^{13}C Isotope Effects as a Probe of the Kinetic Mechanism and Allosteric Properties of *Escherichia coli* Aspartate Transcarbamylase†

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ABSTRACT: ^{13}C kinetic isotope effects have been measured in carbamyl phosphate for the reaction catalyzed by aspartate transcarbamylase. For the holoenzyme, the value was 1.0217 at zero aspartate, but unity at infinite aspartate, with 4.8 mM aspartate eliminating half of the isotope effect. This pattern proves an ordered kinetic mechanism, with carbamyl phosphate adding before aspartate. The same parameters were observed in the presence of ATP or CTP, showing that there is only one form of active enzyme present, regardless of the presence or absence of allosteric modifiers. These data support the Monod model of allosteric behavior in which the equilibrium between fully active and inactive enzyme is perturbed by selective binding interactions of substrates and modifiers, and there are no enzyme forms having partial activity. Isolated catalytic subunits of the enzyme showed similar ^{13}C isotope effects (1.0240 at zero aspartate, 1.0039 at infinite aspartate, 3.8 mM aspartate causing half of the change from one value to the other), but the finite isotope effect at infinite aspartate shows that the kinetic mechanism is now partly random. With the very slow and poorly bound aspartate analog cysteine sulfinate, the ^{13}C isotope effects were 1.039 for both holoenzyme and catalytic subunits and were not decreased significantly by high levels of cysteine sulfinate. The value of 1.039 is probably close to the intrinsic isotope effect on the chemical reaction, while the kinetic mechanism with this substrate is now fully random because the chemistry is so much slower than release of either reactant from the enzyme.

Aspartate transcarbamylase (EC 2.1.3.2, ATCase)[1] is a highly regulated enzyme that 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. ATCase exhibits both homotropic and heterotropic interactions (Gerhart & Pardee, 1962; Bethel et al., 1968). Positive cooperativity, in the form of sigmoidal saturation curves, is seen in the presence of either L-aspartate or carbamyl phosphate. This sigmoidal dependence on substrate concentration is generally felt to be the result of a substrate-induced conformational change which causes the enzyme to undergo a conversion from the unliganded T state to the more highly active R state (Schachman, 1988). Heterotropic inhibition of ATCase is seen in the presence of the pyrimidine pathway end product CTP, and activation of the enzyme is seen in the presence of ATP, the end product of the parallel purine biosynthesis pathway. This type of regulation provides the appropriate balance of nucleotides for nucleic acid synthesis. The ATCase holoenzyme is composed of two catalytic trimers and three regulatory dimers and can be dissociated to yield catalytically active catalytic trimers which are insensitive to the allosteric effectors ATP and CTP. Although ATCase from *Escherichia coli* has been extensively studied for decades (Allewell, 1989; Kantrowitz & Lipscomb, 1990; Schachman, 1988; Cohen et al., 1985; Jacobson & Stark, 1973), a number of controversies regarding the kinetic and allosteric mechanisms remain unresolved in the literature.

Most steady-state kinetic studies of the catalytic subunit indicate an ordered mechanism in which carbamyl phosphate binds to the enzyme first, followed by aspartate, and *N*-carbamylaspartate is released from the enzyme prior to the dissociation of inorganic phosphate (Porter et al., 1969). However, other studies with the catalytic subunit using L-aspartate and either carbamyl phosphate or acetyl phosphate as substrates suggest that the kinetic mechanism is random (Heyde et al., 1973; Heyde & Morrison, 1973). Kinetic studies of the holoenzyme utilizing equilibrium isotope exchange techniques are in support of an ordered mechanism (Hsuanyu & Wedler, 1987).

The allosteric behavior of ATCase has been described, in the simplest case, in terms of a two-state model (Monod et al., 1965) in which the holoenzyme exists in two distinct forms, T and R, which differ in conformation, ligand affinity, and activity. In the absence of substrates, ATCase exists predominantly in the inactive T conformation, but upon substrate addition, all six active sites of each molecule are converted, in a concerted fashion, to the active R conformation. According to this model, the allosteric effectors ATP and CTP affect enzyme activity by shifting the population of ATCase molecules in either of these two states by binding preferentially to one or the other. While to a large extent the allosteric properties of ATCase can be satisfactorily explained in terms of this two-state model (Howlett et al., 1977), some investi-

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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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gators contend that these data are insufficient to rule out intermediate conformational states of the enzyme having intermediate levels of activity or the possibility that enzyme regulation by ATP and CTP can occur via different mechanisms (Hervé et al., 1985; Tauc et al., 1982; Wedler & Gasser, 1974; Wedler et al., 1989).

In the first reported isotope effect study on ATCase, Stark (1971) measured ^{14}C and ^{18}O kinetic isotope effects for carbamyl phosphate. Very small carbon and oxygen isotope effects were found, and Stark proposed that, under optimum conditions, catalysis is not rate limiting. We have measured ^{13}C kinetic isotope effects as a function of aspartate concentration for the reactions catalyzed by the ATCase holoenzyme and by isolated catalytic subunit using the natural abundance of ^{13}C at the carbonyl carbon of carbamyl phosphate as the label; this is a much more precise technique than that used by Stark.[2] Holoenzyme experiments were run in the absence of allosteric effectors as well as in the presence of ATP and CTP to determine whether these allosteric modifiers in any way alter the observed isotope effect. In addition, ^{13}C kinetic isotope effects were also measured for both the holoenzyme and isolated catalytic subunit catalyzed reaction between carbamyl phosphate and the aspartate analog L-cysteine sulfinate, a substrate whose relative activity is ~1.6% that of L-aspartate with the isolated catalytic subunit (Foote et al., 1985). These researchers have proposed that cysteine sulfinate does not promote the allosteric transition from T → R and that the cysteine sulfinate reaction is catalyzed by ATCase in the T quaternary structure. From the present work we conclude that substrate addition to the holoenzyme is strictly an ordered process in which carbamyl phosphate adds to the enzyme first, followed by addition of aspartate. The kinetic mechanism of the catalytic subunit, while predominantly ordered, exhibits a slightly random component, and the kinetic mechanisms of both the holoenzyme and catalytic subunit become random when cysteine sulfinate is used as a substrate. Results obtained with the allosteric modifiers ATP and CTP are in strong support of the two-state model for the allosteric behavior of ATCase.

Materials and Methods

Carbamyl phosphate (dilithium salt), ATP (disodium salt), CTP (sodium salt), L-cysteine sulfinic acid, EDTA (disodium salt), HEPES [*N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid], and HEPPS [*N*-(2-hydroxyethyl)piperazine-*N*′-3-propanesulfonic acid] were purchased from Sigma Chemical Co. L-Aspartic acid was obtained from Aldrich and dithiothreitol from Boehringer-Mannheim. 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. Protein concentration was determined by measuring the absorbance at 280 nm where the extinction coefficient is 0.59 cm^{-1}·mg^{-1}·mL for the holoenzyme and 0.72 cm^{-1}·mg^{-1}·mL for the catalytic subunit (Gerhart & Holoubek, 1967).

[2] For general reviews of the power of isotope effects in elucidating enzyme mechanisms, see Cleland (1982, 1987) and O'Leary (1989).

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 ^{13}C isotope effect on V/K is written $^{13}(V/K)$, and this symbol represents the ratio of V/K for the ^{12}C-containing species relative to that for the ^{13}C-containing species $[(V/K)^{12}C/(V/K)^{13}C]$. To designate the substrate on which the isotope effect is measured, a subscript within the brackets is included. Thus $^{13}(V/K_{CP})$ indicates the ^{13}C isotope effect on V/K for carbamyl phosphate.

^{13}C Isotope Effects on the Holoenzyme Reaction in the Presence of Allosteric Effectors, pH 7.5. ^{13}C isotope effects on the ATCase-catalyzed reaction were determined by analyzing residual carbamyl phosphate after some known fraction of reaction, usually ~50%, and comparing this mass ratio to that of the initial, unreacted carbamyl phosphate. Under acidic conditions, carbamyl phosphate decomposes to CO_2, NH_4^+, and inorganic phosphate (Allen & Jones, 1964), and it is this CO_2 that was collected and analyzed. A ~200 mM carbamyl phosphate solution, pH 5.5, was prepared in water, sealed with a rubber septum, placed in an ice bath, and freed from CO_2 by sparging with CO_2-free N_2 for 1–2 h. This solution was kept on ice throughout the experiment and was calibrated as described below. Reaction vessels fitted with vacuum adaptors and side arms with stopcocks contained 50 mM HEPES, pH 7.5, 2 mM dithiothreitol, and 0.2 mM EDTA in a volume of 15 mL. These solutions were sparged overnight with N_2. The tank N_2 used for sparging was first passed through an Ascarite column to remove CO_2 and then through an acidic water solution to hydrate the N_2 and prevent water loss from solutions being sparged.

L-Aspartate solutions (100 or 200 mM) were prepared in 50 mM HEPES, 2 mM dithiothreitol, and 0.2 mM EDTA, pH 7.5, sealed with septa, and sparged with N_2 for >7 h at room temperature before use. ATP and CTP solutions (70–100 mM) were prepared in 50 mM HEPES, 2 mM dithiothreitol, and 0.2 mM EDTA, pH 7.4 (CTP) or pH 7.6 (ATP), and sparged with N_2 for 1–2 h at room temperature. Concentrations of nucleotide solutions were determined by spectrophotometric assay on the basis of an extinction coefficient of 15.4 cm^{-1}·$mmol^{-1}$·L at 259 nm, pH 7.0, for ATP and 13 cm^{-1}·$mmol^{-1}$·L at 280 nm, pH 2.0, for CTP.

Low Conversion Samples. In experiments involving the allosteric effectors ATP and CTP, appropriate volumes of the sparged nucleotide solutions were added to the sparged reaction solutions via syringe to yield a final nucleotide concentration of 5 mM. Enough ATCase was added (final concentration 0.01–0.09 mg/mL), via syringe, so that the reaction would consume 50% of the added carbamyl phosphate within 2–20 min at room temperature, and then the appropriate volume of sparged aspartate solution was added via syringe to yield the desired starting aspartate concentration (0–200 mM). The reaction was initiated by addition of 1.028 mL of ice-cold carbamyl phosphate solution via syringe and calibrated needle to yield an initial concentration of 12 mM (saturating carbamyl phosphate). In order to maintain a constant concentration of aspartate over the course of the reaction, aspartate was added dropwise via syringe and 6-in. needle to the reaction mixture at a rate calculated to be equal to that at which aspartate was converted to products. Since the reaction rate changes with aspartate concentration and the presence of nuclotides, the procedure for the addition of aspartate was determined experimentally for each set of conditions on the basis of the observed reaction rate for those conditions. Since

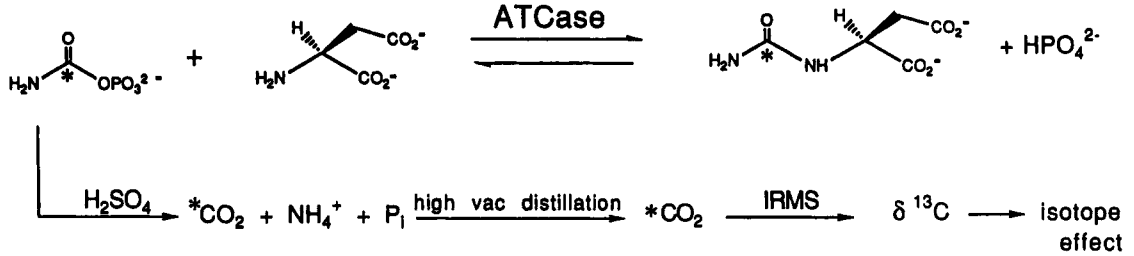


FIGURE 1: Schematic representation of the experimental protocol used for measuring ^{13}C isotope effects on the aspartate transcarbamylase reaction using the competitive method (O'Leary, 1980). Isotope ratios were determined by isotope ratio mass spectrometry (IRMS), and isotope effects were calculated using eq 1.

the experimental protocol involved the consumption of 100 μmol of carbamyl phosphate to achieve 50% reaction, 1 mL of 100 mM aspartate solution would be added dropwise to the reaction mixture over the course of the reaction so that the aspartate concentration remained as constant as possible. 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.

The reaction was quenched with 0.5 mL of concentrated H_2SO_4 to lower the pH below 1 after the appropriate time interval, and the reaction flask was placed in a 37 °C water bath for >1.9 h (10 half-lives of carbamyl phosphate hydrolysis; Allen & Jones, 1964) to allow for complete decomposition of the unreacted carbamyl phosphate. Because the ^{13}C isotope effect for the decomposition of carbamyl phosphate is large [Tipton & Cleland (1988) reported an isotope effect of 1.058 at pH 8.0, 25 °C; our attempts to reproduce this number resulted in a value of 1.045], care must be taken so that the enzymatic reaction of interest proceeds to 50% without a significant spontaneous decomposition of carbamyl phosphate. Control experiments were performed to show that no compounds in the reaction mixture other than carbamyl phosphate decomposed to yield CO_2 under these conditions. The resulting CO_2 was collected by a high-vacuum continuous distillation apparatus using two dry ice/2-propanol traps and one liquid N_2 trap. The CO_2 was further purified by three bulb to bulb distillations. The amount of CO_2 produced was determined by measuring with a calibrated, modified McLeod gauge manometer. Manometric measurements were made in triplicate and had an error less than 3%, which propagates to errors of less than 4–5% in the isotope effect minus one.

The CO_2 samples were then transferred to sample tubes with vacuum-backed stopcocks, and the isotopic content was measured in a Finnigan Delta E or MAT 251 isotope ratio mass spectrometer within 24 h of isolating the sample. Fraction of reaction was determined manometrically, and final aspartate concentrations were calculated on the basis of this value. A schematic representation of the ^{13}C isotope effect methods is shown in Figure 1.

Zero Percent Conversion Samples. A 0.528-mL aliquot of sparged carbamyl phosphate solution was added via syringe and calibrated needle to a reaction flask containing 15 mL of sparged buffer to yield a concentration of ~6 mM. Acidification of this solution by addition of 0.5 mL of concentrated H_2SO_4 (final pH <1) resulted in the decomposition of carbamyl phosphate and the generation of CO_2. The flask was incubated at 37 °C for >1.9 h before isolation of CO_2, as described above. An accurate measurement of the amount of CO_2 produced from a known volume of carbamyl phosphate solution allowed for the determination of the carbamyl phosphate concentration. Control experiments were performed to show that the presence of aspartate, ATP, CTP, or ATCase affected neither the amount of CO_2 produced nor the observed ^{13}C content of the gas. Controls were also done to show that, after sparging with N_2, carbamyl phosphate was the only compound from which any CO_2 was produced.

^{13}C Isotope Effects on the Holoenzyme Reaction, pH 8.3. These experiments were performed as described above except that the reactions were buffered with HEPES, which has a pK_a of 8.0 at 25 °C.

^{13}C Isotope Effects on the Catalytic Subunit Reaction, pH 7.5. ^{13}C isotope effects were measured as a function of aspartate concentration as described above for the holoenzyme. Final catalytic subunit concentration ranged from 0.01 to 0.05 mg/mL and reaction times from 2 to 12 min.

^{13}C Isotope Effects with L-Cysteine Sulfinate. Foote et al. (1985) report that the K_m for cysteine sulfinate at pH 7.0 with the catalytic subunit is 127 mM, whereas that for the holoenzyme is 580 mM. Because the K_m values for this substrates are so high, the cysteine sulfinate concentration was not kept constant over the course of the reaction in these experiments. The relative activity of ATCase with cysteine sulfinate is ~1.6% that with aspartate as substrate (Foote et al., 1985). Solutions of L-cysteine sulfinate were prepared in 50 mM HEPES, pH 7.5, that had been sparged to remove any dissolved CO_2. These substrate solutions were then sparged an additional 1–2 h at room temperature. Carbamyl phosphate solutions were prepared as previously described and sparged at 0 °C for 1–2 h.

Low conversion samples were prepared by adding enough ATCase holoenzyme (0.20–1.37 mg/mL final concentration) or catalytic subunit (0.08–0.21 mg/mL final concentration) to the sparged, buffered substrate solutions so that the reaction proceeded to ~50% completion, based on carbamyl phosphate, within 12–20 min at room temperature. The carbamyl phosphate concentration was saturating in all cases, and the cysteine sulfinate concentration ranged from 30 to 530 mM. Attempts to run the reaction at less than 30 mM cysteine sulfinate were unsuccessful due to the prohibitively large amount of enzyme required to convert 50% of the carbamyl phosphate within a short time. The reaction was initiated by addition of ice-cold carbamyl phosphate solution via syringe and calibrated needle to yield an initial concentration of 12–20 mM.

Zero percent conversion samples were prepared by adding a known volume of carbamyl phosphate to the sparged buffer solution and then acidifying the mixture and isolating the CO_2 as before. Control experiments were performed to show that cysteine sulfinate did not decompose under the reaction conditions to yield CO_2 and that, after sparging, carbamyl phosphate was the only compound from which any CO_2 was produced.

Data Analysis. The ^{13}C isotope effects on the ATCase-catalyzed reaction were measured using the method of internal competition in which changes in the isotopic composition of

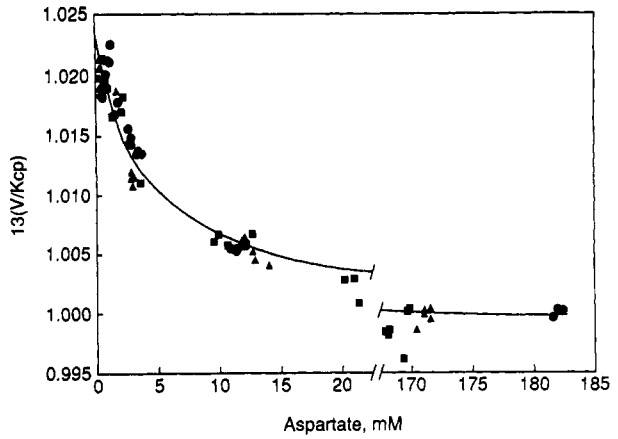


FIGURE 2: ^{13}C isotope effects for the aspartate transcarbamylase holoenzyme-catalyzed reaction at saturating carbamyl phosphate and varying L-aspartate concentrations in 50 mM HEPES, 2 mM dithiothreitol and 0.2 mM EDTA, pH 7.5, 25 °C, with (▲) 5 mM ATP, (■) 5 mM CTP, or (●) no effector. The data were fitted to eq 3. The maximum value of $^{13}(V/K_{CP})$, when aspartate concentration is extrapolated to 0, is 1.0217 ± 0.0005, the concentration of aspartate that half-eliminates the isotope effect is 4.81 ± 0.42 mM, and $^{13}(V/K_{CP})$ at infinite aspartate is unity.

either starting material or product are measured over the course of the reaction (O'Leary, 1980). Equation 1 was used to calculate the ^{13}C kinetic isotope effect. In eq 1, R_s is the

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

^{13}C/^{12}C ratio of a given position in the substrate after fraction of reaction f and R_0 is the initial isotopic composition of the same position in the substrate.

^{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):

$$y = A(1 + x/K_{in})/(1 + x/K_{id}) \quad (2)$$

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 is unity, the data can be fitted to the following equation (HYPRPL; Cleland, 1979):

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

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

RESULTS

^{13}C Isotope Effects on the Holoenzyme Reaction in the Presence of Allosteric Effectors, pH 7.5. The ^{13}C isotope effects for the ATCase holoenzyme-catalyzed reaction were measured by monitoring the ^{13}C content of carbamyl phosphate over the course of the reaction. The observed $^{13}(V/K_{CP})$ values were found to vary considerably with aspartate concentration, as shown in Figure 2. The relationship between these two parameters from reactions that contained ATP, CTP, or no effector resulted in coincident hyperbolas, and the combined data were first fitted to eq 2. Since the asymptote of 0.9989 ± 0.0004 was not significantly different from unity, the data were then fitted to eq 3, where the isotope effect was constrained to unity at an infinite level of aspartate. The limiting $^{13}(V/K_{CP})$ value at an aspartate concentration extrapolated to zero, A, was calculated to be 1.0217 ± 0.0005.

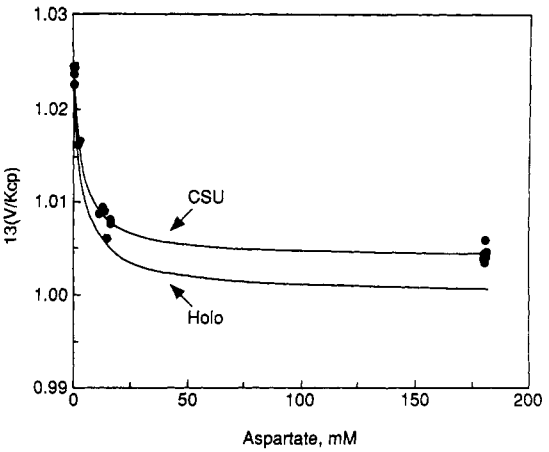


FIGURE 3: ^{13}C isotope effects for the aspartate transcarbamylase catalytic subunit (CSU) compared to those for the holoenzyme (holo) catalyzed reaction from Figure 2 at saturating carbamyl phosphate and varying L-aspartate concentrations in 50 mM HEPES, 2 mM dithiothreitol, and 0.2 mM EDTA, pH 7.5, 25 °C. Experimental points for the holoenzyme have been omitted for clarity. The data for the catalytic subunit were fitted to eq 2. The maximum value of $^{13}(V/K_{CP})$, when aspartate concentration is extrapolated to 0, is 1.0240 ± 0.0005, the concentration of aspartate that reduces the isotope effect by half is 3.82 ● 0.47 mM, and $^{13}(V/K_{CP})$ at infinite aspartate is 1.0039 ± 0.0004.

The amount of aspartate necessary to half-eliminate the isotope effect, K_{id}, is 4.81 ± 0.42 mM.[3] The K_{id} for inhibition of the isotope effect, obtained in this manner, is equal to the K_m for the varied substrate, aspartate, at an extrapolated level of zero carbamyl phosphate. The values for A and K_{id} were identical to those obtained when the data were fitted to eq 2.

^{13}C Isotope Effects on the Holoenzyme Reaction, pH 8.3. The pH optimum for the holoenzyme is reported to be 8.3 (Pastra-Landis et al., 1978), so this experiment was performed at pH 8.3 to see if the ^{13}C isotope effect was different at this pH. The $^{13}(V/K_{CP})$ values ranged from 1.0174 ± 0.0003 at 0.2–1.2 mM aspartate to 1.0053 ± 0.0003 at 11 mM aspartate. These results show that the observed ^{13}C isotope effects do not vary between pH 7.5 and 8.3.

^{13}C Isotope Effects on the Catalytic Subunit Reaction, pH 7.5. Results from ^{13}C isotope effect experiments on the catalytic subunit with saturating carbamyl phosphate and varying amounts of L-aspartate are shown in Figure 3. These data illustrate a hyperbolic relationship between ^{13}C isotope effect and aspartate concentration, as was seen for the ATCase holoenzyme. The $^{13}(V/K_{CP})$ values for the catalytic subunit are somewhat higher than with the holoenzyme, however. When the data were fitted to eq 2, the maximum value of $^{13}(V/K_{CP})$, A, was 1.0240 ± 0.0005 and the concentration of aspartate that half-eliminated the isotope effect, K_{id}, was 3.82 ± 0.47 mM. The isotope effect for the catalytic subunit-catalyzed reaction was not completely suppressed at infinite aspartate; rather, the asymptotic value was 1.0039 ± 0.004.

^{13}C Isotope Effects with L-Cysteine Sulfinate. ^{13}C kinetic isotope effect experiments with saturating carbamyl phosphate and varying amounts of the L-aspartate analog L-cysteine sulfinate were run with both the ATCase holoenzyme and catalytic subunit. $^{13}(V/K_{CP})$ appeared to vary with cysteine sulfinate concentration to only a small extent, and the relationship was not clearly hyperbolic, as shown in Figure 4. This

[3] When the data were analyzed separately, the limiting isotope effects at zero aspartate were 1.0229 ± 0.0008, 1.023 ± 0.001, and 1.0196 ± 0.0004 in the absence of effector or the presence of ATP or CTP, while the levels of aspartate half-suppressing the isotope effect were 4.8 ± 0.6, 4.6 ± 0.4, or 5.3 ± 0.9 mM, respectively.

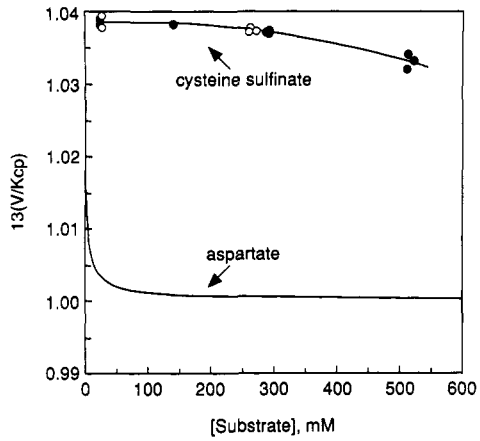


FIGURE 4: ^{13}C isotope effects for the aspartate transcarbamylase reaction catalyzed by holoenzyme (●) or catalytic subunit (○) at saturating carbamyl phosphate and varying L-cysteine sulfinate concentrations in 50 mM HEPES, 2 mM dithiothreitol, and 0.2 mM EDTA, pH 7.5, 25 °C. The calculated curve from Figure 2 for the holoenzyme reaction with L-aspartate is shown for comparison.

relationship was the same for both the holoenzyme and the catalytic subunit despite the significant differences in their K_m values for cysteine sulfinate. The maximum observed ^{13}C isotope effect for the holoenzyme was 1.0387 ± 0.0005 at ~23 mM cysteine sulfinate, and that for the catalytic subunit was 1.0388 ± 0.0008 at ~27 mM cysteine sulfinate. When the cysteine sulfinate concentration was raised to over 500 mM, the ^{13}C isotope effect on the holoenzyme reaction was 1.0331 ± 0.0006.

DISCUSSION

^{13}C Isotope Effects on the Holoenzyme Reaction in the Presence of Allosteric Effectors, pH 7.5. Two very striking observations can be made upon examination of the ^{13}C isotope effect data presented here for the ACTase holoenzyme reaction. First, it is very clear that the ^{13}C isotope effect varies as a function of aspartate concentration. Isotope effects have been used in this way as a very sensitive test of kinetic mechanism (Cook & Cleland, 1981; Duggleby & Northrop, 1989). In particular, a hyperbolic relationship between the isotope effect on substrate A and the concentration of substrate B in which the asymptote of the hyperbola is unity at infinite levels of B is indicative of an ordered kinetic mechanism.[4] The decrease in the observed $^{13}(V/K_{CP})$ value at high aspartate concentrations in the ATCase-catalyzed reaction suggests that there is a large external commitment for carbamyl phosphate, and the synergistic nature of aspartate binding prevents the dissociation of carbamyl phosphate from the enzyme–carbamyl phosphate–aspartate ternary complex. Thus the kinetic mechanism of the holoenzyme is ordered, with aspartate as the second substrate. This is in accord with the kinetic studies of Porter et al. (1969) and of Hsuanyu and Wedler (1987).

The second striking feature of these data is that the hyperbolic relationship between ^{13}C isotope effect and aspartate concentration is the same in the presence of ATP, CTP, or no allosteric effector. The results of these experiments argue strongly in favor of the two-state Monod model for ATCase. The fact that the ^{13}C isotope effect curves are identical with or without the allosteric modifiers indicates that there is no active enzyme form with kinetic properties different from those of the fully active R form and that the reaction in all cases is catalyzed by the R state enzyme. ATP and CTP must then act solely by shifting the equilibrium between T and R states of the enzyme by binding preferentially to one or the other. The kinetic properties of the active enzyme form, including the external commitment induced by a given aspartate level and the dissociation constant of aspartate, are not altered by the allosteric modifiers. This differs from the proposal of Hsuanyu and Wedler (1988) who have suggested that the allosteric effectors differentially perturb aspartate binding rather than the T → R transition. The possibility of multiple inactive enzyme forms cannot be ruled out by these experiments; however, the results presented here do strongly argue against the notion of a catalytically active T state or, in fact, of any catalytically active form other than that which we refer to as the R conformation.

^{13}C Isotope Effects on the Catalytic Subunit Reaction, pH 7.5. The ^{13}C isotope effect data obtained with the isolated catalytic subunit reveal a relationship with aspartate concentration similar to that seen with the holoenzyme. The fact that the isotope effect was not entirely abolished at high aspartate concentrations, however, suggests that the kinetic mechanism of the catalytic subunit is not as ordered as that of the holoenzyme. While the preferred kinetic pathway is still one in which carbamyl phosphate binds before aspartate, the binding of aspartate does not totally prevent the dissociation of carbamyl phosphate from the catalytic subunit as it does in the case of the holoenzyme.

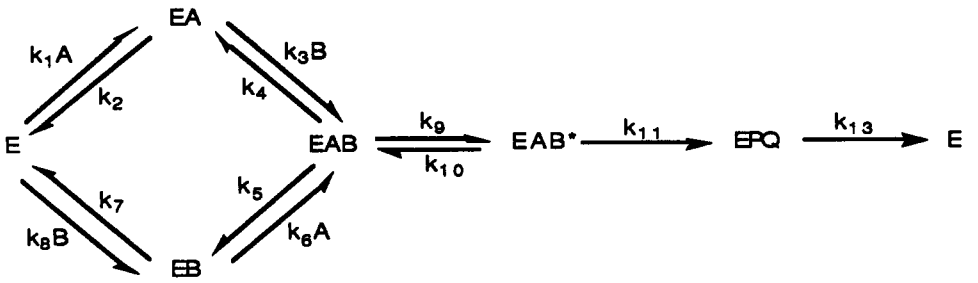
The catalytic subunit, when stripped of the regulatory subunits, is insensitive to ATP and CTP and exhibits hyperbolic substrate saturation curves (Yates & Pardee, 1956; Gerhart & Pardee, 1962). Kinetically it resembles the R form of the holoenzyme, but the T and R nomenclature can strictly be applied only to the different states of the holoenzyme. The isotope effect results presented in this study indicate that the catalytic subunit has similar, but not identical kinetic properties to the R form of the holoenzyme. The maximum value of the observed isotope effect increased from 1.0217 with the holoenzyme to 1.0240 with the catalytic subunit. Assuming the transition states (and therefore the intrinsic isotope effects) of the reaction catalyzed by both enzyme species are the same, which is expected since both species catalyze the same reaction at nearly the same rate, the difference in observed isotope effects must be due to a small change in the commitment for the reaction.

^{13}C Isotope Effects with L-Cysteine Sulfinate. It has been observed that neither the aspartate analog L-cysteine sulfinate (Foote et al., 1985) nor the substrates of the ATCase back-reaction (Foote & Lipscomb, 1981) promote the conformational change from low substrate affinity T state to the high substrate affinity R state of the enzyme. Hyperbolic rather than sigmoidal saturation curves are seen with these substrates, and the catalytic subunit has a much higher specific activity than the holoenzyme. The activity of the holoenzyme can be greatly enhanced by addition of substoichiometric amounts of the bisubstrate analog PALA, which binds at one active site and converts the remaining active sites to the R state (Collins & Stark, 1971).

Foote and co-workers (Foote et al., 1985; Foote & Lipscomb, 1981) explain these observations by proposing that the reaction under these conditions proceeds via the T quaternary structure of ATCase. These same observations, however, can

[4] An ordered kinetic mechanism is one in which the rate constant for dissociation of the first substrate to add is reduced by the presence of the second substrate to an undetectable level with respect to the turnover number. Such a mechanism becomes random when the turnover number is decreased sufficiently to be less than the rate constant for release of the first substrate from the ternary complex, or whenever the second substrate fails to keep the first substrate trapped on the enzyme.

Scheme I



also be explained by the presence of a small amount of catalytically active R state enzyme by which the reaction is catalyzed. Our isotope effect experiments with ATP and CTP support this latter theory.

We have begun to address the question of R vs T state catalysis by measuring the ^{13}C isotope effect on the cysteine sulfinate reaction with the holoenzyme (which, in the absence of the natural substrates carbamyl phosphate and L-aspartate, exists predominantly in the T state) and with the isolated catalytic subunit, which very closely resembles the R conformation of the holoenzyme. ^{13}C isotope effect experiments with both the catalytic subunit and the holoenzyme with cysteine sulfinate yielded identical results—in both sets of experiments a maximum value for $^{13}(V/K_{CP})$ of 1.039 was observed. Therefore, the fact that the holoenzyme, under conditions in which it exists predominantly in the T state, yielded the same isotope effect as the catalytic subunit-catalyzed reaction supports the contention that the catalysis of the ATCase reaction proceeds only through one active form of the enzyme, the R state.

The ^{13}C isotope effects with cysteine sulfinate do not show the variation with substrate concentration that was seen with the physiological substrate L-aspartate. This means that the one atom switch from carbon to sulfur at the β-carboxyl group of aspartate has changed the kinetic mechanism of both the holoenzyme and the catalytic subunit from ordered to fully random.[5] The binding of cysteine sulfinate is very much looser than that of aspartate, as evidenced by the dramatic increase in K_m (which equals the dissociation constant for a slow substrate such as this), and the aspartate analog does not bind synergistically once carbamyl phosphate has bound as is the case with the natural substrates.

While most kinetic analyses of ATCase are in support of an ordered mechanism, Heyde and co-workers (Heyde & Morrison, 1973; Heyde et al., 1973) have suggested that the kinetic mechanism of the catalytic subunit is an equilibrium random one. These earlier studies were performed with either carbamyl phosphate or acetyl phosphate and aspartate as substrates. We provide evidence here that whereas the kinetic mechanism of the holoenzyme is strictly ordered, that of the catalytic subunit is partially random. If the kinetic mechanism were truly equilibrium random, as proposed by Heyde and colleagues, we would not have seen the hyperbolic ^{13}C isotope effect on aspartate concentration. However, we have seen that the kinetic mechanisms of both the holoenzyme and the isolated catalytic subunit change from ordered or predominantly ordered to rapid equilibrium random in the presence of the aspartate analog L-cysteine sulfinate. If, like cysteine sulfinate, acetyl phosphate sufficiently slows down catalysis so that the rates at which the substrates dissociate from the enzyme become significant with respect to the forward rate, this change in substrate would also result in a change in kinetic mechanism. This may explain the apparently anomalous results obtained in the different laboratories.

The maximum ^{13}C isotope effect of 1.039 observed with both holoenzyme and catalytic subunit with L-cysteine sulfinate as substrate is a reasonable value for the intrinsic ^{13}C isotope effect for the aspartate transcarbamylase-catalyzed reaction, since ^{13}C isotope effects on chemical reactions involving nucleophilic attack at carbonyl carbons are generally large. O'Leary and Marlier (1979) have reported a ^{13}C isotope effect of 1.04 for alkaline hydrolysis and hydrazinolysis of methyl benzoate at 25 °C and values of 1.048–1.051 for the hydrolysis of aryl carbonates (Marlier & O'Leary, 1990).

If this value of 1.039 is adopted for the intrinsic isotope effect, the observed $^{13}(V/K_{CP})$ values extrapolated to zero aspartate of 1.0217 and 1.0240 for the holoenzyme and catalytic subunit correspond to commitments of 0.77 and 0.63, respectively, with the commitments increasing to infinity and 9.0 with infinite aspartate. These values are obtained by applying equation 4, where C_f is the forward commitment for

$$^{13}(V/K_{CP}) = \frac{^{13}k_{11} + C_f}{1 + C_f} \quad (4)$$

carbamyl phosphate and $^{13}k_{11}$ is the intrinsic isotope effect on the isotope-sensitive step. The reaction pathways for ATCase can be generalized as shown in Scheme I, where A is carbamyl phosphate, B is L-aspartate, and EAB* is the complex just prior to formation of the tetrahedral intermediate. In Scheme I, the commitment for carbamyl phosphate is

$$C_f = (k_{11}/k_{10})[1 + k_9/(k_5 + k_4k_2/(k_2 + k_3B))] \quad (5)$$

At infinite aspartate this reduces to

$$C_f = (k_{11}/k_{10})(1 + k_9/k_5) = 9.0 \quad (6)$$

where the value given is for the catalytic subunit. At near-zero concentration of aspartate, C_f is given by

$$C_f = (k_{11}/k_{10})[1 + k_9/(k_5 + k_4)] \quad (7)$$

If $k_4 \gg k_9$, as is certainly the case since aspartate is not a sticky substrate (Turnbull et al., 1992), then C_f for the catalytic subunit at zero aspartate is

$$C_f = k_{11}/k_{10} = 0.63 \quad (8)$$

The rate of dissociation of carbamyl phosphate from the initial ternary complex (k_5) relative to the net forward rate constant for product formation ($k_9k_{11}/(k_{10} + k_{11})$) can be calculated from eqs 6 and 8 above as 0.20 for the catalytic subunit. Porter et al. (1969) have estimated the dissociation of carbamyl phosphate from the binary complex of the catalytic subunit to have a rate constant of 2200 s^{-1} compared to the turnover number of 1500 s^{-1}, and this means that the rate of dissociation of carbamyl phosphate from the binary complex is 146% of the rate of product formation. Since carbamyl phosphate dissociates from the ternary complex at 20% the

[5] The initial velocity pattern for carbamyl phosphate and cysteine sulfinate with the catalytic subunit is intersecting (personal communication from Dr. Joanne Turnbull), and not equilibrium ordered as it would be for such a slow substrate if the mechanism remained ordered.

rate of the forward reaction, we conclude that the net effect of aspartate binding is to decrease the rate of dissociation of carbamyl phosphate from the ternary complex by a factor of 1.46/0.2, or 7.3, which is comparable to the value of 8-fold obtained by Turnbull et al. (1992) using isotope trapping techniques. This differs from the case of the holoenzyme reaction, in which aspartate binding completely prevents the dissociation of carbamyl phosphate from the ternary complex.

The application of the heavy atom isotope effect technique to the study of the complex regulatory protein aspartate transcarbamylase has proved very useful in defining the allosteric behavior of the holoenzyme as well as the kinetic mechanisms of both the holoenzyme and the isolated catalytic subunit. We have shown that the kinetic mechanism of the holoenzyme is an ordered one in which carbamyl phosphate binds at the enzyme active site followed by L-aspartate. The kinetic mechanism of the isolated catalytic subunit, on the other hand, has a random component. When the aspartate analog L-cysteine sulfinate is used as a substrate, the kinetic mechanisms of both the holoenzyme and catalytic subunit become random, and the full ^{13}C intrinsic isotope effect of 1.04 is seen. In the paper that follows (Parmentier et al., 1992), we demonstrate the utility of the heavy atom isotope effect technique in unraveling the details of the chemical mechanism of ATCase.

Registry No. ATCase, 9012-49-1; ^{13}C, 14762-74-4; ATP, 56-65-5; CTP, 65-47-4; carbamyl phosphate, 590-55-6; aspartic acid, 56-84-8; cysteine sulfinate, 1115-65-7.

References

Allen, C. M., & Jones, M. E. (1964) *Biochemistry 3*, 1238.

Allewell, N. (1989) *Annu. Rev. Biophys. Chem. 18*, 71.

Bethel, M. R., Smith, K. E., White, J. S., & Jones, M. E. (1968) *Proc. Natl. Acad. Sci. U.S.A. 60*, 1442.

Cleland, W. W. (1979) *Methods Enzymol. 63*, 103.

Cleland, W. W. (1982) *CRC Crit. Rev. Biochem. 13*, 385.

Cleland, W. W. (1987) *Bioorg. Chem. 15*, 283.

Cohen, R. E., Foote, J., & Schachman, H. K. (1985) *Curr. Top. Cell. Regul. 26*, 177.

Collins, K. D., & Stark, G. R. (1971) *J. Biol. Chem. 246*, 6599.

Cook, P. F., & Cleland, W. W. (1981) *Biochemistry 20*, 1790.

Duggleby, R. G., & Northrop, D. B. (1989) *Bioorg. Chem. 17*, 177.

Foote, J., & Lipscomb, W. N. (1981) *J. Biol. Chem. 256*, 11428.

Foote, J., Lauritzen, A., & Lipscomb, W. N. (1985) *J. Biol. Chem. 260*, 9624.

Gerhart, J. C., & Pardee, A. B. (1962) *J. Biol. Chem. 237*, 891.

Gerhart, J. C., & Holoubek, H. (1967) *J. Biol. Chem. 242*, 2886.

Hervê, G., Moody, M., Tauc, P., Vachette, P., & Jones, P. (1985) *J. Mol. Biol. 185*, 189.

Heyde, E., & Morrison, J. F. (1973) *Biochemistry 12*, 4727.

Heyde, E., Nagabhushnam, A., & Morrison, J. F. (1973) *Biochemistry 12*, 4718.

Howlett, G. J., Blackburn, M. N., Compton, J. G., & Schachman, H. K. (1977) *Biochemistry 16*, 5091.

Hsuanyu, Y., & Wedler, F. C. (1987) *Arch. Biochem. Biophys. 259*, 316.

Hsuanyu, Y., & Wedler, F. C. (1988) *J. Biol. Chem. 263*, 4172.

Jacobson, G. R., & Stark, G. R. (1973) in *The Enzymes* (Boyer, P. D., Ed.) 3rd ed., Vol. IX, Part B, p 225, Academic Press, New York.

Kantrowitz, E. R., & Lipscomb, W. N. (1990) *Trends Biochem. Sci. 15*, 53.

Marlier, J. F., & O'Leary, M. H. (1990) *J. Am. Chem. Soc. 112*, 5996.

Monod, J., Wyman, J., & Changeux, J.-P. (1965) *J. Mol. Biol. 12*, 88.

Northrop, D. B. (1977) in *Isotope Effects on Enzyme Catalyzed Reactions* (Cleland, W. W., O'Leary, M. H., & Northrop, D. B., Eds.) p 122, University Park Press, Baltimore.

O'Leary, M. H. (1980) *Methods Enzymol. 64*, 83.

O'Leary, M. H. (1989) *Annu. Rev. Biochem. 58*, 377.

O'Leary, M. H., & Marlier, J. F. (1979) *J. Am. Chem. Soc. 101*, 3300.

Parmentier, L. E., Weiss, P. M., O'Leary, M. H., Schachman, H. K., & Cleland, W. W. (1992) *Biochemistry* (third of six papers in this issue).

Pastra-Landis, S. C., Evans, D. R., & Lipscomb, W. N. (1978) *J. Biol. Chem. 253*, 4624.

Porter, R. W., Modebe, M. O., & Stark, G. R. (1969) *J. Biol. Chem. 244*, 1846.

Schachman, H. K. (1988) *J. Biol. Chem. 263*, 18583.

Stark, G. R. (1971) *J. Biol. Chem. 246*, 3064.

Tauc, P. Leconte, C., Kerbiriou, D., Thiry, L., & Hervê, G. (1982) *J. Mol. Biol. 155*, 155.

Tipton, P. A., & Cleland, W. W. (1988) *Arch. Biochem. Biophys. 260*, 273.

Turnbull, J., Waldrop, G., & Schachman, H. K. (1992) *Biochemistry* (first of six papers in this issue).

Wedler, F. C., & Gasser, F. J. (1974) *Arch. Biochem. Biophys. 163*, 69.

Wedler, F. C., Hsuanyu, Y., Kantrowitz, E. R., & Middleton, S. A. (1989) *J. Biol. Chem. 264*, 17266.

Yates, R. A., & Pardee, A. B. (1956) *J. Biol. Chem. 221*, 757.