Cleavage of Yeast Chromosomal DNA. DNA from yeast strains SEY6210 (no target site) and SEY6210C (+ target site) was isolated in low melting point agarose.[27] Agarose plugs containing yeast strain SEY6210C and SEY6210 were cut to a thickness of approximately 2 mm (volume approximately 80 μL) and placed in 2-mL microcentrifuge tubes. The plugs were washed four times with 900 μL of 1 mM $Co(NH_3)_6^{3+}$, 20 mM HEPES, pH 7.2, and 5 mM EDTA (triplex/alkylation buffer). After removal of the last wash solution, the plugs were incubated with 120 μL of triplex/alkylation buffer, *N*-bromoacetyloligonucleotide **5** was added to a final concentration of 1 μM, and the plugs were incubated at 37 °C. After 20 h, the plugs were washed 4 × 30 min in 900 μL of 10 mM Tris·HCl (pH 9.5), 10 mM EDTA, allowing the *N*-bromoacetyloligonucleotide to fully diffuse out of the agarose. Each of the plugs were then washed with 900 μL of triplex/alkylation buffer (4 × 15 min) and incubated with 120 μL of triplex/alkylation buffer and *N*-bromoacetyloligonucleotide **5** as before. After this second 20-h incubation, the agarose embedded DNA was washed with 900 μL of 0.1% piperidine (3×), 100 mM NaCl, 10 mM Tris·HCl, pH 8.0, 10 mM EDTA. After removal of the final wash solution, the DNA was heated in 900 μL of the piperidine solution described above at 55 °C for 12 h. The plugs were transferred directly without melting to a 0.5 × TBE 1.0% agarose gel and products separated by pulsed field gel electrophoresis using a BioRad Chef-DR II system. Electrophoresis was performed at 200 V for 24 h, with switch times ramped from 10 to 40 s over the first 18 h and from 60 to 90 s over the last 6 h. The gel was stained with ethidium bromide and photographed under UV excitation.

Hybridization. The DNA was fragmented for efficient transfer to a Nytran charge modified Nylon-66 membrane by a 40-s exposure of the gel with a 254-nm UV transilluminator. The gel was soaked in 1.0 M NaCl, 0.5 M NaOH for 30 min to denature the DNA and then equilibrated in 1.5 M NaCl, 0.5 M Tris·HCl, pH 7.4, to neutralize the gel. The DNA was transferred using a Stratagene Pressure Control Station in 6xSSPE. *HIS4* hybridization was performed with a 250-bp SalI/EcoRI fragment derived from HIS4-SUC2 fusion plasmid YCp503.[25] The fragment was labeled using random primer hybridization with degenerate 6mers and α-^{32}P-dCTP.[23,28] The membrane was prehybridized with 5 mL of a solution containing 6× SSPE, 10× Denhardt's, 1% SDS, and 50 mg/mL Salmon sperm DNA solution at 42 °C for 2–4 h. After removal of this solution, the membrane was washed with 2 mL of 6× SSPE, 50% formamide, 1% SDS, and 50 mg/mL salmon sperm DNA solution at 42 °C for 30–60 min. The labeled probe was denatured by incubation at 37 °C for 5 min with 1/10 volume of 0.1 M NaOH and incubated with the membrane in 2 mL of 6× SSPE, 50% formamide, 1% SDS, and 50 mg/mL salmon sperm DNA solution at 42 °C overnight. The blot was washed four times in 25 mL of 1× SSPE buffer, 1% SDS at 42 °C and exposed to film, and cleavage efficiencies were quantitated using PhosphorImaging.

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(28) Feinberg, A. P.; Vogelstein, B. *Anal. Biochem.* **1983**, *132*, 6.
(29) Iverson, B. L.; Dervan, P. B. *Nucleic Acids Res.* **1987**, *15*, 7823.
(30) Maxam, A. M.; Gilbert, W. *Proc. Natl. Acad. Sci. U.S.A.* **1977**, *74*, 560.

^{15}N Isotope Effects on Nonenzymatic and Aspartate Transcarbamylase Catalyzed Reactions of Carbamyl Phosphate

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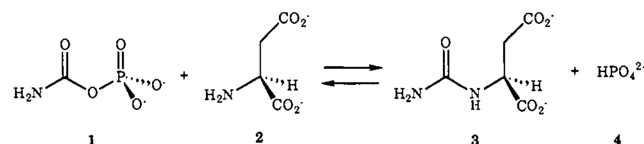
Abstract: Kinetic isotope effects at the amide nitrogen of carbamyl phosphate were measured to determine whether catalysis by aspartate transcarbamylase proceeds through a cyanic acid or tetrahedral intermediate. Decomposition of the mono- and dianion of carbamyl phosphate was used as models for reaction of carbamyl phosphate proceeding through a tetrahedral adduct or cyanic acid intermediate, respectively. The ^{15}N kinetic isotope effects for decomposition of the mono- (1.0028) and dianion (1.0105) of carbamyl phosphate were sufficiently different to permit a distinction to be made between a cyanic acid intermediate or tetrahedral adduct. The intrinsic ^{15}N kinetic isotope effects for the aspartate transcarbamylase catalyzed reaction were determined with an active site mutant of aspartate transcarbamylase in which histidine 134 was replaced with alanine (1.0027) and with the wild-type enzyme with cysteine sulfinate as substrate in place of aspartate (1.0024). In both of these systems a full ^{13}C intrinsic isotope effect has been previously observed (Waldrop et al. *Biochemistry*, in press; Parmentier et al. *Biochemistry*, in press). The similarity of these isotope effects to the isotope effect for the decomposition of the carbamyl phosphate monoanion demonstrates that cyanic acid is not an intermediate in the enzyme catalyzed reaction. The observed ^{15}N kinetic isotope effect for the wild-type enzyme with a low level of aspartate as substrate was 1.0014, a value consistent with the previously measured commitment for carbamyl phosphate (Parmentier et al. *Biochemistry*, in press). ^{31}P NMR analysis of phosphate from reactions run in 50% $H_2{}^{18}O$ showed that catalysis by aspartate transcarbamylase involves C–O bond cleavage, thus ruling out carbamic acid as a potential intermediate. We conclude that catalysis by aspartate transcarbamylase proceeds via a tetrahedral adduct.

Aspartate transcarbamylase (ATCase) catalyzes the transfer of the acyl moiety of carbamyl phosphate (CbmP) (**1**) to the amino group of aspartate (**2**) to yield carbamylaspartate (**3**) and inorganic phosphate (**4**). In *Escherichia coli*, this reaction is the first committed step in pyrimidine biosynthesis and as such ATCase is subject to feedback inhibition by CTP, the end product of the pathway, and activation by ATP, the end product of the parallel purine biosynthetic pathway.[1] ATCase is a dodecamer organized as two catalytic trimers and three regulatory dimers. The regulatory and catalytic subunits can be separated by treatment of the holoenzyme with mercurials.[2] The isolated catalytic trimers remain catalytically competent, and the regulatory dimers retain the ability to bind ATP and CTP.[2] While the activity of the holoenzyme is sigmoidal with respect to the concentrations of both CbmP[3] and aspartate,[1] the catalytic trimer exhibits Michaelis–

Scheme I



(1) Gerhart, J. C.; Pardee, A. B. *J. Biol. Chem.* **1962**, *237*, 891–896.
(2) Gerhart, J. C.; Schachman, H. K. *Biochemistry* **1965**, *4*, 1054–1062.

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Menten kinetics and therefore has been used for investigations of the catalytic mechanism.[2]

Despite over 30 years of research on ATCase (primarily on the allosteric properties) the basic characteristics of the chemistry catalyzed by this enzyme have not been elucidated. There are two plausible chemical mechanisms for the reaction catalyzed by ATCase. One is addition–elimination wherein the amino group of aspartate adds to the carbonyl carbon of CbmP to form a tetrahedral adduct with subsequent elimination of phosphate to form carbamylaspartate. The second mechanism is an S_N1 reaction involving an enzyme catalyzed decomposition of CbmP to cyanic acid and phosphate followed by attack of aspartate on the cyanic acid intermediate. The latter mechanism has provoked considerable discussion because the carbon of cyanic acid is much more electrophilic than that of CbmP. In fact, reaction of CbmP with a variety of nucleophiles has been found to proceed via a cyanic acid intermediate.[4] In contrast, evidence in favor of a tetrahedral adduct for the enzymatic reaction stems from the observation that acetyl phosphate is an alternate substrate for ATCase.[5] If the enzymatic reaction proceeded through cyanic acid, by analogy the reaction with acetyl phosphate would proceed through a ketene intermediate. Since ketenes are very unstable, ATCase catalysis with acetyl phosphate as substrate is unlikely to involve a ketene intermediate, and by inference with CbmP as substrate catalysis does not involve cyanic acid as an intermediate. Catalysis proceeding through a tetrahedral adduct has therefore been the favored mechanism for the enzymatic reaction because it is the most reasonable alternative.

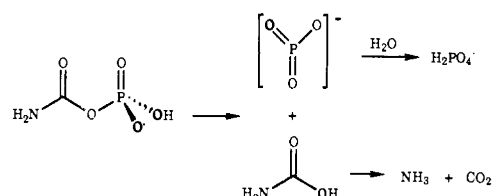
In order to assess directly the identities of intermediates in the reaction catalyzed by ATCase we have determined the ^{15}N kinetic isotope effects at the amide nitrogen of CbmP on ATCase catalysis as well as on the non-enzyme catalyzed decomposition of CbmP. The nitrogen isotope effects on the decomposition of the monoanion and dianion of CbmP in solution served as model reactions for mechanisms involving tetrahedral or cyanic acid intermediates, respectively. The results show unambiguously that the reaction catalyzed by ATCase does not proceed through a cyanic acid intermediate and support the concept that the mechanism employs a tetrahedral adduct.

Experimental Section

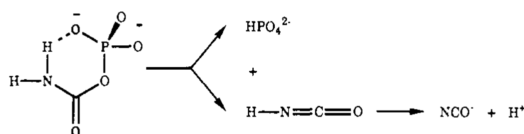
Materials. The dilithium salt of carbamyl phosphate and L-cysteine sulfinic acid were from Sigma and used as received. No free phosphate could be detected (using phosphorus NMR) in a concentrated, freshly prepared solution of the carbamyl phosphate. Analysis for free ammonia showed less than 0.5% in the carbamyl phosphate and none in the cysteine sulfinic acid. Also from Sigma were carbamate kinase, hexokinase, glucose-6-phosphate dehydrogenase, fumaric acid, NADP (nicotinamide adenine dinucleotide phosphate), Hepes (*N*-[2-hydroxyethyl]piperazine-*N'*-[2-ethanesulfonic acid]), and ammonia color reagent. L-Aspartic acid from Aldrich was recrystallized from water before use to remove ammonium ions. Boric acid was from Mallinkrodt. Citric acid was from Amend Drug and Chemical Co. Pipes (piperazine-*N*,*N*-bis(2-ethanesulfonic acid), 1.5 sodium monohydrate) was from Research Organics. DEAE Sephadex A25 was from Pharmacia. $H_2{}^{18}O$ (95.6 atom %) was from EG&G Mound Applied Technologies.

E. coli ATCase was purified according to the procedure of Gerhart and Holoubek from a strain of *E. coli* that overproduces the enzyme.[6] ATCase containing the amino acid replacement alanine for histidine at position 134 (H134A) was constructed and purified as described previously.[7] The catalytic trimer for both the wild-type and mutant enzymes was prepared from ATCase using the procedure of Yang et al.[8] and stored as an ammonium sulfate suspension at 4 °C. For the isotope effect experiments the wild-type and mutant catalytic trimers were dialyzed against 40 mM potassium phosphate, pH 7.0, and 2 mM β-mercaptoethanol. Dialysis was considered complete when the dialysate did not contain any detectable ammonia using Nessler's assay. For the NMR experiments the catalytic trimers were dialyzed against 50 mM Hepes, 2 mM β-mercaptoethanol, and 0.2 mM EDTA (ethylenediaminetetraacetic acid). The concentration of the wild-type catalytic trimer was determined using the extinction coefficient 0.72 cm^{-1} mg^{-1} mL.[6] A previous study found that the wild-type extinction coefficient was satisfactory for determining the concentration of the H134A catalytic trimer.[9]

Scheme II



Scheme III



The concentration of CbmP was measured by enzymatic end-point analysis using carbamate kinase to convert CbmP into carbamic acid and ATP. The production of ATP was coupled to hexokinase and glucose-6-phosphate dehydrogenase so that the production of NADPH could be monitored. A 1-mL assay contained 10 mM glucose, 5 mM ADP, 34 mM $MgCl_2$, 0.2 mM NADP, 10 u of each of the coupling enzymes, and 20 mM Tris, pH 8.3. The concentration of aspartate was also measured by enzymatic end-point analysis.[10]

Nitrogen Isotope Effects. The method of internal competition using the 0.37% natural abundance of ^{15}N was used to measure isotope effects at the amide nitrogen of CbmP.[11] The general experimental strategy was to convert the amide nitrogen of CbmP into NH_4^+ and isolate the NH_4^+ from the reaction mixture by anion exchange chromatography. The change in isotopic composition ($^{15}N/^{14}N$) over the course of the reaction was measured using an isotope ratio mass spectrometer. Since only gases may be introduced into the isotope ratio mass spectrometer, the isolated NH_4^+ was oxidized to N_2. The specific details for measurement of the enzymatic and nonenzymatic isotope effects follow.

Monoanion. The monoanion of CbmP decomposes predominantly by P–O bond cleavage to give carbamic acid (which spontaneously decomposes into NH_3 and CO_2) and phosphate (see Scheme II).[14] A 6-mL solution of 200 mM CbmP with 50 mM fumaric acid as buffer, pH 3.0, was allowed to sit at 25 °C for 1 h. This period of time was sufficient for about 10% of the CbmP to decompose, giving approximately 100 μmol of NH_4^+ (which, after oxidation, gives 50 μmol of N_2 for mass spectral analysis). The pH, which slowly increased during the course of the reaction, was readjusted to 3.0 with H_2SO_4 when necessary. Aliquots (0.4 mL) of the reaction mixture were taken at the beginning and end of the incubation and immediately frozen in liquid N_2. These aliquots were assayed later for CbmP concentration to determine the precise fraction of reaction. After the 1-h incubation the pH of the reaction mixture was raised to 7.5, and the solution was diluted to 20 mL. Raising the pH stops the reaction because the decomposition of the dianion occurs by a different mechanism (see Scheme III) the product of which, cyanate, is easily removed from the NH_4^+ by anion exchange chromatography. The product NH_4^+ was isolated and processed as described below.

Dianion. The dianion of CbmP decomposes predominantly by C–O bond cleavage to give cyanate and phosphate (see Scheme III).[14] A 10-mL solution of 200 mM CbmP in 50 mM borate, pH 8.0, was incubated for 4 h at room temperature. The pH of the reaction mixture gradually decreases as the reaction progresses; therefore, KOH was added

(3) Bethell, M. R.; Smith, K. E.; White, J. S.; Jones, M. E. *Proc. Natl. Acad. Sci. U.S.A.* **1968**, *60*, 1442–1449.
(4) Collins, K. D.; Stark, G. R. *J. Biol. Chem.* **1969**, *244*, 1869–1877.
(5) Porter, R. W.; Modebe, M. O.; Stark, G. R. *J. Biol. Chem.* **1969**, *244*, 1846–1859.
(6) Gerhart, J. C.; Holoubek, H. *J. Biol. Chem.* **1967**, *242*, 2886–2892.
(7) Robey, E. A.; Wente, S. R.; Markby, D. W.; Flint, A.; Yang, Y. R.; Schachman, H. K. *Proc. Natl. Acad. Sci. U.S.A.* **1986**, *83*, 5934–5938.
(8) Yang, Y. R.; Kirschner, M. W.; Schachman, H. K. *Methods Enzymol.* **1978**, *51*, 35–41.
(9) Waldrop, G. L.; Turnbull, J. L.; Parmentier, L. E.; O'Leary, M. H.; Cleland, W. W.; Schachman, H. K. *Biochemistry*, in press.
(10) Parmentier, L. E.; Weiss, P. M.; O'Leary, M. H.; Schachman, H. K.; Cleland, W. W. *Biochemistry*, in press.
(11) O'Leary, M. H. *Methods Enzymol.* **1980**, *64*, 83–104.
(12) Northrop, D. B. In *Isotope Effects on Enzyme-Catalyzed Reactions*; Cleland, W. W., O'Leary, M. H., Northrop, D. B., Eds.; University Park Press: Baltimore, MD, 1977; p 122.
(13) Weiss, P. M. In *Enzyme Mechanism from Isotope Effects*; Cook, P. F., Ed.; CRC Press, Inc.: Boca Raton, FL, 1991; Chapter 11.
(14) Allen, C. M.; Jones, M. E. *Biochemistry* **1964**, *3*, 1238–1247.

periodically to maintain a pH of 8.0. Aliquots of the solution were taken at the beginning and end of the incubation to determine the extent of the reaction as described for the monoanion. At the end of 4 h (which allowed about 50% of the CbmP to decompose to cyanate and phosphate) a 1-mL aliquot of the reaction mixture (containing about 100 μmol of cyanate) was transferred to a 15-mL Corex centrifuge tube and 1.5 mg (in 0.16 mL) of the wild-type catalytic trimer of ATCase and 1.6 mL of a 126 mM solution of aspartate were added. The catalytic trimer and aspartate stopped the reaction by coverting the remaining CbmP into acid stable carbamylaspartate. Ten minutes after adding enzyme and aspartate there was no detectable CbmP left in the solution, as determined by enzymatic end-point analysis, at which point 0.2 mL of 18 N H_2SO_4 was added to precipitate the enzyme and convert the product cyanate into NH_4^+ and CO_2. The precipitate was removed by centrifugation at 12000 × g for 10 min, and the supernatant was carefully removed, diluted to 20 mL, and adjusted to pH 7.5. The isolation and analysis of the NH_4^+ is described below.

Enzymatic Isotope Effects. All experiments utilized the catalytic trimer of ATCase, since all of the work on the catalytic mechanism has been done with this form of the enzyme. Isotopic discrimination in residual CbmP was followed in all experiments involving ATCase catalysis. In these experiments the enzyme concentration and incubation times were adjusted so that approximately 50% of the total CbmP was converted to products. Reactions were stopped by dropwise addition of 0.4 mL of 18 N H_2SO_4 to precipitate the enzyme and the buffer (Pipes). All reactions were carried out in 15-mL Corex centrifuge tubes so that upon acidification the precipitated enzyme and Pipes could be removed by centrifugation at 12000 × g for 10 min. The supernatants were carefully transferred to clean test tubes and incubated for 24 h at 37 °C to convert the residual CbmP into NH_4^+, CO_2, and inorganic phosphate. It was unnecessary to separate the residual CbmP from the products, since carbamylaspartate is acid stable. The NH_4^+ was isolated and processed as described below.

Pipes was chosen as a buffer for the enzymatic reactions because it precipitates upon acidification and therefore can be removed from the reaction mixture by centrifugation. Since Pipes contains nitrogen it was considered important to remove it to prevent any possibility of contamination of the NH_4^+. Control experiments without enzyme or without enzyme and Pipes gave the same $^{15}N/^{14}N$ ratio as the stock CbmP, indicating that the experimental design was adequate and did not allow any nitrogen contamination from the enzyme or Pipes. Even if a small amount of Pipes was not removed by centrifugation, the NH_4^+ was purified by steam distillation which was found not to result in breakdown of the Pipes. The specific conditions for each enzymatic reaction follow.

H134A Catalytic Trimer. Reaction mixtures (5 mL) containing 300 mM aspartate, 40 mM CbmP, 0.6 mg H134A catalytic trimer, and 50 mM Pipes, pH 7.2, were incubated for 10 min. Reactions were initiated by the addition of CbmP. The fraction of reaction was determined by measuring the NH_4^+ produced by acid decomposition of the residual CbmP.

Wild-Type Catalytic Trimer with Cysteine Sulfinate as Substrate. Reaction mixtures (5 mL) containing 100 mM cysteine sulfinate, 40 mM CbmP, 2.26 mg of wild-type catalytic trimer, and 50 mM Pipes, pH 7.2, were incubated for 15 min. Reactions were initiated by the addition of CbmP, and the fraction of reaction was determined as for the H134A catalytic trimer.

Wild-Type Catalytic Trimer with Aspartate as Substrate. To a 3.9-mL solution containing 40 mM CbmP, 0.46 mg of wild-type catalytic trimer and 50 mM Pipes, pH 7.2, 1.1 mL of a 7.69 mM aspartate solution was added dropwise from a needle attached to a syringe over a period of 10 min. The aspartate was added in this manner to keep the concentration as low as possible (see the Results and Discussion section for the rationale for doing this). The fraction of reaction was determined as the number of μmol of aspartate added divided by the total number of μmol of CbmP present prior to addition of aspartate. The total amount of CbmP present was determined as described for the H134A catalytic trimer.

Isotope Effect Nomenclature. The nomenclature used is that of Northrop,[12] in which an isotope effect for a particular atom is designated by the heavier isotope for that atom as a leading superscript. For example, $^{15}(V/K_{CbmP})$ indicates the nitrogen isotope effect on V/K ($[V/K$ with $^{14}N]/[V/K$ with $^{15}N]$) for ATCase catalysis with CbmP as the substrate.

Isolation and Handling of NH_4^+. The NH_4^+ from all reactions was separated from the reaction mixtures by anion exchange chromatography. Reaction mixtures, pH 7.5, were applied to a DEAE Sephadex A25 column (2.6 × 25 cm) in the Cl^- form that had been thoroughly washed with H_2O to remove excess Cl^-. The NH_4^+ was eluted with H_2O, and 20-mL fractions (each containing 0.1 mL of 6 N H_2SO_4 to maintain the protonated form of ammonia) were collected. Fractions containing NH_4^+ (as detected by Nessler's assay) were pooled and concentrated by rotary evaporation to a volume of 10 mL. This solution was made basic by adding 15 mL of 13 N NaOH and then subjected to steam distillation to recover the ammonia. The recovered ammonia was then oxidized to N_2 with NaOBr. Details of the steam distillation and oxidation steps are given by Weiss.[13] The $^{15}N/^{14}N$ ratio in the N_2 was determined using a Finnigan MAT 251 isotope ratio mass spectrometer. The kinetic isotope effects for the nonenzymatic decomposition of CbmP were calculated using eq 1.

$$^{14}k/^{15}k = \log(1-f)/\log[1-fR_p/R_o] \quad (1)$$

R_p is the $^{15}N/^{14}N$ ratio in the product at fraction of reaction f, while R_o is the $^{15}N/^{14}N$ ratio of the starting material. The kinetic isotope effects for the enzymatic reactions were calculated using eq 2

$$^{15}(V/K_{CbmP}) = \log(1-f)/\log[(1-f)R_s/R_o] \quad (2)$$

where R_s is the $^{15}N/^{14}N$ ratio in the residual substrate at fraction of reaction f. The R_o value was determined by incubating stock CbmP in 1 N H_2SO_4 for 24 h at 37 °C. The NH_4^+ was purified by steam distillation and oxidized to N_2. Control experiments revealed the $^{15}N/^{14}N$ ratio was unchanged if the NH_4^+ was subjected to anion exchange chromatography prior to steam distillation.

^{18}O Incorporation Experiment. The details for examining whether ^{18}O from $H_2{}^{18}O$ is incorporated into the phosphate produced from ATCase catalyzed carbamylation of aspartate or the phosphate produced from decomposition of mono- or dianionic CbmP are given below.

Nonenzymatic CbmP Decomposition. To a 400 mM solution of citric acid in D_2O, pD 3.0 (buffer for the monoanion decomposition) or Hepes in D_2O, pD 8.0 (buffer for the dianion decomposition), was added solid CbmP to a final concentration of 200 mM. Immediately after adjusting the pD a 0.25-mL aliquot of each solution was diluted with 0.25 mL of $H_2{}^{18}O$. The solutions were incubated at room temperature for approximately 3 days before the ^{31}P NMR spectra were acquired. Before acqusition of the ^{31}P NMR spectrum, 3.6 mg of disodium EDTA was added to the 0.5-mL sample for the dianion, and 4 mg was added to the monoanion sample.

Enzymatic Reactions. A solution of aspartate (480 mM) in 400 mM Hepes and 0.4 mM EDTA (final pH 7.2) was prepared, and the water was removed from 0.25-mL aliquots by lyophilization. Immediately before use, these aliquots were redissolved in 0.25 mL of $H_2{}^{18}O$. A separate solution of CbmP (357 mM) and Hepes (357 mM) in D_2O was prepared, and the pD was adjusted to 7.2. A 0.14-mL aliquot of this solution was then mixed immediately with the 0.25-mL aspartate solution in $H_2{}^{18}O$ and 0.11 mL of either wild-type catalytic trimer (1 mg) or H134A catalytic trimer (3.15 mg). On the basis of the specific activity of the enzyme solutions, it was predicted that the reaction with wild-type catalytic trimer would be complete in approximately 1 min, while the reaction with the H134A catalytic trimer would take approximately 7 min. The reaction mixtures were allowed to stand at room temperature for 4 h before removal of the enzyme by ultrafiltration (Centricon 30). The solutions were frozen and stored until analysis by ^{31}P NMR.

NMR Analysis. ^{31}P NMR spectra were acquired with a Bruker AM 500 spectrometer operating at 202 MHz with the probe temperature maintained at 298 K. The pulse width was 10 μs, the acquisition time was 10.24 s, and the spectral width was 800 Hz. A total of 12 transients were acquired for each spectrum, and each data set consisted of 16K data points zero-filled to 32K with 0.0–0.2 Hz line broadening.

Results and Discussion

The premise for using the nitrogen isotope effect at the amide of CbmP to distinguish between a chemical reaction involving a tetrahedral adduct or cyanic acid intermediate is based on the assumption that the primary isotope effect for a cyanic acid mediated mechanism will be larger than the secondary isotope effect for a mechanism with a tetrahedral adduct. Thus, prior to determining the isotope effect on the reaction catalyzed by ATCase, a measure of the difference, if any, in the ^{15}N kinetic isotope effects for the reaction of CbmP proceeding through a cyanic acid intermediate and tetrahedral adduct was needed. Allen and Jones[14] showed that decomposition of the monoanion of CbmP occurs by P–O bond cleavage to form carbamic acid and inorganic phosphate. The carbamic acid spontaneously decomposes into NH_3 and CO_2 (Scheme II). Since no bonds are made or broken to the amide group of CbmP, decomposition of the monoanion was used as a model for a reaction of CbmP proceeding through a tetrahedral adduct. Conversely, the decomposition of the dianion of CbmP serves as a model for a mechanism with a cyanic acid intermediate. The decomposition of the dianion of CbmP (Scheme

III) proceeds by extraction of one of the amide hydrogens (presumably by a phosphate oxygen) and C–O bond cleavage to yield inorganic phosphate and cyanic acid, which immediately ionizes to cyanate.[14]

The ^{15}N kinetic isotope effects for the decomposition of the mono- and dianionic forms of CbmP are shown in Table I. The $^{14}k/^{15}k$ value for decomposition of the monoanion was small and normal. Allen and Jones reported that under these conditions about 10% of the CbmP decomposes by C–O bond cleavage.[14] It is not known if this is due to an alternate path for decomposition of the monoanion or due to decomposition of a small amount of dianion present at this pH. Nevertheless, this will only affect the fraction of reaction for the monoanion because the C–O bond cleavage product, cyanate, was removed from the product NH_4^+ (from monoanion decomposition) by chromatography. Correction for the 10% side reaction was found not to change significantly the observed isotope effect.

In comparison to the monoanion, the $^{14}k/^{15}k$ value for decomposition of the dianion was markedly larger. According to Allen and Jones[14] approximately 10% of the CbmP under similar conditions decomposes via P–O bond cleavage; therefore, the observed isotope effect for the C–O bond cleavage path is less than the actual isotope effect. When this was taken into account the calculated isotope effect increased slightly (Table I). The larger isotope effect for the decomposition of the dianion compared with that for the monoanion reflects the fact that the effect is partially primary and involves significant changes in bonding to the nitrogen. This primary ^{15}N kinetic isotope effect for decomposition of the CbmP dianion is in the lower range of primary isotope effects seen for nitrogen. ^{15}N kinetic isotope effects reported for cleavage of C–N bonds range from 1.01 to 1.025.[15] A possible explanation for why the isotope effect for the dianion is not larger is that there is very little motion of the nitrogen atom along the reaction coordinate. By comparison, the carbon atom of CbmP not only undergoes C–O bond cleavage but also experiences a considerable amount of motion along the reaction coordinate to allow for the formation of the linear cyanic acid. This degree of motion is manifested in the very large ^{13}C isotope effect (1.04–1.05) for decomposition of the dianion.[16] Irrespective of the origins of the ^{15}N isotope effects for decomposition of the mono- and dianion of CbmP, the magnitude of the effects are sufficiently different to allow a distinction to be made between a mechanism that proceeds through a cyanic acid intermediate and one that does not.

Most enzymatic reactions are multistep processes where the actual chemical step is often not rate-limiting or only partly rate-limiting. As a consequence, the observed isotope effect for an enzyme catalyzed reaction will be suppressed relative to the actual (intrinsic) isotope effect on the chemical step which is isotopically sensitive. In the case of the catalytic trimer of ATCase, an extensive series of ^{13}C isotope effects (at the carbonyl carbon of CbmP) and ^{15}N isotope effects (at the amino group of aspartate) revealed that the chemical step was indeed only partially rate-limiting.[10] The value for $^{13}(V/K_{CbmP})$ (at near zero concentrations of aspartate) was about half the intrinsic value.[17] Similarly, the ^{15}N kinetic isotope effect (at the amide of CbmP) on the carbamylation of aspartate catalyzed by wild-type ATCase is expected to be suppressed to about half the intrinsic value. Therefore, to obviate any possible ambiguities in interpreting the isotope effect on ATCase catalysis, the ^{15}N kinetic isotope effects at the amide nitrogen of CbmP were measured under circumstances in which the intrinsic isotope effect is observed as well as under normal conditions. For ATCase the chemical step giving rise to the isotope effect can be made rate-limiting by using the alternate substrate cysteine sulfinate in place of aspartate. Cysteine sulfinate reacts at 2% the maximum velocity of aspartate,[18] and when it is used as a substrate the intrinsic ^{13}C isotope effect is observed.[17] The isotopically sensitive step has also been made rate-limiting by site-directed mutagenesis of the catalytic trimer. An altered ATCase catalytic trimer in which an active site histidine was replaced by alanine at position 134 (H134A) resulted in 5% the activity of wild-type catalytic trimer.[7] ^{13}C isotope effect studies found the chemistry of the reaction catalyzed by the H134A catalytic trimer to be solely rate-limiting (i.e., the intrinsic isotope effect was detected).[9]

Table I. Nitrogen Isotope Effects for Reactions of Carbamyl Phosphate

Nonenzymatic Decomposition of Carbamyl Phosphate

species	$(^{14}k/^{15}k) \pm$ SE	no.[a]
monoanion	1.0028 ± 0.0002	4
dianion	1.0105 ± 0.0001	4
	(1.0114 ± 0.0001)[b]	

Enzymatic Reactions of Carbamyl Phosphate

enzyme	substrate	$^{15}(V/K_{CbmP})$	no.
H134A	aspartate	1.0027 ± 0.0002	4
wild-type	cysteine sulfinate	1.0024 ± 0.0001	5
wild-type	aspartate	1.0014 ± 0.0002	4

[a] Number of determinations. [b] Isotope effect corrected for the decomposition of the dianion of carbamyl phosphate as described in the text.

Measurement of the ^{15}N kinetic isotope effects for the H134A catalytic trimer with aspartate as substrate and the wild-type catalytic trimer with cysteine sulfinate as substrate gave the values shown in Table I. The $^{15}(V/K_{CbmP})$ values for both the H134A catalytic trimer and the wild-type catalytic trimer with cysteine sulfinate as substrate are small, normal effects which are not significantly different from the ^{15}N isotope effects for the decomposition of the monoanion of CbmP and are much smaller than those shown for decomposition of the dianion (cyanic acid mechanism). This similarity argues in favor of the idea that at least in the case of the H134A catalytic trimer and the wild-type catalytic trimer with cysteine sulfinate as substrate catalysis does not proceed through a cyanic acid intermediate.[19] However, the validity of this conclusion is subject to the argument that the catalytic mechanisms of mutant enzymes or wild-type enzymes with alternate substrates might be different from those of the wild-type enzyme with the natural substrate. In an effort to address this issue, $^{15}(V/K_{CbmP})$ for the wild-type catalytic trimer with aspartate as substrate was determined. Because the ^{13}C isotope effect decreases with increasing amounts of aspartate, the ^{15}N isotope effects were measured at a very low concentration of aspartate. Since the $^{13}(V/K_{CbmP})$ value at a low concentration of aspartate was approximately half the intrinsic value, the ^{15}N isotope effect for catalysis by the wild-type catalytic trimer with aspartate as substrate is therefore predicted to be about half the value for decomposition of the monoanion of CbmP (1.0014) if cyanic acid is not an intermediate. If cyanic acid is involved in the mechanism, $^{15}(V/K_{CbmP})$ should be about 1.005, or one-half the isotope effect for decomposition of the dianion. The value of $^{15}(V/K_{CbmP})$ for the wild-type catalytic trimer with aspartate as substrate is 1.0014 (Table I), which indicates that catalysis by the wild-type enzyme with the physiological substrate does not proceed through a cyanic acid intermediate. In addition, the data also indicate that the basic chemistry catalyzed by the H134A catalytic trimer and the wild-type catalytic trimer with cysteine sulfinate as substrate is the same as that for the wild-type catalytic trimer with aspartate as substrate.

(15) O'Leary, M. H.; Urberg, M.; Young, A. P. *Biochemistry* **1974**, *13*, 2077–2081.

(16) Tipton, P. A.; Cleland, W. W. *Arch. Biochem. Biophys.* **1988**, *260*, 273–276. Parmentier, L. E. Ph.D. Dissertation, Department of Chemistry, University of Wisconsin, Madison, WI.

(17) Parmentier, L. E.; O'Leary, M. H.; Schachman, H. K.; Cleland, W. W. *Biochemistry*, in press.

(18) Foote, J.; Lauritzen, A. M.; Lipscomb, W. N. *J. Biol. Chem.* **1985**, *260*, 9624–9629.

(19) A reviewer questioned whether the small ^{15}N isotope effect seen in the enzymatic reaction could result from an early transition state for proton removal to give a cyanic acid intermediate. The large size of the ^{13}C isotope effect on the enzymatic reaction catalyzed by the H134A catalytic trimer[9] (1.043) relative to that on the nonenzymatic breakdown of carbamyl phosphate dianion[16] (1.04–1.05) argues strongly against such an interpretation.

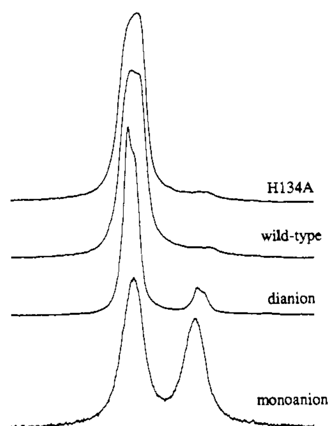


Figure 1. ^{31}P NMR spectra. Monoanion and dianion refer to the phosphate formed from decomposition of the mono- and dianion of CbmP. Wild-type: Reaction catalyzed by the wild-type catalytic trimer with CbmP and aspartate as substrate. H134A: Reaction catalyzed by the H134A catalytic trimer with CbmP and aspartate as substrate.

Although the isotope effect data clearly and effectively demonstrate that ATCase catalysis does not involve a cyanic acid intermediate, the data do not show that the mechanism proceeds through a tetrahedral adduct. Comparison of the isotope effects for the enzymatic reactions with the isotope effect for hydrolysis of the monoanion of CbmP only indicates a similar lack of change in the bonding to the amide nitrogen of CbmP, which is consistent with a mechanism proceeding through a tetrahedral adduct but is also consistent with a carbamic acid intermediate. Therefore, to provide a complete analysis of the chemistry of the reaction catalyzed by ATCase it was necessary to determine experimentally whether or not carbamic acid is an intermediate in the reaction. A carbamic acid intermediate could only be formed from CbmP by P–O bond cleavage with subsequent or concomitant incorporation of an oxygen atom from water to form inorganic phosphate (see Scheme II). Therefore, to distinguish between P–O or C–O bond cleavage, incorporation of ^{18}O from solvent water into the product phosphate was examined. The enzymatic reactions were carried out in water containing approximately 50% ^{18}O and 50% ^{16}O. If P–O cleavage occurs, the ^{31}P NMR spectrum of the phosphate product will show two peaks of approximately equal areas, separated by approximately 0.021 ppm, corresponding to $P^{16}O_4$ and $P^{18}O^{16}O_3$. If C–O bond cleavage occurs, the ^{31}P NMR spectrum will show only one peak corresponding to $P^{16}O_4$. The ^{31}P NMR spectra are shown in Figure 1. To verify that two peaks are seen in the case of P–O bond cleavage and only one peak is seen for C–O cleavage, the decomposition of the mono and dianion of CbmP were also run in 50% $H_2{}^{18}O$. As expected, the spectrum for the phosphate produced from decomposition of the monoanion shows two peaks indicating P–O bond cleavage. The spectrum for the phosphate produced from decomposition of the dianion shows one major peak corresponding to $P^{16}O_4$ and a minor peak corresponding to $P^{18}O^{16}O_3$. The fact that the major peak is $P^{16}O_4$ shows that most of the CbmP decomposed via C–O bond cleavage while the small peak corresponding to $P^{18}O^{16}O_3$ indicates that a small amount of CbmP decomposed by P-O bond cleavage, as reported by Allen and Jones.[14] The results of Allen and Jones also showed that at pH 3.0 a small amount (10%) of C–O cleavage occurs, which is consistent with the fact that the downfield resonance ($P^{16}O_4$) on the spectrum corresponding to decomposition of the monoanion is slightly larger than the upfield ($P^{18}O^{16}O_3$) resonance. Spectra corresponding to catalysis by H134A and wild-type catalytic trimers each show only one peak, indicating no incorporation of ^{18}O from solvent $H_2{}^{18}O$. The hump at the chemical shift corresponding to $P^{18}O^{16}O_3$ indicates a very small amount of P–O bond cleavage. Most likely, this represents nonenzymatic decomposition of the monoanion and reflects the necessity to lower the pH to dissolve the CbmP (and the time involved to do so) before enzyme was added (see Experimental Section). Nevertheless, catalysis by the H134A and wild-type catalytic trimers obviously proceeds through C–O bond cleavage, thereby excluding carbamic acid as a possible intermediate.

The isotope effect and NMR studies presented here conclusively rule out the possibility that catalysis by ATCase occurs by a S_N1 reaction involving either a cyanic acid or carbamic acid intermediate. Although the methods used here cannot directly confirm catalysis proceeding through a tetrahedral intermediate, the only reasonable mechanism for catalysis by ATCase that is consistent with the data and the observation that acetyl phosphate is an alternate substrate is addition–elimination.[20] Thus, given the fact that CbmP is unreactive toward nucleophiles an important question becomes how does the enzyme activate CbmP to render it susceptible to nucleophilic attack? Stark and co-workers postulated over 20 years ago that an active site residue(s) serves either to protonate the carbonyl oxygen of CbmP or to stabilize the oxyanion in the tetrahedral adduct by an ion pair bond.[4] The active site residue that plays this role is unknown. Threonine 55 has been ruled out as playing a role in polarizing the carbonyl by a rigorous kinetic and isotope effect study of the threonine to alanine mutant enzyme.[21] More studies of active site mutant enzymes will have to be done before the residue in question is identified.

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Registry No. ATCase, 9012-49-1; CbmP, 590-55-6; histidine, 71-00-1; cysteine sulfinate, 1115-65-7; aspartic acid, 56-84-8.

(20) The data do not distinguish between a tetrahedral adduct with a finite lifetime or one with a lifetime less than a single vibration.

(21) Waldrop, G. L.; Turnbull, J. L.; Parmentier, L. E.; Lee, S.; O'Leary, M. H.; Cleland, W. W.; Schachman, H. K. *Biochemistry*, in press.