Ionization of Amino Acid Residues Involved in the Catalytic Mechanism of Aspartate Transcarbamoylase†

Joanne L. Turnbull, Grover L. Waldrop,‡ and H. K. Schachman*

Department of Molecular and Cell Biology and the Virus Laboratory, Wendell M. Stanley Hall, University of California, Berkeley, California 94720

Received January 21, 1992; Revised Manuscript Received April 20, 1992

ABSTRACT: The chemical and kinetic mechanisms of the reaction catalyzed by the catalytic trimer of aspartate transcarbamoylase have been examined. The variation of the kinetic parameters with pH indicated that at least four ionizing amino acid residues are involved in substrate binding and catalysis. The pH dependence of K_{ia} for carbamoyl phosphate and the K_i for *N*-(phosphonoacetyl)-L-aspartate revealed that a protonated residue with a p*K* value of 9.0 is required for the binding of carbamoyl phosphate. However, the variation with pH of K_i for succinate, a competitive inhibitor of aspartate, and for cysteine sulfinate, a slow substrate, showed that a single residue with a p*K* value of 7.3 must be protonated for binding these analogues and, by inference, aspartate. The profile of log *V* against pH displayed a decrease in reaction rate at low and high pH, suggesting that two groups associated with the Michaelis complex, a deprotonated residue with a p*K* value of 7.2 and a protonated group with a p*K* value of 9.5, are involved in catalysis. By contrast, the catalytically productive form of the enzyme–carbamoyl phosphate complex, as illustrated in the bell-shaped pH dependence of log $(V/K)_{asp}$, is one in which a residue with a p*K* value of 7.0 must be protonated while a group with a p*K* value of 9.1 is deprotonated. This interpretation is supported by the results from the temperature dependence of the *V* and *V*/*K* profiles and from the pH dependence of pK_i for the aspartate analogues. The pK_i profiles further indicated that the protonated residue (p*K* value of 7.0) is involved in binding aspartate while the deprotonated group (p*K* value of 9.1) participates in catalysis. Based on the results of this investigation, a model is proposed for catalysis by aspartate transcarbamoylase. Isotope trapping studies provide evidence that the kinetic mechanism of the reaction is steady-state ordered with some random character. In addition, these studies along with the results of pH profiles demonstrate that carbamoyl phosphate is a sticky substrate from the binary and ternary complexes while aspartate is not sticky.

Aspartate transcarbamoylase (ATCase,[1] carbamoyl phosphate:L-aspartate carbamoyltransferase, EC 2.1.3.2) catalyzes the carbamoylation of the amino group of aspartate by carbamoyl phosphate (CbmP) in the first committed step of pyrimidine biosynthesis in *Escherichia coli*. The reaction has been shown to proceed via an ordered mechanism in which CbmP binds prior to aspartate followed by the release of carbamoyl aspartate and phosphate (Porter et al., 1969; Parmentier et al., 1992a). The enzyme exists as an oligomer (310 kDa) composed of 12 polypeptide chains organized as two catalytic trimers (100 kDa each) and three regulatory dimers (34 kDa each). Treatment of ATCase with mercurials leads to dissociation of the enzyme into catalytic and regulatory subunits (Gerhart & Schachman, 1965). The activity of the holoenzyme is modulated by both CbmP and aspartate; binding of substrates promotes a conformational change in the enzyme from the low activity (T) state to the highly active (R) state which is manifested kinetically in sigmoidal saturation curves for both substrates (Gerhart & Pardee, 1962; Bethel et al., 1968). The holoenzyme is also subject to allosteric control through interaction of the regulatory subunits with the activator, ATP, and the inhibitor, CTP (Schachman, 1988). In addition, the bisubstrate analogue *N*-(phosphonoacetyl)-L-aspartate (PALA) promotes the allosteric transition (Collins & Stark, 1971; Howlett & Schachman, 1977) and binds in a cooperative fashion to the catalytic chains of the holoenzyme (Newell et al., 1989). In contrast to the holoenzyme, the isolated catalytic trimer conforms to Michaelis–Menten kinetics (Gerhart & Schachman, 1965) and consequently has been the model for steady-state kinetic studies on the catalytic mechanism of ATCase.

The enzymatic reaction of CbmP with aspartate is thought to proceed by addition–elimination with the formation of a tetrahedral intermediate and, as such, would require the participation of acidic and basic catalytic groups (Jacobson & Stark, 1973; Gouaux et al., 1987; Waldrop et al., 1992a,b). Acidic groups on the enzyme may serve to polarize the carbonyl oxygen of CbmP, facilitating a nucleophilic attack by the α-amine of aspartate, and, in addition, may protonate a phosphate oxygen of CbmP, rendering phosphate a better leaving group. Enzyme groups functioning as general base catalysts could participate by deprotonating the attacking amine and by transferring a second proton from the α-amino group of aspartate to the leaving phosphate group. A mechanism involving acid–base chemistry is further supported by the kinetic studies of Leger and Hervé (1988) which have

† This research was supported by National Institute of General Medical Sciences Research Grant GM 12159, National Science Foundation Research Grant DMB 9020605, and American Cancer Society Grant BC 659 (to H.K.S.), by a University of California Cancer Research Coordinating Committee Grant (to J.L.T.), and by Postdoctoral Fellowship DRG 996 (to G.L.W.) from the Damon Runyon–Walter Winchell Cancer Research Fund.

* To whom correspondence should be addressed.

‡ Present address: Institute for Enzyme Research, University of Wisconsin, 1710 University Ave., Madison, WI 53705.

[1] Abbreviations: ATCase, aspartate transcarbamoylase; CbmP, carbamoyl phosphate; PALA, *N*-(phosphonoacetyl)-L-aspartate; MES, 2-(*N*-morpholino)ethanesulfonic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; EDTA, ethylenediaminetetraacetic acid.

0006-2960/92/0431-6562$03.00/0

demonstrated that substrate binding and catalysis are dependent on the protonation states of amino acid residues associated with the catalytic trimer. The identities of these proposed catalytic groups have not been determined, although structural information from crystallographic studies of ATCase liganded to PALA (Krause et al., 1987) along with the results of site-directed mutagenesis studies (Stevens et al., 1991) have implicated a number of residues. In addition, there is little information regarding the relative rates of the various chemical steps in the ATCase-catalyzed reaction.

The present investigation, the first in a series of reports examining the chemical, kinetic, and allosteric properties of ATCase, explores the roles that acidic and basic amino acid side chains play in the catalytic mechanism of ATCase. For this purpose, the variations with pH of the kinetic parameters for the reaction catalyzed by the catalytic trimer were determined. The results extend the findings of Leger and Hervé (1988) and draw attention to an alternative interpretation of the data. In addition, the kinetic mechanism has been investigated further using isotope trapping studies to determine the rate constants for the interactions of CbmP with the trimer. The reports that follow in this issue describe how ^{13}C and ^{15}N isotope effect studies have been employed to probe the kinetic and allosteric mechanisms of ATCase and to determine the relative rates of the chemical steps in the reaction. Furthermore, the roles of two amino acid residues implicated in the catalytic mechanism of ATCase have been more narrowly defined using steady-state kinetics in conjunction with isotope effect studies of two mutant forms.

Experimental Procedures

Materials

L-Aspartic acid was supplied by Calbiochem, and succinic acid was purchased from Mallinckrodt. Cysteine sulfinic acid and dilithium CbmP were obtained from Sigma. Aliquots of a stock solution of CbmP in water, adjusted to pH 8.0 with KOH, were stored at −20 °C and thawed prior to use. [^{14}C]CbmP (12.5 mCi/mmol) was supplied by New England Nuclear, and [^{14}C]aspartate (216 mCi/mmol) was obtained from Amersham. 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. Structures of the substrates and various analogues are shown in Figure 1.

ATCase was isolated from a strain of *E. coli* TR4363 (his$^-$ pyrF701) which contains the multicopy plasmid pPYRB3 (Navre & Schachman, 1983) carrying the intact *pyr*B–*pyr*I operon (Pauza et al., 1982). The enzyme is overproduced 200-fold when the bacteria are grown in a minimal medium (M56) with limiting amounts of uracil (4 μg/mL). ATCase was purified according to the procedure of Gerhart and Holoubek (1967). Catalytic trimer was prepared from ATCase as described by Yang et al. (1978) and could be stored at 4 °C as a precipitate in 3.6 M ammonium sulfate (~pH 7.0) for 2 months without loss of activity. The concentration of the trimer was determined spectrophotometrically using a value of $E^{0.1\%}_{280nm} = 0.72$ (Gerhart & Holoubek, 1967).

Methods

Determination of Enzyme Activity. ATCase activities were measured at 30 °C using the radioactive stopped-time assays of Davies et al. (1970) or Porter et al. (1969) in a three-component buffer system of 0.1 M 2-(*N*-morpholino)ethanesulfonic acid (MES), 0.51 M *N*-ethylmorpholine, and 0.51 M diethanolamine which also contained 0.2 mM EDTA and 2 mM β-mercaptoethanol. Over the pH range of 6.0–10.5, for which the initial velocities were measured, the ionic strength of the buffer mixture remained constant at a value of 0.1 (Ellis & Morrison, 1982). When high concentrations of substrate analogues were utilized, varying amounts of potassium acetate were added to the reaction mixture to correct for any changes in ionic strength. Corrections were also made to account for inhibition by the salt. The pH of the buffer system was adjusted to the appropriate value with KOH or acetic acid.

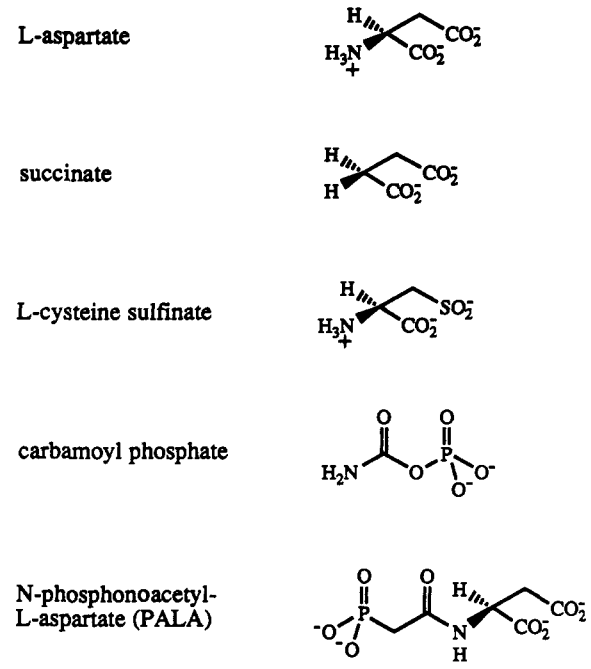


FIGURE 1: Structures of substrates and various active site analogues.

For experiments conducted at 30 °C, the assay mixtures were preincubated for 2 min in a shaking water bath prior to initiation of the reaction with CbmP. When activity was measured at other temperatures, the mixtures were preincubated for 5 min at the given temperature. Under the assay conditions used in this study, the velocity was a linear function of the enzyme concentration, and the method of Selwyn (1965) was used to establish the absence of time-dependent inactivation of the enzyme. The pH of the assay mixture did not change before and after the reaction. 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 Trapping Studies. A solution (0.2 mL) containing 2.6 μM catalytic trimer and 80 μM [^{14}C]CbmP was added to 2 mL of a rapidly stirring solution of 100 mM CbmP and either 4, 7, 10, 20, or 30 mM aspartate. The buffer for both solutions contained 50 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS), 0.2 mM EDTA, and 2 mM β-mercaptoethanol (pH 7.5). The reaction was stopped after 3 s by the addition of 0.5 mL of 2 N acetic acid. The mixture was incubated at 80 °C for 2 h to remove unreacted [^{14}C]CbmP, and the amount of [^{14}C]carbamoyl aspartate in the residual solution (0.5 mL) was determined by liquid scintillation counting. A control was performed at each aspartate concentration by adding 0.2 mL of 2.6 μM catalytic trimer to a reaction mixture of 2 mL containing aspartate, unlabeled CbmP, and [^{14}C]-CbmP and then proceeding with the reaction as described above. The amount of [^{14}C]carbamoyl aspartate detected in the control reaction was subtracted from the experimental value.

Table I: Values of pK and pH-Independent Kinetic Parameters for the Reaction Catalyzed by the 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})	88 ± 10	7.03 ± 0.14	9.13 ± 0.10	
	V (s^{-1})	680 ± 80	7.16 ± 0.11	9.51 ± 0.09	
succinate varied; CbmP saturating	K_i (mM)	0.33 ± 0.02		7.32 ± 0.08	
cysteine sulfinate varied; CbmP saturating	Y_L (M^{-1})	33.3 ± 4.4		7.32 ± 0.08	
	Y_H (M^{-1})	3.06 ± 0.08			
CbmP varied; aspartate nonsaturating	K_{ia} (μM)	7.16 ± 0.31		9.01 ± 0.05	
PALA varied; aspartate varied with CbmP fixed	K_i (nM)	7.23 ± 1.40		8.51 ± 0.07	8.51 ± 0.07

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 substituted in eq 1 to yield values for the maximum velocity

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

(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 concentration of a competitive inhibitor (I) was varied at a fixed concentration of substrate, the data at each pH value were fit to eq 2 to yield a value for the apparent inhibition constant ($K_{i\,app}$).

$$v = V_{app}/[(1 + I/K_{i\,app})] \quad (2)$$

The true inhibition constant (K_i) was determined using the relationship

$$K_i = K_{i\,app}/(1 + A/K) \quad (3)$$

where A is the fixed substrate concentration and K denotes the Michaelis constant for that substrate. Velocity data that conformed to noncompetitive inhibition were fit to

$$v = VA/[K(1 + I/K_{is}) + A(1 + K_{ii})] \quad (4)$$

The concentration of substrate (A) and inhibitor (I) were varied to obtain values for apparent inhibition constants K_{is} and K_{ii}. The variations with pH of the values for V, V/K, $1/K_i$, and $1/K_{ia}$ (where K_{ia} is the dissociation constant of the enzyme–CbmP complex) were fit to the appropriate equations:

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

$$\log y = \log [C/(1 + K_2/[H^+])] \quad (6)$$

$$\log y = \log [C/(1 + K_2/[H^+] + K_2K_3/[H^+]^2)] \quad (7)$$

$$\log y = \log [(Y_L + Y_HK/[H^+])/(1 + K/[H^+])] \quad (8)$$

In these equations y represents the value 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, K_2, and K_3 are acid dissociation constants associated with ionizing groups on the acid limb (K_1) and alkaline limb (K_2 and K_3) of pH profiles. Y_L and Y_H denote limiting, pH-independent values of the velocity at low and high pH, respectively. Apparent pK values obtained at different temperatures (T) were fit to eq 9 to determine values

$$pK = \Delta H_{ion}/(2.303RT) + B \quad (9)$$

for the enthalpy of ionization (ΔH_{ion}). R is the gas constant, 1.987 cal mol^{-1} deg^{-1}.

Results

pH Dependence of V and V/K for Aspartate. The effect of pH on the ATCase-catalyzed reaction was determined over the pH range of 6.0–10.5 by varying aspartate concentrations at a fixed level of CbmP (5 mM) which was saturating at each pH. Under these conditions, the reaction examined was that of aspartate with the enzyme–CbmP complex. As seen in Figure 2, log $(V/K)_{asp}$ decreases at both high and low pH values, indicating that the protonation states of two residues associated with the binary complex or aspartate are important for binding and/or catalysis. The data in Figure 2 also demonstrate a bell-shaped dependence of log V on pH, which suggests that two ionizing groups in the Michaelis complex are important for catalysis. The fit of the data to eq 5 yielded pK values of 7.03 ± 0.14 and 9.13 ± 0.10 for residues in the V/K profile and values of 7.16 ± 0.11 and 9.51 ± 0.09 for groups in the V profile (Table I). The pK values obtained from the V/K profile are in agreement with those previously determined (Leger & Hervé, 1988). In contrast, Leger and Hervé reported that the variation of log V with pH was a half-bell with a limiting slope of +1 at low pH. Presumably a bell-shaped profile was not obtained by these authors since the pK value of the second ionizing reaction (pK of about 9.5) lay outside the limits of the experimental pH range.

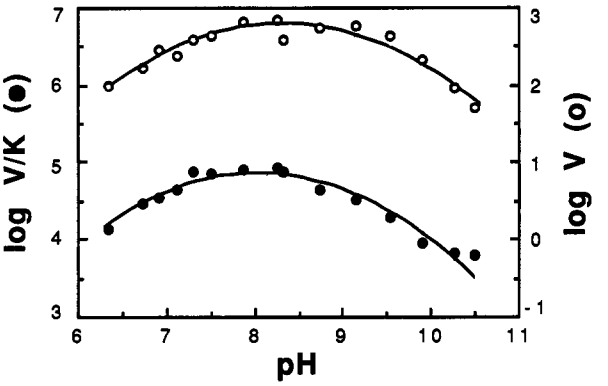


FIGURE 2: Variation with pH of log V and log $(V/K)_{asp}$ for the reaction catalyzed by the catalytic trimers of ATCase. Initial velocity data were obtained by varying aspartate concentrations at 5 mM CbmP. The curves for V (○) and V/K (●) represent best fits of the data to eq 5. The values of the parameters 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.

The temperature dependence of log V and log V/K was determined to investigate the nature of groups whose ionization states affect the activity of ATCase. pH profiles were measured at 18, 30, and 40 °C (data not shown), and the various sets of data were fit to eq 5 to yield the pK values listed in Table II. From plots of the values of pK vs $1/T$, a series of lines were obtained (data not shown) and analyzed by using eq 9 to yield values for ΔH_{ion}, the enthalpy of ionization (Table II). The results indicate that the residue with a pK value of 7.0 in the V/K profile has a ΔH_{ion} of about 14 kcal/mol, which is consistent with a value for a cationic acid (Cleland, 1977). In addition, the group with pK 9.5 in the V profile exhibits a ΔH_{ion} of about 33 kcal/mol. A value of this magnitude suggests that this ionization is accompanied by a conformational change in the protein (Cleland, 1977). The ionizations of groups with pK values of 7.2 in the V profile and 9.1 in the V/K were essentially independent of temperature indicative of residues that are neutral acids.

Table II: Values of pK's and ΔH_{ion} for the Reaction at Various Temperatures

profile	parameter	temperature (°C) 18	30	40	ΔH_{ion} (kcal/mol)
V	pK_1	7.19 ± 0.05	7.25 ± 0.07	7.37 ± 0.07	−3.3 ± 0.8
V	pK_2	10.6 ± 0.15	9.35 ± 0.14	8.85 ± 0.11	33 ± 5
V/K	pK_1	7.56 ± 0.10	7.27 ± 0.13	6.83 ± 0.12	14 ± 3
V/K	pK_2	8.52 ± 0.10	8.88 ± 0.16	8.56 ± 0.11	−1.3 ± 7

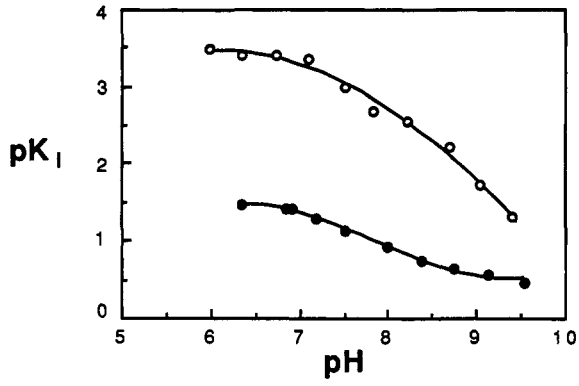


FIGURE 3: Variation with pH for pK_i of succinate and cysteine sulfinate. Initial velocity data were obtained at 5 mM CbmP by varying succinate or cysteine sulfinate concentrations at fixed levels of aspartate. The concentration of aspartate was 15 mM when varying succinate and 5 mM when varying cysteine sulfinate. The curve for inhibition by succinate (○) represents the fit of the data to eq 6 while the curve for inhibition by cysteine sulfinate (●) illustrates the best fit of the data to eq 8. Values of the parameters used to draw the curves are given in Table I. The units for K_i are M.

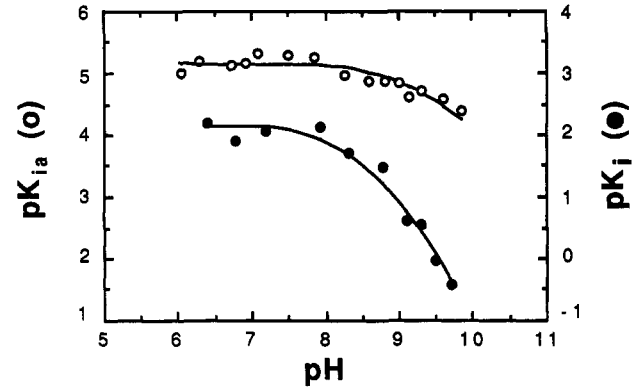


FIGURE 4: Variation with pH of pK_{ia} for CbmP and pK_i for PALA. Initial velocity data for K_{ia} were obtained by varying CbmP concentrations at 0.1 mM aspartate, and velocity data for K_i were obtained by varying the concentration of aspartate at 5 mM CbmP and fixed concentrations of PALA. The velocity data for inhibition by PALA were fit to eq 4, and from the values of K_{is}, the true K_i values were calculated using the relationship $K_i = K_{is}/\{1 + [\text{CbmP}]/(K_{ia}$ for CbmP)$\}$. The curve for the K_{ia} for CbmP (○) represents the best fit of the data to eq 6 while the curve for inhibition by PALA (●) illustrates the fit to eq 7. Curves were drawn by using the parameter values given in Table I. The units for K_{ia} and K_i are M.

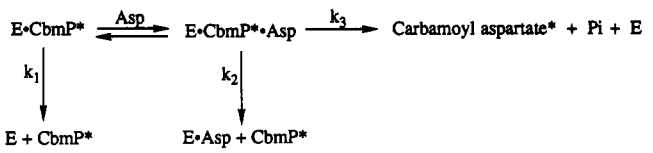
pH Dependence of Binding of Aspartate Analogues. To determine whether the pK values observed in the V/K profile were true or apparent values, we measured the effect of pH on the interaction of succinate with the enzyme–CbmP complex. Succinate, which functions as a competitive inhibitor with respect to aspartate (Porter et al., 1969), gave rise to a pK_i profile over the pH range of 6.0–9.6 that was a half-bell with a limiting slope of −1 at high pH (Figure 3). The data were fit to eq 6 to yield a pK value of 7.32 ± 0.08 (Table I) for the group that must be protonated for binding succinate. A similar pK value has also been reported by Porter et al. (1969) and Leger and Hervé (1988).

The pH dependence of the inhibition constant for cysteine sulfinate was examined to determine if binding is influenced by the ionization state of the α-amino group of aspartate. Cysteine sulfinate (Figure 1) is a substrate analogue that reacts at 2% of the maximum velocity of aspartate (Foote et al., 1985) and therefore can be regarded as a dead-end inhibitor of the reaction (Spector & Cleland, 1981). The data in Figure 3 were fit to eq 8, indicating that the deprotonation of a group on the enzyme or inhibitor with a pK value of 7.32 ± 0.08 causes a reduction in the binding of cysteine sulfinate by an order of magnitude but does not totally prevent binding (Table I). Since the residue can be titrated in the enzyme–inhibitor complex, as demonstrated by a plateau in the curve at high pH (Figure 3), the data also show that the binding of cysteine sulfinate shifts the pK value of the group upward by >2 pH units. Furthermore, this residue must be associated with the enzyme as ionizing groups on the substrates and inhibitor do not exhibit pK values of about 7. It should be noted that the pK values determined from the pK_i profiles for cysteine sulfinate and succinate were identical, indicating that the same group on the enzyme must interact with both inhibitors. However, cysteine sulfinate binds two orders of magnitude more weakly to the protonated residue than does succinate (Table I).

pH Dependence of Binding Carbamoyl Phosphate and PALA. 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 initial velocities were measured by varying CbmP concentrations at a fixed aspartate concentration that was 2% of the Michaelis constant for aspartate. Thus, the apparent Michaelis constant that is determined for CbmP is equal to the K_{ia} (Porter et al., 1969; Cleland, 1986). Over the pH range of 6.0–9.6, the profile of pK_{ia} against pH was a half-bell with binding decreasing with increasing pH (Figure 4). The data were fit to eq 6 to yield a pK value of 9.0 ± 0.05 (Table I) for the group that must be protonated for binding CbmP.

In contrast to the result obtained from the pK_{ia} profile, Leger and Hervé (1988) examined the effects of pH on the binding of a series of CbmP analogues (phosphonoacetate, phosphonoacetamide, and pyrophosphate) and concluded that a group with a pK value of 8.2 must be deprotonated for the interaction of CbmP with the catalytic trimer. In light of this discrepancy, the pH dependence of the binding of PALA was examined. PALA, a bisubstrate analogue (Figure 1) that spans both the CbmP and aspartate binding sites, inhibits competitively with respect to CbmP and noncompetitively with respect to aspartate (Collins & Stark, 1971). In an ordered kinetic mechanism in which CbmP binds first, the aforementioned inhibition pattern can arise if CbmP and PALA compete for the same form of the enzyme while PALA and aspartate compete for different enzyme forms (Spector & Cleland, 1981). Over a pH range of 6.4–10.0, the profile of pK_i against pH was a half-bell with a limiting slope of −2 at high pH (Figure 4), indicating that the binding of PALA requires two enzymic residues in their protonated form. Since PALA spans the CbmP and aspartate binding sites, these two groups may arise from ionizing residues involved in the binding of both

Scheme I



substrates. Fitting the data to eq 7 yielded a pK value of 8.56 ± 0.07 (Table I) which was an average for the two residues whose pK values differed by <2 pH units. Thus, the results obtained for the pH dependence of the binding of CbmP and PALA were in agreement but differed from those reported by Leger and Hervé (1988) for the CbmP analogues.[2]

Isotope Trapping Studies. The partitioning of [^{14}C]CbmP between reaction with aspartate and release from the enzyme was examined using the isotope trapping method of Rose et al. (1974) to determine if CbmP is a sticky substrate in the binary and ternary complexes with the enzyme (i.e., undergoes reaction as rapidly or more rapidly than it dissociates from the enzyme). In a small volume, known amounts of labeled CbmP and catalytic trimer were incubated together to form a binary complex. This solution was added, with rapid mixing, into a larger volume containing a large excess of unlabeled CbmP and a variable level of aspartate. The reaction was stopped after several seconds and the amount of labeled product was determined. As illustrated in Scheme I, labeled CbmP in a binary complex with enzyme can either dissociate or form a ternary complex with aspartate. Similarly, labeled CbmP in the ternary complex can dissociate, if there is any degree of randomness in the kinetic mechanism, or it can react with aspartate to form products. The constants k_1 and k_2 represent the rate of dissociation of CbmP from the binary and ternary complexes, respectively, while k_3 represents the rate constant for the maximum velocity of the enzyme. Since any labeled CbmP that dissociates is diluted by the large excess of unlabeled CbmP, the only parameter examined is the partitioning of CbmP in the binary complex in the first turnover of the enzyme as a function of the concentration of aspartate. The amount of labeled product formed (v) at each aspartate concentration (A) was fit to eq 1 to obtain values for the maximum amount of labeled CbmP trapped as product (V) and the concentration of aspartate required for half-maximal trapping (K). These values along with those of the kinetic parameters listed below were substituted into eqs 10 and 11, derived on the basis of Scheme I (Cleland, 1977).

$$(K'_{asp}/K_{asp})[(E_t/[\text{carbamoyl aspartate*}]_{max})/(1 + K_{ia}/[\text{CbmP*}])] \geq k_1/k_3 \geq K'_{asp}/K_{asp} \quad (10)$$

$$k_2/k_3 = (E_t/[\text{carbamoyl aspartate*}]_{max})/(1 + K_{ia}/[\text{CbmP*}]) - 1 \quad (11)$$

This analysis yielded values for k_1/k_3 and k_2/k_3, which indicate the degree of stickiness of CbmP in the binary and ternary complexes, respectively. In analyzing the data according to eqs 10 and 11, the following values were used: the concentration of active sites, E_t = 8 μM; the maximum concentration of labeled product formed, [carbamoyl aspartate*]$_{max}$ = 5.8 μM; the concentration of free CbmP, [CbmP*] = 72 μM; K_{ia} for CbmP = 8 μM; K_m for aspartate, K_{asp} = 8.1 mM; and the Michaelis constant for trapping, K'_{asp} = 12.7 mM. Analysis of the data produced values of $1.9 \geq k_1/k_3 \geq 1.6$ and 0.23 for the expression k_2/k_3. In addition, multiplication of each of these ratios by the observed k_3 (660 s^{-1}) yielded values of 1290 s^{-1} for k_1 and 160 s^{-1} for k_2.

Discussion

Interpretation of pH Profiles. The results of the pH dependence of log V and log (V/K) for aspartate (Figure 2) indicate that two groups associated with each profile are important for maximum activity. However, the ionization states of the residues cannot be determined solely from the bell-shaped nature of the profiles (Cleland, 1977; Stone & Morrison, 1983). Although it is generally assumed that the decreases in enzyme activity at low and high pH derive from residues that must be deprotonated and protonated, respectively, it is important to note that a bell-shaped pH profile can also arise if the same groups are protonated and deprotonated, respectively. The major difference between the two interpretations is that the latter places a lower percentage of the enzyme population in the catalytically productive form. The phenomenon in which the group with the lower pK value is protonated while the group with the higher pK value is deprotonated has been termed reverse protonation by Cleland (1977) and is well documented in several enzyme systems including fumarase (Brant et al., 1963), phenylalanine ammonia lyase (Hermes et al., 1985), and glutamine synthetase (Colanduoni et al., 1987).

Evidence for the catalytically productive form of the enzyme–CbmP complex, as seen in the V/K profile for ATCase, is obtained from the pH dependence of the inhibition constants for competitive inhibitors of aspartate. The pK_i profiles for succinate and cysteine sulfinate (Figure 3) show clearly that the binding of the inhibitory substrate analogues and, by inference, aspartate involves a single residue with a pK value of 7.32 that must be protonated. The same residue also has a pK value of about 7 in the V/K profile (Figure 2), which displays both binding and catalytic groups, and it is the protonated form of the group which is important for maximal activity. Furthermore, the bell-shaped curve of the V/K profile dictates that the binary complex must also harbor a deprotonated residue with a pK value of 9.13. At pH 8.4 where the enzyme is most active, about 10% of the population of each of the two ionizing residues contributes to a productive binary complex. As a result, only about 1% of the enzyme is in the correctly protonated state for turnover. This finding is consistent with a value for the second-order rate constant for the binding of aspartate with the enzyme–CbmP complex of 8.8 × 10^4 s^{-1} M^{-1}, which is approximately 0.1% of the diffusion-limiting rate.

[2] The conclusion of Leger and Hervé (1988) that a residue on the enzyme with a pK of about 8.2 must be deprotonated for the binding of CbmP is based on the observation that the pH dependence of phosphonoacetic acid binding showed that a group with a pK of about 8.2 must be deprotonated. Leger and Hervé concluded this pK was not the phosphate oxygen of phosphonoacetic acid (pK 8.08) because they observed that the binding of pyrophosphate, which has a phosphate oxygen with a pK of 8.2, was pH independent. An alternative explanation begins with the observation that the phosphate oxygens of CbmP have pK values of 1.1 and 4.9; therefore, CbmP most likely binds as the dianion over the experimentally accessible pH range of 6–10.5. By inference, all that is needed for the binding of pyrophosphate or phosphonoacetic acid is that two oxygens of the phosphate group (for pyrophosphate it could be either phosphate group) must be ionized for binding to the enzyme. In the case of pyrophosphate, 3 out of the 4 pK values for the phosphate oxygens are below pH 6; thus, one of the phosphate groups of pyrophosphate will have both oxygens ionized from pH 6 to 10.5. Since this is all that is required for binding to the CbmP binding site, whether or not the oxygen with pK 8.2 is ionized is irrelevant (i.e., pyrophosphate will bind equally well if the oxygen with pK 8.2 is protonated or ionized); hence, binding is pH independent. Similarly, phosphonoacetic acid must have both phosphate oxygens ionized to bind to the enzyme; however, one of the phosphate oxygens has a pK of 8.08, which is observed experimentally. Thus, the group with pK of about 8.2 that must be deprotonated for phosphonoacetic acid binding is in fact the phosphate oxygen of phosphonoacetic acid with pK 8.08.

Further evidence in support of reverse protonation of ionizing residues in the binary complex is obtained from experiments indicating that aspartate is not a sticky substrate, meaning that the binding of substrate to the enzyme is at equilibrium relative to the catalytic step. According to Cleland (1977), the pH dependence of either V/K in the presence of a nonsticky substrate or of pK_i for a competitive inhibitor will yield the true pK values of ionizing residues in these profiles. If aspartate is not a sticky substrate, then the pK values of 7.03 and 9.13 displayed in the $(V/K)_{asp}$ profile (Figure 2) will represent the intrinsic values. However, if aspartate is sticky, the true pK value of 7.32 for the protonated residue as seen in the pK_i profiles (Figure 3) may be displaced upward to an apparent value of about 9 in the V/K profile. The correct interpretation is derived from the V/K profiles obtained with the slow substrate cysteine sulfinate (Foote et al., 1985) and with a relatively inactive mutant of ATCase (H134A) (Waldrop et al., 1992a). In both of these cases the substrate is not sticky. These profiles displayed bell-shaped curves with pK values of about 7 and 9 for the ionizing residues. Since these values are intrinsic and approximate those obtained from the $(V/K)_{asp}$ profile for the wild-type enzyme, it follows that aspartateis not a sticky substrate. Thus, the aspartate binding group exhibits a true pK value of 7.03 in the V/K profile, but only a small percentage of the enzyme population harbors the residue in its protonated form.

The results of the temperature dependence studies suggest that the productive form of the enzyme in the Michaelis complex, as represented in the V profile (Figure 2), contains residues with pK values of 7.16 and 9.51 that must be deprotonated and protonated, respectively. The ionization of residues with pK values of 9.13 in the V/K profile and 7.16 in the V profile is independent of temperature (Table II). Thus, the most simple interpretation is that a single residue is titrated in both profiles but exhibits different pK values in the binary and Michaelis complexes. Since it is the deprotonated form of the residue with a pK value of 9.13 which is catalytically productive in the binary complex (V/K profile), the same residue must also be deprotonated in the Michaelis complex (V profile), but shows a pK value of 7.16. Consequently, the residue with a pK value of 9.51 in the Michaelis complex must be protonated for maximal activity.

Ionizing residues observed in the V/K profile can participate in binding and/or catalysis (Cleland, 1977). However, the residue with a pK value of 7.03 in the V/K profile (Figure 2) is likely involved in binding aspartate since its ionization is observed in the pK_i profiles for both succinate and cysteine sulfinate (Figure 3). In contrast, the residue with a pK value of 9.13 is not required for binding either inhibitory aspartate analogue, indicating that this group participates in catalysis. Furthermore, the absence of this residue in the pK_i profile for cysteine sulfinate, which also has an α-amino group similar to aspartate, suggests that the group with a pK value of 9.13 is associated with the enzyme and is not the α-amino group of the substrate. The variation of log V with pH (Figure 2) indicates that the residues with pK values of 7.16 and 9.51 are involved in catalysis. In addition, the temperature dependence studies suggest that the pK value of a catalytic group is shifted from 9.13 in the enzyme–CbmP complex (V/K profile) to 7.16 upon the binding of aspartate (V profile).

The pH dependence of the pK_{ia} for CbmP and the pK_i for PALA suggests that a protonated residue on the enzyme with a pK value of about 9.0 is involved in the binding of CbmP. The binding of CbmP to ornithine transcarbamoylase appears similar to that to ATCase in that a residue with a pK value of about 9.6 must be protonated (Zambidis & Kuo, 1990). The pH data are consistent with the crystal structure of the PALA-ligated ATCase, which indicated that salt bridges between the negatively charged phosphate oxygens and arginine residues at positions 54 and 105 participate in binding CbmP at the active site (Krause et al., 1987). In contrast, Hervé and colleagues (Leger & Hervé, 1988; Xi et al., 1990) have argued that the binding of CbmP involves the neutral form of His 134 which exhibits a pK value of 8.2 in the free enzyme and 7.2 in the enzyme–CbmP complex. However, studies involving chemical modification (Cole & Yon, 1986), nuclear magnetic resonance (Kleanthous et al., 1988), and a mutant form of ATCase containing alanine in place of His 134 (Waldrop et al., 1992a) do not completely support this conclusion.

A Plausible Mechanism for Catalysis by ATCase. The results from the pH studies are consistent with the mechanism illustrated in Figure 5. A residue on the enzyme with a pK ~9.0 must be protonated for interaction with CbmP as indicated in Figure 5A. However, upon the binding of CbmP (Figure 5B), the pK value of the group is shifted to a value above 10 since ion pair binding involving a phosphate oxygen and a positively charged residue can lead to the displacement of the pK upward by at least 2 pH units (Cleland, 1982). The binary complex also harbors two residues that interact with aspartate; one residue (pK ~7.3) is involved in binding aspartate and must be protonated, while the other group (pK ~9.1) plays a catalytic role and must be deprotonated. Aspartate binds with the α-amine group protonated (Figure 5C), and the pK value of the binding residue is shifted from about 7.3 in the binary complex to a value greater than 9 upon the formation of an ion pair with one of the carboxylates of aspartate. In this ternary complex, the pK value of the catalytic group is shifted downward to about 7.2. Figure 5D shows that a proton is abstracted from the aspartate amino group by the deprotonated catalytic group (pK ~7.2). The α-amine group of aspartate can now undergo addition to the carbonyl group of CbmP to generate a tetrahedral intermediate (Figure 5E). A group on the enzyme likely acts to polarize the carbonyl group, rendering CbmP more susceptible to nucleophilic attack. This catalytic group may be the protonated residue which displays a pK value of about 9.5 in the ternary complex, although several residues are indicated by structural and kinetic studies to fulfill this role. On the basis of molecular modeling and kinetic studies, the formation of the tetrahedral intermediate involves an intramolecular proton transfer from the amino group of aspartate to a phosphate oxygen (Gouaux et al., 1987; Parmentier et al., 1992b). Finally, the tetrahedral intermediate collapses with elimination of phosphate to generate carbamoyl aspartate (Figure 5F). This model accounts for all of the required proton transfers predicted by the chemistry of the reaction and accommodates conformational changes in the trimer that are associated with the binding of both substrates (Collins & Stark, 1969; Griffin et al., 1972; Hammes et al., 1972; Kirschner & Schachman, 1971).

The validity of this model must be tested by identifying the specific amino acid residues implicated by the pH profiles. Preliminary studies using site-directed mutagenesis (Liu and Schachman, unpublished results) and ^{15}N nuclear magnetic resonance (Heath and Schachman, unpublished results) indicate that Lys 84 is a likely candidate for the residue with a pK value of 9.5 that must be protonated for catalysis. Whether this lysine residue participates in catalysis by polarizing the carbonyl group of CbmP or by aligning the substrates in a favorable orientation for reaction remains to be

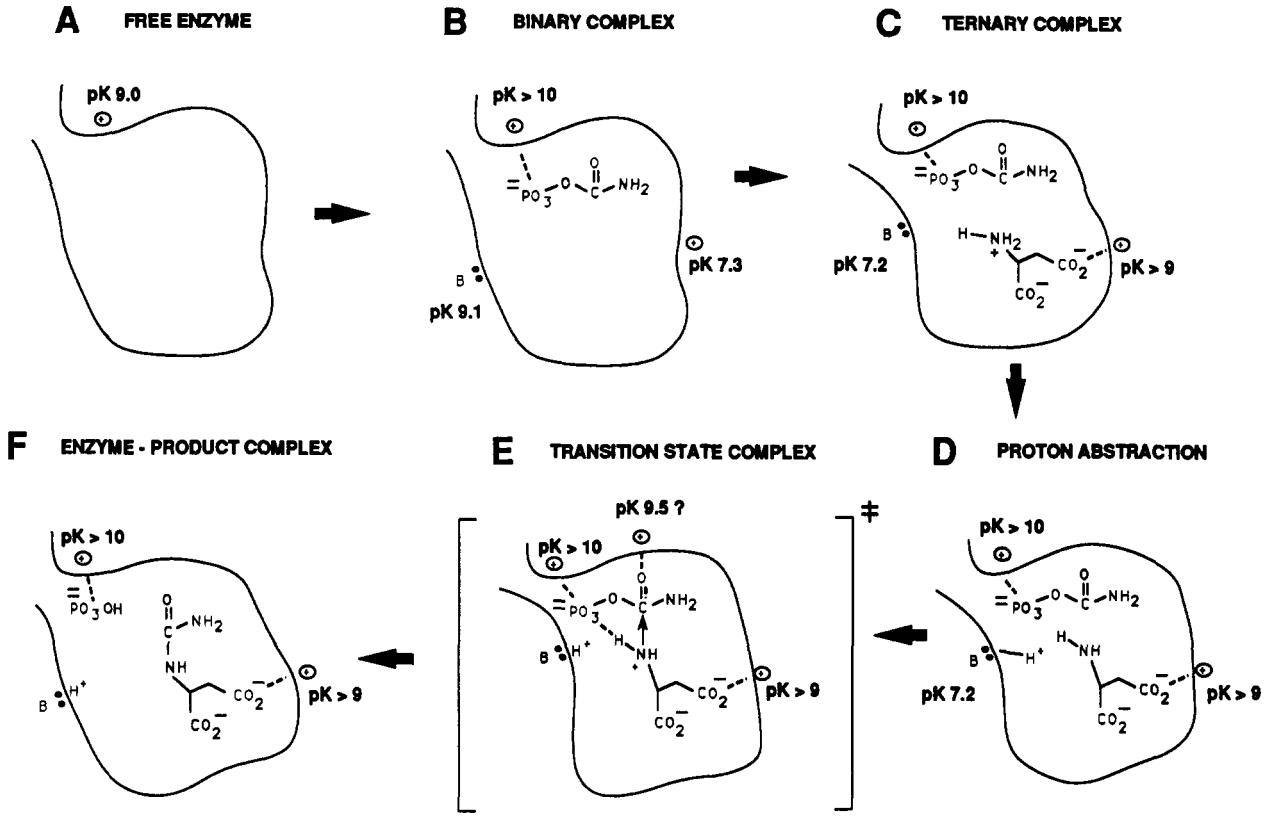


FIGURE 5: A model for the catalytic mechanism of ATCase.

determined. The residue with a pK value of 7.32 involved in aspartate binding must interact with either the α- or β-carboxyl groups of aspartate. This group may be, remarkably, an arginine residue, on the basis of the crystallographic data of Krause et al. (1987) which indicates interactions of Gln 231, Lys 84, Arg 105, Arg 167, and Arg 229 with the aspartate carboxyl groups. Evidence in support of an ion pair bond between a carboxylate and a lysine or arginine (Cleland, 1982) is derived from kinetic data (Figure 2) illustrating that the pK value of the residue that binds cysteine sulfinate, and by inference aspartate, is shifted upward by more than 2 pH units when the inhibitor is bound. Moreover, values of the enthalpy of ionization for the groups with pK values of 7.3 and 9.5 are consistent with arginine or lysine residues (Table II). The identities of the other ionizing residues are being investigated by site-specific replacements in combination with kinetic studies. It should be noted, however, that the present studies were conducted over the experimentally accessible pH range of 6.0–10.5, and it is possible that catalytically important residues whose pK values lie outside this window will be overlooked.

Synergistic Binding of Carbamoyl Phosphate and Aspartate. The isotope partitioning experiment by Rose et al. (1974) was applied to the study of the catalytic trimer of ATCase to determine rate constants for the interaction of CbmP in the binary and ternary complexes. From the values of these constants, conclusions can be drawn regarding the stickiness of the substrate CbmP and how the binding of aspartate affects the interaction of CbmP with the trimer. In addition, results from these measurements are consistent with the proposed kinetic mechanism of catalysis.

The isotope trapping study shows that 70% of the labeled CbmP in the binary complex was trapped as product which is consistent with a steady-state ordered kinetic mechanism for the catalytic trimer that exhibits some random character (Parmentier et al., 1992a; Hsuanyu & Wedler, 1988). In addition, the rate of dissociation of CbmP from the ternary complex (160 s^{-1}), at 25% of the turnover number (660 s^{-1}), agrees well with the value of 20% calculated by Parmentier et al. (1992a) based on carbon isotope effect studies and provides further evidence for the random nature. The results are also in accord with those of Heyde et al. (1973), who concluded that the kinetic mechanism was random on the basis of an intersecting initial velocity pattern coupled with an inhibition profile by succinate that was noncompetitive with respect to CbmP.

By definition, nonsticky substrates dissociate from the enzyme–substrate complex more rapidly than they react to give products. Phosphocreatine, for example, dissociates 80 times more rapidly from the binary complex with creatine kinase than it reacts to give products (Cook et al., 1981), while in the back-reaction for hexokinase, the rate of dissociation of glucose 6-phosphate is 165-fold greater than the turnover number (Viola et al., 1982). By comparison then, a k_1/k_3 ratio of only 1.6–1.9 indicates that CbmP is a relatively sticky substrate in the binary complex with the catalytic trimer of ATCase. The results from pH profiles indicate that aspartate is not a sticky substrate. However, the binding of aspartate greatly enhances the stickiness of CbmP in the ternary complex as illustrated by a value for k_2/k_3 of 0.23. Since the rate of dissociation of CbmP from the binary complex is twice the turnover number, the net effect of binding aspartate is to decrease the dissociation of CbmP by a factor of 8. These findings indicate that although substrate addition is slightly random, the formation of a binary complex involving CbmP and catalytic trimer is preferred and the binding of aspartate serves to strengthen the interaction of CbmP in the ternary complex.

The kinetic mechanism of the catalytic trimer of ATCase closely parallels that observed for yeast hexokinase, an enzyme that catalyzes the phosphorylation of glucose. The first substrate to bind (glucose) is sticky while the second substrate

(ATP) is not sticky (Rose et al., 1974), and the preferred ordered mechanism arises from synergism in substrate binding (Viola et al., 1982). In this system, the binding of glucose enhances the interaction of ATP with the enzyme. In the case of ATCase, the dissociation constant for the enzyme–aspartate complex (~22 mM at pH 8.0; Heyde et al., 1973) is about 5-fold higher than the Michaelis constant for aspartate (~5 mM), suggesting that synergistic binding (Cleland, 1990) also contributes to the preferred order mechanism of the catalytic trimers.

Summary. The present investigation suggests that at least four amino acid side chains on the enzyme participate in the catalytic mechanism of ATCase. Two residues with pK values of 9.01 and 7.03 must be protonated for the binding of CbmP and aspartate, respectively. Catalysis involves a protonated group with a pK value of 9.51 and the deprotonated form of a residue whose pK value is shifted from 9.13 in the enzyme–CbmP complex to 7.16 in the ternary complex.

CbmP is a sticky substrate in both the binary and ternary complexes with the catalytic trimer. However, aspartate is not a sticky substrate but serves to enhance the stickiness of CbmP in the ternary complex.

Acknowledgments

We are grateful to Drs. W. W. Cleland and J. F. Morrison for helpful discussions on various aspects of this work and to Drs. V. Powers and P. White for constructive criticism of the manuscript.

Registry No. cbmP, 590-55-6; PALA, 51321-79-0; ATCase, 9012-49-1; Asp, 56-84-8; succinic acid, 110-15-6; L-cysteinesulfinic acid, 1115-65-7.

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