Dethiobiotin Synthetase: The Carbonylation of 7,8-Diaminononanoic Acid Proceeds Regiospecifically via the N7-Carbamate

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ABSTRACT: Dethiobiotin synthetase (DTBS) catalyzes the penultimate step in biotin biosynthesis, the formation of the ureido ring of dethiobiotin from (7*R*,8*S*)-7,8-diaminononanoic acid (7,8-diaminopelargonic acid, DAPA), CO_2, and ATP. Solutions of DAPA at neutral pH readily formed a mixture of the N7- and N8-carbamates in the presence of CO_2. However, four lines of evidence together indicated that only the N7-carbamate of DAPA was an intermediate in the reaction catalyzed by DTBS. (1) Addition of diazomethane to mixtures of DAPA and [^{14}C]CO_2 yielded a mixture of the N7- and N8-methyl carbamate esters, consistent with carbamate formation in free solution. In the presence of excess DTBS (over DAPA), the ratio of N7:N8-methyl carbamate esters recovered was roughly doubled, suggesting that the enzyme preferentially bound the N7-DAPA-carbamate. (2) Both N7- and N8-DAPA-carbamates were observed directly by ^{1}H and ^{13}C NMR in solutions containing DAPA and [^{13}C]CO_2. In the presence of excess DTBS (over DAPA) only one carbamate was observed, showing that carbamate binding to the enzyme was regiospecific. ^{13}C NMR of mixtures containing enzyme, [7-^{15}N]DAPA, and [^{13}C]CO_2 showed that the enzyme-bound carbamate was at N7 of DAPA. In addition, pulse−chase experiments showed that the binary complex of DTBS and N7-DAPA-carbamate became kinetically committed upon addition of MgATP. (3) The N7-DAPA-carbamate mimic, 3-(1-aminoethyl)nonanedioic acid, in which the carbamate nitrogen was replaced with a methylene group, cyclized to the corresponding lactam in the presence of DTBS and ATP; ADP and P_i were also formed. The K_M and V_{max} for this process were comparable to those for the natural substrate, DAPA. By contrast, the N8-DAPA-carbamate mimic, 4-amino-3-methyldecanedioic acid, was a much poorer substrate ($V/K \leq 0.1\%$ of that for DAPA), and the compound was only weakly inhibitory. These experiments strongly suggest that DTB is formed predominantly through the N7-carbamate of DAPA. (4) Crystallographic analysis at 1.65 Å resolution of several DTBS−DAPA complexes also reveals electron density consistent with the presence of a carbamate on the 7-amino group [Huang, W., Jia, J., Gibson, K. J., Taylor, W. S., Rendina, A. R., Schneider, G., & Lindqvist, Y. (1995) *Biochemistry 34*, 10985−10995].

Dethiobiotin synthetase (EC 6.3.3.3) catalyzes the penultimate step in biotin synthesis (Eisenberg, 1973), the formation of the ureido ring of DTB[1] from DAPA,[2] CO_2, and ATP (Scheme 1). The reaction catalyzed by DTBS presents a number of intriguing mechanistic questions. In their seminal paper, Krell and Eisenberg (1970) established that CO_2, rather than HCO_3^-, is the substrate. Carbamate formation on one of the amino groups of DAPA was suggested as the first step in the reaction. As to the involvement of ATP, Krell and Eisenberg (1970) pointed out that phosphorylation of a carbamate would activate the carbamyl carbon atom for nucleophilic attack by the other amine. Ring closure and displacement of phosphate were suggested to complete the reaction. As indicated in Scheme 2, the reaction can be envisaged as occurring by one of two pathways, depending on which of the two regioisomeric carbamates serves as an intermediate. However, the work of Krell and Eisenberg

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[1] Abbreviations: DTB, dethiobiotin; DTBS, dethiobiotin synthetase; DAPA, 7,8-diaminopelargonic acid; KAPA, 7-keto-8-aminopelargonic acid; PEP, phosphoenolpyruvate; MESG, 2-amino-6-mercapto-7-methylpurine ribonucleoside; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; Bicine, *N,N*-bis(2-hydroxyethyl)glycine; EDTA, ethylenediaminetetraacetic acid; AMP-PNP, adenylyl imidodiphosphate; Rubisco, ribulose bisphosphate carboxylase. CO_2, unless otherwise indicated, refers to the equilibrium mixture of CO_2 and HCO_3^-; N7-DAPA-carbamate and N8-DAPA-carbamate refer to the compounds formed upon addition of CO_2 to the 7-amine of DAPA and to the 8-amine of DAPA, respectively.

[2] The IUPAC nomenclature for biologically active 7,8-diaminopelargonic acid (DAPA) is (7*R*,8*S*)-7,8-diaminononanoic acid, for dethiobiotin is *cis*-(+)-5-methyl-2-oxo-4-imidazolidinehexanoic acid, for diaminobiotin is (2*S*)-(2α,3α,4α)-3,4-diaminotetrahydro-2-thiophenepentanoic acid, and for 7-keto-8-aminopelargonic acid (KAPA) is (8*S*)-8-amino-7-oxononanoic acid.

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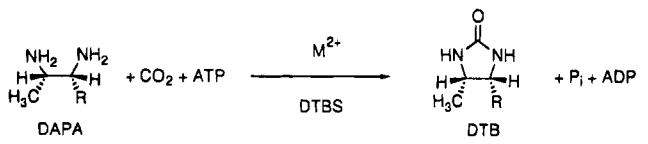
Scheme 1



R = $(CH_2)_5COO^-$

left the regiospecificity of the reaction unresolved. The goal of the work presented here was to determine which of the two pathways was utilized by DTBS. While our work was underway, Baxter et al. (1994) reported that the reaction proceeded through the N8-carbamate of DAPA (**2**). Our experiments, presented below, point instead to the intermediacy of the N7-DAPA-carbamate **1**.

MATERIALS AND METHODS

Analytical Methods. Proton nuclear magnetic resonance (^{1}H NMR) spectra were recorded at 500 MHz on GE Omega 500, at 400 MHz on Varian VXRS400, at 360 MHz on Bruker AMX-360, or at 300 MHz on Varian Unity Plus 300 spectrometers. Carbon nuclear magnetic resonance (^{13}C NMR) spectra and phosphorus nuclear magnetic resonance (^{31}P NMR) spectra were recorded on the GE, Varian VXRS400, or Bruker instruments (above). Trimethyl phosphite was used as a reference for ^{31}P NMR. Fast atom bombardment (FAB), electron ionization (EI), and chemical ionization (CI) mass spectral data were recorded on a Finnigan Mat95 high-resolution mass spectrometer, unless otherwise specified. Except for those described herein, all reagents and chemicals were of the highest commercially available quality.

DTBS Purification. High-purity DTBS was obtained from recombinant *Escherichia coli* cells by chromatography on Q-Sepharose, Phenyl Sepharose Fast Flow, Matrex Green A agarose, and Mono-Q. For details, see supporting information.

Synthesis and Standardization of (7R,8S)-DAPA. DAPA of the biologically active 7*R*,8*S* stereo configuration was synthesized from D-DTB (Sigma) by acid hydrolysis (6 N HCl, 150 °C, 15 h) (Davis et al., 1989) or base hydrolysis [$Ba(OH)_2$, 150 °C, 15 h] (Baker et al., 1947). DAPA·HCl: ^{1}H NMR (D_2O) δ 3.78 (1H, dq), 3.62 (1H, dt), 2.41 (2H, t), 1.8−1.3 (8H, m), 1.43 (3H, d); ^{13}C NMR (D_2O) δ 178.9, 53.6, 48.4, 33.7, 28.7, 27.7, 24.1, 23.8, 13.4. K-DAPA: ^{1}H NMR (D_2O, pD 8.2) δ 3.36 (1H, dq), 3.11 (1H, dt), 2.19 (2H, t), 1.7−1.3 (8H, m), 1.22 (3H, d). Analysis on a Beckman 6300 automatic amino acid analyzer revealed a single ninhydrin-positive peak with a retention time close to that of lysine.

(7*R*,8*S*)-DAPA solutions were standardized by quantitative conversion of DAPA to DTB in the presence of excess DTBS using either [^{14}C]CO_2 of defined specific radioactivity or the coupled spectrophotometric method (see below). A standardized solution of (7*R*,8*S*)-DAPA was used to calibrate the response of the amino acid analyzer; this allowed measurement of the total (7*RS**,8*S*)-DAPA concentration (see synthesis of [7-^{15}N]-DAPA, below), which could not be determined enzymatically.

[^{14}C]CO_2 Fixation Assay of DTBS. The assay is based upon the formation of acid-stable [^{14}C]DTB from acid-labile [^{14}C]CO_2 (New England Nuclear). A 1-mL reaction mixture containing 0.10 M Tris-HCl, pH 7.90, 10 mM $MgCl_2$, 10 mM [^{14}C]$NaHCO_3$ (of defined radiospecific activity, typically about 2500 dpm nmol^{-1}), 112 μM DAPA, 4 μM DTBS, and 270 μM ATP (to start the reaction) was incubated at room temperature. Measured aliquots were transferred at defined times to 250 μL of 10% acetic acid in a glass vial and taken to dryness on a hot plate to remove unreacted [^{14}C]CO_2. The residue was dissolved with a small volume of water, and the ^{14}C radioactivity was determined by scintillation counting. The reaction was linear for at least 1 h.

Coupled Spectrophotometric Assays of DTBS. For kinetic and mechanistic studies with highly purified DTBS, DTBS activity could be measured spectrophotometrically by monitoring either ADP or P_i formation. ADP production was assayed using the pyruvate kinase/lactate dehydrogenase couple (Cleland, 1979a). The disappearance of NADH was continuously monitored at 340 nm (ϵ = 6220 M^{-1} cm^{-1}) at 25 °C using a Uvikon 940 spectrophotometer in 1-cm pathlength self-masking cuvettes. In a total volume of 500 μL, a typical assay contained 100 mM K-HEPES, pH 7.5, 5 mM $MgCl_2$, 3 mM ATP, 15 mM $NaHCO_3$, 0.08 mM NADH, 0.25 mM PEP, 5 units each of pyruvate kinase (Boehringer-Mannheim) and lactate dehydrogenase (Sigma), 0.01−0.05 units of DTBS, and varying amounts of DAPA (or alternate substrates). Inhibitors were analyzed under similar conditions except for 2 mM $MgCl_2$, 1 μM ATP, and 2 μM DAPA.

Alternatively, release of inorganic phosphate was monitored continuously at 360 nm (ϵ = 11 000 M^{-1} cm^{-1}) by a method adapted from Webb (1992). In a total volume of 500 μL, a typical assay contained 100 mM K-HEPES, pH 7.5, 5 mM $MgCl_2$, 3 mM ATP, 3 mM $NaHCO_3$, 0.1 mM PEP, 0.2 mM MESG, 3 units of desalted pyruvate kinase, 0.1 unit of bacterial purine nucleoside phosphorylase (Sigma), 0.01−0.05 unit of DTBS, and varying amounts of DAPA or alternate substrates. Higher levels of bicarbonate produced an unacceptably high background activity. Assays were initiated with DAPA or alternate substrate, and the rates were corrected for the background rate determined in the absence of these substrates.

Data Analysis. Data were fitted to the appropriate rate equations using the FORTRAN programs of Cleland (1979b) modified for a PC. Individual saturation curves were fitted to eq 1, and initial velocity patterns were fitted to eq 2 or the form of this equation where the logarithm was taken of both sides (this changes the assumed error distribution from one with constant errors in initial velocities to one with proportional errors). Competitive inhibition data were fitted to eq 3. In eqs 1−3, v is the initial velocity, V is the maximal

$$v = VA/(K_a + A) \quad (1)$$

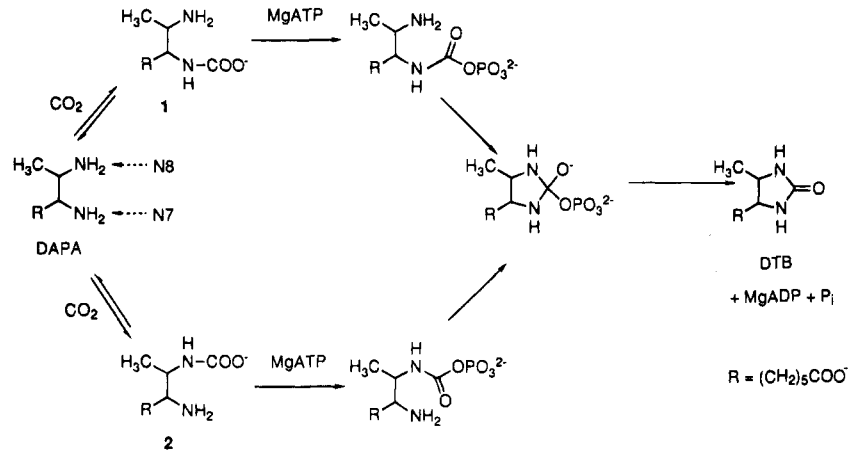
$$v = VAB/(K_{ia}K_b + K_aB + K_bA + AB) \quad (2)$$

$$v = VA/[K_a(1 + I/K_i) + A] \quad (3)$$

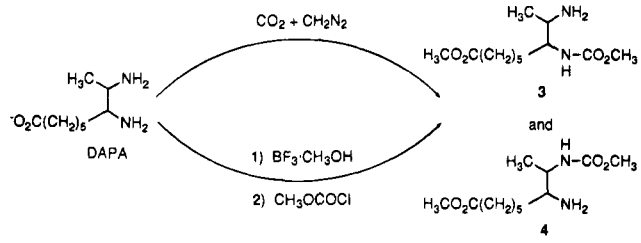
velocity, K_a and K_b are the K_M's for substrates A and B, respectively, K_{ia} is the dissociation constant for A, and K_i is the inhibition constant for inhibitor I.

Synthesis, Separation, and Identification of the N7- and N8-Methyl Carbamate Esters of DAPA. Scheme 3 shows the two different routes used to prepare mixtures of the two isomeric methyl carbamate esters of DAPA (**3** and **4**). For the more efficient two-step route, DAPA·H_2SO_4 (6 mg, 21

Scheme 2



Scheme 3



μmol) was heated at 60 °C for 20 min in 0.25 mL of 14% BF_3·CH_3OH. Conversion of DAPA to its methyl ester was checked by TLC (silica; *tert*-butyl alcohol–butanone–water–concentrated NH_4OH, 4:4:2:0.4; ninhydrin detection; R_f DAPA ~0.05, R_f DAPA methyl ester ~0.5). The ester was not isolated, owing to the difficulty of handling small amounts of compounds bearing free amino groups. Instead, water (~0.1 mL) was added to hydrolyze remaining BF_3, and the mixture was taken almost to dryness. The residue was taken up in ~0.5 mL of an aqueous mixture of 1 N NaOH and 0.5 M Na_2CO_3 to give a final pH of ~8.5 (pH paper) and treated with methyl chloroformate in tetrahydrofuran (31 μL of 1 M reagent, 31 μmol). TLC as above showed that some starting material remained but that there were two new ninhydrin-positive spots, with R_f's of 0.6 and 0.7. The sample was centrifuged and run on a C_{18} HPLC column (Zorbax ODS, 6.2 mm × 8 cm) with a 15 mL linear gradient from 5% to 85% CH_3CN containing 0.1% trifluoroacetic acid; the other component was 0.1% trifluoroacetic acid in water. Ninhydrin-positive fractions were located, and TLC showed that the two new products formed with methyl chloroformate had coeluted. A portion of the new product fraction (estimated to contain <2 mg of solid) was applied in a 5 cm streak to a foil-backed silica TLC plate and developed as before. A strip cut from the side of the plate was stained with ninhydrin. The strip was aligned with the rest of the plate, and the appropriate areas were scraped off. The compounds were eluted with concentrated NH_4OH–methanol (1:95), taken to dryness, and analyzed by GC/MS using an HP5890 GC with a DB-1 capillary column and HP5971 mass-selective detector. The GC/MS data were consistent with the structural assignments shown in Figure 1. The base peak in both 70 eV EI spectra could be accounted for by cleavage of the C7–C8 bond with charge on the fragment containing the free amino group (Silverstein et al., 1981). HR GC/MS (EI): for the N7-methyl carbamate ester **3** (Figure 1A), base peak 44.0529 ± 0.0036 (calculated for C_2H_6N 44.0500); for the N8-methyl carbamate ester **4** (Figure 1B), base peak 158.1181 ± 0.0036 (calculated for $C_8H_{16}NO_2$ 158.1180). Protonated molecular ions for both isomers were observed by HR GC/MS (CI): for **3**, m/z (MH^+) 261.1780 ± 0.0040; for **4**, *m/z* (MH^+) 261.1777 ± 0.0040; calculated for $C_{12}H_{25}N_2O_4$ 261.1814. The same monomethyl carbamate esters were formed in one step by adding diazomethane (~0.5 M) in methoxyethanol (1 mL) to a mixture of $NaHCO_3$ (4.5 μmol), DAPA (0.5 μmol), and $MgCl_2$ (1 μmol) in 0.1 M Na-HEPES, pH 7.2 (50 μL). (CAUTION: Diazomethane, a toxic and potentially explosive gas, was made by NaOH decomposition of 1-methyl-3-nitro-1-nitosoguanidine, a potent mutagen, carcinogen, and irritant. The Aldrich MNNG diazomethane generator was

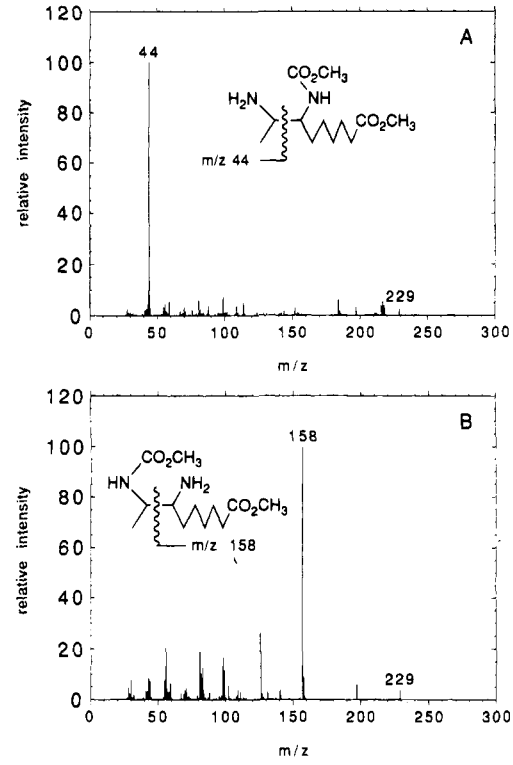


FIGURE 1: Mass spectra of (A) N7-DAPA-methyl carbamate ester **3** and (B) N8-DAPA-methyl carbamate ester **4**. We attribute the peaks at *m/z* = 229 to M − OCH_3. On TLC, the N8-methyl carbamate ester **4** ran ahead of the N7-methyl carbamate ester **3**; on GC, the N7-methyl carbamate ester **3** eluted first (see Materials and Methods).

used as directed, and diazomethane was handled only in a hood.) After ~15 min on ice, the sample was concentrated to ~50 μL, and 5 μL was analyzed by GC/MS.

Carbamate Trapping Experiments. DTBS for these experiments was twice concentrated at least 10-fold over a Centricon-30 membrane (Amicon), and a portion of the last filtrate was used in place of enzyme solution in the no-enzyme control reaction. Typical reaction mixtures (~100 μL) contained 150 mM Na-HEPES, pH 7.2, 0.1 mM EDTA, 22 mM $MgCl_2$, 2 mM DAPA, 2.2 mM DTBS (when present), 4 mM AMP-PNP (when present), and 40 mM [^{14}C]Na_2CO_3 at 4 dpm/pmol, added last. After about 2 min, a large volume (0.8 mL) of methoxyethanol containing diazomethane (~0.5 M) was added to denature the protein and to convert carbamyl and carboxyl groups to their methyl esters. After a few minutes, any remaining diazomethane color was discharged by addition of acetic acid (1 M, 50 μL). Water (3 mL) was added, protein was removed by filtration using a Centricon-10 (Amicon), and the filtrates were taken to dryness using a Speed-Vac. The glassy residue was dissolved in water (200 μL), diazomethane in tetrahydrofuran was added until the color persisted, solvent was evaporated, and the residue was taken up in water (5 mL). After addition of unlabeled DAPA-methyl carbamate esters (~0.2 mg), the mixture was applied to AG 50-X8 (Bio-Rad, 0.6 mL, triethylammonium form, in water) and followed with water (2.5 mL). The DAPA-methylcarbamate esters were eluted with triethylammonium bicarbonate (2.5 mL, 1 M, pH ~7). The aqueous and triethylammonium bicarbonate fractions were taken to dryness and redissolved, and aliquots were counted. The recovery of ^{14}C suggested that about 8−13% of the DAPA present had been recovered as mono-DAPA-methyl carbamate esters. A portion (~5000 dpm) of each sample was fractionated by TLC (plastic-backed silica plate; *tert*-butyl alcohol−butanone−water−concentrated NH_4OH, 4:4:2:0.1). The DAPA-methyl carbamate ester spots were located by autoradiography, cut out, and counted.

Synthesis of [7-^{15}N]-DAPA. (7*RS**,8*S*)-[7-^{15}N]DAPA was made from KAPA[3] via the corresponding oxime, which was made by a modification of the method of Lachman (1943). [^{15}N]Hydroxylamine hydrochloride (0.47 g, 6.73 mmol, C/D/N Isotopes) was added to a solution of KAPA·HCl (0.5 g, 2.24 mmol) in 25 mL of water containing sodium acetate (0.74 g, 8.96 mmol). The reaction was monitored by TLC (silica; 1-butanol−acetic acid−water, 4:1:1). After 24 h at room temperature, an additional 100 mg of [^{15}N]hydroxylamine hydrochloride was added. After being stirred for an additional 24 h, the water was evaporated to afford the crude (8*S*)-8-amino-[7-^{15}N]hydroxyliminenonanoic acid as an off-white solid. Without further purification, the oxime (982 mg, 4.8 mmol) was reduced to the amine at 50 psi H_2 with PtO_2 (0.5 g) in acetic acid (25 mL) and HCl (1 N, 5 mL) for 96 h [modification of Rausser et al. (1966)]. The reaction was filtered through Celite and concentrated. The crude product was dissolved in water and lyophilized to give 0.9 g of a tan solid. (7*RS**,8*S*)-[7-^{15}N]DAPA: HR MS (EI) *m/z* (MH^+ − Cl) 190.1571 (calculated for $C_9H_{21}{}^{15}NNO_2$ 190.1568).

[3] KAPA was made by the route of Nudelman (unpublished experiments); for an alternate synthesis of KAPA, see Suyama and Kaneo (1963).

Since the reductive amination of KAPA gave a mixture of epimers at C7, DTBS itself was used to resolve the mixture of DAPA diastereomers. About 72 μmol of (7*RS**,8*S*)-[7-^{15}N]DAPA was incubated at 23 °C with 100 mL of 3.85 μM DTBS, 0.10 M Tris−acetate, pH 7.9, 0.4 mM EDTA, 1.0 mM ATP, 8 mM magnesium acetate, and 5 mM [^{14}C]-$NaHCO_3$ (211 dpm $nmol^{-1}$). The progress of the reaction was followed by determining the quantity of acid-stable radioactivity (i.e., [^{14}C]DTB). After about 24 μmol of [^{14}C]-DTB had accumulated, the reaction was quenched with AG 50-X8 (Bio-Rad, H^+ form). The suspension was sparged with N_2 to remove unreacted [^{14}C]CO_2 before filtration. The filtrate plus washings were taken to dryness under vacuum. The residue was dissolved in water and applied to a 1 × 10 cm column of AG 1-X8 (Bio-Rad, formate form). The column was developed with a 200 mL linear gradient (0−10%) of formic acid to separate DTB from unreacted DAPA. Fractions containing radioactivity were combined and reduced to dryness as before. Acid hydrolysis of the residue in a sealed ampule (6 N HCl, 150 °C, 15 h) yielded [7-^{15}N]-DAPA. Insoluble material was removed by centrifugation, and excess HCl was removed by evaporation to dryness. The residue was dissolved in water and the pH adjusted to 7 with NaOH. The resolved [7-^{15}N]DAPA was standardized enzymatically and by quantitative amino acid analysis; the values obtained were in good agreement (±5%), indicating that the resolution was successful.[4]

Although the synthesis of the [7-^{15}N]DAPA was unambiguous, the location of the label in the resolved [^{15}N]DAPA used for the NMR experiments was verified by mass spectrometry. A sample (~0.5 mg) of the [^{15}N]DAPA was converted to a mixture of its monomethyl carbamate esters by the chloroformate procedure as described above. Upon analysis by GC/MS, the base peak in the spectrum of the [7-^{15}N]DAPA-8-methyl carbamate ester appeared at m/z = 159, 1 amu higher than for the corresponding fragment in the unlabeled compound (Figure 1B); the m/z = 158 peak had 2.2% of the intensity of the base peak, consistent with high isotopic purity in the ^{15}N. The base peak for the [7-^{15}N]-DAPA-7-methyl carbamate ester appeared at m/z = 44, and the m/z = 45 peak was close to normal in intensity (5.4% of base, versus a typical 3.8% for unlabeled material), suggesting that the label was overwhelmingly at the 7-position. In addition, for neither [^{15}N]DAPA-methyl carbamate ester was there any sign of doubly labeled material; each M − OCH_3 peak appeared at m/z = 230 (relative intensity 2−4% of base), and no peak was detected at m/z = 231 (relative intensity <0.5%).

Enzymatic Formation of cis- and trans-[5-^{13}C]-6. In a total volume of 3.2 mL in a 10 mm wide bore NMR tube (Wilmad) a typical reaction mixture contained 0.1 M K-HEPES, pH 7.8, 5.2 mM $MgCl_2$, 3 mM ATP, 1 mM PEP, 5 units of desalted pyruvate kinase, 25% D_2O (Aldrich), 1 mM [1-^{13}C]-**5**, and 5.5 μM DTBS active sites assuming 2 sites per dimer. ^{13}C NMR spectra were collected each hour over a 16 h time period (1 h acquisition time). The enhanced signals at 182.5 and 182.3 ppm for the starting material

[4] The two diastereomers present in the (7*RS**,8*S*)-[7-^{15}N]DAPA were separable as their methyl esters by TLC [silica; *tert*-butyl alcohol−butanone−water−concentrated NH_4OH, 4:4:2:0.1; R_f of methyl (7*R*,8*S*)-DAPA, 0.44; R_f of methyl (7*S*,8*S*)-DAPA, 0.54]. Comparison of lanes with various amounts of the resolved [7-^{15}N]DAPA enabled us to estimate its diastereomeric purity at >97%.

Table 1: Carbamate Trapping Experiments[a]

expt	no DTBS	DTBS	DTBS + AMP-PNP
I	2.7	4.9	4.8
II	1.3	2.4	3.2
III	1.7	2.8	4.4

[a] Entries in the table are ratios of the N7-DAPA-methyl carbamate ester **3** to the N8-DAPA-methyl carbamate ester **4** recovered from mixtures of DAPA + $[^{14}C]CO_2$, with or without DTBS and the ATP analog, AMP-PNP, after addition of diazomethane in methoxyethanol. See text for further details.

decreased with concomitant increase in enhanced signals at 181.9 and 181.6 ppm, which correspond to the synthetically prepared *cis*- and *trans*-lactams ([5-^{13}C]-**6**, see supporting information). Control samples without DTBS exhibited no change in the enhanced signals of the starting material over the same period. The same experiment was repeated and monitored by ^{31}P NMR. The only changes observed were the decrease in the PEP signal with a corresponding increase in the P_i signal (ADP was cycled back to ATP by the PEP/pyruvate kinase coupled reaction).

RESULTS

Regioselective Interaction of DTBS with the 7-Carbamate of DAPA: Evidence from Diazomethane Trapping Experiments. Since CO_2 exchanged out of DAPA carbamates quickly (see kinetic pulse−chase experiments below), we sought to trap the DAPA carbamates as their methyl esters (i.e., as DAPA-methyl carbamate esters) by reaction with diazomethane. This facile reaction proceeds under mild conditions (Lorimer & Miziorko, 1981). A mixture of the two possible DAPA-methyl carbamate esters was first synthesized. The compounds proved separable by TLC and identifiable by GC/MS (see Materials and Methods and Figure 1). This enabled us to determine if the ratio of the DAPA-methyl carbamate esters recovered from reactions containing DAPA, CO_2, and diazomethane was affected by the presence of DTBS. For quantitation, reaction mixtures labeled with $[^{14}C]CO_2$ were fractionated by TLC, and the appropriate regions of the chromatogram were cut out and counted. The data in Table 1 show that in any given experiment the presence of enzyme approximately doubled the ratio of N7- to N8-DAPA-methyl carbamate ester recovered, though the ratios vary from experiment to experiment. The selectivity of the trapping reaction was only slightly increased by including the relatively inert ATP analog AMP-PNP. The most direct way for the enzyme to induce the observed ratio bias is for it to bind preferentially to the N7-carbamate of DAPA. The lack of absolute specificity in the presence of DTBS could mean that the enzyme was not fully regiospecific or that some of the DAPA carbamates were trapped in solution, where both carbamate isomers were present.

Nonenzymatic Formation of the Carbamates 1 and 2 of DAPA. We considered the carbamate trapping results to be suggestive rather than definitive, in part because they were inconsistent with those of Baxter et al. (1994). We therefore sought other ways of examining the regiochemistry of the DAPA carbamate intermediate utilized by DTBS. The use of ^{1}H and ^{13}C NMR seemed particularly promising. In the ^{1}H NMR spectrum of DAPA without CO_2, the resonance at 3.36 ppm (1H, dq) corresponded to H8 and that at 3.11 ppm

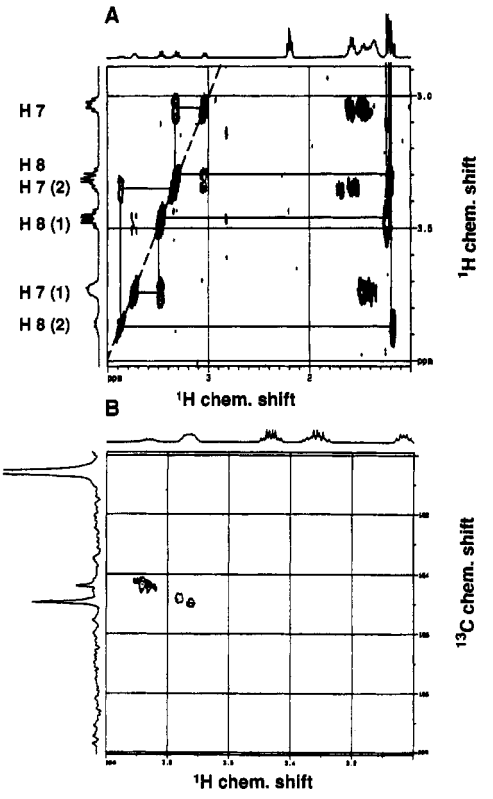


FIGURE 2: (A) ^{13}C-decoupled, COSY ^{1}H NMR spectrum of the mixture of DAPA, [^{13}C]-N7-DAPA-carbamate **1**, and [^{13}C]-N8-DAPA-carbamate **2**. Cross peaks (those off the diagonal dotted line) arise from interactions between protons separated by three bonds. The vertical and horizontal line segments connect the cross peaks to the signals in the 1-D spectrum that are correlated with each other. Use of the C9 methyl proton signals between 1.1 and 1.3 ppm as a starting point allows identification of the H8 signals from the three forms of DAPA present, and the H8 signals in turn point to their corresponding H7 signals. The assignment of the pairs of H7 and H8 resonances to DAPA, **1**, and **2** (left margin) was made using the ^{1}H spectrum of free DAPA (see text) and the HMBC data of panel B. (B) Three-bond correlation of the ^{1}H and ^{13}C spectra of the mixture of [^{13}C]-N7-DAPA-carbamate **1** and [^{13}C]-N8-DAPA-carbamate **2**. Data were obtained on the Bruker AMX-360 instrument using standard pulse sequences on a sample containing 10 mM DAPA and 50 mM $NaH^{13}CO_3$ in 100% D_2O (pD 9.3, measured after analysis).

(1H, dt) to H7. The homonuclear correlated (COSY) spectrum showed correlations between H8 and H9 and between H7 and the adjacent nonequivalent protons at C6. A small correlation occurred between H7 and H8, which is consistent with a small coupling constant (J_{H7-H8} = 4.3 Hz) (data not shown). After addition of $[^{13}C]CO_2$, ^{1}H NMR (Figure 2) and ^{13}C NMR (Figures 2B and 3A) spectra showed a mixture of free DAPA along with a 3.2 to 1 mixture of the two carbamates. COSY and ^{1}H/^{13}C heteronuclear multiple-bond correlation (HMBC) data (Figure 2A,B) allowed specific assignment of proton chemical shifts for each carbamate (Table 2). In addition, clear correlations were seen between the ^{13}C resonance for the major carbamate at 165.3 ppm and the H7 proton signal at 3.73 ppm and between the minor carbamate at 164.8 ppm and the H8 proton signal at 3.86 ppm (Figure 2B). Thus, the major DAPA-carbamate is at N7. We have also examined the ^{13}C NMR spectrum of the DAPA-carbamates prepared with $[^{13}C]CO_2$ and [7-^{15}N]DAPA. A ^{13}C NMR signal will be split by an adjacent ^{15}N nucleus. Figure 3B shows that the major carbamate peak was split, confirming that this was the N7

Table 2: Proton Chemical Shift Assignments

structure	^{1}H NMR chemical shift[a] H7	H8	H9
DAPA	3.11	3.36	1.22
N7-DAPA-carbamate **1**	**3.73**	3.47	1.23
N8-DAPA-carbamate **2**	3.34	**3.86**	1.17

[a] The chemical shifts in bold indicate coupling to the ^{13}C-labeled carbamates.

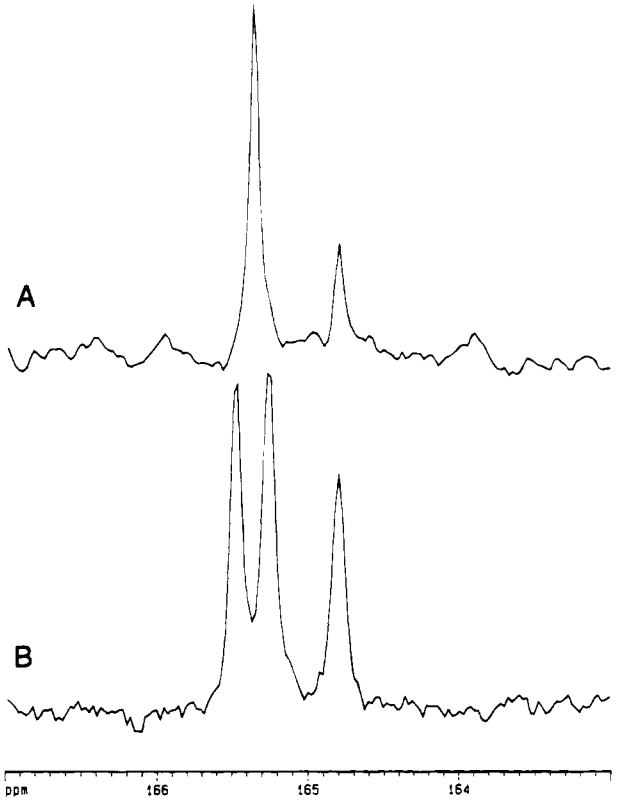


FIGURE 3: 90.55 MHz ^{13}C NMR spectra of DAPA + [^{13}C]CO_2 (A) and [7-^{15}N]DAPA + [^{13}C]CO_2 (B). The conditions were, for spectrum A, the same as in Figure 2; for spectrum B, 5.1 mM resolved [7-^{15}N]DAPA, 90 mM NaH$^{13}CO_3$, and 7% D_2O (pH 9.05). A line broadening of 5 Hz was used; the line widths in spectrum A were 1.8 Hz for the major peak and 2.2 Hz for the minor peak.

regioisomer. The ^{13}C−^{15}N coupling constant of 19.9 Hz is typical of carbamates (Levy & Lichter, 1979; Mueller et al., 1994).

Regiospecific Interaction of DTBS with the N7-Carbamate of DAPA: Evidence from NMR Studies. The ^{13}C NMR spectrum of unenriched DTBS is unremarkable. Among other features it contains a broad carbonyl envelope (170−185 ppm) which represents the contribution of the 224 carbonyl carbon atoms per DTBS monomer. Upon addition of 10 mM [^{13}C]$NaHCO_3$ to the solution of DTBS, four new resonances at 123, 161.0, 164.7, and 164.9 ppm (shoulder) appeared. The resonances at 123 and 161 ppm correspond to [^{13}C]CO_2 and [^{13}C]HCO_3^-, respectively. Those at 164.7 and 164.9 ppm can tentatively be assigned to carbamates formed on DTBS on the ϵ-amino group of a lysine and the α-amino group of the N-terminal serine, by analogy to carbamate formation on hemoglobin (Morrow et al., 1981). Since carbamate formation on proteins is a rather facile reaction (Lorimer, 1983), no particular physiological significance is attached to this observation at the present time.

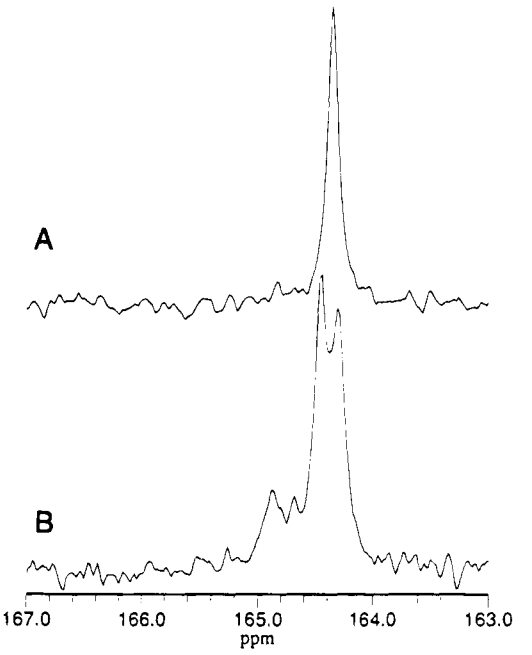


FIGURE 4: ^{13}C NMR spectra of DTBS in the presence of (A) DAPA and [^{13}C]CO_2 and (B) [7-^{15}N]DAPA and [^{13}C]CO_2. 125.76 MHz ^{1}H-decoupled ^{13}C spectra were acquired in nonspinning 12 mm tubes on a GE Omega 500 at 25 °C with 65° pulse widths and 0.47 s recycle times. Low-power, multipulse ^{1}H-decoupling protocols were used. A line broadening of 3.0 Hz was used. More than 20 000 transients were accumulated on solutions containing (A) 1.11 mM DTBS in 50 mM triethanolamine hydrochloride, pH 7.6, 1 mM EDTA, 5 mM [^{13}C]$NaHCO_3$, 673 μM DAPA, and 4% D_2O and (B) 848 μM DTBS in 50 mM triethanolamine hydrochloride, pH 7.6, 1 mM EDTA, 10 mM [^{13}C]$NaHCO_3$, 480 μM [7-^{15}N]-DAPA, and 4% D_2O: J_{C-N} = 19.3 Hz. The small signals at 164.7 and 164.9 ppm in spectrum B are due to interaction between the enzyme and $^{13}CO_2$ (see text) and may have caused the asymmetry of the major doublet.

Addition of an approximately 0.6 molar equiv of DAPA to a solution of DTBS and [^{13}C]CO_2 led to the appearance of a single new resonance line at 164.4 ppm (Figure 4A). The broad line width (about 14 Hz) of this resonance clearly indicates that it originated from a species bound to DTBS. This resonance can be attributed to one or another of the carbamates of DAPA, bound at the active site of DTBS. Since DTBS was in excess over DAPA and at a concentration much greater than K_{ia} (Table 3), virtually all of the DAPA-carbamate should have bound to DTBS. Consistent with this prediction, no sharp resonances at 164.8 and 165.3 ppm, attributable to the free N8- and N7-carbamates of DAPA (Figure 3A), respectively, were detected. Measurement of the quantity of bound [^{13}C]DAPA-carbamate by the method of Morrow et al. (1981) agreed to within 10% of the quantity of DAPA determined on a small aliquot of the solution by the ^{14}C method. Upon addition of a substoichiometric quantity of MgATP, the resonance at 164.38 ppm decreased in intensity, and a new resonance appeared at 166.35 ppm corresponding to [^{13}C]DTB. Addition of a molar excess of MgATP caused the resonance line at 164.38 ppm to disappear altogether. Integration of the ^{13}C resonance lines, together with analysis by the ^{14}C method, confirmed that the species at 166.35 ppm (DTB) increased quantitatively at the expense of that at 164.38 ppm (DAPA-carbamate). Traces of the protein-bound carbamates at 164.7 and 164.9 ppm remained. These results show that DTBS preferentially bound a single carbamate of DAPA.

Table 3: Kinetic Parameters for DTBS Substrates, pH 7.5

substrate	V_{max}[a] (min^{-1})	K[a] (mM)	V/K_a (μM^{-1} min^{-1})	K_{ia} (μM)	K_{MgATP} (μM)	K_{iMgATP} (μM)
DAPA	2.9	0.3 ± 0.04	9.7	4.5 ± 0.5	0.4 ± 0.04	6.3 ± 1.0
5	5.4	12 ± 0.4	0.45	160 ± 5.0	1.5 ± 0.05	20 ± 0.5
diaminobiotin	1.9	9.0 ± 1.3	0.21	500 ± 100	0.8 ± 0.2	40 ± 6.0
7	≈0.4	≈20000	≈0.00002			

[a] Data for V_{max} values for DAPA, **5**, and diaminobiotin were obtained on the same day by determining V and K_M for MgATP from fits of the data to eq 2 at 10 μM DAPA, 50 μM **5**, and 100 μM diaminobiotin. Data for K_M and K_{ia} values for DAPA, **5**, and diaminobiotin were obtained from initial velocity data fitted to the log form of eq 2. DAPA, **5**, and diaminobiotin show substrate inhibition at higher concentrations; however, the levels of substrates used in the analysis were not in the inhibitory range. V_{max} and K_M values for **7** were determined using the P_i-coupled assay, and the V_{max} was normalized to the V_{max} for **5** determined on the same day by the same method. Kinetic values for **7** are approximate due to extrapolation of the data from 3.3 mM, the highest level tested for substrate activity. At pH 7.5 the K_M and K_{ia} values for CO_2 with either DAPA or MgATP as the other varied substrate were both approximately 4 μM (data not shown). When CO_2 was varied, assays were conducted in stoppered cuvettes, and the solutions were sparged with argon prior to use. Free CO_2 concentrations were varied with exogenous $NaHCO_3$ and calculated using a value of 6.12 for the CO_2/HCO_3^- pK (Schloss, 1991). The endogenous levels of HCO_3^- in the assays were estimated as described in Rendina et al. (1988) and included in these calculations. Bicarbonate was not added to the assays for **5** and **7**.

To address the question of the regiochemistry of the DTBS-bound DAPA carbamate, [7-^{15}N]DAPA was employed. When bound to DTBS in the presence of [^{13}C]CO_2/HCO_3^-, a doublet centered at 164.39 ppm was observed (Figure 4B). The ^{13}C−^{15}N coupling constant of this enzyme-bound carbamate was 19.3 Hz. Addition of excess MgATP led to the appearance of a doublet centered at 166.36 ppm (J_{C-N} = 20.6 Hz), corresponding to [^{13}C,^{15}N]DTB. This experiment demonstrated that the DTBS-bound DAPA-carbamate was the N7 regioisomer.

The DTBS·N7-DAPA-Carbamate Complex Is Kinetically Competent. For the above conclusion to have any mechanistic significance, the binary complex of DTBS·N7-DAPA-carbamate must display kinetic competence (the ability to form product at least as fast as the overall reaction). This was tested in pulse−chase experiments. As shown in Figure 5, a burst of [^{14}C]DTB formation was observed when, after preincubation of a mixture of DTBS, DAPA, [^{14}C]CO_2, and $MgCl_2$, the reaction was initiated by simultaneous addition of ATP and excess unlabeled CO_2. Similar results were obtained if $MgCl_2$ was added with the ATP, rather than being included in the preincubation mixture. The burst was proportional to the DTBS concentration and equal to 0.70 active site. No burst was observed when DAPA was omitted from the preincubation mixture (i.e., when the reaction was started with DAPA, ATP, and excess unlabeled CO_2), as expected. There was also no burst when, after preincubation of a mixture of DAPA, [^{14}C]CO_2, and MgATP, the reaction was initiated by the simultaneous addition of DTBS and excess unlabeled CO_2. This shows that the equilibration of CO_2 with DAPA-carbamates in free solution was rapid compared to the rate of enzyme turnover (data not shown). Thus, upon addition of MgATP to the binary complex of DTBS with N7-DAPA-carbamate, the DAPA-carbamate becomes strongly committed to product formation. These observations are consistent with the preincubation results obtained by Baxter et al. (1994). The rate constant associated with the burst was about 8 min^{-1}, which is slightly in excess of the k_{cat} of 2.9 min^{-1}. These results indicate that the DTBS·N7-DAPA-carbamate complex was kinetically competent. This is consistent with a reaction mechanism that proceeds with formation of a mixed carbamic−phosphoric anhydride on the N7-DAPA-carbamate. It is also consistent with a reaction pathway which proceeds by formation of a mixed carbamic−phosphoric anhydride of the N8-DAPA-carbamate, if the two carbamates equilibrate on the enzyme at a rate that is rapid compared to enzyme turnover. The experiments with stable carbamate mimics presented below suggest that the latter pathway is unlikely.

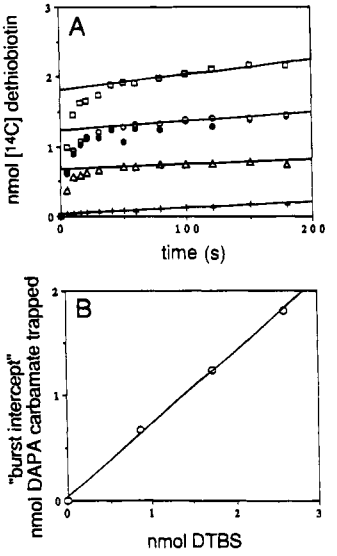
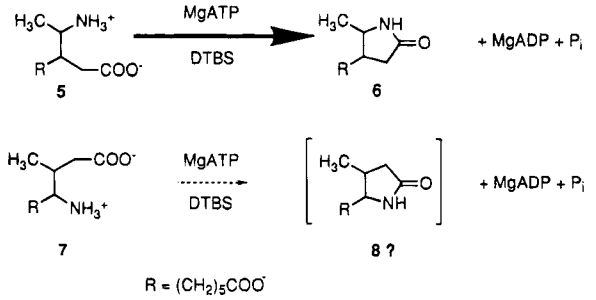


FIGURE 5: Pulse−chase experiments demonstrating that the binary DTBS·DAPA-carbamate complex becomes committed to product formation upon addition of MgATP. (A) DTBS [2.59 nmol (squares), 1.73 nmol (circles and crosses), or 0.86 nmol (triangles)] was preincubated for 10 min at 25 °C in an 80 μL solution of 125 mM Na-Bicine, pH 7.86, 12.5 mM $MgCl_2$ [or no $MgCl_2$ (closed circles)], 6.25 mM [^{14}C]$NaHCO_3$ (4432 dpm $nmol^{-1}$), 3 μg of carbonic anhydrase (Sigma), and 373 μM DAPA [or no DAPA (crosses)]. At time 0 20 μL of 2.7 mM ATP in 0.50 M $NaHCO_3$ was added to start the reactions shown by the open symbols. The reactions shown by the closed circles and crosses were initiated by adding 20 μL of 2.7 mM ATP and 50 mM $MgCl_2$ in 0.50 M $NaHCO_3$ or 20 μL of 2.7 mM ATP and 1.86 mM DAPA in 0.50 M $NaHCO_3$, respectively. The final concentrations were 373 μM DAPA, 540 μM ATP, 10 mM $MgCl_2$, and 0.1 M $NaHCO_3$. The reactions were quenched at the indicated times. The radiospecific activity of the [^{14}C]CO_2 in the preincubation was used to compute the quantity of [^{14}C]DTB formed. The solid lines represent regression analyses on the data from 50 to 180 s. (B) The intercepts of the regression lines in (A), representing the quantities of DAPA-carbamate trapped, are plotted as a function of the quantity of DTBS present. The slope of this line (0.70) is the stoichiometry of N7-DAPA-carbamate that becomes committed to DTB formation upon addition of ATP.

Ureido Ring Formation by DTBS Proceeds Directly from the N7-Carbamate of DAPA: Evidence from Carbamate Analogs. Stable analogs of the two possible carbamates of DAPA were prepared (see supporting information) and tested as inhibitors and substrates (Scheme 4). The analog of the N7-carbamate in which the 7-nitrogen was replaced with a

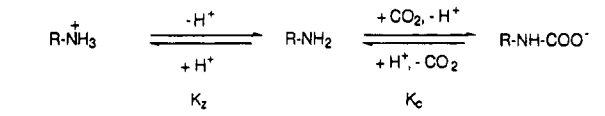
Scheme 4



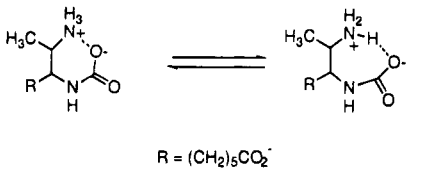
methylene group, (*RS**,*RS**)-3-(1-aminoethyl)nonanedioic acid (**5**), cyclized rapidly in the presence of enzyme and MgATP to give the corresponding lactam **6**, MgADP, and P_i (CO_2 was not required for this reaction). ADP and P_i formation were detected using coupled assays for these two products and by ^{31}P NMR. The ^{13}C NMR spectrum of the enzyme-generated lactam product formed from [1-^{13}C]-**5** was consistent with the spectrum of the synthetically generated ^{13}C-labeled lactams (see Materials and Methods and supporting information). The K_M and V_{max} for lactam formation compared favorably with the values for the diamine substrates, DAPA and diaminobiotin (Table 3). DTBS has previously been shown to form biotin directly from diaminobiotin (Krell & Eisenberg, 1970). All three substrates exhibit a high degree of synergy with ATP ($K_M \ll K_{ia}$). Substrate synergy has also been observed between the nucleotide and sugar substrates of hexokinase (Viola et al., 1982). One explanation for the synergy may be the interaction of the 8-amino groups of these substrates with one of the oxygens of the γ-phosphate of ATP (the distance between the heteroatoms is less than 3 Å; Huang et al., 1995). The more rapid turnover of **5** may be due to the greater reactivity of the postulated acyl phosphate intermediate of **5** compared to the postulated carbamyl phosphate intermediates in the carbonylation of DAPA and diaminobiotin.

Unlike the 7-carbamate mimic discussed above, the methylene analog of the 8-carbamate, (*RS**,*RS**)-4-amino-3-methyldecanedioic acid (**7**), interacted weakly with DTBS. Inhibition of DTBS by **7** was competitive (K_i = 4 mM) with respect to DAPA (K_M = 0.3 μM). Slight substrate activity with **7** was also detected (Table 3). Both ADP release and P_i release dependent on **7** were measured using coupled assays. However, formation of the cyclized product **8** was not directly measured, because the reaction was so slow. It is possible either that cyclization occurred or that **7** induced an ATPase activity similar to the xylose- and lyxose-induced ATPase activity observed with hexokinase (DelaFuente et al., 1970) and the bicarbonate-induced ATPase activity observed with carbamate kinase (Marshall & Cohen, 1966). Nonetheless, the sharp discrimination by DTBS between the two DAPA carbamate analogs suggested strongly that DTBS preferentially catalyzed ureido (or lactam, in the case of **5**) ring formation via nucleophilic attack of the 8-amino group on N7-carbamyl (or acyl) phosphate intermediates.[5]

Scheme 5



Scheme 6



DISCUSSION

The formation of carbamates on simple aliphatic amines (Scheme 5) is a facile equilibrium reaction involving three species, in addition to protons and CO_2 (and the HCO_3^- with which it is in equilibrium) (Ewing et al., 1980; Lorimer, 1983). In Scheme 5, K_C is the equilibrium constant for carbamate formation and K_Z is the equilibrium constant for the interaction of the proton with the amine (Morrow et al., 1981). Monocarbamate formation on DAPA is considerably more complex, involving equilibria between noncarbamylated amino groups and both carbamates, in all possible protonation states. A complete description of the rate constants and equilibria governing the various reactions is beyond the scope of the present investigation. Suffice it to say that both the N7-DAPA-carbamate **1** and the N8-DAPA-carbamate **2** were readily detected in free solution by standard NMR techniques. We attribute the facility with which DAPA-carbamates formed at neutral pH to two factors. (1) Protonation of the second amine of α,β-diamines is less favorable than protonation of the first amine, as exemplified by the pK values for ethylenediamine of 9.93 and 6.85 (Perrin & Dempsey, 1974). The p*K*'s for DAPA were 10.0 and 6.7 (measured potentiometrically; data not shown). Therefore, at neutral to slightly basic pH one of the two amines of DAPA would be largely unprotonated and available for reaction with CO_2. (2) DAPA-carbamates have the potential to be stabilized through interaction with the adjacent ammonium group (Scheme 6).

Although DAPA forms carbamates on both amines with almost equal facility in free solution, the studies reported here and in the accompanying paper (Huang et al., 1995), involving four independent experimental approaches, leave little doubt that the reaction catalyzed by DTBS proceeds regiospecifically on the 7-amine. We do not know whether DTBS catalyzes the formation of the N7-DAPA-carbamate within the active site or merely selects the preformed N7-DAPA-carbamate from the mixture formed in free solution. The efficient enzymatic cyclization of the N7-DAPA-carbamate mimic **5** suggests that carbamate selection is at least possible. In any event, the N7-DAPA-carbamate is stabilized within the active site. Although enzyme-bound carbamates in Rubisco (Lorimer et al., 1976; Andersson et al., 1989) and urease (Park & Hausinger, 1995) are stabilized by coordination to a divalent metal ion, the DTBS-bound N7-DAPA-carbamate may not be so stabilized. The pulse—

[5] Support for the postulated phosphorylated intermediates is provided by the interaction of DTBS with the alternate substrates 7-aminoheptanoic acid and nonanedioic acid. Both compounds afforded phosphorylated products which were stable enough to detect by ^{13}C and ^{31}P NMR (Alan Rendina, unpublished observations). These results are consistent with a mechanism in which DTBS specifically phosphorylates the carbamates of DAPA, diaminobiotin, or 7-aminoheptanoic acid or the carboxyl groups of nonanedioic acid or **5** (at the 1-position). The phosphorylated compounds undergo cyclization (presumably enzyme catalyzed) only when properly positioned internal amino groups are present.

chase experiments showed no requirement for Mg^{2+} to stabilize the DTBS·N7-DAPA-carbamate complex during the preincubation period. In addition, crystallographic analyses of various DTBS complexes with bound N7-DAPA-carbamate suggest instead that the N7-DAPA-carbamate is stabilized by interactions with Lys 15, Lys 37, and Ser 41 (Huang et al., 1995).

DAPA-carbamate formation in the presence and absence of DTBS has recently been investigated by Baxter et al. (1994) using diazomethane trapping methods. Their results and conclusions differ from ours, however. They did not detect carbamate formation in the absence of DTBS, leading them to suggest that this step of the reaction was enzyme-catalyzed. Carbamate trapping in the presence of excess DTBS yielded a 19:1 preponderance of N8- over N7-DAPA-carbamate ester, and they concluded that the 8-carbamate of DAPA was an intermediate in the DTBS reaction. Their procedure for diazomethane trapping differed somewhat from ours; we denatured the protein in solvent accompanying the diazomethane, in the hope that the preexisting, biologically relevant carbamate would become esterified before the CO_2 had dissociated or migrated to another amine. However, in our hands, diazomethane trapping of DTBS-bound DAPA-carbamates proved inefficient and only partly specific, although the N7-DAPA-carbamate was favored. We therefore extended our experimental approaches to include techniques which permitted us to observe the enzyme in its native state. Taken together, our experiments lead us to conclude that the reaction catalyzed by DTBS proceeds by the upper route in Scheme 2, via the N7-DAPA-carbamate **1**.

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SUPPORTING INFORMATION AVAILABLE

Details of the recloning of DTBS and of fermentation and protein purification procedures and syntheses of the two DAPA-carbamate mimics **5** and **7** (7 pages). Ordering information is given on any current masthead page.

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