Allostery and pK_a Changes in Aspartate Transcarbamoylase from *Escherichia coli*: Analysis of the pH Dependence in the Isolated Catalytic Subunits†

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ABSTRACT: The pH dependence of the activity of the isolated catalytic subunits of *Escherichia coli* aspartate transcarbamoylase and also the pH dependence of their inhibition by various nontransformable substrate analogues indicate the implication of three ionizable groups of the enzyme in the binding of the two substrates and in catalysis. The binding of carbamyl phosphate involves a group with a pK_a of 8.2 which is shifted to 7 upon fixation of this substrate. The comparative behavior of several carbamyl phosphate analogues indicates that this group interacts with the carbonyl group of this substrate. From knowledge of the three-dimensional structure of the enzyme, we suggest that His-134 is a good candidate for this interacting group. Similarly, the binding of the second substrate aspartate involves a group whose pK_a is 7.2 which is shifted to 9.4 upon binding of this substrate. In addition, catalysis involves a group with a pK_a of 7.22. There is an interesting possibility that this last group could be the same as one of the two that are involved in the binding of substrates. This study is currently being extended to native aspartate transcarbamoylase in which the allosteric transition is accompanied by an important shift of the pH dependence for activity.

Aspartate transcarbamoylase (ATCase) from *Escherichia coli* (EC 2.1.3.2) catalyzes the first reaction of the pyrimidine nucleotide pathway, that is, the carbamoylation of the amino group of aspartate by carbamyl phosphate. Numerous observations indicate that this reaction proceeds via an ordered mechanism in which carbamyl phosphate binds first followed by aspartate (Collins & Stark, 1969, 1971; Hammes et al., 1971; Schaeffer & Stark, 1972; Wedler & Gasser, 1974; Issaly et al., 1982; Hsuanyu & Wedler, 1987). ATCase activity is inhibited by the end product CTP and stimulated by ATP. Although ATCase is now one of the best known allosteric enzymes [see Kantrowitz et al. (1980a,b) and Hervé (1988) for review], many aspects of its regulatory properties are still obscure. This enzyme consists of an association of two catalytic trimers and three regulatory dimers; it shows homotropic cooperative interactions between the catalytic sites for the utilization of aspartate and heterotropic interactions between catalytic and regulatory sites which allow for the modulation of its activity by the two effectors ATP and CTP. These two kinds of interactions are interpreted through different mechanisms (Thiry & Hervé, 1978; Tauc et al., 1982), a conclusion that was more recently confirmed by X-ray solution scattering experiments (Hervé et al., 1985) and equilibrium isotope exchange kinetics (Hsuanyu & Wedler, 1988). Catalytic and regulatory subunits can be easily and reversibly dissociated (Gerhart & Schachman, 1965; Gerhart & Holoubeck, 1967). The isolated catalytic subunits are fully active but do not show any regulatory properties and have a hyperbolic response to aspartate. The isolated regulatory subunits are catalytically inactive but bear the regulatory sites still able to bind ATP and CTP (Changeux et al., 1968).

The homotropic cooperative interactions between the catalytic sites are explained by a transition from a conformation of the enzyme which possesses a low affinity for aspartate (T state) to a conformation which has a high affinity for this substrate (R state). These two states can be experimentally stabilized through modification of the protein–solvent interactions (Dreyfus et al., 1984). The three-dimensional structure of these two extreme conformations are known with a resolution of 2.5 Å (Honzatko et al., 1982; Krause et al., 1987), the high-affinity conformation being obtained in the presence of *N*-(phosphonoacetyl)-L-aspartate (PALA), a bisubstrate analogue which is able to promote the allosteric transition in ATCase (Collins & Stark, 1971).

It has long been known that the apparent pH dependence of the reaction catalyzed by ATCase depends upon the concentration of its substrate aspartate (Gerhart & Pardee, 1964). The optimum pH is 6.8 at low aspartate concentrations, when the enzyme is essentially in the T form, whereas it is 8.2 at high concentrations of aspartate, when the enzyme is essentially in the R form (Thiry & Hervé, 1978; Tauc et al., 1982). Significantly, the optimum pH for activity of the isolated catalytic subunits is 8.2 whatever the concentration of aspartate. The exact values of these maxima vary slightly with the buffer system used (Ladjimi et al., 1985). It was shown by Pastra-Landis et al. (1978) that, at high concentrations of aspartate, the apparent pH dependence of native ATCase is altered by substrate inhibition. However, correction of this effect does not abolish the difference in the pH dependence of the reaction between high and low aspartate concentrations. Through the use of a series of aspartate analogues, it was shown that the variation of pH dependence in the native enzyme is actually linked to the homotropic cooperative interactions between the catalytic sites, suggesting that the two extreme forms of ATCase differ in the pK_a of at least one group involved in substrate binding and/or in catalysis (Thiry & Hervé, 1978). This change of pK_a might be the physicochemical basis of the variation of substrate affinity that accompanies the allosteric transition. In order to investigate this possibility, as a first step, the pH dependence of the reaction catalyzed by the isolated catalytic subunits was analyzed. It is noteworthy that a mutant form of ATCase was recently obtained in which one observes the uncoupling of the shift in pH dependence, cooperativity, and strength of subunit interactions (Ladjimi & Kantrowitz, 1987). The results, presented

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here, indicate the involvement of three titrable groups in substrate binding and catalysis.

Materials and Methods

Chemicals. Carbamyl phosphate (lithium salt), L-aspartate, succinate (sodium salt), pyrophosphate, phosphonoacetic acid, 3-(cyclohexylamino)propanesulfonic acid (CAPS), [bis(2-hydroxyethyl)amino]tris(hydroxymethyl)methane (Bis-Tris), diethanolamine, *N*-ethylmorpholine, and 2-(*N*-morpholino)-ethanesulfonic acid (MES) were purchased from Sigma Chemical Co, tris(hydroxymethyl)aminomethane (Tris) was from Merck, and L-[U-^{14}C]aspartate (300 mCi/mmol) was from C.E.A.-Saclay. Phosphonoacetamide was a generous gift from E. Gouaux and N. Lipscomb.

Enzyme Preparation. E. coli ATCase was prepared and dissociated into catalytic and regulatory subunits, according to the methods of Gerhart and Holoubeck (1967).

Enzyme Assays. ATCase activity was determined by the method of Porter et al. (1969) under the conditions previously described (Perbal & Hervé, 1972), but in the presence of the tribuffer system diethanolamine (0.051 M), MES (0.1 M), and *N*-ethylmorpholine (0.051 M).

pH Measurements. pH measurements were performed with a Knick 654 pH meter and an Ingold microelectrode. For the calibration, three standard buffers were used, and the precision was about 0.01 pH unit at constant temperature. To obtain a pH range from 6 to 10, the tribuffer system was titrated with HCl or NaOH.

Data Analysis. The substrate saturation curves were fitted to the Michaelis–Menten equation by an iterative method, and the kinetic parameters were calculated from Lineweaver and Burk representations with programs devised in this laboratory by P. Tauc for the Apple IIe computer. The Dixon plots of log V_M versus pH and log (V_M/K_M) versus pH were fitted to the theoretical equations with programs written by J. Laporte and P. Tauc (Dixon, 1953a).

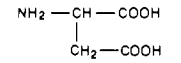
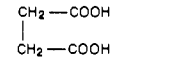
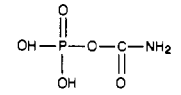
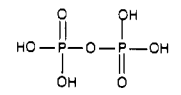
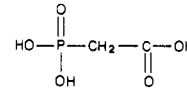
The dissociation constants K_i of the substrate analogues were determined from a Dixon plot of $1/V$ against analogue concentration (Dixon 1953b).

Determination of Phosphonoacetic Acid pK_a's. A 5-mL sample of 0.5 M phosphonoacetic acid was titrated with 0.1 M NaOH. The variation of pH was followed as described above. The pK_a values obtained are shown in Table I.

Results

Determination of Adequate Buffer System and Experimental Conditions. (A) Buffer System. Previous studies of the influence of pH on the catalytic properties of ATCase and on its isolated catalytic subunits were performed in the presence of two different buffer systems. In some cases Tris-HCl and cacodylate were used in turn to cover the entire range of pH over which the investigations were performed (Gerhart & Pardee, 1964; Kerbiriou & Hervé, 1972). More recently, it was proposed by Pastra-Landis et al. (1978) to use the ternary mixture of Tris, Bis-Tris, and CAPs, which allows the entire pH range from 6 to 12 to be covered. However, it appears that these two procedures are not adequate for accurate determinations of the influence of pH on the catalytic properties of the enzyme. In the first case, cacodylate and Tris might have different nonspecific influences on the activity. In the second case, it is easy to show, with the formalism developed by Ellis and Morrison (1982), that the adjustment of the pH of such a mixture by either HCl or NaOH leads to important variations of the ionic strength. Consequently, the three-buffer system MES–*N*-ethylmorpholine–diethanolamine (MND) was used, according to Kuo et al. (1985), for studies of ornithine transcarbamylase. This mixture allows the pH range from 6 to 10 to be covered without significant change of the ionic strength. The present analysis was limited to this range of pH.

Table I: pK of Substrates and Analogues Used in These Experiments

name	formula	pK_a
aspartate[a]		2.09 3.86 9.82
succinic acid[b]		4.16 5.61
carbamyl phosphate[a]		1.4 4.9 12.4
pyrophosphate[b]		0.85 1.49 5.77 8.22
phosphonoacetic acid[c]		1.76 4.84 8.08

[a] From Allen and Jones (1964) and Pastra-Landis et al. (1978). [b] *Handbook of Chemistry and Physics* (1969). [c] Determined in this work as described under Materials and Methods.

It was verified that ATCase is stable at each pH tested in the presence of this three-buffer system and that none of the three components inhibits the enzyme when tested above or below its pK_a.

(B) Ionic Nature of Substrates and Substrate Analogues. The pH dependence of an enzyme-catalyzed reaction will depend on the pK_a values of groups belonging to the protein, and possibly on the pK_a's of groups in the substrates. The pK_a values of the substrates and substrate analogues used in this study are reported in Table I. Over the 6–10 range of pH investigated in this study, the amino group of aspartate (pK_a = 9.82) and one phosphate pK_a of both pyrophosphate and phosphonacetate (pK_a = 8.22 and 8.08, respectively) could potentially interfere with the pK_a determinations for groups belonging to the protein. The way in which these possibilities were taken into account will be reported in the following sections. The β-carboxyl group of succinate (pK_a = 5.61) and the third pK_a of pyrophosphate (5.77) are located in a region of pH where no variation of the catalytic parameters of the catalytic subunits could be detected.

pH Dependence of ATCase and Its Isolated Catalytic Subunits in the Presence of the Three-Buffer System MND. The pH dependence of ATCase activity and of its isolated catalytic subunits was investigated. ATCase shows the previously described shift of pH dependence when the aspartate concentration is varied, the optimum pH of activity going from 6.4 to 7.8 when the aspartate concentration is raised from 1 to 30 mM (Figure 1A). Under the same conditions, the pH dependence of the isolated catalytic subunits does not change significantly in response to the increase in aspartate concentration and exhibits a maximum at pH 8 (Figure 1B).

These results are in accordance with what has been previously reported, except for a small variation in the absolute values of optimum pH. The exact values of the pH optimum are know to depend on the nature of the buffer (Ladjimi et al., 1985). Thus, in the buffer system used in this study, we still observe the shift in pH dependence between the two extreme conformations of ATCase (T and R).

Influence of pH on the Saturation Curve of the Catalytic Subunits by Aspartate. The aspartate saturation curves of

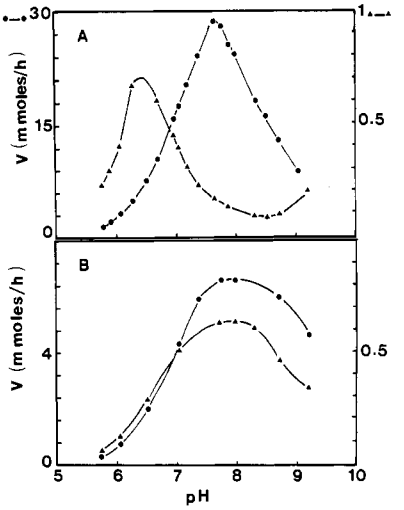


FIGURE 1: pH dependence of the activity of ATCase and of its isolated catalytic subunits. The activity was determined as described under Materials and Methods in the presence of 5 mM carbamyl phosphate and 1 (▲) or 30 mM (●) aspartate, with 0.25 and 0.1 μg of enzyme, respectively, at 37 °C and 0.1 ionic strength in the tribuffer system MES–*N*-ethylmorpholine–diethanolamine (0.1 M/0.051 M/0.051 M). (A) ATCase; (B) catalytic subunits.

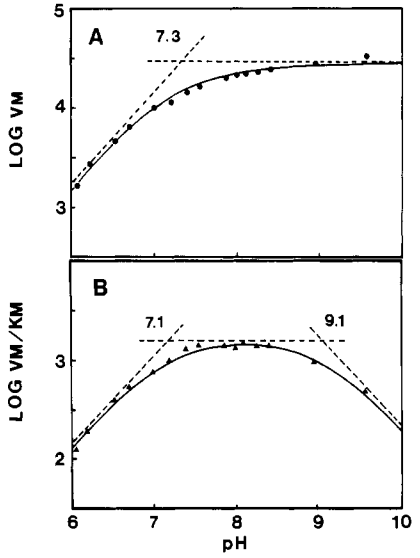


FIGURE 2: Variation of maximum velocity and V_M/K_M of catalytic subunits with pH. (A) Logarithmic plot of V_M against pH. The dotted lines are the asymptotes with slopes of 0 and +1. According to Dixon interpretation, the intersection point indicates a pK_a = 7.3. The theoretical line calculated from eq 1 as indicated under Materials and Methods gives the best fit of the experimental points with pK_a of 7.22 (solid line). (B) Logarithmic plot of the V_M/K_m against pH. The dotted lines are the asymptotes with slopes of +1, 0, and −1. According to Dixon interpretation, the intersection points indicate pK_a's of 7.1 and 9.1. The theoretical line calculated from eq 2 as indicated under Materials and Methods gives the best fit of the experimental points with pK_a's of 7.10 and 9.10 (solid line).

the isolated catalytic subunits in the presence of a saturating concentration of carbamyl phosphate (5 mM) were determined from pH 6 to pH 10 by steps of 0.2 unit of pH. It was verified that carbamyl phosphate was saturating at each pH used and that the pH remained constant throughout the experiment. For each pH, maximum velocity (V_M) and K_M for aspartate were determined with the computer program described under Materials and Methods. The values obtained were analyzed according to the methods described by Dixon (1953a, 1979).[1]

[1] A table presenting these results was submitted for the scrutiny of the reviewers and would be furnished to any interested reader by writing directly to the authors.

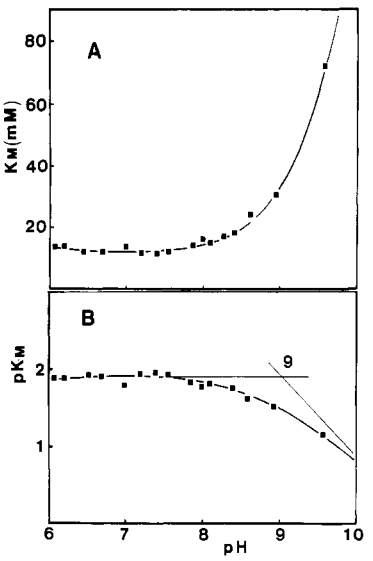


FIGURE 3: Effect of pH on K_M and pK_M of aspartate for catalytic subunits. (A) Variation of K_M of aspartate with pH, indicating the dissociation of the enzyme–substrate complex. (B) Representation of pK_M against pH, showing the involvement of a pK_a = 9 belonging to one of the free reactants. The analysis takes in account two asymptotes of slope 0 and 1. A slope of −2 would not fit the result.

(*A*) *Variation of* V_M. The plot of log V_M against pH is shown in Figure 2A. The experimental curve was analyzed on the basis that the slopes can have only integral values corresponding to the number of ionized groups involved. The intersection of the asymptotes, whose slopes are 0 and +1, indicates the involvement of a single group whose pK_a is about 7.3. An additional control of this interpretation is provided by the fact that the experimental curve passes 0.3 unit (log 2) below the intersection. In order to improve the accuracy of this determination, the experimental data were fitted to

$$V_M^{obsd} = V_M^{lim}/(1 + 10^{pK_a - pH}) \quad (1)$$

giving a best fit pK_a value of 7.22 (Figure 2A). This pK_a is attributable to a group present in the enzyme–substrate complex and involved in the catalysis.

(*B*) *Variation of* V_M/K_M. Interpretation of a plot of V_M/K_M versus pH was made in the same way as under Variation of V_M. The results, obtained on the basis of three asymptotes whose slopes are +1, 0, and −1, respectively, give two pK_a values of 7.10 and 9.10. As shown in Figure 2B, values of 7.1 and 9.1 provide the best fit of the experimental results to

$$(V_M/K_M)^{obsd} = (V_M/K_M)^{lim}/(1 + 10^{pK_{a1} - pH} + 10^{pH - pK_{a2}}) \quad (2)$$

According to Dixon et al. (1979), these pK_a's must be attributed to chemical groups belonging either to the unliganded protein or to free substrates. The pK_a values of the substrates which are reported in Table I exclude the possibility that these groups could belong to carbamyl phosphate. As far as the pK_a = 9.10 value is concerned, the possible implication of the amino group of aspartate will be discussed below.

(*C*) *Variation of* K_M. The variation of K_M as a function of pH is generally more difficult to interprete, due to the implication of both substrate binding and catalysis in this parameter. In the case of the isolated catalytic subunits of ATCase, it has been concluded that the catalytic constant is small compared to the rate of dissociation of the enzyme–aspartate complex and that in this case K_M is a good approximation of the dissociation constant for this substrate (Porter et al., 1969). Therefore, the representation of K_M against pH gives information about pK_a's present in the en-

zyme–substrate complex. Figure 3A shows that the affinity of aspartate for the catalytic sites decreases drastically above pH 8.5, indicating the involvement in the enzyme–aspartate complex of a group whose pK_a must be around 9.5. The corresponding plot of pK_M against pH is shown in Figure 3B. Again, in this representation, each unit of slope results from the involvement of one ionizable group. In addition, an upward curvature indicates the pK_a of a group belonging to the enzyme–substrate complex (aspartate in this case), and a downward curvature indicates the pK_a of a group belonging to one of the free reactants. This analysis gives a pK_a of about 9 which must be attributed to a group belonging either to the unliganded enzyme or to aspartate, on the basis of the downward concavity of the curve. This particular point was further investigated through the use of substrate analogues.

Influence of pH on the Dissociation Constant of Substrate Analogues. The use of substrate analogues that are purely competitive inhibitors and that cannot be used as pseudo-substrates provides information on the pK_a's of groups involved specifically in the binding of the substrates to the enzyme. The influence of pH on the K_i of analogues of the two substrates was determined for the catalytic subunits of ATCase.

(A) Carbamyl Phosphate Analogues. Three ATCase inhibitors, phosphonoacetate, phosphonoacetamide, and pyrophosphate, which have been shown to be competitive with respect to carbamyl phosphate (Porter et al., 1969), were used in this study. The most important structural difference between these compounds is the lack of carbonyl group in pyrophosphate (Table I). Inhibition constants were determined as a function of pH: at each value of pH, K_i was determined according to Dixon (1953b), three concentrations of carbamyl phosphate being used. In each case, the results confirmed the competitive nature of the inhibition.

Figure 4A shows that the K_i of pyrophosphate is virtually independent of pH over the range which was investigated. In contrast, a deprotonation process increases the affinity of phosphonoacetate for the catalytic site by a factor of 20 between pH 6.5 and pH 8.0 (Figure 4A). Phosphonoacetamide behaves in the same way. A plot of pK_i versus pH can be analyzed in the same way as a plot of pK_M versus pH (Figure 4B). Therefore, the binding of phosphonoacetate involves a single ionizing group in this pH range (slope = 1) whose pK_a is 7 in the enzyme–substrate complex (upward curvature) and 8.2 in the unliganded enzyme (downward curvature). The variation of pK_a equals the variation of pH, reinforcing this conclusion (Dixon et al., 1979; Fersht, 1977). The lack of variation of affinity for pyrophosphate in this range of pH means that the 8.08 pK_a of phosphonoacetate cannot be involved here. This is confirmed by the +1 value of the slope which implies that only one group is concerned.

(B) Aspartate Analogues. Succinate is competitive with respect to aspartate (Porter et al., 1969); at each pH, the K_i of succinate was determined by the method of Dixon (1953b) with three concentrations of aspartate. In each case, the results confirmed the competitive nature of the inhibition. Figure 4C shows the sharp decrease in the affinity of the catalytic site for succinate above pH 8.

The corresponding plot of pK_i against pH (Figure 4D) indicates the presence on the protein of a single group whose pK_a is 9.4 in the enzyme–substrate complex and 7.2 in the unliganded enzyme.

Discussion

The present study of the influence of pH on the catalytic constant of the isolated catalytic subunits of ATCase, and on the binding of its two substrates, points to pK_a's of groups

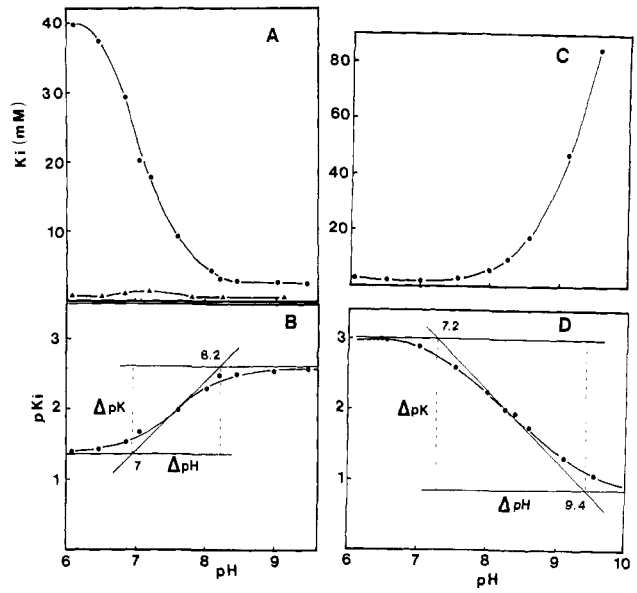


FIGURE 4: Effect of pH on the inhibition constant of carbamyl phosphate and aspartate analogues. (A) Variation of the K_i of phosphonoacetate (●) and pyrophosphate (▲). The inhibition constant of this analogue was determined from Dixon plot of $1/V$ versus inhibitor concentration (0–10 mM), with three concentrations of carbamyl phosphate (0.1, 0.2, and 0.4 mM); the competitive nature of the inhibition was confirmed. (B) Representation of pK_i versus pH, for phosphonoacetate. Interpretation of pK_i versus pH shows asymptotes of 0 and +1, giving pK_a's of 7 and 8.2. (C) The K_i of succinate was determined for each pH with 0–25 mM succinate in the presence of three aspartate concentrations (20, 30, and 40 mM). (D) Interpretation of pK_i versus pH shows asymptotes with slopes of 0 and −1, giving pK_a's of 7.2 and 9.4.

which are involved in these processes. These results are analyzed here on the basis of Dixon's theory (Dixon, 1953a; Dixon et al., 1979), and especially by taking into account the following rules:

(1) Depending on the type of graphic representation which is used, the pK_a values that are obtained are characteristic either of the unliganded enzyme or of the enzyme–substrate complex. In the case of the pK_M versus pH plot, downward curvature corresponds to unliganded enzyme or substrate, and upward curvature corresponds to the enzyme–substrate complex.

(2) Each unit of slope of the logarithmic representation of the data corresponds to the involvement of one ionizable group.

The variation of V_M as a function of pH indicates the involvement of a group whose pK_a is 7.22, and which must be deprotonated to ensure the maximal catalytic activity. The logarithmic plot of V_M/K_M indicates the presence on the free enzyme or on the substrates of two groups, having pK_a's of 7.10 and 9.10. The fact that a pK_a of 7.22–7.10 is seen in both log V_M and log (V_M/K_M) against pH plots suggests that these two pK_a values belong to the same group, corresponding to its pK_a in the enzyme–substrate complex and the unliganded enzyme, respectively.

As far as aspartate binding is concerned, and on the basis that, in this case, K_M is a good approximation of K_D (Porter et al., 1969), the variation of K_M and pK_M as a function of pH points to the involvement of a group having a pK_a of about 9. This pK_a could correspond either to a group belonging to the protein or to the amino group of aspartate. The influence of pH on the binding of succinate provides additional information; succinate, a good competitive inhibitor with respect to aspartate, has no such amino group. Comparison of the effect of pH on the pK_i of succinate and the pK_M of aspartate shows that the binding of the latter is influenced by a group

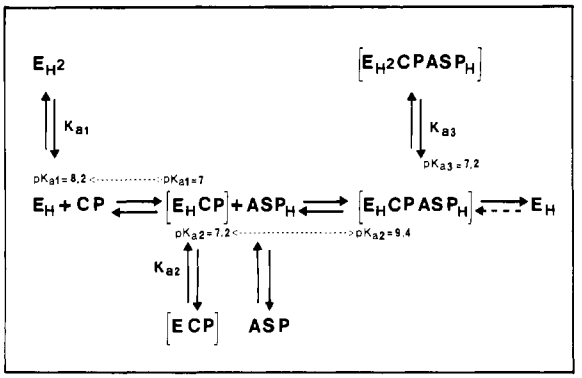


FIGURE 5: Summary of involvement of ionizable groups of the isolated catalytic subunits in substrate binding and during catalysis.

whose pK_a is about 9, which must be present either in the free enzyme or in aspartate, whereas the binding of succinate does not seem to be altered by such a group on the free enzyme. In addition, the results reported in Figure 3 show that, contrary to what is observed in the case of succinate, this pK_a is identical in the enzyme–aspartate complex and in the free reactants (K_m and pK_M, respectively). These results suggest that this pK_a is that of the amino group of aspartate. Alternatively, it could correspond to a group of the enzyme which would be involved in the binding of aspartate but not succinate.

Our data (Figure 4C,D) implicate in the binding of succinate a group whose pK_a is shifted from 7.2 to 9.4 upon binding of this compound. This is consistent with the observation made by Allewell et al. (1979) that in ATCase the binding of succinate is accompanied by a proton uptake. It should also be mentioned that, on the basis of steady-state kinetic experiments, Porter et al. (1969) concluded that a group with a pK_a of 7.1 is involved in the binding of succinate to the isolated catalytic subunits.

The influence of pH on the binding of phosphonoacetate, a competitive inhibitor with respect to carbamyl phosphate (Figure 4A,B), indicates the involvement of a group on the enzyme whose deprotonation increases the affinity for this compound. As a consequence of this binding, the pK_a of this group is shifted from 8.2 to 7. The comparison of the influence of pH on the affinity of the catalytic site for phosphonoacetate, phosphonoacetamide, and pyrophosphate indicates that this group is involved only when these analogues contain a carbonyl group equivalent to that of carbamyl phosphate.

Taking in account the ordered binding of substrates, these results are assembled in Figure 5. On the unliganded enzyme, a group with a pK_a of 8.2 (pK_{a1}) must be deprotonated to favor the binding of carbamyl phosphate; this binding changes this pK_a to 7. A group whose pK_a is 7.2 (pK_{a2}) is then involved in the binding of aspartate, and its pK_a is shifted to 9.4 upon fixation of this substrate, whose binding is probably also dependent on the protonation of its own amino group. The catalysis then involves the presence of a group with a pK_a of 7.22 (pK_{a3}).

Since the three-dimensional structure of ATCase is known with a resolution of 2.5 Å (Honzatko et al., 1982; Ke et al., 1984; Krause et al., 1987), providing a description of the amino acid side chains which are located at the catalytic site, it is interesting to hypothesize as to which of these amino acids the pK_a's determined in this study can be attributed (Figure 6). His-134 is a very good candidate to account for the group which is involved in the interaction with the carbonyl group of carbamyl phosphate. This interpretation is consistent with the deduction made from the structural information (Krause et al., 1987), from the use of a series of carbamyl phosphate

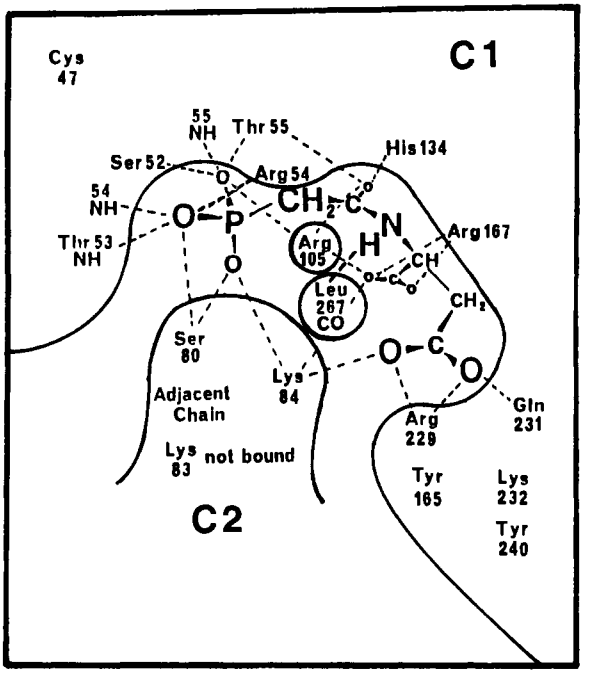


FIGURE 6: Amino acid side chains involved in the binding of the bisubstrate analogue PALA to the catalytic site of ATCase [adapted from Krause et al. (1987) with permission from W. Lipscomb]. Dotted lines indicate salt links, hydrogen bonds, and other polar contacts less than 3.5 Å.

analogues in competition studies (Dennis et al., 1986), and from chemical inactivation of the enzyme (Greenwell et al., 1973; Cole & Yon, 1986) or site-directed mutagenesis (Robey et al., 1986). The subsequent involvement of this group in the catalysis is also consistent with the proposed mechanism of this reaction, that is, an increase of the polarization of the carbonyl group of carbamyl phosphate, through its interaction with a group of the protein, favoring its nucleophilic attack by the amino group of aspartate (Jacobson & Stark, 1973; Krause et al., 1987; Gouaux et al., 1987). Figure 6 shows that the pK_a that is important for the binding of succinate and that shows an important shift upon binding of this substrate analogue could belong to one of the following residues: Arg-105, Arg-167, and Arg-229 on one catalytic chain; Lys-84 on the adjacent chain. This would require that in the unliganded enzyme the pK_a of this group would be considerably shifted toward neutrality by its environment.

The changes in pK_a's observed in this work might be correlated with the ordered mechanism of the ATCase reaction, in which carbamyl phosphate binds first and induces a structural change that allows the binding of the second substrate, aspartate (Collins & Stark, 1969, 1971; Hammes et al., 1971; Schaeffer & Stark, 1972; Wedler & Gasser, 1974; Issaly et al., 1982; Hsuanyu & Wedler, 1987). Carbamyl phosphate binding shifts from 8.2 to 7 the pK_a of a group involved in its binding. This group could be the one that is then shifted to 9.4 upon succinate binding. However, it is more likely that this pK_a of 7, present in the carbamyl phosphate–enzyme complex, corresponds to the group also involved in the catalysis (pK_a = 7.22). These hypotheses are currently being further investigated, especially by the use of in vitro mutagenesis.

In addition, in view of the change of pH dependence which accompanies the allosteric transition in ATCase, the values of the pK_a's described above and their putative variations during this transition are currently being investigated. In this regard it is interesting to note that some of the amino acid side chains potentially involved in this process belong to the two

structural loops which undergo an important movement during the quaternary structure transition (Krause et al., 1985, 1987; Middleton & Kantrowitz, 1986).

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