Steady-State Kinetics and Isotope Effects on the Mutant Catalytic Trimer of Aspartate Transcarbamoylase Containing the Replacement of Histidine 134 by Alanine†

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ABSTRACT: A detailed kinetic analysis of the catalytic trimer of aspartate transcarbamoylase containing the active site substitution H134A was performed to investigate the role of His 134 in the catalytic mechanism. Replacement of histidine by alanine resulted in decreases in the affinities for the two substrates, carbamoyl phosphate and aspartate, and the inhibitor succinate, by factors of 50, 10, and 6, respectively, and yielded a maximum velocity that was 5% that of the wild-type enzyme. However, the pK values determined from the pH dependence of the kinetic parameters, log V and log (V/K) for aspartate, the pK_i for succinate, and the pK_{ia} for carbamoyl phosphate, were similar for both the mutant and the wild-type enzymes, indicating that the protonated form of His 134 does not participate in binding and catalysis between pH 6.2 and 9.2. ^{13}C and ^{15}N isotope effects were studied to determine which steps in the catalytic mechanism were altered by the amino acid substitutions. The $^{13}(V/K)$ for carbamoyl phosphate exhibited by the catalytic trimer containing alanine at position 134 revealed an isotope effect of 4.1%, probably equal to the intrinsic value and, together with quantitative analysis of the ^{15}N isotope effects, showed that formation of the tetrahedral intermediate is rate-determining for the mutant enzyme. Thus, His 134 plays a role in the chemistry of the reaction in addition to substrate binding. The initial velocity pattern for the reaction catalyzed by the H134A mutant intersected to the left of the vertical axis, negating an equilibrium ordered kinetic mechanism. However, inhibition of activity by the bisubstrate analogue *N*-(phosphonoacetyl)-L-aspartate was competitive with respect to aspartate, demonstrating that the kinetic mechanism of the H134A mutant is random. The random mechanism was confirmed by the observation that the carbon isotope effect did not vary with aspartate concentration. Possible roles for His 134 in catalysis are discussed.

Over the past ten years, site-directed mutagenesis has become the sine qua non for elucidating the role of active site residues in enzymatic catalysis. Indeed, several enzymes such as triose phosphate isomerase (Knowles, 1991), dihydrofolate reductase (Benkovic et al., 1988), and aspartate aminotransferase (Goldberg et al., 1990; Inoue et al., 1991) have been studied extensively using site-directed mutagenesis together with rigorous kinetic analyses. However, out of 13 active site mutations (Stevens et al., 1991) that have been generated for aspartate transcarbamoylase (ATCase),[1] none have been sufficiently well characterized kinetically to assess the role of a particular active site residue in the catalytic mechanism. This report focuses on the kinetic characterization of a mutant catalytic trimer of ATCase harboring the substitution alanine for histidine at position 134.

His 134 is conserved in bacterial and mammalian ATCases, suggesting that this residue plays a significant role (Lerner & Switzer, 1986; Simmer et al., 1989). Its involvement in the catalytic mechanism was first implicated by the crystal structure of ATCase ligated to the bisubstrate analogue *N*-(phosphonoacetyl)-L-aspartate (PALA) (Krause et al., 1987). Figure 1 shows amino residues that appear to be in contact with PALA in the crystal structure. Lipscomb and colleagues proposed that His 134 acts as either a general acid to polarize the carbonyl group of carbamoyl phosphate (CbmP) or as a general base to deprotonate the α-amino group of aspartate (Gouaux et al., 1987). This hypothesis was tempered by the observation that the alanine mutant retained 5% of the activity of the wild-type enzyme (Robey et al., 1986). Moreover, Kleanthous et al. (1988) reported that the pK value of His 134 was less than 6 and concluded that this residue did not participate in acid–base catalysis. In contrast, Hervé and coworkers studied the pH dependence of the binding of CbmP

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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; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetate; H134A and H134N, mutant catalytic trimer from ATCase with His 134 in the catalytic polypeptide chains replaced by Ala and Asn, respectively.

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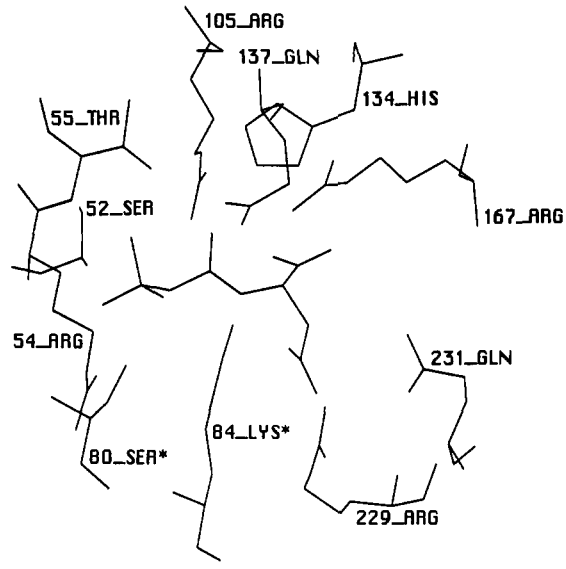


FIGURE 1: Positioning of the bisubstrate analogue PALA in the catalytic site of ATCase. Active site residues which are contributed by an adjacent catalytic chain are designated (*). The tracing was generated using X-ray crystallographic coordinates from the Brookhaven Protein Data Bank, based on the structure of Krause et al. (1989).

analogues to the asparagine mutant and concluded that His 134 had a pK of about 8 in the unliganded enzyme and about 7 in the enzyme–CbmP complex (Xi et al., 1990). These authors proposed that this histidine assisted in binding CbmP by interacting with the carbonyl group. Clearly, these conflicting observations warrant additional studies.

Several lines of evidence indicate that the H134A mutant catalytic trimer is an appropriate model for detailed mechanistic studies. Enzymes with five amino acid replacements have been constructed at position 134, and of these, the alanine mutation is the most active, displaying 5% residual activity (Silversmith and Schachman, unpublished results). This mutant is suitable for measurements of heavy atom isotope effects, a technique used in this study which requires considerably more protein than initial velocity studies (O'Leary, 1980). The nuclear magnetic resonance spectra obtained for the wild-type and H134A catalytic trimers labeled with [^{13}C]histidine were very similar, illustrating that global conformational changes in the protein did not occur because of the amino acid substitution (Kleanthous et al., 1988). Finally, the alanine side chain does not possess the capacity to hydrogen bond to CbmP or aspartate, thus obviating any potential ambiguities in interpreting the role of His 134 in substrate binding. The objective of this study is to gain insight into the role of His 134 in the catalytic mechanism through a detailed mechanistic analysis.

Experimental Procedures

Materials

L-Aspartic acid was supplied by Calbiochem, and succinic acid was purchased from Mallinkrodt. Dilithium CbmP was from Sigma. [^{14}C]CbmP (12.5 mCi/mmol) was supplied by New England Nuclear. PALA was provided by Dr. Robert Engle, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute, National Institutes of Health. The concentration of PALA was determined by phosphorus analysis (Ames & Dubin, 1960). All other chemicals were obtained commercially and were of the highest purity available.

The plasmid pAF1, encoding the H134A mutation, was constructed previously by Robey et al. (1986) and was transformed into *Escherichia coli* strain EK1104 kindly provided by Dr. E. R. Kantrowitz. This strain of *E. coli* harbors a leaky mutation in *pyr*F, thereby allowing the mutant enzyme to be overproduced when the bacteria are grown in a medium described by Nowlan and Kantrowitz (1985). The chromosomal copy of *pyr*B is deleted in the strain EK1104 to prevent contamination with wild-type enzyme. ATCase containing the H134A substitution in the catalytic chains was purified according to the procedure of Wall et al. (1981), and catalytic trimer was prepared from holoenzyme as described by 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) and 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). The H134A mutant enzyme was stable between pH 6.2 and 9.2 for 15 min at 30 °C. Above and below this pH range, the enzyme exhibited time-dependent inactivation after 15 min as determined by the method of Selwyn (1965). pH profiles were constructed as described by Turnbull et al. (1992) over a pH range of 6.2–9.2. 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.

Nomenclature for Isotope Effects. The nomenclature used is that of Northrop (1977) in which the leading superscript denotes the isotope responsible for the effect on a given kinetic or thermodynamic parameter. For example, the ^{13}C isotope effect on V/K is written $^{13}(V/K)$, and this symbol represents the V/K of the ^{12}C-containing species relative to the rate of the ^{13}C-containing species $[(V/K)^{12}C/(V/K)^{13}C]$. Multiple isotope effects are written as a combination of superscripts and subscripts. Thus, $^{15}(V/K)_{D_2O}$ is the ^{15}N isotope effect on V/K with D_2O as solvent. Substrates on which the isotope effect is measured are designated by a subscript within the brackets. The notation $^{15}(V/K_{asp})_{D_2O}$ represents the ^{15}N isotope effect on V/K for the substrate aspartate for the reaction in deuterium oxide.

Isotope Effects. ^{13}C and ^{15}N isotope effects were determined according to the procedure described by Parmentier et al. (1992a,b).

Data Analysis. The data were fit to the appropriate equations using the nonlinear regression computer programs described by Cleland (1979). Initial velocities (v) obtained at each pH by varying the concentration of substrate (A) were fit to to yield values for the maximum velocity (V), the Michaelis constant for that substrate (K), and the apparent first-order rate constant for the interaction of enzyme and substrate (V/K). When the concentrations of both substrates A and B were varied, velocity data were fit to eq 2, where v

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

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

is the experimentally determined velocity, V is the maximum velocity, A and B are the substrate concentrations, K_a and K_b are the respective Michaelis constants, and K_{ia} is the dissociation constant of A. Inhibition data obtained by varying the concentrations of A and of a competitive inhibitor of B were fit to eq 3, where K is the apparent Michaelis constant of

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

substrate A, I is the inhibitor concentration, and K_{is} the slope inhibition constant. The true inhibition constant K_i was determined using the relationship given in eq 4, where K_{ib} denotes

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

the dissociation constant of the nonvaried substrate. Inhibition data obtained by varying the concentration of a competitive inhibitor of substrate A at a fixed concentration of A were analyzed as previously described (Turnbull et al., 1992) to obtain values for the inhibition constant (K_i). The variation with pH of the values for V, V/K, $1/K_i$, and $1/K_{ia}$ were fit to the appropriate equations (eqs 5–8). y represents the value

$$\log y = \log [C/(1 + [H^+]/K_1 + K_3/[H^+] + [H^+]^2/K_1K_2)] \quad (5)$$

$$\log y = \log [C/(1 + [H^+]/K_1)] \quad (6)$$

$$\log y = \log [C/(1 + K_3/[H^+])] \quad (7)$$

$$\log y = \log [\{(Y_H + Y_L)[H^+]/K\}/(1 + [H^+]/K)^2] \quad (8)$$

of V, V/K, $1/K_i$, or $1/K_{ia}$ at a 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 side of the pH profiles, and K_3 denotes acid dissociation constants on the alkaline side. Y_H and Y_L denote the limiting pH-independent values of the parameter at high and low pH, respectively.

Results

pH Dependence of V and V/K for Aspartate. Because of the instability of the H134A mutant trimer at pH values below 6.2 and above 9.2, V and $(V/K)_{asp}$ were examined over the limited pH range of 6.2–9.2. The variation of log (V/K) with pH was bell-shaped with limiting slopes of +2 at low pH and −1 at high pH (Figure 2). The data fit to eq 5 yielded pK values of 6.49 ± 0.46 and 7.15 ± 0.16 on the acid limb and 8.76 ± 0.14 on the alkaline limb (Table I). These results differed from the bell-shaped V/K profile obtained for the wild-type enzyme (Turnbull et al., 1992) only in the appearance of a new residue with a pK value of about 6.5. The V/K profile for the asparagine mutant (H134N) also showed activity decreasing at low and high pH values with limiting slopes of +1 and −1 (Xi et al., 1990).

The variation of log V with pH for H134A trimer gave rise to a half-bell profile with a limiting slope of +1 at low pH (Figure 2). Fitting the data to eq 6 yielded a pK of 7.15 ± 0.03 (Table I), in agreement with the value determined previously for the wild-type enzyme (Turnbull et al., 1992). Since the activity of H134A trimer could not be measured at high pH values, it was not possible to determine whether the log V profile would display the ionization of the catalytic group with a pK of about 9.5 as observed in the profile for the wild-type enzyme. This residue was not observed in the V profile for H134N trimer although activities were recorded up to pH 10 (Xi et al., 1990). It is likely that the pK value of the catalytic group was shifted beyond the experimentally accessible pH range as a result of the asparagine substitution since the pK value of the group on the acid limb of the profile (pK ~6.2) was also displaced outward relative to the value obtained for the wild-type enzyme (pK ~7.2).

pH Dependence of the K_i for Succinate. The true pK values of residues involved in binding aspartate were determined by examining the variation with pH of the inhibition constant (K_i) for succinate, which is a competitive inhibitor with respect to aspartate (Porter et al., 1969). As seen in Figure 3, the binding of succinate decreases at both low and high pH values due to the protonation and deprotonation, respectively, of two residues, although some binding of inhibitor occurs despite deprotonation. The residue whose ionization is shown on the alkaline side of the profile can be titrated in the enzyme–succinate complex, as demonstrated by a plateau in the curve at high pH (Cleland, 1982). By fitting the data to eq 8, a single pK value of 7.26 ± 0.05 (Table I) was determined for the two ionizable groups associated with the enzyme–CbmP complex whose pK values are too close to be determined separately by this analysis. A similar pK value was reported for both the wild-type enzyme (Turnbull et al., 1992) and the H134N mutant (Xi et al., 1990) although in these cases the pK_i profiles indicated that succinate binding decreases only at higher pH values.

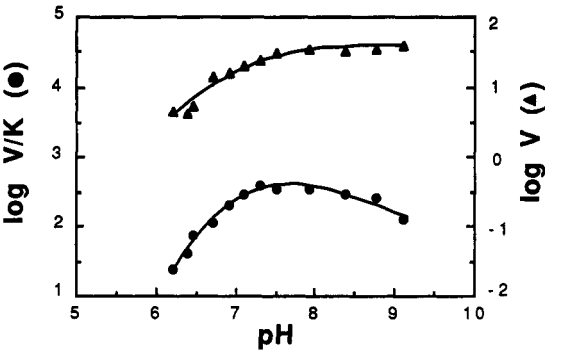


FIGURE 2: Variation with pH of log V and log $(V/K)_{asp}$ for the reaction catalyzed by H134A catalytic trimer. Initial rates were measured by varying the concentration of aspartate of saturating levels of CbmP. The concentrations of CbmP were 7.5 mM from pH 6.2 to 8.4, 10 mM at pH 8.8, and 15 mM at pH 9.2. The curves for V (▲) and V/K (●) represent the best fits of the data to eqs 6 and 5, respectively. The values of the parameters used to draw the curves are given in Table I. The units for V and V/K are s^{-1} and M^{-1} s^{-1}, respectively.

pH Dependence of the Binding of Carbamoyl Phosphate. The effect of pH on the dissociation constant (K_{ia}) of the enzyme–CbmP complex was examined to determine the pK values of residues involved in binding CbmP. The plot of pK_{ia} against pH was a half-bell with a limiting slope of −1 on the alkaline side (Figure 3). The data when fit to eq 7 yielded a pK of 8.67 ± 0.03 (Table I). A similar value was observed by Turnbull et al. (1992) for the wild-type enzyme. Although the profiles for both wild-type enzyme and H134A trimer indicate that a residue must be protonated for binding CbmP, the interaction is reduced by a factor of 50 in the mutant.

Inhibition by PALA. The inhibition of ATCase activity by PALA was examined to establish the order of substrate addition to the mutant catalytic trimer. If CbmP binds prior to aspartate, then the pattern of inhibition for the bisubstrate analogue PALA will be competitive with respect to CbmP and noncompetitive with respect to aspartate (Collins & Stark, 1971). However, PALA gave rise to linear competitive inhibition with respect to aspartate in the H134 mutant enzyme (data not shown). This result is consistent with a mechanism involving either a random binding of substrates or an ordered addition in which binding of CbmP and aspartate to the enzyme are at equilibrium. The inhibition constant for PALA, obtained by fitting the data to eqs 3 and 4, was 1.12 ± 0.08 μM for H134A trimer, which is about 2 orders of magnitude greater than the value reported for the wild-type enzyme at pH 7.5 (Trunbull et al., 1992).

Table I: Values of pK and pH-Independent Kinetic Parameters for the Reaction Catalyzed by the H134A Catalytic Trimer of ATCase

conditions	parameter determined	pH-independent value of parameter	pK_1	pK_2	pK_3
aspartate varied; CbmP saturating	$(V/K)_{asp}$ (mM^{-1} s^{-1})	0.45 ± 0.06	7.15 ± 0.16	6.49 ± 0.46	8.76 ± 0.14
	V (s^{-1})	39.1 ± 1.5	7.15 ± 0.03		
succinate varied; CbmP saturating	Y_L (mM^{-1})	0.52 ± 0.01	7.26 ± 0.05		7.26 ± 0.05
	Y_H (mM^{-1})	0.024 ± 0.002			
CbmP varied; aspartate nonsaturating	K_{ia} (μM)	350 ± 10			8.67 ± 0.03

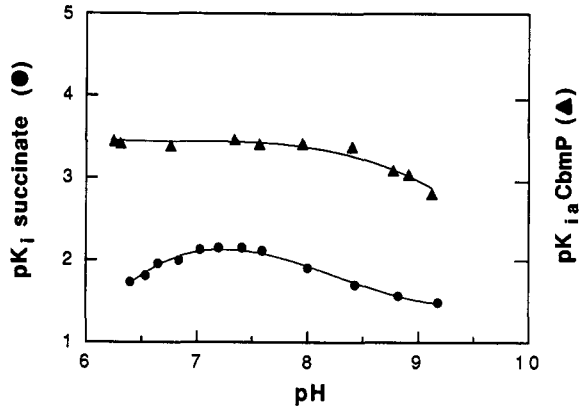


FIGURE 3: Variation with pH of the pK_i for succinate and K_{ia} for CbmP. Initial velocity data for K_i were obtained by varying the concentration of succinate at a fixed level (180 mM) of aspartate and at concentrations of CbmP that were saturating at each pH (7.5 mM from pH 6.2 to 8.4, 10 mM at pH 8.8, and 15 mM at pH 9.2). Initial velocity data for K_{ia} were measured by varing the concentration of CbmP at 1 mM aspartate. The curve for the K_i succinate (●) represents the fit of the data to eq 8 while the curve for the K_{ia} for CbmP (▲) illustrates the fit of the data to eq 7. Curves were drawn using the values of the parameters given in Table I. The units for K_i and K_{ia} are M.

Initial Velocity Pattern. An initial velocity pattern was obtained for the reaction catalyzed by the mutant to distinguish between a random and an equilibrium ordered kinetic mechanism. Velocities measured by varying the concentration of CbmP (between 0.1 and 2.0 mM) at several fixed levels of aspartate (between 2.0 and 15.0 mM) gave rise to a double reciprocal plot that was linear and intersected to the left of the vertical axis (data not shown). An interesting plot was also obtained when aspartate was varied at fixed levels of CbmP (data not shown). The data clearly negate an equilibrium ordered mechanism, for which the initial velocity pattern would intersect on the vertical axis (Cleland, 1977). These results, together with the inhibition pattern for PALA, indicate that the kinetic mechanism of the H134A mutant is random.

Isotope Effects. The relative rates of steps in the catalytic mechanism for the wild-type catalytic trimer have been determined using ^{13}C and ^{15}N isotope effects (Parmentier et al., 1992b). ^{13}C and ^{15}N isotope effects were measured on the H134A mutant using the method of internal competition (O'Leary, 1980) to determine if the mutation alters the rates of the steps in the reaction. The results from these experiments are summarized in Table II. All experiments were performed at saturating levels of CbmP. At near 0 mM aspartate and at ~270 mM aspartate the values for $^{13}(V/K_{CbmP})_{H_2O}$ were 1.0399 ± 0.0006 and 1.0423 ± 0.0019, respectively. Since the ^{13}C isotope effect did not change with aspartate concentration, subsequent ^{13}C isotope effect experiments were performed at high levels of aspartate where the reaction proceeds more rapidly. The average $^{13}(V/K_{CbmP})_{H_2O}$ value for all aspartate concentrations was 1.0413 ± 0.0011 (Table II). These data

Table II: ^{13}C and ^{15}N Kinetic Isotope Effects for the H134A Mutant Catalytic Trimer

parameter	value	no.[c]
$^{13}(V/K_{CbmP})_{H_2O}$[a]	1.0413 ± 0.0011	8
$^{13}(V/K_{CbmP})_{D_2O}$[a]	1.0434 ± 0.0004	4
$^{15}(V/K_{asp})_{H_2O}$[b]	1.0053 ± 0.0003	7
$^{15}(V/K_{asp})_{D_2O}$[b]	1.0121 ± 0.003	4

[a] Reactions run at 12 mM carbamoyl phosphate and aspartate concentrations ranging from 0 to 270 mM, pH(D) 7.5, 25 °C. [b] Reactions run at 12 mM carbamoyl phosphate and an initial aspartate concentration at 12 mM, pH(D) 7.5, 25 °C. [c] Number of determinations.

are represented graphically and compared with data for the wild-type enzyme in Figure 4.

Since proton transfers are implicated in the catalytic mechanism of ATCase (Turnbull et al., 1992), the effect of substituting deuterium for protium on the ^{13}C isotope effects was determined by measuring isotope effects in D_2O. The observed $^{13}(V/K_{CbmP})_{D_2O}$ value for this reaction at ~260 mM aspartate was 1.0434 ± 0.0004 (Table II).

^{15}N isotope effects for H134A trimer were measured in both H_2O and D_2O (Table II). The observed values for $^{15}(V/K_{asp})_{H_2O}$ and $^{15}(V/K_{asp})_{D_2O}$ were 1.0053 ± 0.0003 and 1.0121 ± 0.0003, respectively.

Discussion

The present investigation has involved a detailed kinetic characterization of the H134A mutant and provides answers to the following questions.

Did the Amino Acid Substitution Affect Catalysis and Binding of Substrates? Replacement of His 134 by alanine clearly alters the binding of substrates and inhibitor, as indicated by a reduction in the binding constant for CbmP by a factor of 50 and decreases in the affinities for aspartate and succinate to one-tenth and one-sixth, respectively, those of wild-type enzyme. In addition, a maximum velocity approaching that observed for the wild-type enzyme is never achieved, indicating that the histidine to alanine substitution affects the chemistry of the reaction.

As a result of the amino acid substitution, the activity of ATCase between pH 6.2 and 9.2 becomes dependent upon the ionization of an additional residue with a pK value between 6.5 and 7.0 that was not observed for the wild-type enzyme (Turnbull et al., 1992). This residue is involved in aspartate binding rather than catalysis, as indicated by its presence in the profiles for $(V/K)_{asp}$ and K_i for succinate, which display binding groups, and its absence in the V profile, which exhibits only catalytic groups. This residue, which must be deprotonated for binding, is likely associated with the enzyme since the pK values of ionizing groups of aspartate (Weast, 1986), CbmP (Allen & Jones, 1964), and succinate (Weast, 1986) lie outside the experimentally accessible pH range. The role of this residue in aspartate binding and its location relative to the active site are not clear; however, we propose two explanations for its appearance. First, the residue could be involved in binding aspartate in both mutant and wild-type

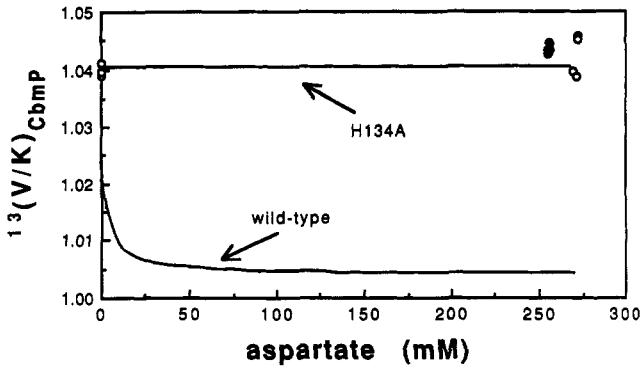


FIGURE 4: ^{13}C isotope effects for the H134A mutant-catayzed reaction at a saturating level (12 mM) of CbmP and varying aspartate concentrations in 50 mM HEPES, 2 mM DTT, and 0.2 mM EDTA, pH (D) 7.5, 25 °C, in H_2O (○) and D_2O (●). Shown for comparison is the corresponding curve for the wild-type catalytic trimer in H_2O (Parmentier et al., 1992b) for which experimental points have been omitted for clarity.

enzymes, but the mutation could have shifted the pK value of the group into the experimentally accessible pH range. Alternatively, the ionization might reflect the participation of a new binding group in the mutant that is not functional in the wild-type enzyme. This residue may be unique to the alanine substitution at position 134 since it was not observed in the V/K profile and succinate binding profile for the H134N mutant (Xi et al., 1990).

Hervé and co-workers noted that the substitution of asparagine for histidine at position 134 also reduces the binding of both aspartate and CbmP but to a lesser degree than the alanine replacement, by factors of 3 and 4, respectively (Xi et al., 1990). However, the maximum velocities of the reaction catalyzed by H134N and H134A were similar, at 4–5% of wild-type enzyme.

Has the H134A Substitution Altered the Kinetic Mechanism? The observation of an inhibition pattern for PALA that is competitive with respect to aspartate together with an intersecting initial velocity pattern demonstrates that the kinetic mechanism has changed from steady-state ordered to random as a result of this substitution. This conclusion is further substantiated by the observation that the $^{13}(V/K_{CbmP})$ is invariant with aspartate concentration, which is consistent with a random mechanism (Cook & Cleland, 1981; Parmentier et al., 1992a).

The predominantly ordered mechanism of the catalytic trimers of wild-type ATCase is due, in part, to the tighter binding of CbmP compared to aspartate (Turnbull et al., 1992). The decrease in CbmP binding observed as a result of the alanine substitution may release the constraint that the tight interaction with CbmP imposes on the ordered addition of substrates resulting in the random mechanism of H134A trimer.

Is the Protonated Form of His 134 Involved in the Catalytic Mechanism? The results from the pH profiles suggest that the protonated form of His 134 does not participate in the catalytic mechanism between pH 6.2 and 9.2. The pK values of residues in the H134A mutant determined from the pH dependence of log V, log (V/K) for aspartate, and the pK_{ia} from CbmP (Figures 2 and 3, Table I) were also displayed in the pH profiles for the wild-type enzyme (Turnbull et al., 1992). The catalytic group (pK value of about 9.5) whose ionization is shown in the V profile for the wild-type enzyme but is absent in both H134A and H134N (Xi et al., 1990) mutant enzymes is unlikely to be His 134, as this would suggest histidine exhibits an elevated pK value in an active site environment enriched with positively charged residues (Krause et al., 1987). The ^{13}C nuclear magnetic resonance studies of the catalytic trimer liganded to PALA also indicated that His 134 does not titrate between pH 6.2 and 8.2 (Kleanthous et al., 1988). Moreover, the pH dependence of the modification of His 134 in ATCase by diethyl pyrocarbonate yielded an estimated pK value of 5.4 (Cole & Yon, 1986). Hence, His 134 may have a depressed pK value which is consistent with its location in an active site enriched with arginine residues and at the amino terminus of an α-helix (Ke et al., 1988). Indeed, there are several examples of enzymes with active site residues that are located at the termini of α-helices where the helix dipole influences the pK value (Hol, 1985). These results do not support the conclusion presented by Hervé and coworkers that His 134 exhibits a pK value as high as 8.2 in the free enzyme and 7.2 in the enzyme–CbmP complex (Xi et al., 1990).

Have the Various Steps in the Enzymatic Reaction Been Altered by the Histidine to Alanine Substitution? A striking observation from the ^{13}C isotope effects is that substitution of alanine for histidine at position 134 appears to make the chemistry of the enzyme-catalyzed reaction completely rate-limiting. The $^{13}(V/K_{CbmP})_{H_2O}$ value increased from 1.0240 ± 0.0005 in the wild-type catalytic trimer (Parmentier et al., 1992a) to 1.0413 ± 0.0011 in the H134A catalytic trimer, which is presumably the ^{13}C intrinsic isotope effect for the reaction. A value of about 1.04 was also obtained using the slow substrate L-cysteine sulfinate, which makes the chemistry of the reaction rate-limiting (Parmentier et al., 1992b). Thus, the studies with the H134A mutant enzyme and the slow substrate L-cysteine sulfinate confirm the conclusion that 1.04 is the intrinsic ^{13}C isotope effect for the chemical reaction catalyzed by ATCase. Furthermore, the insensitivity of $^{13}(V/K_{CbmP})$ to D_2O/H_2O substitution in the mutant, unlike the native catalytic trimer, suggests that the intrinsic ^{13}C isotope effect is being observed (O'Leary, 1989).

The specific chemical step that is rate-determining in the H134A mutant enzyme can be determined by quantitative analysis of the ^{13}C and ^{15}N isotope effects. The overall mechanism of the H134A mutant can be analyzed according to the model shown in Scheme I, as described for the wild-type enzyme (Parmentier et al., 1992b). This model applies to either an ordered or random kinetic mechanism since the substrate binding steps are presumably isotope insensitive. In this model, k_7, k_8, and k_9 are ^{13}C sensitive, k_5, k_6, k_7, k_8, and k_9 are ^{15}N sensitive, k_5 and k_6 are deuterium sensitive, and all commitments are internal (i.e., only steps subsequent to the formation of the Michaelis complex are analyzed). Analysis of the isotope effects for the H134A mutant can be performed as described for the wild-type enzyme (Parmentier et al., 1992b). The appropriate equations are

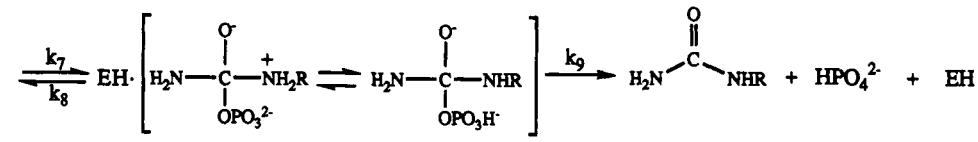
$$^{13}(V/K)_{CbmP} = \frac{^{13}k_7 + ab(1 + c)/(1 + b)}{1 + ab(1 + c)/(1 + b)} \quad (9)$$

$$^{15}(V/K_{asp})_{H_2O} = \frac{^{15}K_{eq5H}{}^{15}K_{eq7}{}^{15}k_9 + {}^{15}K_{eq5H}{}^{15}k_7 b + {}^{15}k_{5H} ab + abc}{1 + b + ab + abc} \quad (10)$$

$$^{15}(V/K_{asp})_{D_2O} = \frac{^{15}K_{eq5D}{}^{15}K_{eq7}{}^{15}k_9 + {}^{15}K_{eq5D}{}^{15}k_7 b + {}^{15}k_{5D} ab + abc}{1 + b + ab + abc} \quad (11)$$

where $a = k_7/k_6$, $b = k_9/k_8$, and $c = k_5/k_4$. The same assumptions made for the wild-type enzyme are used in analyzing the data for the H134A mutant. These assumptions are as follows: $^{13}k_8 = {}^{13}k_9$, $^{15}K_{eq5H} = 1.016$, $^{15}K_{eq5D} = 1.0201$, $^{15}K_{eq7}$

Scheme I



= 0.970, $^{15}k_9$ = 1.0137, $^{15}k_7$ = 1.0, and $^{15}k_{5H(D)} = {}^{15}K_{eq5H(D)}$ [see Parmentier et al. (1992b) for a discussion of the rationale for these assumptions].

For the H134A mutant, D_2O causes a 0.68% increase in the observed value of $^{15}(V/K_{asp})$. This change presumably results from the fractionation factor difference between nitrogen bonded to protium and deuterium which appears to be 0.4% per hydrogen (Parmentier et al., 1992b; Hermes et al., 1985). During the steps leading up to the actual attack of aspartate on carbamoyl phosphate, a proton is removed from the nitrogen of aspartate and thus a NH_3^+ group changes to NH_2. In D_2O the change is from ND_3^+ to ND_2, and because of the extra deuterium in the ND_3^+ group, the equilibrium constant for this step will decrease by 1.004. While the measured change of 0.68% is larger than the 0.41% difference in ^{15}N equilibrium isotope effects measured for the wild-type enzyme (Parmentier et al., 1992b), probably the result of experimental errors, the value suggests that for the H134A mutant the steps leading to catalysis have come fully to equilibrium. Thus, the parameter c must approach zero. Since the full ^{13}C intrinsic isotope effect is expressed with the H134A mutant, the commitment term a must also approach zero. Therefore, for the H134A mutant where a and c are both zero, eq 10 becomes

$$^{15}(V/K_{asp})_{H_2O} = 1.0053 = \frac{1.016(0.983 + b)}{1 + b} \quad (12)$$

Solving this equation yields a value of $b = 0.62 \pm 0.03$.

Mechanistically, these calculations indicate that formation of the tetrahedral intermediate (reflected by term a, k_7/k_6) has become rate-limiting for the H134A mutant. Once formed, however, the tetrahedral adduct partitions in a manner similar to that seen with the wild-type enzyme, as evidenced by the similar value of b (k_9/k_8), which is 0.30 ± 0.11 for the wild-type catalytic trimer (Parmentier et al., 1992b).

Possible Roles for His 134 in the Catalytic Mechanism. The results presented in this investigation together with the studies of Ke et al. (1988), Kleanthous et al. (1988), and Cole and Yon (1986) suggest that His 134 exists in the imidazole rather than the imidazolium form over the pH range in which the enzyme is active. The finding that the formation of the tetrahedral intermediate is rate-limiting when His 134 is removed, however, implies that this residue does play an important role in the chemistry of the reaction.

There are two possible mechanisms by which the imidazole form of H134 could participate in the chemical mechanism. First, the neutral form of His 134 could interact with the carbonyl group of CbmP by either forming a strong hydrogen bond or completely protonating the carbonyl oxygen. The resulting anionic imidazole could be stabilized by the electronic environment of the active site and by a positive charge generated through a helix dipole since His 134 is located at the amino terminus of helix 12 in the catalytic chain (Ke et al., 1988). In accord with this hypothesis, the pK value of the neutral imidazole (normally around 14) could be markedly depressed by the helix dipole effect, facilitating protonation of the carbonyl group of CbmP. This system is analogous to that of triose phosphate isomerase (Knowles, 1991). Knowles and co-workers concluded that the imidazole form of a histidine can function as an electrophile to polarize the carbonyl group of dihydroxyacetone phosphate or glyceraldehyde 3-phosphate (Komives et al., 1991). As in the case of ATCase, the ionization of the electrophilic histidine in triose phosphate isomerase was not displayed in the pH–rate profiles (Plaut & Knowles, 1974), and nuclear magnetic resonance studies indicated that the histidine has a pK less than 4.5 (Lodi & Knowles, 1991), which is consistent with the location of this residue at the amino terminus of an α-helix (Alber et al., 1981). As a corollary to the above hypothesis, His 134 may polarize the carbonyl group of CbmP indirectly through a water molecule. This idea is in accord with the crystallographic studies of PALA-liganded ATCase (Krause et al., 1987) which indicate that the active site contains 10–15 water molecules.

An interaction of His 134 solely with the carbonyl group of CbmP, however, is inconsistent with the reduction in affinities for both substrates produced by the alanine substitution at position 134. As a second possible mechanism, the presence of His 134 may promote an active site conformation that is optimal for CbmP and aspartate binding and align the substrates favorably for catalysis. A high-resolution crystal structure of the H134A mutant would be instrumental in distinguishing between these two possibilities.

This detailed kinetic analysis of the H134A mutant has narrowed down the possible roles of this histidine residue in catalysis. However, analyses of other mutant enzymes with replacements at position 134, using the techniques described in this study, plus structural information of the mutants are required to define the precise function of His 134.

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