Isolation and Chemistry of the Mixed Anhydride Intermediate in the Reaction Catalyzed by Dethiobiotin Synthetase

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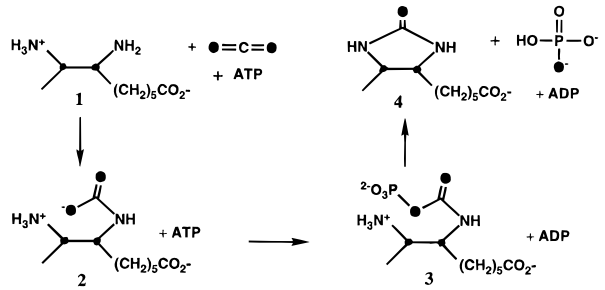
ABSTRACT: Dethiobiotin synthetase (DTBS) catalyzes the formation of the cyclic urea, dethiobiotin (DTB), from (7*R*,8*S*)-diaminononanoic acid (DAPA), CO_2, and ATP; the other products of the reaction are ADP and P_i. The first intermediate in the reaction sequence is the 7-carbamate of DAPA [Huang, W., et al. (1995) *Biochemistry 34*, 10985−10995; Gibson, K. J., et al. (1995) *Biochemistry 34*, 10976−10984; Alexeev, D., et al. (1995) *Structure 3*, 1207−1215]. The existence of the second postulated intermediate, a mixed carbamic−phosphoric anhydride formed when the carbamate is phosphorylated by ATP, is consistent with the cleavage of the γ-phosphoryl group of ATP seen in DTBS reaction mixtures [Baxter, R. L., & Baxter, H. C. (1994) *J. Chem. Soc., Chem. Commun.*, 759−760]. Two more direct lines of evidence for the mixed anhydride intermediate have now been obtained. First, a DTBS reaction mixture containing [^{18}O]CO_2 produced ^{18}O-enriched DTB and P_i, as the existence of such an intermediate would require. Second, a moderately stable intermediate that could be labeled with either $^{14}CO_2$, [γ-^{33}P]ATP, [9-^{3}H]DAPA, or [1,7-^{14}C]DAPA was trapped by quenching DTBS reactions at pH 4 and isolated by thin-layer chromatography. As expected for the proposed mixed anhydride, this species underwent acid hydrolysis to DAPA, CO_2, and P_i; under basic conditions, the intermediate cyclized, yielding DTB and P_i. When returned to fresh enzyme at pH 7.5, the intermediate underwent cyclization at a rate comparable to that of normal turnover.

Dethiobiotin synthetase (EC 6.3.3.3) catalyzes the penultimate step in biotin biosynthesis, the formation of the cyclic urea, DTB.[1] The substrates of the reaction are DAPA (**1**), ATP, and CO_2; the products are DTB (**4**), ADP, and P_i (Krell & Eisenberg, 1970) (upper line of Scheme 1). These workers proposed the existence of two key intermediates: a DAPA-carbamate and a mixed carbamic−phosphoric anhydride formed by phosphorylation of the carbamate. Subsequent work established the regiochemistry of the carbamate intermediate **2** (Huang et al., 1995; Gibson et al., 1995; Alexeev et al., 1995). Baxter and Baxter (1994) detected cleavage of the γ-phosphorus to β,γ-bridge oxygen bond of ATP in DTBS reaction mixtures, which is consistent with the intermediacy of a mixed carbamic−phosphoric anhydride. The crystal structure of the enzyme has recently been solved to 1.65 Å (Huang et al., 1994). The protein is a dimer (24 kDa/subunit) with a fold like that of the GTP-binding protein H-*ras*-p21. Crystallography of DTBS bound to N7-DAPA-carbamate and β,γ-methylene-ATP suggests that ATP would be well-placed for γ-phosphoryl transfer to the 7-carbamate of DAPA (Huang et al., 1995), to give the corresponding mixed anhydride. In addition, the parent compound of the mixed anhydride intermediate is carbamyl phosphate, which is reasonably stable through the pH range of 2−8 (Allen & Jones, 1964); this suggested that the putative mixed anhydride intermediate **3** might be isolable. These considerations

Scheme 1



motivated a search for direct chemical evidence for the mixed anhydride, which has now been obtained. These studies are a step toward a thorough understanding of the mechanism and kinetics of the enzyme-catalyzed reaction.

MATERIALS AND METHODS

Materials. Escherichia coli DTBS was purified from overproducing strains, and unlabeled DAPA was made from DTB, as previously described (Gibson et al., 1995). The enzymatic syntheses of [^{14}C]- and [^{3}H]DAPA are outlined in Scheme 2. [1,7-^{14}C]Pimelic acid (American Radiolabeled Chemicals) was converted to DAPA in a reaction mixture containing *Bacillus sphaericus* pimeloyl-CoA synthetase (Ploux et al., 1992), *E. coli* KAPA synthase (K. J. Gibson, unpublished), and DAPA synthase (Stoner & Eisenberg, 1976), plus appropriate substrates and cofactors. These enzymes were purified from overproducing *E. coli* strains (K. J. Gibson, unpublished). The mixture was fractionated by silica TLC using 4:1:1 butanol/acetic acid/water. After autoradiography, DAPA ($R_f \sim 0.4$) was eluted from the plate using methanol containing 2.5% NH_4OH. Better incorpora-

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[1] Abbreviations: DTB, dethiobiotin; DTBS, dethiobiotin synthetase; DAPA, (7*R*,8*S*)-diaminononanoic acid; KAPA, 8-amino-7-oxononanoic acid; HEPES, (hydroxyethyl)piperazineethanesulfonic acid; MOPS, 4-morpholinepropanesulfonic acid; PEI TLC, polyethyleneimine cellulose thin-layer chromatography.

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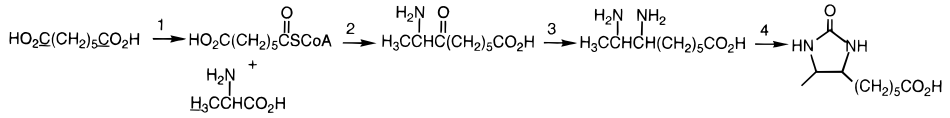
Scheme 2[a]



[a] (1) Pimeloyl-CoA synthetase, ATP, CoASH; (2) KAPA synthase; (3) DAPA synthase, AdoMet; (4) DTBS, ATP, CO_2. Radiolabeled atoms in the starting materials are underlined.

tion of [*methyl*-^{3}H]alanine was obtained if DTBS, MgATP, and $NaHCO_3$ were added to the reaction mixture along with KAPA and DAPA synthases and their substrates; preliminary data suggest that alanine bound poorly to *E. coli* KAPA synthase, as it does to the *B. sphaericus* enzyme (Ploux & Marquet, 1992). The resulting [*methyl*-^{3}H]DTB ($R_f \sim 0.7$) was isolated on silica TLC in 70:30:0.25 ethanol/water/concentrated NH_4OH and hydrolyzed in 6 N HCl, using a sealed tube and microwave heating. Excess acid was removed by repeated coevaporation with water.

^{18}O-Transfer Experiment. DTBS reaction at room temperature was initiated by addition of 864 μL of a mixture containing 200 nmol of ATP, 5 μmol of $MgCl_2$, and 34 nmol of EDTA in 20 mM HEPES-NaOH at pH 7.5 to 180 μL of a pre-equilibrated solution containing 12.5 μmol of HEPES-NaOH (pH 7.5), 25 nmol of EDTA, 200 nmol of DTBS, 400 nmol of DAPA, and 55 μmol of $NaHC^{18}O_3$ in nominally 84% ^{18}O-enriched water. At 30 and 60 s, 0.5 mL aliquots were quenched with EDTA (20 μL, 0.5 M). Protein was removed by ultrafiltration (Centricon 30, Amicon), and small molecules were fractionated on a Brownlee C-8 HPLC column using a 99.9% water to 99.9% methanol gradient containing 0.1% HCO_2H. DTB-containing fractions were dried and esterified with diazomethane in tetrahydrofuran. The HPLC breakthough peak containing P_i was either dried down, dissolved in water (120 μL), and bubbled with CH_2N_2 or fractionated on a Bond-Elut SAX cartridge (Analytichem) with a triethylammonium bicarbonate step gradient, followed by drying and methylation of P_i-containing fractions in tetrahydrofuran/diazomethane. Both DTB methyl ester and trimethyl phosphate were analyzed by GC/MS using an HP5890GC instrument with a DB-1 capillary column and HP5971 mass-selective detector; spectra and retention times were compared with those of unlabeled standards. The degree of enrichment was estimated from the heights of the M and M + 2 peaks; enrichments were large enough that a correction for the unlabeled M + 2 peaks was unnecessary.

Isolation of the Mixed Anhydride by TLC. Time courses at 0 °C were taken under the following reaction conditions: 100 μM DTBS monomer (determined by UV absorbance, using an ϵ_{280} of 3.75×10^4 M^{-1} cm^{-1}; quantitative amino acid analysis supported this extinction coefficient), 150 μM ATP, 200 μM DAPA, 5 mM $MgCl_2$, 5 mM $NaHCO_3$, 100 mM MOPS-NaOH buffer (pH 7.5), and 0.1 mM EDTA. Aliquots were taken on a 10−300 s time scale. For tracking mixed anhydride formation, aliquots were quenched in 0.5 M ammonium acetate at pH 4 and fractionated on silica TLC plates in 25:15:0.75 isobutyric acid/water/concentrated NH_4OH. ADP formation was monitored by spotting aliquots of reaction mixture containing [α-^{33}P]ATP directly onto PEI TLC plates (Macherey-Nagel), which were then developed in 0.3 M KP_i at pH 7. Spots on TLC plates were quantitated either using autoradiography, followed by cutting of the plate and scintillation counting, or with a phosphorimager. In $^{14}CO_2$ fixation experiments, aliquots were quenched in 10 volumes of either 1 N HCl or 1 N NaOH. After at least 10 min, base-quenched samples were acidified with HCl and taken to dryness on a hot plate at ∼50−60 °C. The residue was dissolved in water and counted. Acid-quenched samples were dried and counted similarly.

To test its stability at neutral pH with and without DTBS, mixed anhydride was generated at 0 °C in a mixture containing 100 μM [γ-^{33}P]ATP, 150 μM DAPA, 250 μM DTBS, 5 mM $MgCl_2$, 5 mM $NaHCO_3$, 25 mM MOPS-NaOH (pH 7.5), and 0.1 mM EDTA. The reaction was stopped at 90 s by addition of ammonium acetate (pH 4) to 0.25 M. Protein was removed by ultrafiltration (Microcon 30, Amicon), and the solution was diluted 10-fold into 0.2 M MOPS-NaOH at pH 7.5 and 0 °C containing 5 mM $MgCl_2$, 5 mM $NaHCO_3$ and 0.1 mM EDTA, with or without 50 μM DTBS. Time points were taken in 0.5 M ammonium acetate at pH 4 and analyzed by TLC as usual. ^{3}H-labeled mixed anhydride was generated similarly, except that ATP was present at 150 μM and [9-^{3}H]DAPA at 100 μM.

Chemistry of the Mixed Anhydride. Reaction mixtures at 0 °C contained ATP, DAPA, and DTBS at 50 μM, $MgCl_2$ and $NaHCO_3$ at 5 mM, and 0.1 mM EDTA in 50 mM MOPS-NaOH at pH 7.5. Label was introduced as $^{14}CO_2$, [9-^{3}H]DAPA, or [γ-^{33}P]ATP, and reaction mixtures were brought after 60 s to pH 4, as above. Aliquots were either (a) stored as quenched at −80 °C, (b) diluted with 5 volumes of 1 N HCl, heated at 60 °C for 2 h, and taken to dryness using a Speed-Vac, or (c) diluted with 5 volumes of triethylamine and treated as in part b. Dry residues were taken up in water, and all samples were fractionated on silica TLC and quantitated, as above. A portion of the samples labeled with ^{33}P was also fractionated on PEI TLC, as above, because the silica TLC system does not separate P_i from ATP.

Time Course Analysis. Experimental points were plotted using KaleidaGraph. Curves were drawn either point-to-point or by least-squares fitting to the sum of a linear and an exponential process.

RESULTS

^{18}O Transfer from $C^{18}O_2$ to DTB and Inorganic Phosphate. The DTBS mechanism in Scheme 1 predicts that oxygen atoms derived from CO_2 (filled atom symbols) should appear equally in the ureido oxygen of DTB and in P_i. The prediction was tested by GC/MS analysis of DTB and P_i (after methylation with diazomethane) formed in reactions labeled with [^{18}O]CO_2. The base peak in the spectrum of unlabeled DTB appeared at $m/z = 99$. High-resolution mass spectrometry indicated that the composition of this fragment was $C_4H_7N_2O$ ($m/z = 99.0603 \pm 0.005$, calculated 99.0559); it therefore represented the ureido ring after loss of the $(CH_2)_5CO_2H$ side chain. For DTB recovered from the ^{18}O reaction, the base peak appeared at $m/z = 101$, indicating that ^{18}O was present in the ureido oxygen of DTB. The

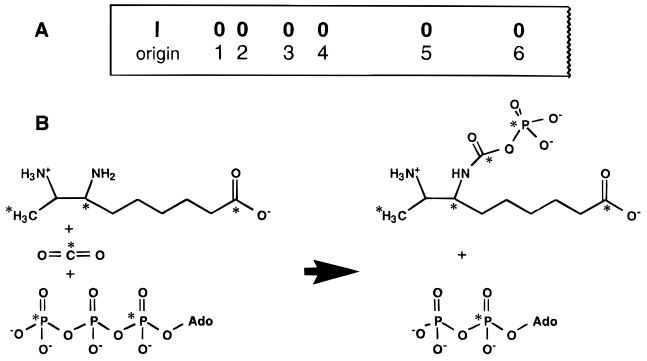


FIGURE 1: (A) Schematic representation of the TLC (silica, 25:15:0.75 isobutyric acid/water/NH_4OH) separation of ATP and P_i (1), ADP (2), the mixed anhydride intermediate of the DTBS reaction (3), AMP (4), DAPA (5), and DTB (6). (B) Left of arrow, positions of substrate radiolabeling (indicated by asterisks); right of arrow, expected positions of label in mixed anhydride and ADP.

enrichment was 77% (92% of the theoretical value). Likewise, the $C^{18}O_2$ reaction gave a much stronger $m/z = 112$ peak than the unlabeled trimethyl phosphate control (base peak $m/z = 110$). However, the enrichment of P_i was 45% (54% of theory). Adventitious contamination with unlabeled phosphate of either glassware or components of the reaction mixture, or ADP hydrolysis during the mildly acidic chromatography, could have contributed to the unexpectedly low enrichment of P_i. However, even with discrepant quantitation, this experiment strongly supports the existence of the mixed anhydride intermediate.

Identification of the Mixed Anhydride Intermediate. This intermediate was first observed in DTBS reactions labeled with [γ-^{33}P]ATP that were quenched at pH 4 and fractionated on TLC. The separation is shown schematically in Figure 1A. A ^{33}P-labeled spot migrating between ADP and AMP was identified as the mixed anhydride by repeating the experiment with either $^{14}CO_2$ or [9-^{3}H]- or [1,7-^{14}C]DAPA as the source of label. In each case, a radioactive spot was obtained at the same R_f, showing that the new spot contained the carbon atom of CO_2 and both ends of the DAPA molecule, in addition to the γ-phosphorus of ATP (Figure 1B). Although these experiments did not explicitly reveal the fate of the β,γ-bridge oxygen, the ^{18}O-transfer experiment (above) was consistent with transfer of only the γ-phosphoryl group of ATP to the new spot. Assignment of the N7-regioisomeric structure shown is based on previous work with stable N7- and N8-carbamate mimics, which ruled out the possibility of group migration to N8 in the course of the reaction (Gibson et al., 1995). The chemical identification of the new spot as the mixed anhydride intermediate was also supported by two kinetic results. Figure 2A shows the predicted concomitant initial formation of the spot and of ADP; in addition, the spot was missing from reaction mixtures allowed to run to completion (not shown).

The experiments in Figure 2 suggest that formation of the mixed anhydride was rapid compared to the overall reaction, but they do not distinguish between enzymatic and nonenzymatic cyclization of the intermediate. The following evidence for enzymatic cyclization was obtained. First, when a ^{33}P-labeled reaction mixture at 0 °C (pH 7.5) was filtered (Microcon 30) and analyzed by TLC, no mixed anhydride was detected in the small molecule fraction (<0.7% of the level of mixed anhydride present above the membrane), suggesting either that this intermediate remained enzyme-

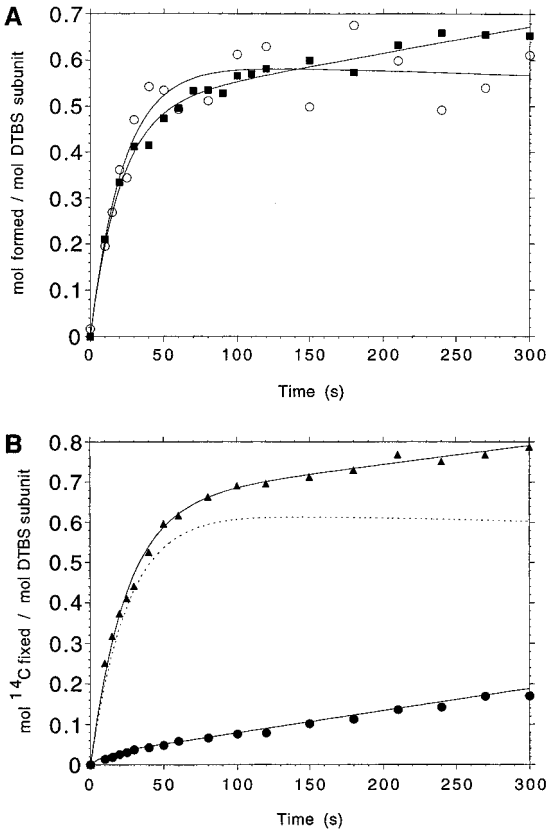


FIGURE 2: (A) Formation of mixed anhydride (open circles) and of ADP (squares) in DTBS reactions at 0 °C. (B) Fixation of $^{14}CO_2$ in DTBS reactions at 0 °C using 1 N HCl (filled circles) or 1 N NaOH (triangles) as the quench. Symbols are experimental points; the curves were calculated. The dotted line in panel B is the calculated difference between the fits to the experimental points.

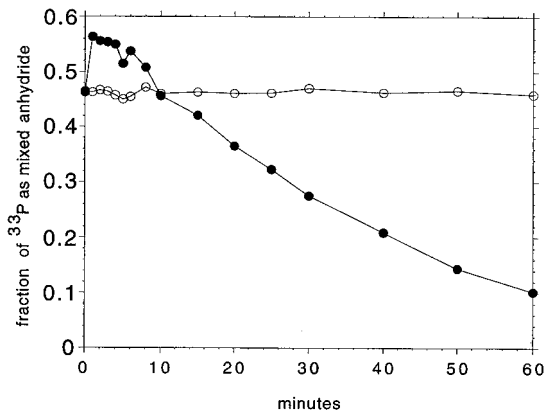


FIGURE 3: DTBS-catalyzed loss of mixed anhydride at pH 7.5 and 0 °C. Protein-free ^{33}P-labeled mixed anhydride and ADP at pH 4 were added back to buffer (open circles) or buffer plus 50 μM DTBS (closed circles). The concentration of mixed anhydride and ADP when $t = 0$ was about 5 μM.

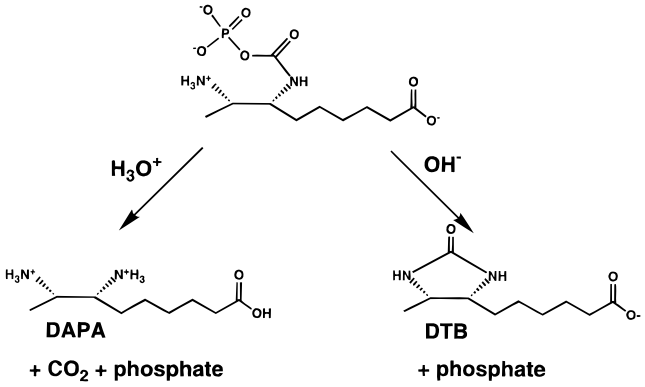
bound or that it cyclized rapidly upon release. The second possibility was ruled out as follows. A deproteinized mixture containing ^{33}P-labeled mixed anhydride and ADP was obtained by ultrafiltration of a DTBS reaction quenched at pH 4. The sample was diluted into buffer at pH 7.5 and 0 °C. The mixed anhydride was stable under these conditions for at least 1 h (Figure 3). However, if the mixture was supplemented with a molar excess of fresh enzyme, there was a small transient increase in the mixed anhydride, followed by an apparently exponential decay. Two experiments gave rate constants for this decay of ~0.03 and ~0.04

Table 1[a]

species	[γ-^{33}P]ATP			[9-^{3}H]DAPA			$^{14}CO_2$		
	pH 4	base	acid	pH 4	base	acid	pH 4	base	acid
DTB	–	–	–	1386 (4.9)	27935 (58)	1098 (3.4)	294 (6.4)	6439 (98)	400 (77)
DAPA[b]	–	–	–	14479 (51)	19723 (41)	31183 (96)	–	–	–
mixed anhydride	41394 (39)	301 (0.7)	61 (0.1)	12333 (44)	564 (1.2)	230 (0.7)	4297 (94)	137 (2)	120 (23)
ATP/Pi[c]	64294 (61)	44758 (99)	71102 (99)	–	–	–	–	–	–

[a] Top row, source of label for each group of three samples. Second row, workup (see the text). Left column, species on a TLC plate. Numerical entries are disintegrations per minute on a TLC square bearing spot of interest; parenthetical values are the percentage of counts in the lane in that spot. – = not applicable. [b] In both without DTBS controls and with DTBS samples, there was some decomposition of DAPA to a slightly slower-running spot upon heating, under both acidic and basic conditions. Disintegrations per minute values are the sum of the DAPA spot and the byproduct spot. [c] Part of the sample was also analyzed on polyethyleneimine cellulose TLC, which indicated that label lost from mixed anhydride was recovered as P_i or polyphosphates, not as ATP (not shown).

Scheme 3



min^{-1}, indistinguishable from the turnover rates of ~0.033 to ~0.04 min^{-1} typically seen in steady-state experiments under similar conditions (e.g., Figure 2A). A separate experiment with mixed anhydride formed from [9-^{3}H]DAPA verified that the loss of mixed anhydride was accompanied by DTB formation (not shown). Taken together, these experiments suggest that cyclization of the mixed anhydride was normally enzyme-catalyzed. The results also suggest that exogenous mixed anhydride was at least nearly kinetically competent, perhaps because the relatively solvent-exposed active site of DTBS (Huang et al., 1994) allowed the intermediate to bind rapidly.

Chemistry of the Mixed Anhydride. As a substituted carbamyl phosphate, the mixed anhydride would be expected to undergo acid hydrolysis to DAPA, P_i, and CO_2. Under basic conditions, however, one would expect the mixed anhydride to cyclize to DTB (Scheme 3), either by attack of the deprotonated 8-amino group on the carbamyl carbon followed by expulsion of phosphate or possibly via the isocyanate (Allen & Jones, 1964). The results shown in Table 1 are qualitatively consistent with these predictions. A separate experiment showed that the half-life of the putative mixed anhydride in 1 N NaOH at room temperature was about 1 min (not shown). In practice, direct quantitation of this intermediate was best done with ^{33}P from [γ-^{33}P]ATP. However, the results of the acid and base treatment of the mixed anhydride suggested an alternative approach. If parallel $^{14}CO_2$-labeled reactions were quenched into either acid or base, with subsequent acidification of the base-quenched reactions to drive off excess $^{14}CO_2$, the difference in ^{14}C fixed by the two quenches should represent the mixed anhydride present. Figure 2B shows time courses obtained for base- and acid-trappable $^{14}CO_2$ fixation and their calculated difference. The difference corresponds reasonably well to the curve for mixed anhydride formation in Figure 2A.

DISCUSSION

The experiments presented above provide direct evidence for the mixed carbamic−phosphoric anhydride intermediate in the DTBS reaction, which was first proposed by Krell and Eisenberg (1970). Although these experiments do not address the regiochemistry of this intermediate, the work with stable carbamate mimics (Gibson et al., 1995) shows that it must be the N7-regioisomer. The analytical methods developed here will make it possible to examine partial reactions involving the mixed anhydride in pre-steady-state kinetic experiments under a variety of conditions, leading to a better understanding of the overall reaction catalyzed by DTBS.

The experiments above indicate that both the formation and the cyclization of the mixed anhydride intermediate were DTBS-mediated. It follows that, although DTBS is a slow enzyme, it does the work of two. In making the mixed anhydride, which is a substituted carbamyl phosphate, DTBS acts as a specialized carbamyl phosphate synthetase; in forming the cyclic urea by intramolecular carbamyl transfer, DTBS serves as a transcarbamylase. As a carbamyl phosphate synthetase, DTBS is energetically more efficient than the glutamine- or ammonia-dependent enzymes that synthesize (unsubstituted) carbamyl phosphate. The latter two enzymes use HCO_3^- as the source of the carbon that they fix; this forces them to use 1 equiv of ATP as a condensing agent to drive, via phosphocarbonate, the formation of enzyme-bound carbamate (Powers & Meister, 1976; Abeles et al., 1992). DTBS, by contrast, benefits from the stabilization of DAPA monocarbamates by the (protonated) neighboring amino group (Gibson et al., 1995). This enables DTBS to use CO_2, rather than HCO_3^-, as its carbon source (Krell & Eisenberg, 1970). The 1 equiv of ATP used by DTBS is used for DAPA-carbamate phosphorylation, which corresponds to the second ATP-requiring step of the carbamyl phosphate synthetase reaction. The subsequent conversion of the DAPA-derived mixed anhydride to the cyclic urea, DTB, plus inorganic phosphate, is formally analogous to the carbamyl phosphate-dependent formation of the

substituted ureas citrulline and *N*-carbamylaspartate catalyzed by ornithine and aspartate transcarbamylases, respectively.

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