^{13}C Isotope Effect Studies of *Escherichia coli* Aspartate Transcarbamylase in the Presence of the Bisubstrate Analog *N*-(Phosphonoacetyl)-L-aspartate†

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ABSTRACT: ^{13}C isotope effects have been measured for the aspartate transcarbamylase holoenzyme (ATCase) and catalytic subunit catalyzed reactions in the presence of the bisubstrate analog *N*-(phosphonoacetyl)-L-aspartate (PALA). For holoenzyme-catalyzed reactions in the physiological direction with very low levels of L-aspartate as substrate, or with L-cysteine sulfinate as substrate, or in the reverse direction with carbamyl-L-aspartate and phosphate as substrates, the isotope effect data show a slight dependence on PALA concentration. Under these conditions, PALA first stimulates the rate and then inhibits it at higher concentrations. The observed isotope effect at maximum stimulation by PALA is slightly smaller than in the absence of the analog, but as the PALA concentration is increased to reduce the rate to its original value, the observed isotope effect also increases and approaches the value of the isotope effect determined in the absence of PALA. These data suggest that the kinetic properties of the active enzyme are affected by the number of active sites occupied by PALA, indicating communication between subunits, and a mathematical model is proposed which explains our experimental observations. In contrast to these results with the holoenzyme, isotope effects measured for the reaction catalyzed by the isolated catalytic subunits are not altered in the presence of PALA. Taken together, these data are consistent with the two-state model for the homotropic regulation of ATCase.

Aspartate transcarbamylase (EC 2.1.3.2, ATCase)[1] is a complex allosteric enzyme that catalyzes the reaction between carbamyl phosphate and L-aspartate in the biosynthesis of pyrimidine nucleotides. The stable bisubstrate analog *N*-(phosphonoacetyl)-L-aspartate (PALA) (Collins & Stark, 1971) binds very tightly to the active site of ATCase in a manner similar to the binding of carbamyl phosphate and the competitive inhibitor succinate (Gouaux & Lipscomb, 1988) and effects the conformational change of ATCase from the inactive T state to the active R state. In addition to changes in quaternary structure (Ladner et al., 1982), this transformation allows the domains of the catalytic chains to move together, thus forming the active site pocket (Krause et al., 1987; Ke et al., 1988).

The two-state model of Monod et al. (1965) is the generally accepted one regarding the homotropic allosteric behavior of ATCase (Schachman, 1988). The enzyme exists in two states, an active R form and an inactive T form, with all six active sites changing at once during the transition between R and T forms. The T/R ratio in unliganded enzyme is large (50–250). Carbamyl phosphate shows selective binding to the R form, so that the T/R ratio is only 7 in the presence of saturating carbamyl phosphate (Howlett et al., 1977). Aspartate binds only the enzyme in the R form, while ATP induces an increase in the proportion of the R form and CTP induces an increase in the proportion of T. Consistent with this theory is the observation that the binding of one molecule of PALA, under certain conditions, can convert all the subunits of an ATCase molecule into the active R state (Foote & Schachman, 1985).[2] In addition, sedimentation studies have demonstrated that, in the presence of substoichiometric quantities of PALA, ATCase exists as a mixture of T and R state molecules rather than as a uniform population of molecules of intermediate conformation (Werner & Schachman, 1989). Others, however, dispute the idea that homotropic interactions are consistent with the two-state model (Hervé, 1988) and contend that, under conditions in which the T state is the predominant species, for instance, at low aspartate concentrations, in the presence of aspartate analogs L-alanosine

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[1] Abbreviations: ATCase, aspartate transcarbamylase holoenzyme; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*'-2-ethanesulfonic acid; HEPPS, *N*-(2-hydroxyethyl)piperazine-*N*'-3-propanesulfonic acid; PALA, *N*-(phosphonoacetyl)-L-aspartate; MES, 2-(*N*-morpholino)-ethanesulfonic acid.

[2] Foote and Schachman (1985) showed that addition of PALA under conditions of high enzyme concentration where essentially all added PALA became bound produced a stimulation in the rate of arsenolysis of *N*-carbamylaspartate of ~17 after addition of just over 3 PALA per enzyme hexamer, with the rate then decreasing to zero as all six active sites became filled with PALA. ATP stimulated both the rate in the absence of PALA and the maximum rate in the presence of PALA by factors of 1.7 and 1.4, while CTP decreased these rates by a factor of 1.7. These authors estimated the number of active sites apparently converted from T to R for each PALA originally bound to be 2 in the absence of nucleotides, but 4 in the presence of ATP and 1 in the presence of CTP. However, the data of Foote and Schachman (1985) are readily modeled by assuming that all six sites are either R (active) or T (inactive), with a T/R ratio of ~50 in the absence of nucleotides, and 75-fold tighter binding of PALA to R than to T. The effects of ATP and CTP are most easily explained by changes in the V/K values for P_i or *N*-carbamylaspartate, which were below saturation in these experiments.

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(Baillon et al., 1985) or L-cysteine sulfinate (Foote et al., 1985), and in the nonphysiological direction of reaction with carbamyl aspartate and phosphate as substrates (Foote & Lipscomb, 1981), ATCase in the T state contributes significantly to catalysis.

Local conformational changes are often proposed to explain cooperative ligand binding in oligomeric enzymes. Structural alterations at the active site of one polypeptide chain are thought to be communicated to the unliganded sites on other chains, converting these open sites to the more active conformation and thereby resulting in sigmoidal saturation curves. Using hybrid ATCase molecules containing trinitrophenyl chromophores at some of the active sites, Lahue and Schachman (1986) have demonstrated that communication occurs between regulatory and catalytic chains, between catalytic trimers, and between polypeptide chains within catalytic trimers. We provide here additional evidence for communication between polypeptide chains in the ATCase holoenzyme.

Isotope effects are very powerful in studying a complex, regulated system like ATCase because they provide a dynamic probe of only the active enzyme form. In the second article in this series we used ^{13}C isotope effect studies to examine the kinetic properties of ATCase under conditions in which the allosteric modifiers ATP and CTP were bound to the regulatory subunits and concluded that only the R form is active under these conditions of heterotropic control (Parmentier et al., 1992). We now report a series of ^{13}C isotope effects in the presence of PALA designed to study the transcarbamylation reaction under conditions in which the enzyme exists predominantly in the T state (in the absence of PALA) or predominantly in the R state (in the presence of PALA).

Materials and Methods

Carbamyl phosphate (dilithium salt), L-cysteine sulfinic acid, EDTA (disodium salt), HEPES [*N*-(2-hydroxyethyl)-piperazine-*N*'-2-ethanesulfonic acid], HEPPS [*N*-(2-hydroxyethyl)piperazine-*N*'-3-propanesulfonic acid], glutamic oxalacetic transaminase from porcine heart, malate dehydrogenase from porcine heart, NADH (disodium salt), α-ketoglutaric acid, and *N*-carbamyl-DL-aspartic acid were purchased from Sigma Chemical Co. L-Aspartic acid, 2,3-butanedione monoxime, and antipyrine (1,2-dihydro-1,5-dimethyl-2-phenyl-3-pyrazolone) were obtained from Aldrich, and dithiothreitol was obtained from Boehringer-Mannheim. Imidazole, from Aldrich, was recrystallized from benzene before use. DEAE-Sephadex A-25 anion-exchange resin was purchased from Pharmacia. All other chemicals used were reagent grade. Spectroscopic assays were performed using a Beckman DU spectrophotometer. ATCase holoenzyme and isolated catalytic subunits were stored and dialyzed for reactions in the forward direction as described by Parmentier et al. (1992). For reactions in the reverse direction ATCase was dialyzed at 4 °C against 50 mM HEPES, pH 7.0, 2 mM dithiothreitol, and 0.2 mM EDTA. *N*-(Phosphonoacetyl)-L-aspartate (PALA) was provided by Dr. H. K. Schachman.

Synthesis and Purification of N-Carbamyl-L-aspartate. *N*-carbamyl-L-aspartate was prepared by enzymatic synthesis (Porter et al., 1969) and isolated by DEAE anion-exchange chromatography using a 1-L 0–3 N acetic acid gradient to elute aspartate, followed by isocratic elution with 500 mL of 3 N acetic acid to elute carbamyl aspartate which was detected by colorimetric assay (Pastra-Landis et al., 1981). Fractions containing carbamylaspartate were pooled and reduced in volume by rotary evaporation. The oily residue was reconstituted in H_2O and lyophilized several times to remove all traces of acetic acid. An aliquot of the reconstituted solution was analyzed for aspartate, and none was found by either end-point assay with glutamate oxalacetate transaminase or ^1H NMR.

Isotope Effect Nomenclature. The nomenclature used throughout this work is that of Northrop (1977) and has been outlined previously (Parmentier et al., 1992).

Enzyme Assays. ATCase activity in the forward direction with carbamyl phosphate and L-aspartate as substrates was measured by determination of carbamylaspartate (Pastra-Landis et al., 1981) at 1 mM L-aspartate, 5 mM carbamyl phosphate, and 1 μg/mL ATCase or catalytic subunits at 23 °C. For the holoenzyme reaction, stimulation by PALA was greater at pH 8.4 than at pH 7.0 or 7.5; therefore, all reactions in the forward direction were run in 50 mM HEPPS buffer, pH 8.4. The activity of the holoenzyme in the presence of carbamyl phosphate and the aspartate analog L-cysteine sulfinate was measured by the method of Davies et al. (1970) at 10 mM carbamyl phosphate, 300 mM L-cysteine sulfinate, and 0.15 mg/mL ATCase in a tri-part buffer containing 51 mM *N*-ethylmorpholine, 100 mM MES, 51 mM diethanolamine, 0.2 mM EDTA, and 2 mM β-mercaptoethanol at 23 °C. No stimulation due to PALA was observed at pH 7.6 under these conditions, but a 6-fold increase in rate was seen at pH 8.4. ATCase activity in the reverse direction was measured by the method of Foote and Lipscomb (1981).

In all cases with the ATCase holoenzyme, isotope effects were determined in the absence of PALA, at the concentration of PALA giving maximum stimulation of rate, and at the concentration of PALA giving a rate equal to that in the absence of PALA. In the catalytic subunit reaction where PALA is inhibitory at all concentrations, isotope effects were determined in the absence of PALA and at the concentrations of PALA that gave 2/3 and 1/3 the original rate.

^{13}C Isotope Effects in the Forward Direction in the Presence of Varying Amounts of PALA. ^{13}C isotope effects on the ATCase-catalyzed reaction were determined by analyzing residual carbamyl phosphate as described by Parmentier et al. (1992). For low conversion samples, reaction vessels contained 50 mM HEPPS, pH 8.4, 2 mM dithiothreitol, 0.2 mM EDTA, and an initial concentration of 5 mM carbamyl phosphate in a volume of 20 mL. One milliliter of 50 mM L-aspartate in 50 mM HEPPS, 2 mM dithiothreitol, and 0.2 mM EDTA, pH 8.4, was added to the reaction mixture dropwise as described by Parmentier et al. (1992). Holoenzyme reactions contained 0.03–0.21 mg/mL ATCase and 0, 3, 60, or 100 μM PALA, and fraction of reaction ranged from 0.28 to 0.51. For reactions with the isolated catalytic subunit, enzyme concentrations were 0.04 mg/mL at 0 μM PALA, 0.06 mg/mL at 3 μM PALA, and 0.07 mg/mL at 20 μM PALA, and fraction of reaction ranged from 0.45 to 0.52.

^{13}C Isotope Effects with L-Cysteine Sulfinate and PALA. ^{13}C isotope effects on the ATCase holoenzyme-catalyzed reaction were determined as described by Parmentier et al. (1992) in 50 mM HEPPS, 2 mM dithiothreitol, and 0.2 mM EDTA, pH 8.4, with initial concentrations of carbamyl phosphate and L-cysteine sulfinate of 17 and 300 mM, respectively, in a final volume of 6 mL. The concentration of ATCase was 1.1 mg/mL for reactions at 0 and 400 μM PALA and 0.22–0.35 mg/mL for reactions at 40 μM PALA. Fraction of reaction ranged from 0.30 to 0.68.

^{13}C Isotope Effects in the Reverse Reaction. As originally described by Foote and Lipscomb (1981), the reverse reaction was studied under conditions of rapid aspartate removal in which the aspartate produced was coupled off with glutamate

oxalacetate transaminase and malate dehydrogenase. ^{13}C isotope effects were determined by analyzing the product carbamyl phosphate formed after some known fraction of reaction (<10%) and comparing this mass ratio to that of the initial, unreacted *N*-carbamyl-L-aspartate.

HEPES buffer solutions were sparged with N_2 for 1–2 h prior to pH adjustment (unadjusted pH ~5.5) and then adjusted to the proper pH with saturated NaOH and brought to the correct volume. A 150 mM *N*-carbamyl-L-aspartate solution, pH 7.0, and a 150 mM solution of NADH, pH 7.7, were prepared in 30 mM sparged, CO_2-free HEPES buffer and sparged for 2 h, protected from light, at 0 °C. Reaction vessels (100 mL) fitted with vacuum adaptors and equipped with side arms contained 10 mM KH_2PO_4, 10 mM α-ketoglutarate, and 30 mM HEPES, pH 7.0, and were sparged 2 h at room temperature.

ATCase was dialyzed as described versus sparged, CO_2-free HEPES buffer, pH 7.0. Malate dehydrogenase was diluted into this CO_2-free buffer to yield a stock solution of 548 units/mL. The glutamate oxalacetate transaminase solution was purchased as 1667 units/mL in 3 M $(NH_4)_2SO_4$, 0.05 M maleate, and 2.5 mM α-ketoglutarate, pH 6. For use in isotope effect studies, this solution was centrifuged and the pellet resuspended in sparged 30 mM HEPES buffer, pH 7.0, to remove excess ammonium sulfate, which was found to inhibit the ATCase reaction. None of the enzyme solutions were sparged prior to use. In controls, no CO_2 resulted from either substrate, enzyme, or PALA solutions.

Low conversion samples contained 10 mM *N*-carbamyl-L-aspartate, 10 mM KH_2PO_4, 10 mM α-ketoglutarate, 5 mM NADH, 66 units/mL glutamate oxalacetate transaminase, 5.5 units/mL malate dehydrogenase, and 0.1 mg/mL ATCase at 0 and 2.5 μM PALA and 0.07 mg/mL ATCase at 0.22 μM PALA in a total volume of 30 mL in 30 mM HEPES, pH 7.0. The reaction was initiated by addition of ATCase and quenched after the appropriate time (6.6 h for 0 μM PALA, 3.8 h for 0.22 μM PALA, and 6.0 h for 2.5 μM PALA) by addition of 0.5 mL of concentrated H_2SO_4. Resulting CO_2 was isolated and analyzed as described by Parmentier et al. (1992). Fraction of reaction ranged from 0.05 to 0.10.

Total conversion samples were prepared by chemical hydrolysis of *N*-carbamyl-L-aspartate (Stark & Smyth, 1963). *N*-Carbamyl-L-aspartate (100 μmol) was dissolved in CO_2-free 0.2 N NaOH. The samples were made up in 50-mL round-bottom flasks fitted with reflux condensers, the tops of which were sealed with rubber septa and vented to an N_2 bubbler to maintain a CO_2-free environment. The samples were then refluxed in an oil bath at 110 °C for 24 h to hydrolyze the *N*-carbamyl-L-aspartate to carbamate and aspartate.

After the hydrolysis step had gone to completion, as monitored by glutamate oxalacetate transaminase end-point assay for aspartate, the mixture after cooling was acidified with 0.5 mL of concentrated H_2SO_4 to decompose carbamate to CO_2 and NH_4^+, and the condensers were replaced with vacuum adaptors. The samples were distilled on a high-vacuum line, and the resulting CO_2 was isolated and analyzed. The amounts of CO_2 and aspartate produced in this reaction were in good agreement with the amount of *N*-carbamyl-L-aspartate used initially.

Data Analysis. Equation 1 was used to calculate the kinetic isotope effect when the isotopic composition of residual starting material was analyzed. R_s is the isotope ratio ($^{13}C/^{12}C$) of

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

a given position in the substrate after fraction of reaction f, and R_0 is the initial isotope ratio of the same position in the substrate. When analyzing products to determine the isotope effect, eq 2 was used. In this case, R_p is the isotope ratio in

$$^{13}(V/K) = \frac{\log (1 - f)}{\log [1 - (fR_p/R_0)]} \quad (2)$$

the product after some fraction of reaction f and R_0 is the isotope ratio of the same position in the product after complete reaction, or in the initial substrate.

Results

The effect of the bisubstrate analog PALA on the ATCase reaction rate depends on pH and the buffer used. At pH 7.0 PALA acted only as an inhibitor of the forward reaction when low levels of aspartate (1 mM) were used with the holoenzyme and the buffer was HEPES. The Hill number when aspartate was varied was only 1.4. With imidazole buffer at pH 7.0, however, the Hill number was 2.3, and 1 μM PALA stimulated the rate by 2-fold. The rate in the absence of PALA at 1 mM aspartate was 50% higher with HEPES than with imidazole, however. At higher pH, the rate in the presence of HEPES at pH 7.5 or HEPPS at pH 8.4 decreased relative to that at pH 7, but the stimulation by PALA increased to 2-fold or 5-fold, respectively, with the maximum stimulated rates being about equal. Thus at pH 7 the holoenzyme presumably exists more in the R form than at high pH, and the nature of the buffer affects the R–T equilibrium.

A similar situation was seen with cysteine sulfinate and holoenzyme (Figure 1). At pH 7.6 PALA was only an inhibitor, but at pH 8.4 it stimulated at least 5-fold. In the reverse reaction at pH 7, PALA stimulated the reaction with the holoenzyme 3-fold. The holoenzyme in this case is presumably largely in the T state in the absence of carbamyl phosphate, which shows preferential binding to the R form.

In contrast to the data above, the reaction with the catalytic subunit was only inhibited by PALA even at pH 8.4.

Table I summarizes ^{13}C isotope effects measured under different conditions by following the change in the natural abundance of ^{13}C in carbamyl phosphate during the reaction.

Discussion

A wealth of information is available on the structural changes that the ATCase holoenzyme undergoes upon ligand and nucleotide binding (Gerhart & Schachman, 1968; Howlett & Schachman, 1977; Howlett et al., 1977; Moody et al., 1979; Ke et al., 1984; Krause et al., 1987; Gouaux & Lipscomb, 1988), but very little is known about the effects of the allosteric processes on catalysis. We have previously demonstrated that the active form of ATCase that is responsible for catalysis shows the same isotope effects in the absence of allosteric effectors or in the presence of ATP or CTP (Parmentier et al., 1992). The present study addresses the question of catalysis in the presence of the bisubstrate analog PALA, which binds at the enzyme active site and promotes the T → R transition (Howlett & Schachman, 1977).

All the isotope effect data obtained with the ATCase holoenzyme and PALA follow a common pattern: the observed isotope effect at maximum stimulation by PALA is slightly smaller than that in the absence of PALA. However, as PALA concentration is increased further to decrease the reaction rate to a value equal to that in the absence of the analog, the observed isotope effect also increases, approaching the value of the isotope effect measured in the absence of PALA. For the holoenzyme reactions in the forward direction with L-as-

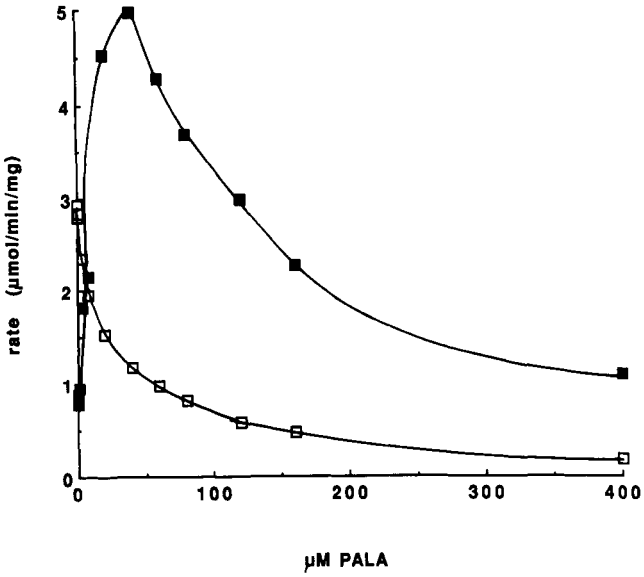

FIGURE 1: ATCase-catalyzed reaction at pH 7.6 (□) and pH 8.4 (■) with 300 mM L-cysteine sulfinate, 10 mM carbamyl phosphate, and 0.15 mg/mL ATCase, 23 °C, in 51 mM *N*-ethylmorpholine, 100 mM MES, 51 mM diethanolamine, 0.2 mM EDTA, and 2 mM β-mercaptoethanol. Activity was determined by the method of Davies et al. (1970).

Table I: ^{13}C Isotope Effects on the ATCase-Catalyzed Reaction in the Presence of PALA

reaction studied	[PALA] (μM)	relative rate	$^{13}(V/K_{CP})$	no. of values
a	0	(1.00)	1.0199 ± 0.0004	6
	3	3.64	1.0183 ± 0.0007	6
	60	1.19	1.0203 ± 0.0004	6
	100	0.63	1.0209 ± 0.0007	3
b	0	(1.00)	1.0216 ± 0.0002	3
	3	0.67	1.0224 ± 0.0003	3
	20	0.33	1.0223 ± 0.0003	3
c	0	(1.00)	1.0396 ± 0.0004	8
	40	3.19	1.0365 ± 0.0005	10
	400	0.90	1.0373 ± 0.0005	3
d	0	(1.00)	1.0214 ± 0.0005	7
	0.22	3.04	1.0173 ± 0.0010	7
	2.5	1.00	1.0214 ± 0.0005	7

[a] ATCase (holoenzyme) catalyzed reaction in the forward direction with limiting L-aspartate and 5 mM carbamyl phosphate, pH 8.4. [b] Catalytic subunit-catalyzed reaction in the forward direction with limiting L-aspartate and 5 mM carbamyl phosphate, pH 8.4. [c] ATCase-catalyzed reaction in the forward direction with 300 mM L-cysteine sulfinate and 17 mM carbamyl phosphate, pH 8.4. [d] ATCase-catalyzed reaction in the reverse direction with 10 mM *N*-carbamyl-L-aspartate and 10 mM KH_2PO_4, pH 7.0.

partate and in the reverse direction with phosphate and carbamyl aspartate as substrates this pattern is very pronounced. In the forward reaction with L-cysteine sulfinate as substrate, the same trend is evident, but the isotope effect of 1.0373 at 400 μM PALA has not completely returned to the original value of 1.0396, presumably due to the high carbamyl phosphate concentration used in this experiment.

The fact that the observed isotope effect in the absence of PALA, under conditions in which the enzyme exists predominantly in the T state (at very low aspartate concentrations, in the presence of cysteine sulfinate, or in the presence of carbamylaspartate and phosphate), is different from that under conditions in which the enzyme exists predominantly in the R state (at maximum stimulation by PALA) may appear to suggest that both R and T states of the enzyme have activity and that the kinetic properties of these two states are different. However, the fact that the observed isotope effect at higher PALA concentrations is the same as that in the absence of PALA refutes this theory and rather suggests that the kinetic behavior of the active enzyme is affected by the number of active sites occupied by PALA, with small differences in the kinetic properties made manifest in the observed isotope effect. There thus must be some communication between subunits (Lahue & Schachman, 1986). The binding of PALA to the isolated catalytic subunit does not result in a change in observed isotope effect, presumably because the isolated catalytic subunit exists in a form that kinetically resembles the R form of the holoenzyme (Parmentier et al., 1992), and the kinetic properties of the catalytic chains are not affected by analog binding. Thus, the data presented here are consistent with the two-state Monod model for the homotropic regulation of ATCase in which catalysis occurs via the R state of the enzyme, and the T state is catalytically inactive.

The observation that the ^{13}C isotope effect changes with PALA binding can be explained mathematically by the following analysis. Consider a model in which the constant governing the equilibrium between unliganded T state and unliganded R state enzyme is K_0. The total distribution of states with PALA bound (ranging from 0 to 6 PALA/6 active sites) is given by the expression:

$$\Delta = 1 + K_0 + 6P/K_p + 30P^2/K_p^2 + 48P^3/K_p^3 + 108P^4/K_p^4 + 72P^5/K_p^5 + 72P^6/K_p^6 \quad (3)$$

where P is PALA concentration, the exponents correspond to the number of PALA molecules bound, and K_p is the dissociation constant for PALA from an active site. This model assumes that PALA binds equally to all open R sites on a catalytic trimer.[3] The activity of the enzyme, if all open sites react with substrates at equal rates, is given by

$$\text{activity} = (6 + 30P/K_p + 120P^2/K_p^2 + 144P^3/K_p^3 + 216P^4/K_p^4 + 72P^5/K_p^5)/\Delta \quad (4)$$

The proportion of catalytic trimers within the holoenzyme with PALA bound in 0, 1, or 2 sites is given by

$$p0\ (0\ \text{sites}) = (1 + 3P/K_p + 6P^2/K_p^2 + 6P^3/K_p^3)/\Delta \quad (5)$$

$$p1\ (1\ \text{site}) = (3P/K_p + 18P^2/K_p^2 + 18P^3/K_p^3 + 18P^4/K_p^4)/\Delta \quad (6)$$

$$p2\ (2\ \text{sites}) = (6P^2/K_p^2 + 18P^3/K_p^3 + 72P^4/K_p^4 + 36P^5/K_p^5)/\Delta \quad (7)$$

and the fraction of the rate contributed by ATCase catalytic trimers with 0, 1, or 2 molecules of PALA bound is given by

$$\text{fp0} = 3p0/(3p0 + 2p1 + p2) \quad (8)$$

$$\text{fp1} = 2p1/(3p0 + 2p1 + p2) \quad (9)$$

$$\text{fp2} = p2/(3p0 + 2p1 + p2) \quad (10)$$

A plot of the rate and the fraction of enzyme containing 0, 1, or 2 PALA molecules per catalytic trimer versus PALA concentration is shown in Figure 2. In the absence of PALA, the only contributing enzyme form is p0, and at a concentration of PALA that restores the rate to that seen in the absence of PALA, the principal contributor to the rate is p2. Maximum rate, however, is achieved when the contribution from trimers containing one bound PALA (p1) is 44%. This

[3] The binding curve for PALA (Newell et al., 1989) appears cooperative because PALA binds preferentially to the R form of the holoenzyme and displaces the T–R equilibrium.

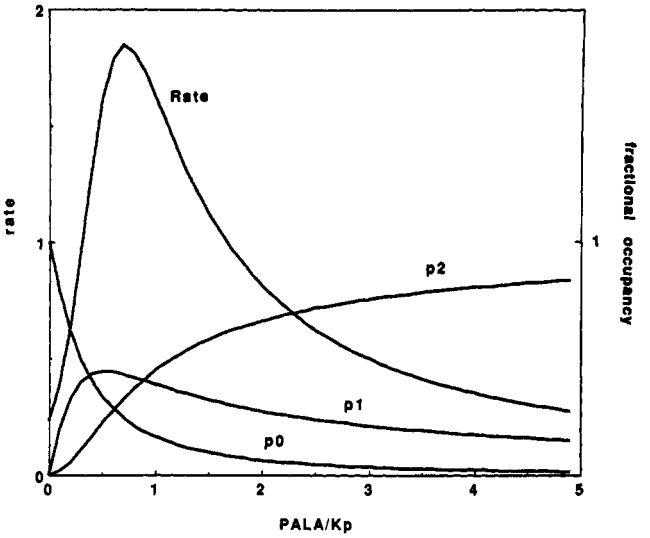

FIGURE 2: ATCase rate and fraction of enzyme containing 0, 1, or 2 PALA molecules per catalytic trimer (p0, p1, p2) versus PALA concentration based on calculations in text. The fraction of p3 (3 PALA molecules per trimer) is not shown, since this form has no activity.

model predicts that if enzyme forms p0, p1 and p2 have different kinetic properties, the observed isotope effect would reflect the change in contribution to the overall rate by the different enzyme forms.

Our data suggest that while p0 and p2 have similar kinetic properties, p1 has slightly higher commitments and thus gives a lower observed isotope effect.[4] The above analysis is in accord not only with the data presented here, but also with the observations of Lahue and Schachman (1986), who have demonstrated that PALA binding to native catalytic subunits affects the spectral properties of adjacent, derivatized catalytic subunits. However, Lahue and Schachman also report that the binding of PALA at one site of an isolated catalytic trimer results in a conformational change that is communicated to the active sites of the neighboring unliganded catalytic chains within the same trimer. The ^{13}C isotope effect data obtained in the present study show that the kinetic properties of the isolated catalytic subunit do not change with the binding of PALA at the active site. These seemingly anomalous findings can be reconciled if it is assumed that the binding of PALA to the catalytic trimer results in a structural change of the unliganded sites (thus altering the spectral response of the derivatized catalytic chains), but does not alter the kinetic properties of the trimer (the isotope effect remains the same). This proposal is not unreasonable given the fact that the kinetic parameters K_m and V_{max} for the native trimer and the trimer with one derivatized catalytic chain do not differ greatly (Lahue & Schachman, 1986).

The power of isotope effect studies as a means of understanding a highly complex enzyme such as ATCase lies in the fact that the observed isotope effects reflect only catalysis by active enzyme. Small changes in the observed isotope effects for the holoenzyme catalyzed reaction are seen in the presence of the bisubstrate analog PALA. Interestingly, since these changes are only seen at maximum stimulation by PALA and not at higher analog concentrations, these results not only provide additional evidence in support of the two-state model of allosteric behavior (Monod et al., 1965), but further suggest that communication occurs between subunits of ATCase as a result of substrate or analog binding.

[4] If 1.04 is taken as the intrinsic isotope effect in the forward reaction, the commitment for p1 is ~1.5 compared to a value of ~1.0 for p0 and p3 when low aspartate is used, while with cysteine sulfinate p2 has a commitment of ~0.2 compared to little or no commitment for p0 or p2. In the reverse reaction where the intrinsic isotope effect should be only slightly higher than 1.04, the commitment for p1 is ~2.3, while that for p0 and p3 would be ~0.9. The data in Table I are also consistent with changes in the intrinsic isotope effects for p1 vs p0 and p2, rather than changes in commitments.

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REFERENCES

Baillon, J., Tauc, P., & Hervé, G. (1985) *Biochemistry 24*, 7182.
Collins, K. D., & Stark, G. R. (1971) *J. Biol. Chem. 246*, 6599.
Davies, G. E., Vanaman, T. C., & Stark, G. R. (1970) *J. Biol. Chem. 245*, 1175.
Foote, J., & Lipscomb, W. N. (1981) *J. Biol. Chem. 256*, 11428.
Foote, J., & Schachman, H. K. (1985) *J. Mol. Biol. 186*, 175.
Foote, J., Lauritzen, A., & Lipscomb, W. N. (1985) *J. Biol. Chem. 260*, 9624.
Gerhart, J. C., & Schachman, H. K. (1965) *Biochemistry 4*, 1054.
Gerhart, J. C., & Schachman, H. K. (1968) *Biochemistry 7*, 538.
Gouaux, J. E., & Lipscomb, W. N. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 4205.
Hervé, G. (1988) in *Enzyme Dynamics and Regulation* (Chock, P. B., Huang, C. Y., Tsou, C. L., & Wang, J. H., Eds.) p 155, Springer, Berlin.
Howlett, G. J., & Schachman, H. K. (1977) *Biochemistry 16*, 5077.
Howlett, G. J., Blackburn, M. N., Compton, J. G., & Schachman, H. K. (1977) *Biochemistry 16*, 5091.
Ke, H., Honzatko, R. B., & Lipscomb, W. N. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 4037.
Ke, H., Lipscomb, W. N., Cho, Y., & Honzatko, R. B. (1988) *J. Mol. Biol. 204*, 725.
Krause, K. L., Volz, K. W., & Lipscomb, W. N. (1987) *J. Mol. Biol. 193*, 527.
Ladner, J. E., Kitchell, J. P., Honzatko, R. B., Ke, H.-M., Volz, K. W., Kalb, A. J., Ladner, R. C., & Lipscomb, W. N. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 3125.
Lahue, R. S., & Schachman, H. K. (1986) *J. Biol. Chem. 261*, 3079.
Monod, J., Wyman, J., & Changeux, J.-P. (1965) *J. Mol. Biol. 12*, 88.
Moody, M. F., Vachette, P., & Foote, A. M. (1979) *J. Mol. Biol. 133*, 517.
Newell, J. O., Markby, D. W., & Schachman, H. K. (1989) *J. Biol. Chem. 264*, 2476.
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 Press, Baltimore.
O'Leary, M. H. (1980) *Methods Enzymol. 64*, 83.
Parmentier, L. E., O'Leary, M. H., Schachman, H. K., & Cleland, W. W. (1992) *Biochemistry* (second of six papers in this issue).
Pastra-Landis, S. C., Evans, D. R., & Lipscomb, W. N. (1978) *J. Biol. Chem. 253*, 4624.
Pastra-Landis, S. C., Foote, J., & Kantrowitz, E. R. (1981) *Anal. Biochem. 118*, 358.
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., & Smyth, D. G. (1963) *J. Biol. Chem. 238*, 214.