^{13}C and ^{15}N Isotope Effects as a Probe of the Chemical Mechanism of *Escherichia coli* Aspartate Transcarbamylase†

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ABSTRACT: ^{13}C and ^{15}N isotope effects have been measured for the aspartate transcarbamylase (ATCase) reaction in an effort to elucidate the chemical mechanism of this highly regulated enzyme. The observed $^{15}(V/K_{asp})_{H_2O}$ value for the ATCase holoenzyme at saturating carbamyl phosphate and 12 mM L-aspartate is 1.0045 at pH 7.5, and this value remains unchanged in the presence of 5 mM ATP (activator) or 5 mM CTP (inhibitor). The fact that the isotope effect is not changed by the allosteric modifiers supports the conclusion that the kinetic properties of the active form of ATCase are not influenced by ATP or CTP. The observed $^{15}(V/K_{asp})$ values for the catalytic subunit of ATCase are also the same as those determined for the holoenzyme, suggesting that the chemical mechanisms of both enzyme species are the same. Quantitative analysis of ^{13}C and ^{15}N isotope effects in both H_2O and D_2O has led to the proposal of a chemical model for the ATCase reaction which involves a precatalytic conformational change to form an activated complex that facilitates deprotonation of L-aspartate by an enzyme functional group. Nucleophilic attack on the carbonyl carbon of carbamyl phosphate by the α-amino group of L-aspartate results in the formation of a tetrahedral intermediate. An intramolecular proton transfer leads to formation of products *N*-carbamyl-L-aspartate and inorganic phosphate.

The highly regulated oligomeric enzyme aspartate transcarbamylase (EC 2.1.3.2, ATCase)[1] from *Escherichia coli* catalyzes the formation of *N*-carbamyl-L-aspartate and inorganic phosphate from L-aspartate and carbamyl phosphate in the first committed step of pyrimidine biosynthesis [see Allewell (1989), Kantrowitz and Lipscomb (1990), Schachman (1988), Cohen et al. (1985), and Jacobson and Stark (1973)]. The native enzyme (holoenzyme) consists of two types of subunits, catalytic and regulatory. The catalytic subunits, which retain catalytic activity even when separated from the regulatory subunits, interact with active site ligands, whereas the regulatory subunits interact with the allosteric inhibitor CTP and the allosteric activator ATP.

Although ATCase has been extensively studied, little is actually known about the mechanistic details of the transcarbamylation reaction. Collins and Stark (1969) have proposed a general model for the catalytic action of ATCase which involves the proper positioning of carbamyl phosphate in the active site such that the carbonyl group is susceptible to nucleophilic attack by the amino group of L-aspartate. This model invokes an enzymic acid to polarize the carbonyl group of carbamyl phosphate, and enzymic base to deprotonate the reacting amine, and compression of the enzyme–substrate complex, driven by the initial binding, to facilitate catalysis. The carbamyl group transfer between carbamyl phosphate and L-aspartate was postulated by Jacobson and Stark (1973) to occur directly without formation of either cyanate or carbamylated enzyme, and kinetic isotope effect studies with ^{14}C- and anhydride ^{18}O-labeled carbamyl phosphate (Stark, 1971) were interpreted in terms of a tetrahedral intermediate. Waldrop et al. (1992c) have demonstrated by measurement of ^{15}N isotope effects in carbamyl phosphate, as well as ^{18}O experiments that establish C–O cleavage during the reaction, that neither a carbamic acid nor a cyanic acid intermediate is possible and, thus, that the mechanism involves a tetrahedral intermediate, or possibly a tetrahedral transition state in a concerted reaction.

The previous paper shows the power of heavy atom isotope effect studies as a probe of the kinetic and allosteric mechanisms of ATCase (Parmentier et al., 1992). Here we present a series of ^{13}C and ^{15}N isotope effect experiments on both the ATCase holoenzyme and isolated catalytic subunit that have been undertaken in an effort to define more narrowly the nature and relative rates of the various chemical steps in the ATCase-catalyzed reaction.

Materials and Methods

Glutamic oxalacetic transaminase from porcine heart, malate dehydrogenase from porcine heart, carbamyl phosphate (dilithium salt), L-cysteine sulfinic acid, NADH (disodium salt), α-ketoglutaric acid, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (HEPES), EDTA (disodium salt), ATP (disodium salt), CTP (sodium salt), *N*-carbamyl-DL-aspartic acid, and Ammonia Color Reagent were purchased from Sigma Chemical Co. L-Aspartic acid, KOD (98+ atom % D), D_2SO_4 (99.5+ atom % D), 2,3-butanedione monoxime, and antipyrine (1,2-dihydro-1,5-dimethyl-2-phenyl-3-pyrazolone)

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[1] Abbreviations: ATCase, aspartate transcarbamylase; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid); HEPPS, *N*-(2-hydroxyethyl)piperazine-*N*′-3-propanesulfonic acid.

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were obtained from Aldrich. D_2O (99.9 %D) was obtained from Cambridge Isotope Laboratories, and dithiothreitol was from Boehringer-Mannheim. DEAE-Sephadex A-25 anion-exchange resin was purchased from Pharmacia. All other chemicals used were reagent grade. Buffer solutions were prepared with distilled, deionized water and Millipore filtered before use. pH measurements were made using a Beckman 11 pH meter, and spectroscopic assays were performed using a Beckman DU spectrophotometer. ATCase holoenzyme and isolated catalytic subunits, from the lab of one of the authors (H.K.S.), were stored as a suspension in 3.6 M ammonium sulfate and 2 mM β-mercaptoethanol, pH 7.0, 4 °C, and dialyzed against 50 mM KH_2PO_4, 2 mM dithiothreitol, and 0.2 mM EDTA, pH 7.0, at 4 °C before use. For equilibrium isotope effect experiments the enzyme was dialyzed against 50 mM HEPES, 2 mM dithiothreitol, and 0.2 mM EDTA, pH 7.0, at 4 °C. Protein concentration was determined by measuring the absorbance at 280 nm where the extinction coefficient is 0.59 $cm^{-1}\cdot mg^{-1}\cdot mL$ for the holoenzyme and 0.72 $cm^{-1}\cdot mg^{-1}\cdot mL$ for the catalytic subunit (Gerhart & Holoubek, 1967).

Isotope Effect Nomenclature. The nomenclature used throughout this work is that of Northrop (1977) in which the leading superscript denotes the isotope responsible for the effect on a given kinetic or thermodynamic parameter. Thus the ^{15}N isotope effect on V/K is written $^{15}(V/K)$, and this symbol represents the rate of the ^{14}N-containing species under V/K conditions relative to the rate of the ^{15}N-containing species $[(V/K)^{14}N/(V/K)^{15}N]$. Multiple isotope effects are written as a combination of superscripts and subscripts, thus $^{15}(V/K)_{D_2O}$ is the ^{15}N isotope effect on V/K with D_2O as solvent. To designate the substrate on which the isotope effect is measured, a subscript within the parentheses is included. Thus $^{15}(V/K_{asp})$ indicates the isotope effect on V/K for aspartate.

Normalization of Deuterium Oxide. Commercially available D_2O is generally enriched in the heavy isotopes of oxygen, and since CO_2 equilibrates with the oxygen in D_2O, D_2O used as a solvent for ^{13}C isotope effect experiments was exchanged with excess CO_2 to bring the ^{17}O and ^{18}O contents nearer to natural abundance in order to avoid a large correction for ^{17}O which would result in a less accurate determination of the ^{13}C isotope effect [Weiss et al., 1988b; see also O'Leary et al. (1981)]. This normalization was accomplished by treating the D_2O with a large quantity (~0.5 g/L) of carbonic anhydrase and bubbling dry CO_2 of natural abundance through the solution for several hours. The D_2O solution was then filtered in an Amicon filtration apparatus to remove the protein and purged of CO_2 by sparging with dry N_2. ^{13}C contents for control CO_2 samples incubated with H_2O or D_2O treated as described above were identical.

Preparation of Sodium Hypobromite. Sixty milliliters of Br_2 was added slowly to 150 mL of 17 N NaOH in an ice bath with efficient stirring. An additional 150 mL of 17 N NaOH was added, and the mixture was stirred for 15 min. The flask was then sealed and stored in the dark at 4 °C for 5 days. After this time the solution was filtered through a medium sintered glass funnel. Equal volumes of filtrate and cold 12 mM KI were mixed, and the resulting hypobromite solution was stored in the dark at 4 °C.

^{13}C Kinetic Isotope Effects in Deuterium Oxide. To determine the effect of deuterated solvent on the ^{13}C isotope effects for the ATCase holoenzyme and isolated catalytic subunit-catalyzed reactions, $^{13}(V/K_{CP})$ was measured as a function of aspartate concentration in D_2O. The experimental procedure follows that outlined previously (Parmentier et al., 1992) for ^{13}C isotope effects, except that, in order to conserve normalized D_2O, reaction vessels contained 50 mM HEPES, 2 mM dithiothreitol, and 0.2 mM EDTA, pH 7.5, in a volume of 10–15 mL. All solutions were prepared in D_2O except for ATCase solutions, which were in H_2O. The H_2O contribution from addition of ATCase ranged from 0.2% to 1.0% of the final volume of each sample. For the low conversion samples, enough ATCase holoenzyme or catalytic subunit was added to the sparged reaction solutions so that the reaction proceeded to 50% (based on carbamyl phosphate) within the specified time at 25 °C. A typical experiment involved the consumption of 60 μmol of carbamyl phosphate at 50% completion; therefore, 0.6 mL of 100 mM aspartate solution was added dropwise over the course of the reaction to maintain a constant aspartate concentration. Additional aspartate was not added to reactions run at saturating levels of this substrate because it was found that the observed isotope effect did not change as a function of substrate concentration under these conditions.

Buffer solutions were sparged with N_2 for 1–2 h at pD < 6, and then the pD was raised with saturated NaOD in D_2O and the solutions were sparged for another 1–2 h. Aspartate solutions were prepared in this CO_2-free buffer, and the pD was then adjusted and the solution sparged an additional 1–2 h. Carbamyl phosphate solutions in D_2O were sparged as described by Parmentier et al. (1992). Controls were run to ascertain that solutions sparged in this manner were CO_2 free. pD values were taken as the pH meter reading +0.4 (Glasoe & Long, 1960). Initially samples were quenched at the appropriate time with D_2SO_4, but since deuterium substitution at quenching does not affect the ATCase reaction, subsequent samples were quenched with H_2SO_4.

^{15}N Kinetic Isotope Effects. The $^{15}(V/K_{asp})$ isotope effects on both holoenzyme and catalytic subunit reactions were determined by isotope ratio mass spectral analysis from zero or partial (41–60% based on aspartate) conversion samples using the natural abundance of ^{15}N as the label. The $^{15}N/^{14}N$ mass ratio of unreacted aspartate compared with that of aspartate remaining after partial conversion reflects the isotope discrimination. A schematic representation of the experimental protocol for determining ^{15}N isotope effects is shown in Figure 1.

Partial conversion samples contained 12 mM L-aspartate, 12 mM carbamyl phosphate, 0.003–0.03 mg/mL ATCase holoenzyme or isolated catalytic subunit, and 5 mM effectors in 50 mM HEPES, 2 mM dithiothreitol, and 0.2 mM EDTA, pH 7.5, in 15–20 mL. Reactions were initiated by the addition of carbamyl phosphate. After 2–16 min the reaction was quenched with 0.1 mL of concentrated H_2SO_4 which lowered the pH to <1.4. After determining the extent of reaction by end-point assay for aspartate (described below), the pH was raised to 7.5 with KOH. The sample was diluted ~100-fold to lower the ionic strength (specific conductance <800 $\mu mho/cm^3$, usually ~400) and loaded onto a 2.6 × 25-cm DEAE-Sephadex A-25 Cl^--form anion-exchange column that had been thoroughly flushed with water to remove any unbound chloride ion. After loading, the column was flushed with water (twenty 8-mL fractions collected) and then eluted with 0.1 M KCl (sixty 8-mL fractions collected). Under these conditions aspartate eluted from the column in fractions 43–60 and *N*-carbamylaspartate remained bound. *N*-Carbamylaspartate could be eluted with 0.3 M KCl and was detected by the procedure of Pastra-Landis et al. (1981).

Aspartate concentrations were determined enzymatically by coupling the glutamic oxalacetic transaminase-catalyzed

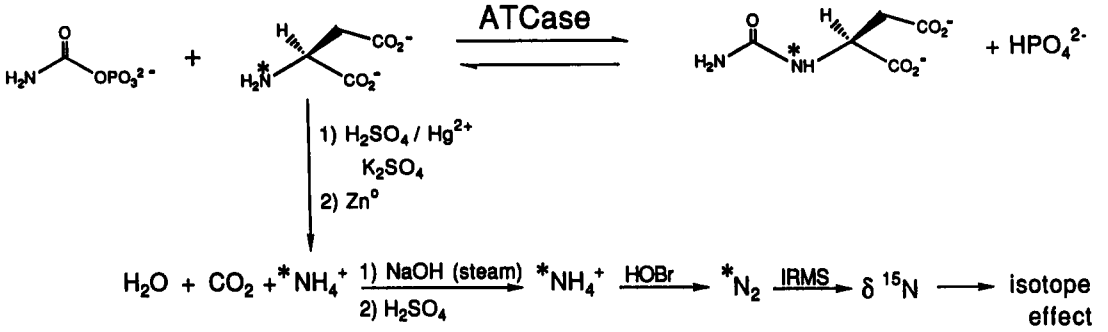


FIGURE 1: Schematic representation of the experimental protocol used for measuring ^{15}N isotope effects on the aspartate transcarbamylase reaction using the competitive method (O'Leary, 1980). Isotope ratios were determined by isotope ratio mass spectrometry (IRMS), ^{15}N kinetic isotope effects were calculated using eq 1, and ^{15}N equilibrium isotope effects were calculated using eq 2.

conversion of aspartate to oxalacetic acid with the malate dehydrogenase-catalyzed conversion of oxalacetic acid to malate and measuring spectrophotometrically the decrease in absorbance at 340 nm due to oxidation of NADH. Each 1-mL cuvette contained 5 mM α-ketoglutarate, 0.2 mM NADH, 40 units of glutamic oxalacetic transaminase, and 4 units of malate dehydrogenase in 100 mM HEPES, pH 8.0. Fractions found to contain aspartate by the above end-point assay were pooled, reduced in volume to 3–5 mL by rotary evaporation, and digested by the Kjeldahl procedure. The sample was added to a 100-mL Kjeldahl flask containing 3 mL of concentrated H_2SO_4, 1.5 mL of mercuric sulfate [prepared by diluting 24 mL of concentrated H_2SO_4 to 200 mL in H_2O and dissolving 20 g of red mercury(II) oxide], 1.5 g K_2SO_4, and several glass boiling beads. The mixture was refluxed on a digestion rack overnight. After digestion was complete, the solution was cooled to room temperature and 20 mL of H_2O was added, with swirling. After cooling slightly, 0.6 g of Zn dust was added to precipitate Hg(II), and the solution was filtered through a coarse sintered glass funnel. The resulting ammonia was liberated by alkaline steam distillation of the filtrate with 15 mL of 13 N NaOH, and 40 mL of the distillate was collected in 10 mL of 0.1 N H_2SO_4. Nessler's assay of the purified ammonia showed that the recovery of nitrogen was quantitative. The volume of the distillate was reduced to ~1 mL by rotary evaporation, and the sample was transferred to a Y-tube for oxidation to N_2 with 2 mL of hypobromite solution. Exogenous molecular nitrogen was removed from both sample and hypobromite solutions by two freeze–thaw cycles under vacuum. After warming to room temperature, the hypobromite solution was added to the sample solution under vacuum, and the reaction was allowed to proceed for 15 min. After this time, the reaction mixture was frozen in liquid nitrogen.

The N_2 produced upon oxidation was transferred under vacuum to a sample tube where it was adsorbed onto 3-Å molecular sieves, 8–12 mesh, at liquid N_2 temperature. The sample was then sealed for isotope ratio analysis. These procedures are further described by Weiss (1991).

The isotopic content of the initial aspartate was determined by Kjeldahl digestion of either solid, unreacted aspartate or aspartate isolated from 0% conversion samples lacking carbamyl phosphate and enzyme by DEAE chromatography; the latter method yielded the best results. All samples were steam distilled, oxidized with hypobromite, and analyzed with a Finnigan Delta E or MAT 251 isotope ratio mass spectrometer as described above.

^{15}N kinetic isotope effects on both the holoenzyme- and isolated catalytic subunit-catalyzed reactions were also run in D_2O as solvent. The experimental procedure followed that outlined above for ^{15}N kinetic isotope effects except that all solutions except enzyme were prepared in D_2O (D_2O for ^{15}N isotope effects need not have its ^{18}O content normalized). The H_2O contribution from addition of holoenzyme was 0.16% and that from catalytic subunit was 0.26% of the final volume of each sample. Fraction of reaction was determined by end-point assay for aspartate.

^{15}N Equilibrium Isotope Effects. To determine the $^{15}K_{eq}$ value in both H_2O and D_2O for the aspartate $\rightleftharpoons$ *N*-carbamylaspartate equilibrium, the following were incubated at 25 °C: 65 mM L-aspartate, 55 mM carbamyl phosphate, and 50 mM HEPES, pH(D) 7.0. These initial concentrations were based on an equilibrium constant of 5900 ± 600 at pH 7.0, 30 °C, reported by Hsuanyu and Wedler (1987). Holoenzyme reactions in H_2O were run in 20–60-mL volumes with 0.38–0.48 mg/mL enzyme. Catalytic subunit reactions were run in 20 mL with 0.17 mg/mL enzyme in H_2O or with 0.22 mg/mL enzyme in D_2O (enzyme used for $^{15}K_{eq}$ experiments in D_2O was dialyzed first against buffer prepared in H_2O to reduce salt concentration and then against buffer prepared in D_2O to minimize the addition of H_2O to the reaction). After equilibrium was established, the pH(D) was lowered to 1.3 by addition of concentrated H_2SO_4 (0.1 mL per 10-mL reaction volume), and the denatured protein was removed from the solution either by Amicon filtration with a YM30 43-mm membrane or by pelleting by table top centrifugation for 5 min. Isotopic equilibrium is usually thought to be achieved after 10 times the time required for a system to reach chemical equilibrium, which for the ATCase system under the described conditions was 4 min, as determined by monitoring the aspartate concentration by end-point assay over time and assuming a first-order approach to equilibrium. However, preliminary experiments revealed that the observed ^{15}N equilibrium isotope effect measured after 40 min of total reaction time was unity, a value presumably between the kinetic and true equilibrium isotope effects for this reaction. The observed $^{15}K_{eq}$ values changed slightly over time and became constant between 5 and 10 h. For this reason, solutions for ^{15}N equilibrium isotope effects were allowed to incubate for 5–10 h before isotopic analysis.

The filtered solution or supernatant was divided into three portions, adjusted to pH 7.5, and diluted so that the specific conductance was <800 μmho/cm^3. The nitrogen of aspartate was isolated and analyzed as described above. The $^{15}N/^{14}N$ ratio of this sample was compared to that of the initial aspartate to calculate the equilibrium isotope effect, using eq 2.

Data Analysis. The ^{13}C and ^{15}N isotope effects on the ATCase-catalyzed reaction were measured using the method of internal competition in which changes in the isotopic composition of either starting material or product are measured over the course of the reaction (O'Leary, 1980). The following

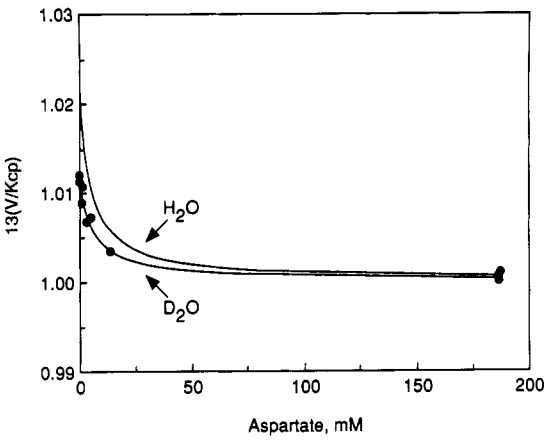


FIGURE 2: ^{13}C isotope effects for the aspartate transcarbamylase holoenzyme-catalyzed reaction at 12 mM (saturating) carbamyl phosphate and varying L-aspartate concentrations in 50 mM HEPES, 2 mM dithiothreitol, and 0.2 mM EDTA, pD 7.5, 25 °C, in D_2O (●). Shown for comparison is the corresponding curve in H_2O (Parmentier et al., 1992) for which experimental points have been omitted for clarity. The data were fitted to eq 4. The maximum value of $^{13}(V/K_{CP})_{D_2O}$, when aspartate concentration is extrapolated to 0, is 1.0116 ± 0.0004, the concentration of aspartate that half-eliminates the isotope effect is 5.81 ± 0.93 mM, and $^{13}(V/K_{CP})_{D_2O}$ at infinite aspartate approaches unity.

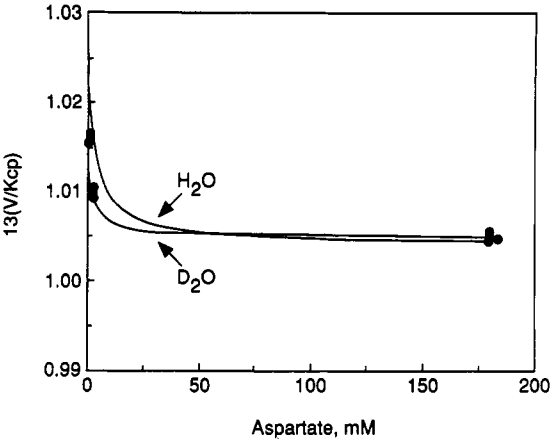


FIGURE 3: ^{13}C isotope effects for the aspartate transcarbamylase catalytic subunit-catalyzed reaction at 12 mM (saturating) carbamyl phosphate and varying L-aspartate concentrations in 50 mM HEPES, 2 mM dithiothreitol, and 0.2 mM EDTA, pD 7.5, 25 °C, in D_2O (●). Shown for comparison is the corresponding curve in H_2O (Parmentier et al., 1992) for which experimental points have been omitted for clarity. The data were fitted to eq 3. The maximum value of $^{13}(V/K_{CP})_{D_2O}$, when aspartate concentration is extrapolated to 0, is 1.0172 ± 0.0007, the concentration of aspartate that reduces the isotope effect by half is 1.76 ± 0.41 mM, and $^{13}(V/K_{CP})_{D_2O}$ at infinite aspartate is 1.0047 ± 0.0004.

equation was used to calculate the ^{13}C or ^{15}N kinetic isotope effects:

$$^{13\text{ or }15}(V/K) = \frac{\log (1-f)}{\log [(1-f)(R_s/R_0)]} \quad (1)$$

R_s is the $^{13}C/^{12}C$ or $^{15}N/^{14}N$ ratio of a given position in the substrate after fraction of reaction f, and R_0 is the initial ratio of the same position in the substrate.

^{15}N equilibrium isotope effects were calculated from the equation:

$$^{15}K_{eq} = x/[(1 + x)(R_0/R_{eq}) - 1] \quad (2)$$

where x is the [carbamyl aspartate]/[asparate] ratio at equilibrium, R_0 is the mass ratio of the initial aspartate, and R_{eq} is the mass ratio of aspartate at equilibrium.

^{13}C kinetic isotope effects were plotted versus final aspartate concentration, and the data were fitted to the following equation for a hyperbola (HYPRP; Cleland, 1979):

$$y = A(1 + x/K_{in})/(1 + x/K_{id}) \quad (3)$$

where y is the observed ^{13}C isotope effect, x is the final aspartate concentration, A is the isotope effect at an aspartate concentration extrapolated to zero, K_{id} is the aspartate concentration that half-eliminates the isotope effect, and the quantity $(A)(K_{id})/K_{in}$ is the isotope effect at infinite aspartate (the asymptote of the hyperbola).

In cases where the asymptote of the hyperbola was unity, the data were fitted to the following equation (HYPRPL; Cleland, 1979):

$$y = (A + x/K_{id})/(1 + x/K_{id}) \quad (4)$$

where x, y, K_{id}, and A are as described above.

RESULTS

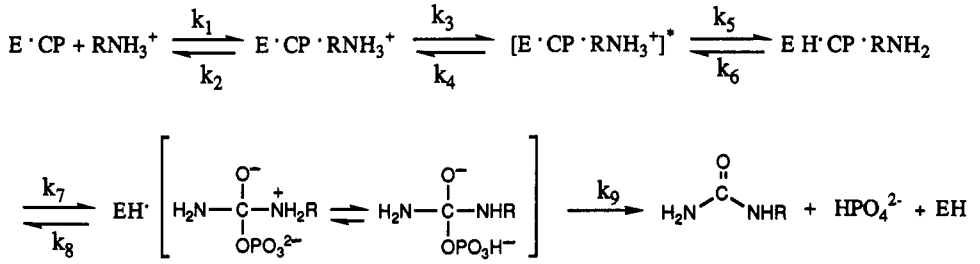
^{13}C Kinetic Isotope Effects in Deuterium Oxide. The observed $^{13}(V/K_{CP})_{D_2O}$ values for the holoenzyme-catalyzed reaction were found to vary with aspartate concentration in a hyperbolic fashion as shown in Figure 2. The data were fitted to eq 4 where the isotope effect was constrained to unity at an infinite level of aspartate. The limiting $^{13}(V/K_{CP})_{D_2O}$ value at an aspartate concentration extrapolated to zero, A, was calculated to be 1.0116 ± 0.0004. The amount of aspartate necessary to half-eliminate the isotope effect, K_{id}, was 5.81 ± 0.93 mM. Identical values for A and K_{id} were obtained when the data were fitted to eq 3 that no longer forced the asymptote to unity. The asymptote in this case was 1.0001 ± 0.0004.

The ^{13}C isotope effect data obtained with the isolated catalytic subunit in D_2O also obey a hyperbolic relationship with aspartate concentration, as illustrated in Figure 3, but, as was seen with the catalytic subunit reaction in H_2O (Parmentier et al., 1992), the asymptote of the hyperbola is not unity. When the data were fitted to eq 3, the maximum value of $^{13}(V/K_{CP})_{D_2O}$ was 1.0172 ± 0.0007 and the concentration of aspartate that half-eliminated the isotope effect was 1.76 ± 0.41 mM. The asymptote had a value of 1.0047 ± 0.0004 at infinite aspartate.

^{15}N Kinetic Isotope Effects. ^{15}N kinetic isotope effects for the ATCase holoenzyme and isolated catalytic subunit reactions were measured in both H_2O and D_2O at 12 mM L-aspartate (about twice the K_d value) and 12 mM carbamyl phosphate (saturating), pH(D) 7.5. For the holoenzyme-catalyzed reaction, the observed $^{15}(V/K_{asp})_{H_2O}$ value was 1.0045 ± 0.0004 (12 determinations) in the absence of allosteric effectors. This experiment was repeated with the allosteric modifiers ATP and CTP, and the observed $^{15}(V/K_{asp})_{H_2O}$ values were 1.0046 ± 0.0005 (3 determinations) in the presence of 5 mM ATP and 1.0047 ± 0.0003 (3 determinations) in the presence of 5 mM CTP. These results show that the presence of the allosteric modifiers does not alter the observed isotope effect. This observation supports our contention (Parmentier et al., 1992) that ATP and CTP exert their effects by binding preferentially to the R or T state of the enzyme without altering the kinetic properties of the active form. The observed $^{15}(V/K_{asp})_{D_2O}$ value was 1.0063 ± 0.0002 (6 determinations) for the holoenzyme.

For the isolated catalytic subunit, $^{15}(V/K_{asp})_{H_2O}$ was 1.0044 ± 0.0003 (8 determinations), which is not significantly different from the isotope effect obtained with the holoenzyme. When the catalytic subunit reaction was run in D_2O, the

Scheme I



observed value of $^{15}(V/K_{asp})_{D_2O}$ was 1.0076 ± 0.0003 (4 determinations).

^{15}N Equilibrium Isotope Effects. $^{15}K_{eq}$ for the aspartate/*N*-carbamylaspartate equilibrium, pH 7.0, for the holoenzyme was found to be 0.9986 ± 0.0001 from 4 determinations (this value is for the protonated amino group of aspartate). When aspartate and *N*-carbamylaspartate were brought to equilibrium at pH 7.0 in the presence of the ATCase catalytic subunit, an ^{15}N equilibrium isotope effect of 0.9994 ± 0.0003 from 6 determinations was observed. The average of all 10 determinations is 0.9990 ± 0.0002. The fact that this value is inverse suggests that ^{15}N tends to enrich in *N*-carbamylaspartate, where it is more stiffly bonded, relative to the NH_3^+ group of aspartate.

$^{15}K_{eq}$ was also determined for the catalytic subunit in D_2O, pD 7.0. In this case, $^{15}K_{eqD}$ was found to be 1.0071 ± 0.0002 from six determinations. This value is 0.81% larger than the ^{15}N equilibrium isotope effect in H_2O as a result of the stiffening effect of deuterium on bond vibrations and the fact that the nitrogen of aspartate is bonded to three deuterium atoms, while that of *N*-carbamylaspartate is bonded to only one.

Discussion

The ATCase-catalyzed transfer of the carbamyl moiety of carbamyl phosphate to L-aspartate has been shown by Waldrop et al. (1992c) to occur via a tetrahedral intermediate or transition state. We will thus analyze the ^{13}C and ^{15}N isotope effects for the reaction in terms of a chemical model involving a tetrahedral intermediate to provide information about the relative rates of the various steps in the overall chemical transformation.

Consider the model shown in Scheme I, in which carbamyl phosphate binds to free enzyme first to form the enzyme–carbamyl phosphate binary complex, and aspartate binds in the protonated form (Turnbull et al., 1992) to form the ternary complex E·CP·RNH_3^+. A precatalytic conformation change of this ternary complex (k_3) poises the aspartate within the active site in such a way as to facilitate deprotonation of the α-amino group by an enzymic base (k_5). Once deprotonated, aspartate becomes a suitable nucleophile and attacks carbamyl phosphate to form the tetrahedral intermediate (k_7). All the steps for the breakdown of the tetrahedral intermediate and for product release are combined into one irreversible step with rate constant k_9. In this model, k_7, k_8, and k_9 are ^{13}C sensitive, k_5, k_6, k_7, k_8, and k_9 are ^{15}N sensitive, k_5 and k_6 are deuterium sensitive, and all commitments are internal.

As shown in Scheme I above, a proton has to move from the nitrogen of aspartate directly or indirectly to the phosphate group in order for the tetrahedral intermediate to break down in the forward direction. This conversion might be deuterium sensitive, but as we will show below, it appears not to be, either because the proton shift is at equilibrium or because the proton is in a low-barrier hydrogen bond between the nitrogen and oxygen, and thus any isotope effect is equal on k_8 and k_9.

The equation for the observed ^{13}C kinetic isotope effect for carbamyl phosphate thus can be written as

$$^{13}(V/K_{CP}) = \frac{^{13}K_{eq_7}{}^{13}k_9 + {}^{13}k_7 b + ab + abc}{1 + b + ab + abc} \quad (5)$$

where $a = k_7/k_6$, $b = k_9/k_8$, and $c = k_5/k_4$. We will assume that the ^{13}C isotope effect on breaking a C–O bond is comparable to that for breaking a C–N bond and therefore $^{13}k_8 = {}^{13}k_9$.[2] Since $^{13}K_{eq_7} = {}^{13}k_7/{}^{13}k_8$, eq 5 becomes

$$^{13}(V/K_{CP}) = \frac{^{13}k_7 + {}^{13}k_7 b + ab + abc}{1 + b + ab + abc} \quad (6)$$

Equation 6 can be rearranged to combine the partition ratios into one term:

$$^{13}(V/K_{CP}) = \frac{^{13}k_7 + ab(1 + c)/(1 + b)}{1 + ab(1 + c)/(1 + b)} \quad (7)$$

where $^{13}k_7$ is the ^{13}C intrinsic isotope effect on formation of the tetrahedral intermediate. We have previously proposed that 1.039 is a reasonable value for the intrinsic isotope effect for this nucleophilic addition to the carbonyl carbon on the basis of the observed $^{13}(V/K_{CP})$ value for the ATCase reaction with cysteine sulfinate (Parmentier et al., 1992). We have also measured a $^{13}(V/K_{CP})_{D_2O}$ value of 1.0434 ± 0.0007 for the reaction catalyzed by the ATCase catalytic subunit in which His 134 was replaced with Ala by site-directed mutagenesis (Waldrop et al., 1992a). If 1.043 is adopted for $^{13}k_7$ in eq 7,[3] values of 0.81 in H_2O or 1.52 in D_2O can be calculated for the commitment term $ab(1 + c)/(1 + b)$.

Exact solutions for the parameters *a*–*c*, which reflect the relative rates of the various steps in the overall reaction, can be obtained by analysis of the above equations for $ab(1 + c)/(1 + b)$ in conjunction with eqs 8 and 9 for the observed ^{15}N

$$^{15}(V/K_{asp})_{H_2O} = \frac{^{15}K_{eq5H}{}^{15}K_{eq7}{}^{15}k_9 + {}^{15}K_{eq5H}{}^{15}k_7 b + {}^{15}k_{5H} ab + abc}{1 + b + ab + abc} \quad (8)$$

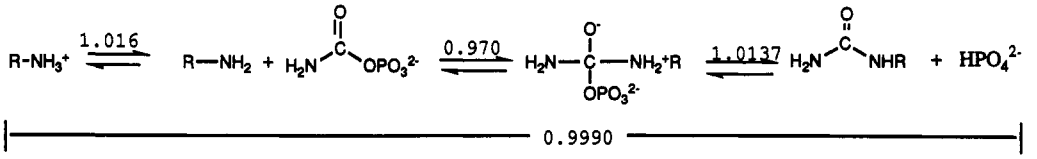
$$^{15}(V/K_{asp})_{D_2O} = \frac{^{15}K_{eq5D}{}^{15}K_{eq7}{}^{15}k_9 + {}^{15}K_{eq5D}{}^{15}k_7 b + {}^{15}k_{5D} ab + abc}{1 + b + ab + abc} \quad (9)$$

kinetic isotope effects in H_2O and D_2O. Values for $^{15}K_{eq5H(D)}$ (that value in H_2O or D_2O), $^{15}K_{eq7}$, and $^{15}k_9$ can be estimated

[2] We have no experimental evidence concerning the relative ^{13}C isotope effects on C–N and C–O cleavage of the tetrahedral intermediate, and the assumption of equal values is for convenience and simplicity of analysis. We doubt, however, that the values will be very different, since both are primary isotope effects involving conversion of tetrahedral to trigonal carbon.

[3] We have chosen 1.043 rather than 1.039 as the intrinsic value of $^{13}k_7$ because it was determined with aspartate, rather than cysteine sulfinate, as the substrate. The differences in the intrinsic isotope effects with the two substrates may be real.

Scheme II

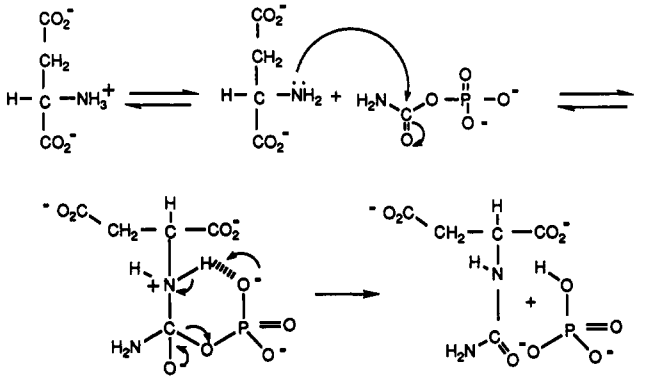


by the following analysis of the observed overall equilibrium isotope effect and by analogy with known systems.

The observed $^{15}K_{eq}$ value of 0.9990 ± 0.0002 in H_2O is the product of several ^{15}N equilibrium isotope effects, as shown in Scheme II. Reasonable values for the first two ^{15}N equilibrium isotope effects, based on analogy with the reaction catalyzed by phenylalanine ammonia lyase (Hermes et al., 1985), are 1.016 (deprotonation of phenylalanine) and 0.970 (slightly more inverse than 0.979, which was the value obtained for nucleophilic addition of unprotonated phenylalanine to the prosthetic group of phenylalanine ammonia lyase). We expect that the equilibrium isotope effect for the nucleophilic attack on a carbon bonded to oxygen will be more inverse than that for a carbon bonded to carbon due to the stiffening effect of the oxygen. Dividing the observed isotope effect of 0.9990 by 1.016 and 0.970 gives a value of 1.0137, which encompasses both proton abstraction and product formation. Therefore, in Scheme I, $^{15}K_{eq5}$ in H_2O = 1.016, $^{15}K_{eq7}$ = 0.970, and $^{15}K_{eq9}$ = 1.0137.[4] We will assume that the isotope effect on the reversal of the step corresponding to k_9 is unity,[5] and therefore, $^{15}k_9 = {}^{15}K_{eq9}$. Thus $^{15}k_9$ = 1.0137.

Similarly, $^{15}k_7$, which reflects a primary bond making step, is assumed to be unity. $^{15}K_{eq5}$ in D_2O is assumed to be 1.0201, which is 0.41% larger than that equilibrium isotope effect in H_2O. The ^{15}N equilibrium isotope effect measured for the overall ATCase reaction in D_2O is 0.81% greater than that obtained in H_2O. In this chemical transformation, two protons (deuterons) are lost from the reacting amino group of aspartate. Dividing the observed difference in equilibrium isotope effect by 2 results in a value of 0.41% for the stiffening effect of a single deuteron. This value is in accord with the findings of Hermes et al. (1985), who have reported an increase of 0.5% for the $ND_4^+ \rightleftharpoons ND_3$ equilibrium relative to $NH_4^+ \rightleftharpoons NH_3$ under identical reaction conditions. In addition, ^{15}N kinetic isotope effect results with the His 134 → Ala mutant ATCase (Waldrop et al., 1992a) reveal a 0.68% increase in D_2O relative to H_2O. However this value is a comparison of two kinetic isotope effects and is probably less reliable than the comparison of equilibrium isotope effects given above. $^{15}k_5$ will be assumed to be equal to $^{15}K_{eq5}$ (which implies that $^{15}k_6$ for a primary bond making step is unity). We will further assume that neither b nor k_7 is changed in D_2O,[6] but that k_6 is reduced by a factor of 1.88 (since $[ab(1 + c)/(1 + b)]_{D_2O}/[ab(1 + c)/(1 + b)]_{H_2O}$ = 1.88). This relationship requires that the partition ratio a is increased in D_2O by a factor of 1.88. k_5 is also reduced in D_2O, but we will assume that k_4 is likewise

Scheme III



reduced so that c remains constant.

Equations 8 and 9 now become

$$^{15}(V/K_{asp})_{H_2O} = 1.0044 = \frac{1.016[0.983 + b(1 + a)] + abc}{1 + b(1 + a) + abc} \quad (10)$$

$$^{15}(V/K_{asp})_{D_2O} = 1.0076 = \frac{1.0201[0.983 + b(1 + 1.88a)] + 1.88abc}{1 + b(1 + 1.88a) + 1.88abc} \quad (11)$$

Solving the above equations under the described conditions for the ATCase catalytic subunit yields values of a = 1.43 ± 0.58, b = 0.30 ± 0.11, and c = 1.46 ± 0.32. If the maximum value of 0.68% is adopted for the fractionation factor difference between protium and deuterium bonded to nitrogen, the above value of a decreases 3.2-fold, b increases 2.1-fold, and c increases 2.6-fold. The fact that b (k_9/k_8) is less than unity suggests that partitioning of the tetrahedral intermediate to form products may be slightly slower than partitioning backward, but since this ratio is not very different from unity, both tetrahedral adduct formation and breakdown are partially rate limiting.

The isotope effect data obtained with the holoenzyme can be analyzed in the same fashion. The relevant equations for the holoenzyme are

$$[ab(1 + c)/(1 + b)]_{H_2O} = 1.00 \quad (12)$$

$$[ab(1 + c)/(1 + b)]_{D_2O} = 2.74 \quad (13)$$

which are both obtained from eq 7. In addition:

$$^{15}(V/K_{asp})_{H_2O} = 1.0045 = \frac{1.016[0.983 + b(1 + a)] + abc}{1 + b(1 + a) + abc} \quad (14)$$

$$^{15}(V/K_{asp})_{D_2O} = 1.0063 = \frac{1.0201[0.983 + b(1 + 2.74a)] + 2.74abc}{1 + b(1 + 2.74a) + 2.74abc} \quad (15)$$

Simultaneous solution of these equations yields values of a = 0.77 ± 0.23, b = 0.53 ± 0.16, and c = 2.72 ± 0.39. The mechanisms of the ATCase holoenzyme and catalytic subunit are governed by slightly different commitments, although the partitioning of the tetrahedral intermediate is not drastically changed (see Table I for a summary of these calculations).

[4] The value of 1.016 for $^{15}K_{eq5}$ is well determined as is the overall $^{15}K_{eq}$ value, but the value of $^{15}K_{eq7}$ is less certain. If 0.979 is used for this parameter, $^{15}K_{eq9}$ = 1.005. This makes little difference in any of the calculations presented here.

[5] The step represented by k_9 includes transfer of the proton from the $-NH_2^+-$ group to phosphate, as well as the elimination of phosphate to give carbamyl phosphate. Previously measured ^{15}N kinetic isotope effects on C–N cleavage are equal to or slightly greater than the value of $^{15}K_{eq}$ (Weiss et al., 1988a, 1987; Hermes et al., 1985), and we feel that a similar situation should occur here; that is, the major portion of the equilibrium isotope effect should be expressed on the N–H cleavage, rather than on N–H bond formation. $^{15}k_{10}$ is unlikely to be inverse, since this step involves a primary isotope effect.

[6] We address the question of the deuterium sensitivity of b below.

Table I: Summary of ^{13}C and ^{15}N Isotope Effects and Partition Ratios for the ATCase Holoenzyme and Catalytic Subunit Reactions, pH(D) 7.5, 25 °C[a]

value	catalytic subunit	holoenzyme
$^{13}(V/K_{CP})_{H_2O}$[b]	1.0240 ± 0.0005	1.0217 ± 0.0005
$^{13}(V/K_{CP})_{D_2O}$[c]	1.0172 ± 0.0007	1.0116 ± 0.0004
$^{15}(V/K_{asp})_{H_2O}$[c]	1.0044 ± 0.0003	1.0045 ± 0.0004
$^{15}(V/K_{asp})_{D_2O}$[c]	1.0076 ± 0.0003	1.0063 ± 0.0002
$^{15}K_{eqH}$[c]	0.9990 ± 0.0002[e]	0.9990 ± 0.0002[e]
$^{15}K_{eqD}$[c]	1.0071 ± 0.0002	
$^{D_2O}(V/K_{asp})$[d]	0.73 ± 0.02	
$^{D_2O}V_{max}$[d]	1.56 ± 0.18	
$[ab(1 + c)/(1 + b)]_{H_2O}$	0.81 ± 0.03	1.00 ± 0.04
$[ab(1 + c)/(1 + b)]_{D_2O}$	1.52 ± 0.08	2.74 ± 0.11
a	1.43 ± 0.58	0.78 ± 0.23
b	0.30 ± 0.11	0.53 ± 0.16
c	1.46 ± 0.32	2.72 ± 0.39

[a] See text for equations and assumptions necessary for the calculation of terms a–c, which relate to the chemical model presented in Scheme I. [b] Parmentier et al., 1992. [c] Present study. [d] J. L. Turnbull and G. L. Waldrop (personal communication, 1990). [e] Average of values from four determinations with the holoenzyme and six determinations with the catalytic subunit.

The chemical mechanisms of the two species are the same.

The isotope effect data obtained in the present study are consistent with the chemical mechanism shown in Scheme III, which is adapted from that proposed by Gouaux et al. (1987) in a molecular modeling study and verified by Waldrop et al. (1992c) on the basis of ^{15}N isotope effects in carbamyl phosphate.

The tetrahedral intermediate can exist within the active site as a six-membered ring in a chair conformation. This orientation allows the phosphate group of carbamyl phosphate, which needs to be protonated in order to become a better leaving group, to abstract a proton from the charged amino group of L-aspartate. This intramolecular proton transfer leads to collapse of the tetrahedral intermediate and formation of products.

Since breakdown of the tetrahedral intermediate involves a proton transfer, the question arises whether this step should be sensitive to D_2O. If it were, the assumption that b does not vary in D_2O would not be valid. The question of whether or not the parameter b is solvent sensitive can be addressed by no longer holding this term constant and rather letting the parameter c vary in the above equations. For the catalytic subunit reaction, if b is reduced by a factor of 1.88 and a is increased by this same factor, the resulting combination of equations yields a quadratic equation in b that has two negative roots. When b is decreased by only half as much as a is increased, two negative roots are again obtained. From these sample calculations, it is clear that the overall reaction is very sensitive to changes in b and that the intramolecular proton transfer is not rate limiting in the breakdown of the tetrahedral intermediate nor is it very D_2O sensitive.

The evaluation of solvent isotope effects on enzyme-catalyzed reactions is very complex [see reviews by O'Leary (1989) and Venkatasubban and Schowen (1984)]. For reactions run in D_2O, for instance, not only can multiple groups on both the enzyme and the substrates exchange with the medium, but conformational changes in the protein can occur as well. In short, many steps in the overall catalytic process can be affected by solvent. For the ATCase catalytic subunit reaction, J. L. Turnbull and G. L. Waldrop (1990, personal communication) have found an inverse D_2O solvent isotope effect on V/K_{asp} of 0.73 ± 0.02 (which reflects steps up through the first irreversible step) and a normal solvent effect on V_{max} of 1.56 ± 0.18 (which includes steps for product release). Foote et al. (1985) have also reported an inverse $^{D_2O}(V/K_{asp})$ value for the catalytic subunit reaction with L-aspartate as substrate, but with L-cysteine sulfinate as substrate no D_2O solvent isotope effect on V/K_{CS} was seen. These findings are consistent with the model presented in Scheme I where k_3, the rate constant governing the conformational change which leads to the formation of the catalytic complex [E·CP·RNH_3^+]*, is increased in D_2O. No isotope effect was seen with cysteine sulfinate because all steps prior to tetrahedral adduct formation are presumably at equilibrium.

Conclusions. The measurement of several isotope effects on the same enzyme system allows for a detailed, quantitative analysis of the overall reaction mechanism. By measuring a series of ^{13}C and ^{15}N isotope effects on the ATCase-catalyzed reaction and observing how these isotope effects change when measured in deuterium oxide as solvent, we have begun to elucidate the nature of the various chemical steps in the catalytic process. We have proposed a model that accounts for the experimental data and from which we can calculate reasonable values for the partition ratios of intermediates in the overall reaction. From this analysis, we have shown that the overall mechanism of ATCase involves the binding of protonated L-aspartate to the binary E·CP complex to form the E·CP·RNH_3^+ ternary complex. This ternary complex undergoes a conformational change to form the activated ternary complex [E·CP·RNH_3^+]*. A proton is removed from the α-amino group of L-aspartate in this ternary complex, presumably by an active site base. Nucleophilic attack of the carbonyl carbon of carbamyl phosphate by the deprotonated amino group of L-aspartate leads to formation of a tetrahedral intermediate. The carbonyl group of carbamyl phosphate is most likely polarized by an enzymic acid, which would make the carbon center more susceptible to nucleophilic attack. The identity of this active site acid is still to be determined. An intramolecular proton transfer between the positively charged amino group of aspartate and one of the negatively charged phosphate oxygens of carbamyl phosphate accompanies collapse of the tetrahedral intermediate to form the products *N*-carbamyl-L-aspartate and inorganic phosphate. In subsequent papers (Waldrop et al., 1982a,b), we begin to examine the roles of specific amino acid residues in the catalytic mechanism of ATCase.

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