The Contribution of Threonine 55 to Catalysis in Aspartate Transcarbamoylase†

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ABSTRACT: Heavy-atom isotope effects and steady-state kinetic parameters were measured for the catalytic trimer of an active site mutant of aspartate transcarbamoylase, T55A, to assess the role of Thr 55 in catalysis. The binding of carbamoyl phosphate to the T55A mutant was decreased by 2 orders of magnitude relative to the wild-type enzyme whereas the affinities for aspartate and succinate were not markedly altered. This indicates that Thr 55 plays a significant role in the binding of CbmP. If, as had been suggested previously, Thr 55 assists in the polarization of the carbonyl group of CbmP, the carbon isotope effect for the T55A mutant should increase relative to that observed for the wild-type enzyme. However, the opposite is seen, indicating that Thr 55 is not involved in stabilizing the oxyanion in the transition state. Quantitative analysis of a series of ^{13}C and ^{15}N isotope effects suggested that the rate-determining step in the reaction catalyzed by T55A trimer may be a conformational change in the protein subsequent to formation of the Michaelis complex. Thus, Thr 55 may facilitate a conformational change in the enzyme that is a prerequisite for catalysis. An altered active site environment in the binary and Michaelis complexes with T55A trimer is reflected in the pH profiles for log V, log $(V/K)_{asp}$, and $pK_{i\ succinate}$, which show a displacement in the pK values of ionizing residues involved in aspartate binding and catalysis relative to the wild-type enzyme. Inhibition of the activity of T55A trimer by the bisubstrate analogue *N*-(phosphonoacetyl)-L-aspartate was competitive with respect to aspartate, and the initial velocity pattern was of the intersecting type. These results indicate that the threonine to alanine mutation alters the kinetic mechanism from steady-state ordered to rapid-equilibrium random.

On the basis of the crystal structure of aspartate transcarbamoylase (ATCase)[1] ligated to the bisubstrate analogue *N*-(phosphonoacetyl)-L-aspartate (PALA), it has been inferred that the active site residue, Thr 55, interacts with both the carbonyl oxygen and phosphate moieties of carbamoyl phosphate (CbmP) (Krause et al., 1987). This hypothesis led Lipscomb and co-workers to speculate that Thr 55 may participate in catalysis by assisting in polarizing the carbonyl group of CbmP (Gouaux et al., 1987). Xu and Kantrowitz (1989) concurred with this proposal after noting that the replacement of Thr 55 by alanine resulted in a reduction of the maximal activity by a factor of 5 relative to the wild-type enzyme.

In the previous paper (Waldrop et al., 1992a), it was shown that the application of heavy-atom isotope effects could provide valuable information in determining how an active site mutation affects the individual steps of an enzyme-catalyzed reaction. In a mutant trimer of ATCase in which His 134 was replaced by alanine, the value for the $^{13}(V/K_{CbmP})$ was 1.04, which is probably the intrinsic isotope effect for the reaction between CbmP and aspartate. In addition, quantitative analyses of ^{13}C and ^{15}N isotope effects showed that formation of the tetrahedral intermediate is the rate-limiting step in the reaction catalyzed by the H134A mutant trimer which is only about 5% the activity of the wild-type enzyme. If Thr 55 plays a role in polarizing the carbonyl group, then the $^{13}(V/K_{CbmP})$ of T55A trimer should increase relative to the value for the wild-type enzyme. To test this prediction and examine further the role of Thr 55 in the catalytic mechanism, we measured ^{13}C and ^{15}N isotope effects for the mutant catalytic trimer of ATCase containing alanine in place of Thr 55. In addition, pH–rate profiles and initial velocity patterns were constructed and the pattern of inhibition by PALA was determined to assess if alterations occur in the kinetic mechanism of the reaction and in the ionization of residues involved in substrate binding and catalysis as a result of the amino acid substitution.

Experimental Procedures

Materials

Substrates, substrate analogues, and buffer components were obtained and prepared in solution as described previously (Turnbull et al., 1992).

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[1] Abbreviations: ATCase, aspartate transcarbamoylase; CbmP, carbamoyl phosphate; PALA, *N*-(phosphonoacetyl)-L-aspartate; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*'-2-ethanesulfonic acid; T55A, mutant *Escherichia coli* ATCase catalytic trimer with Thr 55 replaced by Ala.

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Thr 55 was replaced by Ala using the site-directed mutagenesis procedure of Kunkel (1985). The oligonucleotide (5′-AGAGAGGCGGGC ACGGGTAGA-3′) was used to prime second-strand synthesis using the Klenow fragment of DNA polymerase with the template M13mp8pyrB2 (Robey et al., 1986). The resulting dsDNA was transformed into JM103, and plaques coding for potential mutants were screened by DNA sequencing. Replicative from DNA encoding the alanine replacement was cleaved with the restriction enzymes *Eco*RI and *Bss*HII. The 835-bp fragment was ligated to the 5.4-kb fragment of the expression vector pPYRB11 (Robey & Schachman, 1984) cut with the same two restriction enzymes, and the resulting plasmid, pGW1, was transformed into HB101. The subcloned DNA was subjected to sequence determination using the method of Toneguzzo et al. (1988) to confirm the presence of this single mutation. Alanine does not disrupt α-helical secondary structure (Marqusee et al., 1989) and hence is an appropriate replacement for Thr 55 which is located in helix 2 of the catalytic trimer of ATCase (Krause et al., 1987).

The T55A mutant enzyme was overproduced by growing *Escherichia coli* strain EK1104, transformed with pGW1, under the conditions reported by Nowlan and Kantrowitz (1989). The holoenzyme form of the mutant enzyme was purified according to the procedure of Wall et al. (1981), and the catalytic trimer was prepared according to Yang et al. (1978). The concentration of enzyme was determined spectrophotometrically using the extinction coefficient determined for the wild-type enzyme of $E^{0.1\%}_{280nm} = 0.72$ (Gerhart & Holoubek, 1967). Concentration of protein determined by this method agrees with values measured by the colorimetric assay of Smith et al. (1985).

Methods

Determination of Enzyme Activity. ATCase activities were measured using the stopped-time assay of Davies et al. (1970). pH profiles were constructed as described by Turnbull et al. (1992) over a pH range of 6.4–9.6. Values of V and V/K were calculated per active site using a value of 33 000 for the molecular weight of each catalytic chain.

Isotope Effects and Nomenclature. ^{13}C and ^{15}N isotope effects were determined according to the procedure described by Parmentier et al. (1992b) with reactions performed in the presence of 20 mM CbmP. The nomenclature used to describe the isotope effects is that of Northrop (1977) and has been outlined in an earlier paper (Parmentier et al., 1992a).

Data Analysis. Initial velocity data were plotted graphically in double-reciprocal form to check the linearity of the curves and to determine the patterns of the plots. A fit of each set of data was then made to the appropriate equation using the nonlinear regression computer programs described by Cleland (1979). Initial velocities obtained at each pH were fit to eq 1 when the concentration of one substrate was varied at a constant level of the other substrate and to eq 2 when the

$$v = VA/(K + A) \quad (1)$$

$$v = VAB/(K_{ia}K_b + K_aB + K_bA + AB) \quad (2)$$

concentrations of both substrates were varied. The inhibition constant (K_i) for succinate, a competitive inhibitor of aspartate, was determined as previously described (Turnbull et al., 1992). Data conforming to competitive inhibition were fit to eq 3, and

$$v = VA/[K(1 + I/K_{is}) + A] \quad (3)$$

$$K_{is} = K_i(1 + B/K_{ib}) \quad (4)$$

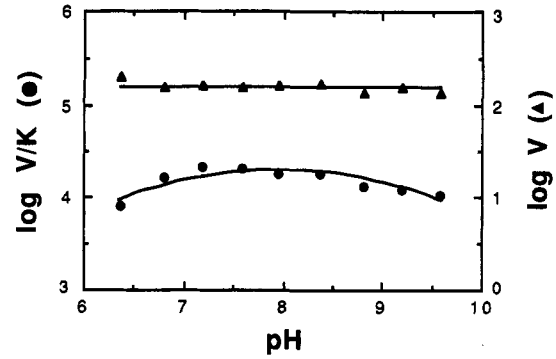

FIGURE 1: Variation with pH of log V and log $(V/K)_{asp}$ for the reaction catalyzed by T55A trimer. Initial velocities were measured by varying the concentration of aspartate at levels of CbmP that were saturating at each pH value. The concentrations of CbmP were 40 mM at pH 6.4 and 9.6, 30 mM at pH 6.8 and 9.2, 20 mM at pH 8.8 and 15 mM at pH 7.2, 7.6, 8.0, and 8.4. The curve for V/K (●) represents the best fit of the data to eq 5 whereas the data for V (▲) were fitted to the equation for a straight line. The values of the parameters used to draw the curves are given in Table I. The units of V/K and V are M^{-1} s^{-1} and s^{-1}.

the true inhibition constant (K_i) was determined using the relationship given in eq 4. The variation with pH of the values for V/K, $1/K_i$, and $1/K_{ia}$ were fit to the appropriate equation (eq 5 and 6). y represents the value of the parameters at a

$$\log y = \log [C/(1 + [H^+]/K_1 + K_2/[H^+])] \quad (5)$$

$$\log y = \log [(Y_H + Y_LK/[H^+])/(1 + K/[H^+])] \quad (6)$$

particular pH, and C is the pH-independent value of the parameter. K_1 and K_2 are acid dissociation constants of ionizable groups on the acid and alkaline sides, respectively, of the pH profiles. Y_H and Y_L denote the limiting pH-independent values of the parameter at high and low pH, respectively.

Results

Previous studies of the T55A mutant catalytic trimer at pH 8.3 (Xu & Kantrowitz, 1989) indicated that the binding of aspartate and CbmP were both markedly reduced as a result of the amino acid replacement, and no determination was made of kinetic constants from substrate saturation curves where the nonvaried substrate is saturating. Kinetic analysis was limited further by the finding that the mutant trimer displayed a sigmoidal dependence of activity as a function of aspartate concentration. In contrast, preliminary kinetic characterization of the T55A mutant produced in this laboratory under the same assay conditions described by Xu and Kantrowitz (1989) indicated that the catalytic trimer exhibited Michaelis–Menten kinetics and the data were amenable to a detailed kinetic analysis. Velocity data obtained for the mutant trimer at pH 8.3 by varying aspartate concentrations (1–100 mM) at a fixed concentration (15 mM) 15 times the dissociation constant (K_{ia}) of CbmP were hyperbolic with respect to aspartate concentration. The data (not shown) fit well to eq 1, yielding a Michaelis constant for aspartate of 15.7 ± 1.1 mM and a maximum velocity of 120 ± 3 s^{-1}. Although CbmP binding was reduced by 2 orders of magnitude by the amino acid substitution (data not shown), the affinity for aspartate was similar to that of the wild-type enzyme, which is consistent with the site-specific replacement lying exclusively in the CbmP binding domain (Krause et al., 1987).

pH Dependence of the Reaction Catalyzed by T55A Trimer. Studies of the effect of pH on the reaction catalyzed by T55A were limited to a range of pH values from 6.4 to 9.6 because

Table I: Values of pK and pH-Independent Kinetic Parameters for the Reaction Catalyzed by the T55A Catalytic Trimer of ATCase

conditions	parameter determined	pH-independent value of parameter	pK_1	pK_2
aspartate varied; CbmP saturating	$(V/K)_{asp}$ (mM^{-1} s^{-1})	2.04 ± 0.21	6.45 ± 0.17	9.49 ± 0.17
	V (s^{-1})	155		
succinate varied; CbmP saturating	Y_L (mM^{-1})	3.03 ± 0.09		6.93 ± 0.11
	Y_H (mM^{-1})	0.023 ± 0.002		
CmbP varied; aspartate nonsaturating	K_{ia} (mM)	1.32 ± 0.47	7.15 ● 0.27	8.75 ± 0.22

of the large increase in the dissociation constant (K_{ia}) for CbmP outside this range. The results (Figure 1) indicate that the variation with pH of log $(V/K)_{asp}$ differs considerably from that of log V. The V/K profile is bell-shaped and illustrates the decrease in the rate of reaction of aspartate with the enzyme–CbmP complex at low and high pH values. Although the curve was very shallow, the data represented in the profile were fit to eq 5 to yield pK values of 6.45 ± 0.17 and 9.49 ± 0.17 for the ionizing groups that are involved in binding aspartate and catalysis (Table I). These values were displaced outward by at least 0.5 pH unit relative to those reported for the wild-type enzyme (Turnbull et al., 1991). By contrast, the V profile for T55A trimer showed that the maximum velocity of the reaction was independent of pH. Since the mutant is considerably active, exhibiting 25% of the activity of the wild-type enzyme, it is likely that one or both of the residues whose ionizations are displayed in the V profile for the wild-type enzyme (Turnbull et al., 1992) still participate in the reaction catalyzed by the mutant, but the pK values of the groups are shifted outside the experimentally accessible pH range.

pH Dependence of Succinate Binding. The true pK values of residues involved in binding aspartate were determined by examining the effects of pH on the binding of succinate to the T55A mutant. This substrate analogue, which functions as a competitive inhibitor with respect to aspartate (Porter et al., 1969), gave rise to a profile (Figure 2) indicating that succinate binds to both the protonated and deprotonated forms of the enzyme although the binding to the protonated enzyme is abount 2 orders of magnitude stronger (Table I). The data represented in the curve of Figure 2 were fit to eq 6 to yield a pK for the residue of 6.94 ± 0.11 (Table I). Comparison of the kinetic parameters obtained from the $pK_{i\,succinate}$ profiles for the T55A trimer and the wild-type enzyme (Turnbull et al., 1992) indicated that the amino acid substitution shifts the pK value of the binding group downward by about 0.4 pH unit, although the affinity of the enzyme for succinate, as reflected in the pH-independent value of K_i, is not significantly altered.

pH Dependence of CbmP Binding. The effect of pH on the dissociation constant (K_{ia}) of the T55A mutant was examined to determine the pK values of residues involved in binding CbmP. The profile of pK_{ia} against pH was a bell-shaped curve (Figure 3) and fitting the data to eq 5 yielded pK values (Table I) for the ionizing residues observed on the acid (7.15 ± 0.28) and alkaline (8.75 ± 0.22) limbs of the profile. These results constrasted with those of the wild-type enzyme (Turnbull et al., 1992) where CbmP binding requires the participation of a single protonated residue with a pK value of about 9. In addition, the pH-independent value of the K_{ia} for T55A trimer was reduced by 2 orders of magnitude relative to the value for the wild-type enzyme, indicating that the alanine mutation clearly affects the degree of CbmP binding. This is in agreement with Xu and Kantrowitz (1989), who reported that both the apparent K_{ia} for CbmP and the apparent inhibition constant for PALA were reduced in this mutant catalytic trimer by an order of magnitude relative to the wild-type enzyme at pH 8.3.

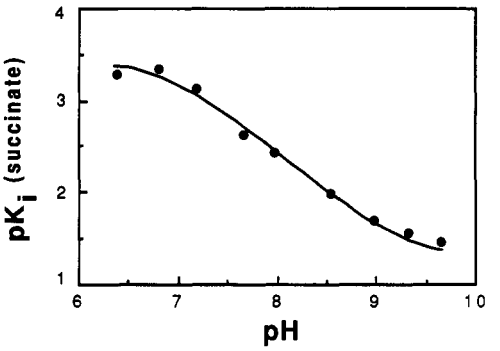

FIGURE 2: Variation with pH of the pK_i for succinate. Initial rates were obtained by varying the concentration of succinate at a fixed level (15 mM) of aspartate and at concentrations of CbmP that were saturating at each pH (40 mM at pH 6.4 and 9.6, 30 mM at pH 6.8 and 9.2, 20 mM at pH 8.8, 15 mM at pH 7.2, 7.6, 8.0, and 8.4). The curve represents the fit of the data to eq 6 and was drawn using the values of the parameters given in Table I. The units of K_i are M.

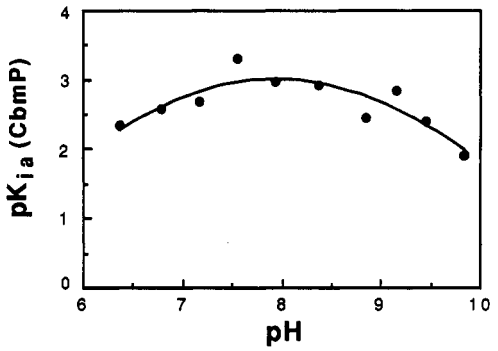

FIGURE 3: Variation with pH of the K_{ia} for CbmP. Initial velocities were measured by varying the concentration of CbmP at 0.1 mM aspartate. The curve represents the fit of the data to eq 5. Values for the parameters used to draw the curves are given in Table I. The units for K_{ia} are M.

Inhibition by PALA. It has been shown previously that PALA inhibits competitively with respect to CbmP and noncompetitively with respect to aspartate (Collins & Stark, 1971) in the reaction catalyzed by the wild-type catalytic trimer. This pattern is consistent with a steady-state ordered mechanism of substrate addition (Porter et al., 1969; Parmentier et al., 1992a; Hsuanyu & Wedler, 1988). However, velocity data obtained for T55A trimer by varying the concentration of aspartate at a constant amount of CbmP and fixed concentrations of PALA gave rise to a family of lines that intersected on the vertical axis (data not shown). This pattern, indicating that PALA acts as a linear competitive inhibitor with respect to aspartate, is consistent with a kinetic mechanism for either a random addition of substrates or an equilibrium ordered mechanism where CbmP binds prior to aspartate. A similar inhibition pattern has also been reported for the H134A mutant which conforms to a random kinetic mechanism (Waldrop et al., 1992a). The value of the inhibition constant for PALA, obtained by fitting the velocity data to eqs 3 and 4, was about 125 ± 25 nM. Comparison of this

Table II: Isotope Effects, Commitment Factors, and Partition Ratios for Wild-Type, H134A, and T55A Catalytic Trimers of Aspartate Transcarbamoylase

value	wild type[c]	H134A[d]	T55A
$^{13}(V/K_{CbmP})_{H_2O}$[a]	1.0240 ± 0.0005	1.0413 ± 0.0011	1.0134 ± 0.0006
$^{13}(V/K_{CbmP})_{D_2O}$[a]	1.0172 ± 0.0007	1.0434 ± 0.0004	1.0150 ± 0.0001
$^{15}(V/K_{asp})_{H_2O}$[b]	1.0044 ± 0.0003	1.0053 ± 0.0003	1.0007 ± 0.0002
$^{15}(V/K_{asp})_{D_2O}$[b]	1.0076 ± 0.0003	1.0121 ± 0.0004	1.0040 ± 0.0003
$[ab(1 + c)/1 + b]_{H_2O}$	0.81 ± 0.03	approaches 0	2.24 ± 0.11
$[ab(1 + c)/1 + b]_{D_2O}$	1.52 ± 0.08	approaches 0	1.89 ± 0.03
a	1.43 ± 0.58	approaches 0	~1
b	0.30 ± 0.11	0.62 ± 0.03	~0.11
c	1.46 ± 1.32	approaches 0	~21.6

[a] Reactions run at saturating carbamoyl phosphate and an aspartate concentration extrapolated to zero (wild type) or maintained as near zero as possible (mutants), pH(D) 7.5, 25 °C. [b] Reactions run at saturating carbamoyl phosphate and an initial aspartate concentration of 12 mM, pH(D) 7.5, 25 °C. [c] Data taken from Parmentier et al. (1992a,b). [d] Data taken from Waldrop et al. (1992).

result to the value of about 10 nM for the wild-type enzyme (Turnbull et al., 1992) indicated that the binding of PALA at pH 7.5 was reduced by a factor of 16 by the replacement of Thr 55 by alanine.

Initial Velocity Pattern. The reaction catalyzed by T55A trimer was examined at various concentrations of substrates in order to distinguish between an equilibrium ordered and a random kinetic mechanism. Double-reciprocal plots obtained by measuring velocities at various concentrations of CbmP and fixed amounts of aspartate as well as at various concentrations of aspartate and fixed levels of CbmP were linear and intersected to the left of the vertical axis (data not shown). The data conform to a sequential initial velocity pattern described by eq 2, which is consistent with either a random addition of substrates, as reported for the H134A mutant (Waldrop et al., 1992a), or a steady-state ordered mechanism, exhibited by the wild-type catalytic trimer (Porter et al., 1969; Hsuanya & Wedler, 1988; Parmentier et al., 1992a). The data clearly did not conform to an equilibrium ordered mechanism where the initial velocity pattern intersects on the vertical axis (Cleland, 1986).

Isotope Effects. The ^{13}C and ^{15}N isotope effects for the reaction catalyzed by the T55A trimer were measured in both H_2O and D_2O, and the results are summarized in Table II. At levels of aspartate that were very low and at saturating levels of aspartate, the average values for $^{13}(V/K_{CbmP})_{H_2O}$ from 4 determinations each were 1.0134 ± 0.0006 and 1.0071 ± 0.0003, respectively. The values for the ^{13}C isotope effects measured at intermediate concentrations of aspartate ranging from 3 to 16 mM (8 determinations in total) were similar to those obtained at very low amounts of aspartate (Figure 4). The ^{13}C isotope effects for this reaction in D_2O at very low aspartate concentrations and at a saturating concentration of aspartate yielded average values for $^{13}(V/K_{CbmP})_{D_2O}$ of 1.0150 ± 0.0001 and 1.0100 ± 0.0003, respectively, from 3 determinations each. The reaction of carbamoyl phosphate with the slow substrate L-cysteine sulfinate (300 mM) in H_2O gave an average $^{13}(V/K_{CbmP})_{H_2O}$ value of 1.0353 ± 0.0006 from 6 determinations.

^{15}N isotope effects for the T55A mutant measured in H_2O and D_2O yielded average values for $^{15}(V/K_{asp})_{H_2O}$ and $^{15}(V/K_{asp})_{D_2O}$ of 1.0007 ± 0.0002 (3 determinations) and 1.0040 ± 0.0003 (4 determinations), respectively.

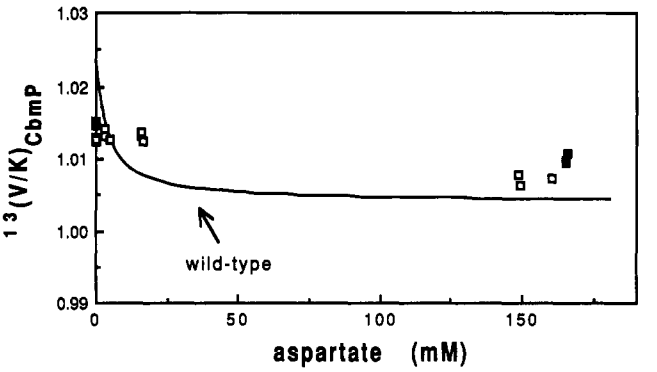

FIGURE 4: ^{13}C isotope effects for the T55A mutant-catalyzed reaction at saturating levels (20 mM) of CbmP and varying concentrations of aspartate in 50 mM HEPES, 2 mM DTT, and 0.2 mM EDTA, pH (D) 7.5 at 25 °C, in H_2O (□) and D_2O (■). Shown for comparison is the corresponding curve for the wild-type catalytic trimer in H_2O (Parmentier et al., 1992a) for which experimental points have been omitted for clarity.

Discussion

Function of Thr 55 in the Catalytic Mechanism. Conservation of Thr 55 in bacterial and mammalian ATCases provides an indication that this residue plays an important role in the function of the enzyme (Lerner & Switzer, 1986; Simmer et al., 1989). The crystal structure (Krause et al., 1987) of the PALA-liganded enzyme [see Figure 1 from Waldrop et al. (1992a)] and previous studies on the T55A mutant (Xu & Kantrowitz, 1989) have led to the hypothesis that Thr 55 plays two major roles in the catalytic mechanism of ATCase. On the one hand it has been postulated that Thr 55 assists in CbmP binding through hydrogen bond formation with one of the phosphate oxygens, and on the other hand it was proposed that Thr 55 participates, in concert with His 134 and Arg 105, in the polarization of the carbonyl group of CbmP. The finding that the alanine substitution at position 55 reduces the affinity of the enzyme for CbmP by 2 orders of magnitude without markedly altering the affinity for aspartate and succinate implicates Thr55 in the binding of CbmP.

In contrast, the ^{13}C isotope effects, which monitor the chemistry affecting the carbonyl carbon of CbmP, yielded results indicating that Thr 55 does not assist in polarizing the carbonyl group. If Thr 55 fulfilled this catalytic role, then replacement of the critical amino acid should render nucleophilic attack by aspartate more rate-limiting and the value of $^{13}(V/K_{CbmP})_{H_2O}$ would increase as observed for the H134A mutant (Waldrop et al., 1992a) where formation of the tetrahedral intermediate is the rate-determining step. On the contrary, the value of $^{13}(V/K_{CbmP})_{H_2O}$ for the T55A mutant trimer, 1.0134 ± 0.0006, was reduced relative to that of the wild-type enzyme, 1.0240 ± 0.0005 (Table II) (Parmentier et al., 1992a). This result indicates that the chemistry of the T55A trimer-catalyzed reaction is less rate-limiting than that of the wild-type enzyme. Hence, an alternate step in the catalytic process has become more rate-determining as a consequence of the alanine substitution for Thr 55.

Additional support for this conclusion is provided by the results of the ^{13}C isotope effects obtained with the slow substrate L-cysteine sulfinate. Since L-cysteine sulfinate reacts at about 2% of the maximum velocity of aspartate (Foote et al., 1985), the chemical steps of the mutant-catalyzed reaction should become rate-limiting and yield a $^{13}(V/K_{CbmP})_{H_2O}$ value equal to the intrinsic isotope effect as reported for the wild-type enzyme under these conditions (Parmentier et al., 1992a). However, a value was obtained for the T55A mutant enzyme which, at 1.0353 ± 0.0006, was greater than the ^{13}C isotope effect with aspartate (1.0134 ± 0.0006) but less than the full ^{13}C intrinsic isotope effect 1.04 (Parmentier et al., 1991a), indicating that the chemistry of the reaction has become slower but not rate-limiting with L-cysteine sulfinate as a substrate. Therefore, a step prior to the chemistry, such as a confor-

mational change, is partially rate-limiting.

The ^{13}C isotope effects can be analyzed together with the ^{15}N isotope effects, which reflect the chemistry associated with the α-amino nitrogen of aspartate, in order to identify the rate-limiting step in the reaction catalyzed by the T55A mutant. A quantitative analysis of the combined ^{13}C and ^{15}N isotope effects in H_2O and D_2O for the T55A mutant can be performed based on the model for the wild-type enzyme presented in Scheme I of Parmentier et al. (1992b).

In this model, the rate constants of steps associated with the formation and breakdown of the tetrahedral intermediate, k_7, k_8, and k_9, are ^{13}C sensitive, while ^{15}N-sensitive rate constants include not only k_7, k_8, and k_9 but also those involved in the deprotonation of the α-amino group of aspartate, k_5 and k_6. The constants k_5 and k_6 are deuterium sensitive, and all commitments are internal (i.e., only steps subsequent to the formation of the Michaelis complex, E·CbmP·RNH_3^+, are analyzed). The following assumptions made for the wild-type enzyme regarding this model (Parmentier et al., 1991b) will be employed in analyzing the data for the mutant enzyme: $^{13}k_8 = {}^{13}k_9$, $^{15}K_{eq5H} = 1.016$, $^{15}K_{eq5D} = 1.0201$, $^{15}K_{eq7} = 0.970$, $^{15}K_9 = 1.0137$, $^{15}K_7 = 1.0$, and $^{15}K_{5H(D)} = {}^{15}K_{eq5H(D)}$. The equation for the observed ^{13}C kinetic isotope effect can be written as

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

where $a = k_7/k_6$, $b = k_9/k_8$, and $c = k_5/k_4$.

The appropriate equations for the observed ^{15}N kinetic isotope effects in H_2O and D_2O are shown:

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

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

With the assumptions given above, these equations become

$$^{15}(V/K_{asp})_{H_2O} = 1.0007 \pm 0.0002 = \frac{1.016[0.983 + b(1 + a_H)] + a_H bc_H}{1 + b(1 + a_H) + a_H bcH} \quad (10)$$

$$^{15}(V/K_{asp})_{D_2O} = 1.0040 \pm 0.0003 = \frac{1.020[0.983 + b(1 + a_D)] + a_D bc_D}{1 + b(1 + a_D) + a_D bc_D} \quad (11)$$

Most likely the partition factor for the tetrahedral intermediate, b, is not affected by changing H_2O to D_2O, since this did not appear to happen with wild-type enzyme (Parmentier et al., 1992b), but clearly the other partition factors ($a = k_7/k_6$ and $c = k_5/k_4$) will change because k_5 and k_6 involve proton transfer and should be decreased in D_2O.

The product ac would not vary in D_2O if the effect of deuteration were equal on k_5 and k_6, and no other rate constants were affected. However, this seems not be to be the case, since substitution of 1.0434 (Waldrop et al., 1992a) for the ^{13}C intrinsic isotope effect, $^{13}k_7$, in eq 7 gives

in H_2O: $$a_H b(1 + c_H)/(1 + b) = 2.24 \pm 0.11 \quad (12)$$

in D_2O: $$a_D b(1 + c_D)/(1 + b) = 1.89 \pm 0.03 \quad (13)$$

If k_5 and k_6 were affected equally by deuteration, a would increase and c would decrease by an equal ratio, and the expression in eq 13 would be greater than, rather than less than, that in eq 12. Thus, c is decreasing by a greater factor than a is increasing in D_2O.

We have explored possible solutions to eqs 10–13 (allowing a and c to vary in D_2O, with b held constant) and conclude that the most reasonable set of values is close to the following: $a_H = 1$, $a_D = 1.9$, $c_H = 21.6$, and $c_D = 9$, with b around 0.11 (comparison of these values with those for the wild-type enzyme and H134A mutant enzyme can be found in Table II). These values fit eqs 10, 12, and 13 very well, but give slightly too small a value in eq 11 unless $^{15}K_{eq5}$ is raised from 1.020 to 1.023. In any case, the major change from the wild-type enzyme is an increase in k_5/k_4 (c), presumably as the result of a decrease in k_4. Thus, the major effect of the threonine to alanine mutation appears to be on the conformational change that sets up the beginning of the catalytic reaction. This is the first study providing evidence that a conformational change associated with ATCase is kinetically significant.

The results of the pH profiles also support the conclusion that Thr 55 participates in a conformational change associated with the Michaelis complex prior to the chemical steps in the reaction. The log V profile for T55A (Figure 1) was flat, indicating that the pK values of ionizing residues in the Michaelis complex were displaced outward by the substitution. The conformation of the active site in the binary complex may also be altered since the pK values of residues titrated in the profiles for log $(V/K)_{asp}$ (Figure 1) and $pK_{i\ succinate}$ (Figure 2) were also shifted in the mutant. In addition, the ionization of a second residue was observed in the pK_{ia} profile for CbmP (Figure 3) that was absent in the profile for the wild-type enzyme (Turnbull et al., 1992). This observation may arise from a conformational change in T55A trimer that shifts the pK value of a group involved in binding CbmP in both mutant and wild-type enzymes into the experimentally accessible pH range. Alternatively, the result may reflect the participation of a new binding group unique to the mutant. Further evidence supporting the involvement of Thr 55 in a conformational change is documented by Xu and Kantrowitz (1989), who reported a difference in the circular dichroism spectrum for T55A trimer and the wild-type catalytic trimer. Although it is clear that Thr 55 is involved in catalysis, its precise role in the conformational change associated with catalysis has not been established.

Kinetic Mechanism of T55A Trimer. Previous investigations on ATCase (Waldrop et al., 1992a) and ornithine transcarbamoylase (Kuo et al., 1988) have shown that specific amino acid replacements at the active site of either enzyme can alter the kinetic mechanism of the reactions. The results of the present investigation suggest that the kinetic mechanism of the reaction catalyzed by the catalytic trimer has also change as a result of the threonine to alanine substitution at position 55. The pattern of inhibition of T55A activity by PALA was competitive with respect to aspartate, precluding a steady-state ordered mechanism noted previously for the wild-type enzyme (Parmentier et al., 1992a; Turnbull et al., 1992; Hsuanyu & Wedler, 1988), but instead reflects cases of either rapid-equilibrium random or equilibrium ordered. However, as the initial velocity pattern was of the intersecting type, indicating that the mutant does not conform to an equilibrium ordered mechanism, it must be concluded that the T55A catalytic trimer follows a rapid-equilibrium random mechanism for the addition of substrates. The mechanism is not fully random as reported for the H134A mutant (Waldrop et al., 1992a), however, since the ^{13}C isotope effects measured in H_2O and D_2O at saturating concentrations of aspartate were less than those measured at very low levels of aspartate (Figure 4).

Concluding Remarks. The results of the present study and those of the previous paper in this series have demonstrated the utility of the technique of heavy-atom isotope effects in the study of active site mutants of ATCase. The crystallographic data of ATCase containing bound PALA have indicated that three amino acids, His 134, Thr 55, and Arg 105, may play a catalytic role in the reaction through the polarization of the carbonyl group of CbmP. However, the isotope effect studies performed on the T55A mutant enzyme suggest that Thr 55 is not involved in polarizing the carbonyl group of CbmP but, rather, is associated with CbmP binding and a conformational change in the enzyme that initiates catalysis. On the other hand, isotope effects indicate that His 134 may participate in the chemical mechanism by one of two ways. Histidine, present in the imidazole form, may assist in catalysis directly by serving as an electrophile to polarize the carbonyl group of CbmP. Alternatively, His 134 may participate indirectly by promoting the binding of aspartate and CbmP such that the substrates are properly oriented for the subsequent chemistry. Although the isotope effect studies cannot distinguish between the two mechanisms, the observation that the H134A mutant retains considerable activity yet shows a reduction in the binding affinities for both substrates is evidence in favor of the latter mechanism. The structural data of ATCase liganded with PALA also implicate Arg 105 as an enzymic acid. Hence, it would be of interest to assess the contribution of this residue to catalysis using isotope effect techniques.

It is worth noting that the binding of the trigonal inhibitor PALA may be different from that of the two substrates, which are believed to adopt a tetrahedral configuration in the transition state (Waldrop et al., 1992b). Molecular modeling (Gouaux et al., 1987) has revealed that the oxyanion of the putative tetrahedral intermediate could be positioned to interact with either His 134, Thr 55, Arg 105, or Arg 54. In the latter case, the guanidinium side chain of Arg 54 could stabilize the oxyanion by an ion pair interaction. This hypothesis is consistent with the observation that the maximum velocity for the R54A mutant is decreased by 4 orders of magnitude while the binding of both substrates remains unaltered (Stebbins et al., 1989). Ultimately, enzymes genetically altered at positions 54 and 105 will have to be characterized using the kinetic analyses outlined in this study combined with structural data before a final conclusion is drawn regarding the residue that activates the carbonyl group of CbmP.

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