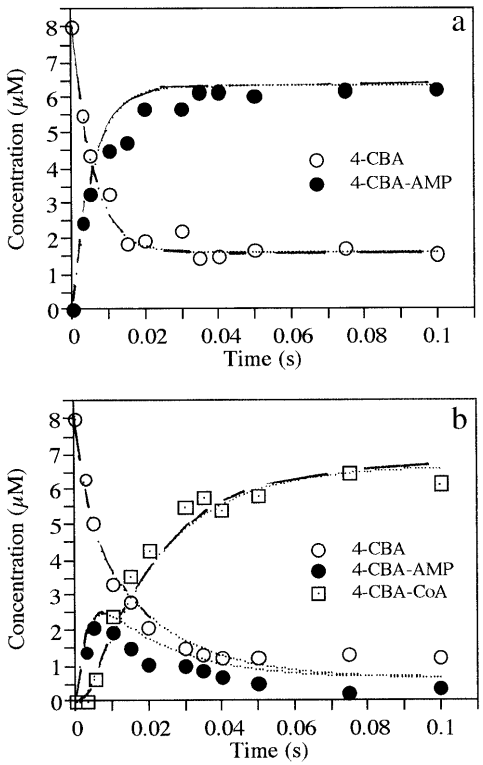


FIGURE 2: Time course for a single turnover of 4-CBA + MgATP catalyzed by 4-CBA:CoA ligase at 25 °C. A 43 μL solution of the enzyme (60 μM) in 50 mM K^+Hepes (pH 7.5) was mixed in the rapid quench instrument with a 43 μL solution containing 16 μM [^{14}C]-4-CBA (0.01 μCi), 7 mM ATP, and 10 mM $MgCl_2$ in 50 mM K^+Hepes (pH 7.5) without CoA (A) and with 2 mM CoA (B). The final concentrations of reactants in the 86 μL reaction mixture were 30 μM enzyme, 8 μm 4-CBA, 3.5 mM ATP, and 5 mM $MgCl_2$ (A) and same as in A (B) plus 1 mM CoA. After various reaction periods (ms), the solution was quenched with 164 μL of 0.1 N HCl. The 4-CBA, 4-CBA-AMP, and 4-CBA-CoA present in the reaction mixtures were analyzed by HPLC as described in Methods. The curves shown in the figure were generated using the kinetics simulation program KINSIM and the kinetic model presented in eq 1 for (A) and eq 2 for (B).

reaction (eq 1) and the computer program KINSIM (Barshop

$$\text{E·4-CBA·ATP} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{E·4-CBA-AMP·PP}_i \qquad (1)$$

et al., 1983) to fit the single-turnover time course data obtained from the rapid quench experiments. The simulated curves shown in Figure 2A defined the rate constants k_1 and k_{-1} in eq 1 to be 135 and 35 s^{-1}, respectively. We note that these values are estimates based on the assumption that the ratio of 4-CBA to 4-CBA-CoA, observed at the end of the reaction, reflects the true internal equilibrium constant (k_1/k_{-1}). As discussed in the previous section, the enzyme concentration used in the experiment does not exceed 10 times that of limiting reactant/product (4-CBA/PP_i), and, therefore, a source of error is introduced into the analysis as the observed equilibrium deviates from the true internal equilibrium.

Single-Turnover Profile for the 4-CBA:CoA Ligase-Catalyzed Full Reaction: E + 4-CBA + MgATP + CoA → E·4-CBA-CoA·AMP·PP$_i$. The single-turnover reaction described above was repeated, this time including a saturating level of CoA along with the other reactants. The 4-CBA, 4-CBA-AMP, and 4-CBA-CoA thus generated were analyzed by HPLC. As shown in Figure 2B, 4-CBA-AMP accumulated, reaching a maximum within 5−10 ms corresponding to approximately 25% of the radiolabeled mixture and then gradually disappearing with the formation of 4-CBA-CoA (4-CBA-CoA, in turn, reaching a maximum within 100 ms). The rate constants for 4-CBA decay and 4-CBA-CoA formation were determined in the same manner as described before to be 99 ± 4 and 73 ± 8 s^{-1}, respectively. These values were used along with those of k_1 and k_{-1}, eq 2, and the simulation program KINSIM to simulate the

$$\text{E·4-CBA·ATP·CoA} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{E·4-CBA-AMP·PP}_i\text{·CoA} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \text{E·4-CBA-CoA·AMP·PP}_i \qquad (2)$$

progress curves. The values of k_2 and k_{-2} obtained are 100 and 10 s^{-1}. The progress curves shown in Figure 2B were generated by relaxing the constraint placed on the value of k_{-1}. This step improved the fit to the 4-CBA-AMP data while not significantly affecting the 4-CBA and 4-CBA-CoA progress curves. The values of k_1, k_{-1}, k_2, and k_{-2} defined by the simulation are 135, 135, 100, and 10 s^{-1}, respectively. The *ca*. 4-fold increase seen in the value of k_{-1} simulated from the two respective sets of data (i.e., in Figure 2A,B) may or may not represent a real change in the rate constant for return of 4-CBA-AMP back to substrate in presence of bound CoA. It has been our experience that the values of rate constants simulated to a single set of time course rate data are reasonably well defined [usually no more than 50% variation in the value of an individual rate constant value is tolerated in making a good fit of the progress curve(s) to the time course data]. A seemingly less well defined rate constant may emerge when comparing values obtained from fits to duplicate or triplicate sets of time course measurements. Even greater variation in the value of the defined rate constant can be expected from simulations made using different types of time course measurements sets, subject to different constraints, as they are in the case of the time courses of Figure 2A and B.

Single-Turnover Profile for the Ligase-Catalyzed Reaction of 4-CBA + MgATP + Pantetheine. The rate constant for formation of 4-CBA-AMP measured in the presence of CoA (k_{obs} = 140 ± 10 s^{-1}; k_1 = 135 s^{-1}) is not largely different from that measured in its absence (k_{obs} = 99 ± 4 s^{-1}; k_1 = 100 s^{-1}), suggesting that the adenylation and thioesterification partial reactions are essentially kinetically independent.[2] Thus, substitution of CoA with an alternate, slow reacting substrate should decrease k_2 without effecting k_1 and hence allow greater accumulation of the intermediate 4-CBA-AMP to be seen during the single-turnover event since the level of 4-CBA-AMP formed will be more closely defined by k_1/k_{-1}. Pantetheine, a CoA analog missing the nucleotide moiety, is a slow substrate for the ligase, k_{cat} = 0.7 s^{-1} and K_m = 4 mM, as compared to CoA, k_{cat} = 40 s^{-1} and K_m = 80 μM (Chang, 1994). The profile for the single turnover reaction of 4-CBA, MgATP, and pantetheine (Pan) is shown in Figure 3. In this reaction, 4-CBA-AMP is formed at the expense of 4-CBA in a rapid phase peaking at 100 ms to a

[2] The value of k_{-1}, on the other hand, appears to increase from 35 s^{-1}, measured in the absence of CoA, to 135 s^{-1}, measured in the presence of CoA. This difference, however, is likely to fall within the error limit associated with defining the rate constant for the reverse reaction by fitting two different types of data sets by iterative curve simulation.

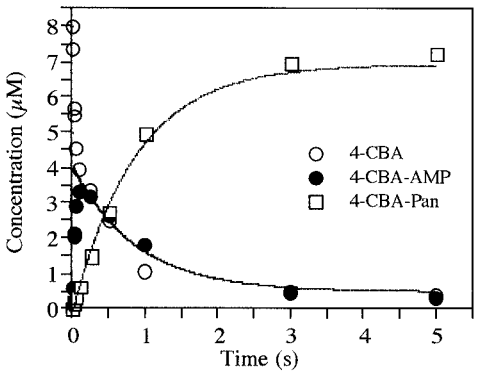


FIGURE 3: Time course for a single turnover of 4-CBA + MgATP + pantetheine catalyzed by 4-CBA:CoA ligase at 25 °C. A 43 μL solution of the enzyme (60 μM) in 50 mM K^+Hepes (pH 7.5) was mixed with a 43 μL solution containing 16 μM [^{14}C]-4-CBA (0.01 μCi), 7 mM ATP, 10 mM $MgCl_2$, and 20 mM pantetheine in 50 mM K^+Hepes (pH 7.5). The final concentrations of reactants in the 86 μL reaction mixture were 30 μM enzyme, 8 μM 4-CBA, 3.5 mM ATP, 5 mM $MgCl_2$, and 10 mM pantetheine. After various reaction periods, the solution was quenched with 164 μL of 0.1 N HCl. The 4-CBA, 4-CBA-AMP, and 4-CBA-Pan present in the reaction mixture were quantitated by HPLC as described in Methods. The curves shown in the figure were generated by computer simulation (KINSIM) using the kinetic model depicted in eq 2.

maximum corresponding to roughly half of the radiolabeled mixture. A slow phase follows in which 4-CBA and 4-CBA-AMP are consumed and 4-CBA-Pan is formed. The curves

$$\text{E·4-CBA·ATP·Pan} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{E·4-CBA-AMP·PP}_i\text{·Pan} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \text{E·4-CBA-Pan·AMP·PP}_i \quad (3)$$

simulated to fit the rate data (Figure 3) define the rate constants k_2 and k_{-2} equal to 2 and 0.15 s^{-1}, respectively. The ratio of 4-CBA to 4-CBA-AMP at the burst maximum is *ca*. 1, consistent with the $k_1 = k_{-1} = 135$ s^{-1} assumed in the simulation.

CONCLUSIONS

The 4-CBA:CoA ligase belongs to a large group of structurally homologous enzymes which function in ester, thioester, or amide synthesis. Common to these enzymes is the use of pantetheine as an intermediary acyl acceptor in ester or amide synthesis or CoA as a final acyl acceptor in thioester synthesis. To our knowledge the three-dimensional structure and mechanism of action have not yet been determined for any one of these enzymes. However, the present studies of 4-CBA:CoA ligase catalysis may provide some insight into the functioning of its structural relatives, especially in the particular cases of the carboxylic acid:CoA ligases (e.g., fatty acid:CoA, coumarate:CoA, acetyl:CoA ligase). First, the intermediacy of the acyl adenylate assumed for each of these enzyme-catalyzed reactions was convincingly demonstrated for the 4-CBA:CoA ligase reaction. This was accomplished by first isolating the 4-CBA-AMP adduct formed by the ligase-catalyzed reaction of 4-CBA and MgATP in the absence of CoA, establishing its structure by NMR analysis and then demonstrating its kinetic competence in the overall reaction using transient kinetic techniques. Pre-steady state kinetic analysis of the 4-CBA:CoA ligase adenylation and thioesterification partial reactions defined the forward and reverse rate constants for the adenylation half-reaction as 135 and 35−135 s^{-1} and the forward and reverse rate constants for the thioesterification partial reactions as 100 and 10 s^{-1}. Reaction at the 4-CBA/MgATP site appears to occur with essentially the same efficiency in the absence of CoA as in its presence. As we might expect, release of the 4-CBA-AMP/PP_i from the enzyme, measured in the absence of CoA, is slow (0.013 s^{-1}), thus minimizing wasteful consumption of ATP.

A particularly interesting finding is that pantetheine substitutes for CoA in the thioesterification step, albeit with less reactivity ($k_2 = 2$ s^{-1} vs 100 s^{-1}). This is in stark contrast to the 4-CBA-CoA dehalogenase (the enzyme which acts on the ligase product, 4-CBA-CoA) which requires the nucleotide moiety of the CoA unit for tight and productive binding, deriving minimal binding energy from the pantoic acid moiety (Taylor, 1996).

SUPPORTING INFORMATION AVAILABLE

^{1}H- and ^{31}P-NMR and absorbance spectra of 4-CBA-AMP (4 pages). See any current masthead page for ordering information.

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BI961284W