The Catalytic Site of *Escherichia coli* Aspartate Transcarbamylase: Interaction between Histidine 134 and the Carbonyl Group of the Substrate Carbamyl Phosphate†

Xu Guang Xi,‡ Françoise Van Vliet,§ Moncef M. Ladjimi,‡ Raymond Cunin,§ and Guy Hervé*,‡

Laboratoire d'Enzymologie, CNRS, 91198 Gif-sur-Yvette, France, and Laboratorium voor Microbiologie, Vrije Universiteit Brussel, 1070 Brussels, Belgium

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ABSTRACT: Previous pK_a determinations indicated that histidine 134, present in the catalytic site of aspartate transcarbamylase, might be the group involved in the binding of the substrate carbamyl phosphate and, possibly, in the catalytic efficiency of this enzyme. In the present work, this residue was replaced by an asparagine through site-directed mutagenesis. The results obtained show that histidine 134 is indeed the group of the enzyme whose deprotonation increases the affinity of the catalytic site for carbamyl phosphate. In the wild-type enzyme this group can be titrated only by those carbamyl phosphate analogues that bear the carbonyl group. In the modified enzyme the group whose deprotonation increases the catalytic efficiency is still present, indicating that this group is not the imidazole ring of histidine 134 (pK_a = 6.3). In addition, the pK_a of the still unknown group involved in aspartate binding is shifted by one unit in the mutant as compared to the wild type.

Aspartate transcarbamylase (ATCase) from *Escherichia coli* (EC 2.1.3.2) is extensively studied as a model for allosteric regulation [for review, see Allewell (1989) and Hervé (1989)]. This enzyme catalyzes the first reaction of the pyrimidine pathway, that is, the carbamylation of the amino group of aspartate by carbamyl phosphate. This reaction is synergistically feedback inhibited by CTP and UTP (Wild et al, 1989) and is stimulated by ATP, an antagonism that, in the cell, contributes to balance the production of pyrimidines and purines. In terms of structure ATCase is composed of two catalytic trimers (catalytic subunits) that are in contact and linked together through interaction with three regulatory dimers (regulatory subunits). On these regulatory subunits are located the regulatory sites that bind the effectors CTP and ATP. The crystallographic structure of this complex enzyme is known with a resolution of about 2.5 Å (Honzatko et al., 1982; Ke et al., 1984).

ATCase operates through an ordered Bi–Bi mechanism in which carbamyl phosphate binds first followed by aspartate (Porter et al., 1969; Collins & Stark, 1969; Wedler & Gasser, 1974; Issaly et al., 1982; Hsuanyu & Wedler, 1987). This enzyme shows homotropic cooperative interactions between the catalytic sites for the binding of the second substrate aspartate. These cooperative effects are explained by the transition of the protein from a conformation that has a low affinity for aspartate (T state) to a conformation that has a high affinity for this substrate (R state) (Howlett & Schachman, 1977; Moody et al., 1979; Krause et al., 1985). The crystallographic structures of these two extreme conformations are known with a resolution of about 2.5 Å (Honzatko et al., 1982; Ke et al., 1984; Krause et al., 1987). This transition of quaternary structure (Ke et al., 1984; Krause et al., 1987; Ladjimi et al., 1988; Ladjimi & Kantrowitz, 1988) is coupled to a change of the tertiary structure of the catalytic chains (Krause et al., 1987; Ladjimi & Kantrowitz, 1988) which, in turn, is coupled to changes in the configuration of the catalytic sites. This change in configuration must account for the variation of the affinity that these sites have for aspartate. This complex transition is accompanied by an important shift of the pH dependence for activity of the enzyme (Gerhart & Pardee, 1964; Kerbiriou & Hervé, 1972; Thiry & Hervé, 1978), suggesting that the two extreme conformations differ by the pK_a of one or several groups involved in substrate binding and/or catalysis. Mutants were obtained in which a sigmoidal saturation curve for aspartate is still observed without shift of pH dependence (Ladjimi & Kantrowitz, 1987). Experimental work and theoretical calculations predict various pK_a changes associated with ligand binding and conformational changes (Allewell et al., 1979; Glackin et al., 1989).

ATCase can be reversibly dissociated into catalytic and regulatory subunits (Gerhart & Holoubek, 1967). The isolated catalytic subunits (trimers of catalytic chains) can still catalyze the carbamylation of the amino group of aspartate, but they lack the regulatory properties; their saturation curve by aspartate is hyperbolic and their activity is insensitive to the effectors ATP and CTP. The catalytic sites in ATCase are located at the interface between two catalytic chains within a catalytic trimer and involve amino acid residues that belong to both chains. Figure 1 shows the amino acid residues from neighboring catalytic chains (C1 and C2) that appear to be in contact with the bisubstrate analogue *N*-(phosphonacetyl)-L-aspartate in the crystal structure (Volz et al., 1986).

As a first step in the study of the pK_a changes associated with the T → R transition, the pH dependence for activity of the isolated catalytic subunits was previously investigated (Lêger & Hervé, 1988). The results obtained led to the following conclusions. The binding of the first substrate carbamyl phosphate involves a group on the protein whose pK_a is shifted from 8.2 to 7 upon this binding. The binding of the second substrate aspartate or its analogue succinate involves

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‡ CNRS.

§ Vrije Universiteit Brussel.

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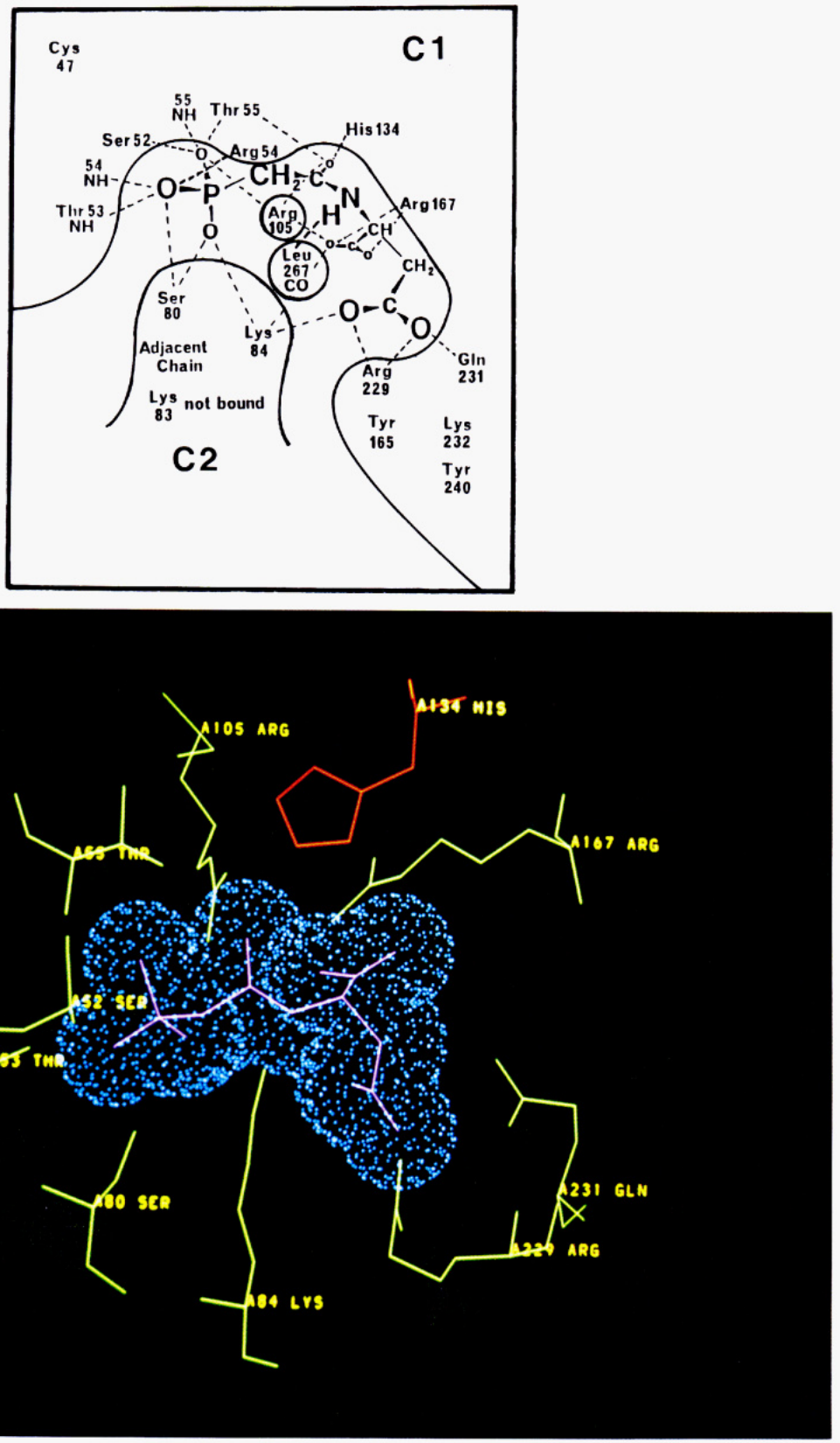

FIGURE 1: Positioning of the bisubstrate analogue PALA in the catalytic site of ATCase. (Top) Schematic view of the amino acid side chains involved in the binding of PALA [adapted from Volz et al. (1986) with permission from W. Lipscomb]. Dotted lines indicate salt links, hydrogen bonds, and other polar contacts less than 3.5 Å. (Bottom) Corresponding view based on the atomic coordinates. The atomic coordinates of the PALA-bound ATCase were generously provided by W. Lipscomb. The PALA backbone is shown in purple with its carbonyl pointing toward histidine 134, which is shown in red.

a group on the protein whose pK_a is shifted from 7.2 to 9.4 upon binding. A third group whose pK_a is 7.2 is involved in the catalytic process. Among the different putative residues to which these pK_a's could be attributed, histidine 134 is a good candidate for being the group involved in the binding of carbamyl phosphate, since it appears to be in contact with the carbonyl of this substrate (Figure 1). It could as well be a good candidate for being the residue involved in catalysis, since

the postulated mechanism for the reaction involves an interaction between the carbonyl group of carbamyl phosphate and a group on the protein that would increase the polarization of this carbonyl, thus favoring its nucleophilic attack by the amino group of aspartate (Jacobson & Stark, 1973; Gouaux et al., 1987).

In order to test these hypotheses, site-directed mutagenesis was used to obtain a modified form of ATCase in which histidine 134 is replaced by an asparagine residue. The analysis of the properties of the catalytic subunits isolated from this mutant shows that histidine 134 is indeed the group whose deprotonation is involved in carbamyl phosphate binding.

Materials and Methods

Chemicals

Carbamyl phosphate (lithium salt), L-aspartate, succinate (sodium salt), pyrophosphate, phosphonacetic acid, [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, L-[U-^{14}C]aspartate (300 mCi/mmol) was from CEA-Saclay, and [^{14}C]carbamyl phosphate (13.8 mCi/mmol) was from NEN.

Enzyme Preparation

Wild-Type ATCase. This enzyme was prepared and dissociated into catalytic and regulatory subunits according to Gerhart and Holoubek (1967).

Asn134-ATCase.[1] This His_c 134 → Asn mutation was created by oligonucleotide-directed mutagenesis, following the method developed by Kramer et al. (1984). Mutagenesis was performed on the *pyrBI* genes (coding respectively for the catalytic and regulatory polypeptide chains) cloned on a 2740-bp *Pst*I–*Sal*I DNA fragment into the polylinker region of bacteriophage M13mp9 (Messing & Vieira, 1982). The presence of the mutation as well as the absence of unwanted modifications was ascertained by dideoxy terminator nucleotide sequencing (Sanger et al., 1977). The mutation was then transferred to an ATCase expression vector on a 630-bp *Bst*EII–*Bgl*II DNA cassette. The cassette harboring the mutation was substituted to the corresponding wild-type DNA fragment in a pUC18 plasmid (Yanisch-Perron et al., 1985) carrying the *pyrBI* genes. The resulting plasmid was used to transform strain ATC25 to ampicillin resistance. ATC25 is a derivative of KMBL 1510G4 (*pyrBI*, *thi*; Perbal and Hervé, unpublished), which harbors a partial deletion of the *pyrBI* genes, preventing the production of wild-type ATCase, and a *pyrF* bradytrophic mutation, which causes physiological derepression of the pyrimidine biosynthetic genes by limiting the rate of pyrimidine synthesis. The combination of the physiological derepression and high plasmid copy number results in overproduction of aspartate transcarbamylase. This modified form of the enzyme was purified and dissociated into catalytic and regulatory subunits by the same method as in the case of the wild-type enzyme.

Enzyme Assay

Test Using [^{14}C]Aspartate. In most experiments the ATCase activity was determined as previously described (Perbal & Hervé, 1972) but in the presence of the tribuffer system 0.051 M diethanolamine, 0.1 M MES, and 0.051 *N*-ethylmorpholine (Léger & Hervé, 1988). The enzyme concentrations were determined by the method of Lowry et al. (1951), using bovine serum albumin as a standard and taking into account the 20% overestimate which is given by this method (Kerbiriou et al., 1977). The specific activity of the different enzyme species is expressed as micromoles of carbamyl aspartate formed per hour per milligram of protein.

Test Using [^{14}C]Carbamyl Phosphate. In order to obtain more accurate carbamyl phosphate saturation curves, [^{14}C]-carbamyl phosphate was used as the labeled substrate. The assay was performed as previously described (Robin et al., 1989). Prior to use, both labeled and unlabeled carbamyl phosphate were recrystallized according to Adair and Jones (1972).

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 going from 6 to 10, the tribuffer system was titrated with HCl or NaOH.

Data Analysis

The substrate saturation curves were fitted either to the Hill equation (native ATCase) or to the Michaelis–Menten equation (isolated catalytic subunits), and the kinetic parameters were calculated by an iterative nonlinear Gauss–Newton least-squares method using computer programs developed in this laboratory by P. Tauc. According to Dixon (1953a) the plots of log V_m versus pH and log (V_m/K_M) versus pH were fitted to the theoretical equations with programs written in this laboratory by J. Laporte. The dissociation constants K_i of the substrate analogues were determined from Dixon plots of $1/V$ against analogue concentration (Dixon, 1953b).

Results

Catalytic and Regulatory Properties of Asn134-ATCase

The modified form of ATCase in which His134 is replaced by an asparagine residue was purified to homogeneity as indicated under Materials and Methods. Although this modified enzyme has a much lower specific activity than the wild-type enzyme, its aspartate saturation curve is sigmoidal, indicating they are still homotropic cooperative interactions between the catalytic sites. The fit of a series of saturation curves to the Hill equation indicates a maximum velocity of 420 units/mg (micromoles of carbamyl aspartate formed per hour per milligram of enzyme), i.e., about 2% of that of the wild-type enzyme under the same conditions. The $S_{0.5}$ value is 50 mM and the Hill number is 1.5 as compared to 16 mM and 2.8, respectively, in the case of the wild-type enzyme.

The pH dependence of the reaction catalyzed by Asn134-ATCase was determined at two extreme concentrations of aspartate, 3 and 60 mM, conditions under which this enzyme should be predominantly in the T and R states, respectively. These conditions are similar to those where the wild-type enzyme shows an important shift of pH dependence (6.8 and 8.2 at 3 and 20 mM aspartate, respectively). Figure 2 shows that, unlike wild-type ATCase, the modified enzyme exhibits the same pH dependence at both aspartate concentrations. The optimum value is at pH = 6.8, the value of the apparent optimum pH of the wild-type enzyme in the presence of a low aspartate concentration.

Figure 3 shows that the modified enzyme is as sensitive to the feedback inhibitor CTP as the wild-type enzyme.

[1] Abbreviations: Asn134-ATCase, modified form of ATCase in which histidine 134 of the catalytic chain was replaced by an asparagine residue; Asn134-Cat, catalytic subunits isolated from Asn134-ATCase.

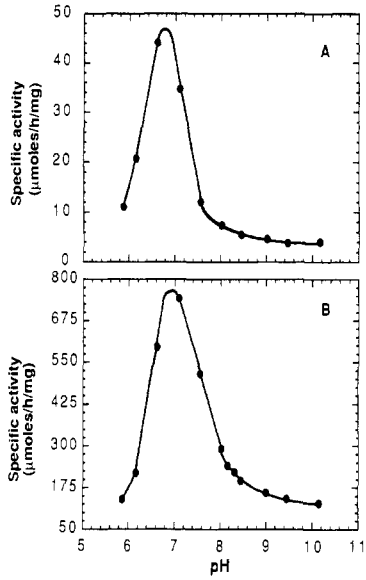

FIGURE 2: pH dependence for activity of Asn134-ATCase at low and high aspartate concentrations. The activity of 10-μg samples of Asn134-ATCase was measured at various pH values in the conditions described under Materials and Methods. (A) In the presence of 3 mM aspartate; (B) in the presence of 60 mM aspartate.

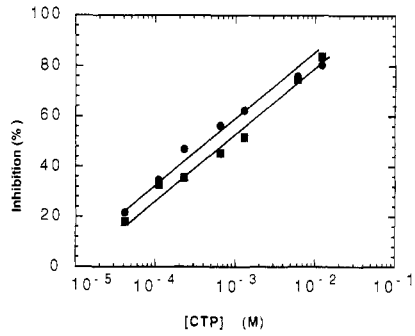

FIGURE 3: Feedback inhibition of the activity of Asn134-ATCase by CTP. The activity of ATCase and Asn134-ATCase was determined as indicated under Materials and Methods in the presence of increasing concentrations of CTP. The aspartate concentration was 2 mM in the case of ATCase and 3.3 mM in the case of Asn134-ATCase. The percent inhibition is calculated as $[(V_0 - V_n)/V_0] \times 100$, where V_0 is the rate of reaction in the absence of CTP and V_n the rate of reaction in its presence. (●) ATCase; (■) Asn134-ATCase.

Kinetic Properties of Asn134 Catalytic Subunits (Asn134-Cat)

Asn134-ATCase was dissociated into its catalytic and regulatory subunits as described under Materials and Methods, and the kinetic properties of Asn134-Cat were investigated. Its aspartate saturation curve was determined in the presence of 5 mM carbamyl phosphate. The fit of a series of saturation curves to the Michaelis–Menten equation gives a maximum velocity of 590 μmol of carbamyl aspartate formed h^{-1} (mg of enzyme)$^{-1}$, a value which is about 4% of that of the wild-type catalytic subunits under the same conditions. The apparent K_M for aspartate is 60 mM as compared to 20 mM for wild-type catalytic subunits under the same conditions.

Since the crystallographic data indicate that histidine 134 is in contact with the carbonyl group of carbamyl phosphate (Volz et al., 1986), it could be expected that Asn134-Cat would have a decreased affinity for this substrate. We therefore determined the K_M for carbamyl phosphate of Asn134-Cat. Because this determination cannot be performed at saturating aspartate (Porter et al., 1969), the apparent K_M was determined at various aspartate concentrations, and the values

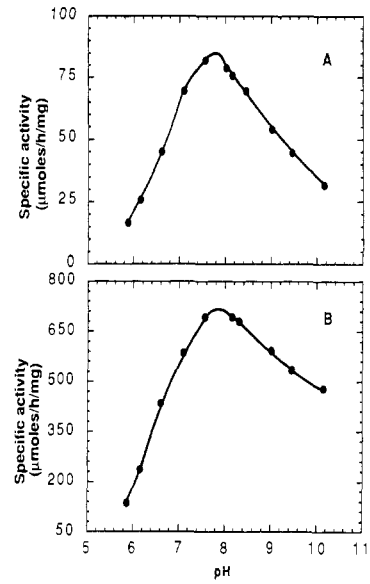

FIGURE 4: pH dependence for activity of Asn134 catalytic subunits. The pH dependence for activity of Asn134-Cat was determined as indicated under Materials and Methods in the presence of 3.3 mM aspartate (A) or 60 mM aspartate (B).

obtained were extrapolated to an infinite concentration of this substrate. The intercept replot gives a K_M of 2.4 mM as compared to 0.62 mM for the wild-type catalytic subunits under the same conditions.

Influence of pH on the Kinetic Parameters of Asn134-Cat

pH Dependence of Activity. The influence of pH on the overall rate of the reaction catalyzed by Asn134-Cat was investigated at several concentrations of aspartate. This reaction shows an optimum pH at 7.8 whatever the concentration of this substrate (Figure 4). It was verified that the enzyme is stable at the extreme values of pH used in these experiments (pH = 5.8 and 10.2). This is shown by the fact that at these pH values the reaction rate is constant over more than 20 min.

Influence of pH on Maximal Velocity. In order to obtain some information about the influence of pH on the catalytic efficiency of Asn134-Cat and on its apparent K_M for aspartate, the saturation curve for this substrate was established at varying pH. The maximal velocity, expressed as micromoles of carbamyl aspartate synthesized per hour per milligram of enzyme, decreases from 980 to 250 when the pH is shifted from 7.88 to 5.87 (Figure 5A). It was verified that the rate of the reaction is constant at all pH values for at least 30 min. This variation of the catalytic efficiency is much smaller than that observed with the wild-type enzyme under the same conditions, that is, from 21 800 to 1690. The possible significance of this difference will be discussed further. The Dixon logarithmic plot of the results is shown in Figure 5B. This result 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 suggested the involvement of a single group of pK_a about 6.3. In order to improve the accuracy of this determination, the experimental data were fitted to the equation:

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

The best fit was obtained for a pK_a value of 6.3 (Figure 5B). According to Dixon's theory (Dixon et al., 1979) this pK_a is attributable to a group present in the enzyme–substrate com-

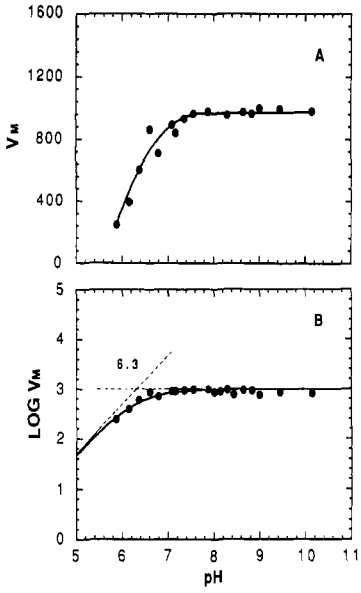

FIGURE 5: Variation of the catalytic efficiency of Asn134 catalytic subunits with pH. The saturation curve by aspartate of Asn134-Cat was determined as indicated under Materials and Methods with 16 μg of enzyme for pH = 5.87–6.37 and pH = 9.0–10.15 and 10 μg of enzyme for the intermediary pH values. (A) Variation of V_m as a function of pH. (B) Variation of log V_m as a function of pH. The dotted lines are the asymptotes with slopes of 0 and +1. According to Dixon interpretation, the intersection point indicates a pK_a value of about 6.3. The solid line is the theoretical fit to eq 1 as indicated under Materials and Methods. It gives the best fit of the experimental points with pK_a = 6.3.

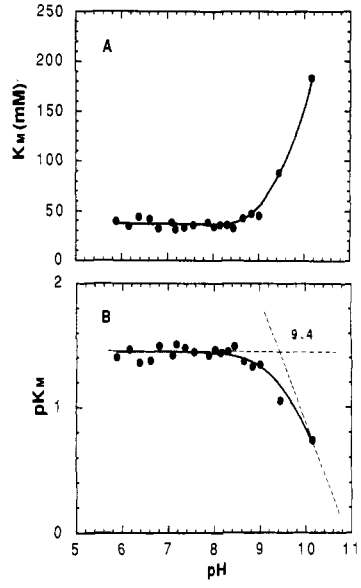

FIGURE 6: Effect of pH on K_M and pK_M of aspartate for Asn134 catalytic subunits. (A) Variation of K_M of aspartate with pH indicating the dissociation of the enzyme–substrate complex. (B) Representation of pK_M against pH showing the involvement of a pK_a of about 9.4 of a group belonging to one of the free reactants. The analysis takes in account two asymptotes of slope 0 and 1.

plex and involved in the catalysis.

Influence of pH on the Apparent K_M for Aspartate. Although the influence of pH on K_M is more difficult to interpret since K_M implies both affinity for the substrate and catalytic efficiency, this parameter was measured as a function of pH. The results obtained are presented in Figure 6A. The corresponding plot of pK_M against pH is shown in Figure 6B. In terms of Dixon's theory this graph is interpreted on the basis of two asymptotes whose slopes are 0 and −1, indicating, as

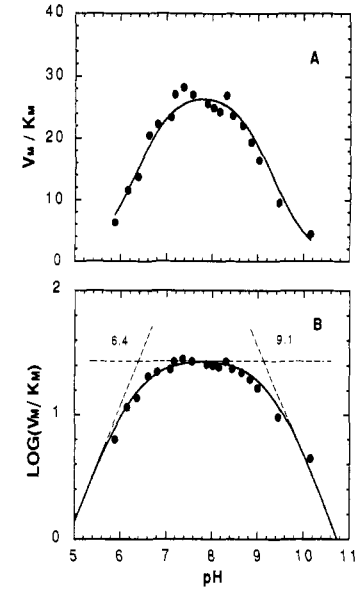

FIGURE 7: Variation of V_m/K_M of Asn134 catalytic subunits with pH. (A) Direct representation. (B) Logarithmic plot. The dotted lines are the asymptotes with slopes +1, 0, and −1. According to Dixon interpretation the intersection points indicate pK_a's of 6.4 and 9.1. The solid line is the theoretical fit to eq 2 as indicated under Materials and Methods. It gives the best fit of the experimental points with pK_a's of 6.4 and 9.1.

in the case of the wild-type catalytic subunits (Léger & Hervé, 1988), the binding of aspartate involves a group belonging to the unliganded enzyme, and whose pK_a is about 9.4.

Influence of pH on the Ratio V_m/K_M. The influence of pH on the ratio of V_m/K_M for aspartate is shown in Figure 7A. The corresponding logarithmic plot was analyzed according to Dixon's theory on the basis of three asymptotes whose slopes are +1, 0, and −1 (Figure 7B). This analysis indicates the involvement of two groups whose pK_a's are respectively 6.4 and 9.1. These values are confirmed (Figure 9B) by the fact that they provide the best fit of the experimental data to the equation:

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

As for the wild-type catalytic subunits (Léger & Hervé, 1988), these two pK_a's must be attributed to chemical groups belonging either to the unliganded protein or to the free substrates.

Influence of pH on the Dissociating Constants of Substrate Analogues

The pK_a's involved in substrate binding and catalytic activity of the Asn134-Cat were further investigated through the use of substrate analogues as was done previously in the case of the wild-type catalytic subunits (Léger & Hervé, 1988).

Carbamyl Phosphate Analogues. Phosphonacetate and pyrophosphate are inhibitors of ATCase, acting competitively with respect to carbamyl phosphate. They were previously used to demonstrate that there is a group on the protein that is involved in carbamyl phosphate binding through interaction with the carbonyl group of this substrate. The pK_a of this group is 8.2 in the free enzyme but is shifted to 7 upon carbamyl phosphate binding (Léger & Hervé, 1988). Since this group could well be histidine 134 (Figure 1), the variation of the dissociation constant of phosphonacetate as a function of pH was investigated for Asn134-Cat. At each value of pH, the inhibition constant K_i was determined according to Dixon

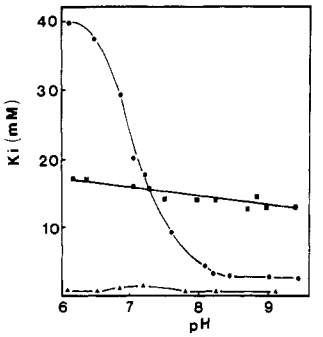

FIGURE 8: Effect of pH on the inhibition constants of carbamyl phosphate analogues toward Asn134 catalytic subunits and the wild-type catalytic subunits. The inhibition constants were determined from Dixon plots of $1/V$ versus inhibitor concentration at three concentrations of carbamyl phosphate: (●) K_i of phosphonacetate toward the wild-type catalytic subunits using 0–10 mM phosphonacetate and 0.1, 0.2, and 0.4 mM carbamyl phosphate; (■) K_i of phosphonacetate against Asn134-Cat using 0–50 mM phosphonacetate and 1.35, 2.7, and 5.4 mM carbamyl phosphate; (▲) K_i of pyrophosphate toward the wild-type catalytic subunits using 0–10 mM pyrophosphate and 0.1, 0.2, and 0.4 mM carbamyl phosphate. The competitive nature of the inhibition was confirmed in all cases.

(1953b), with three concentrations of carbamyl phosphate being used. The results obtained are presented in Figure 8 along with those from the wild-type catalytic subunits. These results show clearly that no group can be titrated in this way in Asn134-Cat, contrary to what is observed in the case of the wild-type enzyme. As a control, pyrophosphate does not allow to titrate this group in the wild-type catalytic subunits, confirming that it is indeed the carbonyl group of carbamyl phosphate that is involved in the interaction investigated here.

Aspartate Analogue. Succinate is an inhibitor of ATCase that is competitive with respect to aspartate (Porter et al., 1969). It was shown previously that in the wild-type catalytic subunits the binding of this inhibitor involves a group on the protein whose pK_a is 7.2 in the carbamyl phosphate–enzyme complex and is shifted to 9.4 in the tertiary complex. The same analysis was performed with Asn134-Cat. Figure 9A shows the variation of the K_i of succinate as a function of pH. The conditions used allow the complete titration of the group belonging to the protein that is involved in the binding of this substrate analogue and presumably in the binding of aspartate. The plot of pK_i versus pH can be analyzed as previously indicated (Figure 9B). On the basis of three asymptotes whose slopes are 0 and −1, this analysis indicates that the binding of succinate to Asn134-Cat involves a group on the protein whose pK_a is 7.4 in the carbamyl phosphate–enzyme complex and is shifted to 8.4 in the ternary complex. Additional support for this interpretation is provided by the fact that, according to the theory, the experimental curve passes about 0.3 unit (log 2) below the intersection. The amplitude of the pK_a shift provoked by the binding of succinate is smaller than in the wild-type catalytic subunits. This observation will be discussed further.

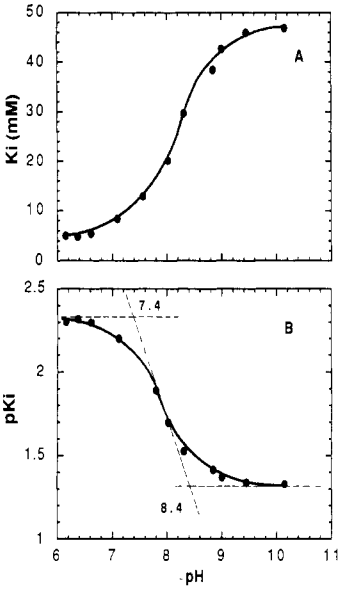

FIGURE 9: Effect of pH on the inhibition constant of succinate toward Asn134 catalytic subunits. The K_i of succinate was determined for each pH with 0–50 mM succinate in the presence of three concentrations of aspartate, 20, 40, and 60 mM. (A) Variation of K_i as a function of pH; (B) interpretation of pK_i versus pH with asymptotes of slopes 0 and −1 giving pK_a's of 7.4 and 8.4.

Discussion

Carbamyl Phosphate Binding. The results presented above show unambiguously that in the wild-type catalytic subunits histidine 134 is the group whose deprotonation increases the affinity for the carbamyl phosphate analogue, phosphonacetate. In contrast with what is observed with the wild-type catalytic subunits, the affinity for this analogue does not vary significantly over the range of pH studied in the mutant in which histidine 134 is replaced by an asparagine residue. This is in accordance with the fact that there is no decrease of the apparent K_M for carbamyl phosphate of Asn134-Cat when the pH goes from 6 to 8. In addition, in the wild-type catalytic subunits, the affinity for pyrophosphate, another efficient competitive inhibitor toward carbamyl phosphate, does not vary with pH. Taken together these results indicate that it is the carbonyl group of carbamyl phosphate that interacts with the imidazole ring of histidine 134, a conclusion that is consistent with the crystallographic data (Figure 1; Volz et al., 1986). In Asn134-Cat the dissociation constant for phosphonacetate is intermediary between those of the wild-type enzyme in which histidine 134 is protonated and deprotonated. In Asn134-Cat the dissociation constant for phosphonacetate is 15 mM at pH = 8, as compared to 3 mM for the wild-type catalytic subunits at the same pH, conditions under which histidine 134 is virtually completely deprotonated. This difference is in accordance with that observed for the apparent K_M for carbamyl phosphate which, under the same conditions, is 2.4 mM in the case of Asn134-Cat and 0.62 mM in the case of the wild-type catalytic subunits.

The titration of histidine 134 indicates a pK_a value of 8.2 in the unliganded enzyme, which is shifted to 7 in the enzyme–carbamyl phosphate complex (Lêger & Hervé, 1988). It must be noted that this value differs from that of 5.4, which was suggested by the results of the reaction of diethyl pyrocarbonate with histidine 134 (Cole & Yon, 1986) and from that of 6 suggested by the NMR study of this residue (Kleanthous et al., 1988). This discrepancy deserves attention. The possible involvement of arginine 105 in the pH dependence of carbamyl phosphate binding will be investigated.

Catalytic Efficiency. The replacement of histidine 134 by asparagine reduces the catalytic efficiency of the enzyme by only a factor 25, indicating that the presence of this residue is not an absolute requirement for activity. This result is consistent with the fact that there is still a group on Asn134-Cat whose deprotonation increases the catalytic efficiency. This group has a pK_a of 6.3 in the liganded Asn134-Cat instead of 7.2 in the wild-type catalytic subunits. Thus, histidine 134 does not seem to be involved in a general acid mechanism for the transfer of the carbamyl group of

carbamyl phosphate, a conclusion similar to that attained through the NMR study of the histidine residues of the enzyme (Kleanthous et al., 1988). Moreover, it must be noted that the deprotonation of the group(s) titrated in the wild-type catalytic subunits increases the catalytic efficiency of this enzyme by a factor 15, whereas the deprotonation of the group titrated in Asn134-Cat increases the catalytic efficiency by only a factor 4 over the range of pH studied. This phenomenon might have several explanations:

(i) Since this group could not be entirely titrated toward the acidic pH with the buffering system used, this difference might only reflect the one unit shift of the pK_a involved. This possibility is currently being investigated.

(ii) There is a group distinct from His134 whose deprotonation increases the catalytic efficiency; however, due to a small distortion of the catalytic site as the result of the replacement of histidine 134 by asparagine, the deprotonation of this group would have a smaller effect on the catalytic efficiency than it has in the wild-type catalytic subunits. The identity of this group is currently being determined through site-directed mutagenesis.

(iii) Although not essential, the deprotonation of histidine 134 might have a partial effect on the catalytic efficiency. The knowledge of the other group involved will allow us to test this possibility.

Aspartate Binding. The influence of pH on the dissociation constant for succinate indicates the involvement in the binding of this substrate analogue to Asn134-Cat, of a group on the protein whose pK_a is 7.4 in the carbamyl phosphate–enzyme complex and is shifted to 8.4 upon succinate binding. This situation is similar to that observed for the wild-type catalytic subunits, except that in this case the pK_a of this group is shifted to 9.4 upon succinate binding. Interestingly, this difference allowed the complete titration of this group in the modified enzyme. This group must interact with either the α- or the β-carboxyl group of aspartate. The crystallographic data (Volz et al., 1986; Krause et al., 1987) indicate possible interactions of Lys84, Arg229, and Gln231 with the α-carboxylate and of Arg105, Arg167, and Lys84 with the β-carboxylate. In any case the group involved must be drastically influenced by its electronic environment since its pK_a is close to neutrality in the carbamyl phosphate–enzyme complex. The identity of this group is currently being determined by site-directed mutagenesis.

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Registry No. L-Asn, 70-47-3; L-Asp, 56-84-8; L-His, 71-00-1; CTP, 65-47-4; ATCase, 9012-49-1; carbamyl phosphate, 590-55-6; succinic acid, 110-15-6; pyrophosphate, 14000-31-8; phosphonoacetic acid, 4408-78-0.

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Mycobacteria Glycolipids as Potential Pathogenicity Effectors: Alteration of Model and Natural Membranes

Annie Sut, Sylvie Sirugue, Sophie Sixou, Faouzi Lakhdar-Ghazal, Jean-François Tocanne, and Gilbert Lanêelle*
Centre de Biochimie et Gênêtique Cellulaires du CNRS and Universitê Paul Sabatier, 118 Route de Narbonne, 31062 Toulouse Cêdex, France

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ABSTRACT: Four mycobacterial wall glycolipids were tested for their effects on phospholipidic liposome organization and passive permeability and on oxidative phosphorylation of isolated mitochondria. From fluorescence polarization of diphenylhexatriene performed on liposomes it was concluded that the two trehalose derivatives (dimycoloyltrehalose and polyphthienoyltrehalose) rigidified the fluid state of liposomes, the triglycosyl phenolphthiocerol slightly fluidized the gel state, while the peptidoglycolipid ("apolar" mycoside C) just shifted the phase transition temperature upward. Dimycoloyltrehalose was without effect on liposome passive permeability, as estimated from dicarboxyfluorescein leak rates, and polyphthienoyltrehalose and triglycosyl phenolphthiocerol slightly decreased leaks, while mycoside C dramatically increased leaks. Activity of these lipids on mitochondrial oxidative phosphorylation was examined. The two trehalose derivatives have been tested previously: both had the same type of inhibitory activity, dimycoloyltrehalose being the most active. Triglycosyl phenolphthiocerol was inactive. Mycoside C was very active, with effects resembling those of classical uncouplers: this suggested that its activity on mitochondria was related to its effect on permeability. All these membrane alterations were called nonspecific because it is likely that they result from nonspecific lipid-lipid interactions, and not from recognition between specific molecular structures. Such nonspecific interactions could be at the origin of some of the effects of mycobacteria glycolipids on cells of the immune system observed in the last few years.

Mycobacteria wall glycolipids are potential effectors of pathogenicity. For instance, it was shown that two mycolic acid derivatives, dimycoloyltrehalose (Cord factor) and mycoloyl diarabinoside, were active against mitochondrial oxidative phosphorylation (Kato, 1970; Rouanet & Lanêelle, 1983); a species-specific glycolipid, the triglycosyl phenolphthiocerol from *Mycobacterium leprae*, could have specific or nonspecific effects on lymphocytes (Mehra et al., 1984; Brett et al., 1984); a polar mycoside C of *Mycobacterium avium* (serovar 4) was also shown to be active on lymphocytes (Brownback & Barrow, 1988).

Lipids can represent 60% of mycobacteria walls, and some glycolipids are profusely produced in infected organs (Draper & Rees, 1973; Hunter & Brennan, 1981). As mycobacteria are causative agents of long-lasting diseases (tuberculosis, leprosy), and as pathogenic mycobacteria are intracellular parasites, glycolipids have enough time to diffuse and act. It is likely that membranes of host cells are the first target of these glycolipids, and as mycobacteria glycolipids have structures greatly differing from that of animal glycolipids, it is possible that they disturb the host membrane structure and function. However, very few studies have been devoted to effects of mycobacteria lipids on membranes, and they were performed with crude solvent extracts (Stewart-Tull et al., 1978; Roozemond et al., 1985).

We have looked for effects on model and natural membranes of four glycolipids (Figure 1): dimycoloyltrehalose (Cord factor, present in most mycobacteria), the diglycosyl peptidolipid (mycoside C) from *Mycobacterium smegmatis* (Daffê et al., 1983), whose structure is representative of the most abundant mycosides C present in *M. avium*, and two new glycolipids isolated from *Mycobacterium tuberculosis* (strain Canetti), namely, a triglycosyl phenophthiocerol (Daffê et al., 1987) and a polyphthienoyltrehalose (Minnikin et al., 1985; Daffê et al., 1988).

Materials and Methods

Mycobacteria Lipids. Polyphthienoyltrehalose and triglycosyl phenolphthiocerol, from *M. tuberculosis* (strain Canetti), were generous gifts of Drs. M. Daffê, C. Lacave, and M. A. Lanêelle. Mycoside C and dimycoloyltrehalose (Cord factor) were isolated from *M. smegmatis* (ATCC 607). Crude dimycoloyltrehalose was obtained in the methanol-precipitated fraction of lipid extracts, and mycoside C, in the supernatant. Both fractions were further purified by adsorption chromatography on silicic acid or on Florisil. The purity of the compounds was checked by thin-layer chromatography.

Molecular weights of the natural compounds were calculated from published formulae of the major compounds and from the relative percentages of their homologues as determined by mass spectrometry or gas-liquid chromatography: dimycoloyltrehalose (Cord factor), 2700 Da; polyphthienoyltrehalose, 1950 Da; triglycosyl phenolphthiocerol, 1820 Da; mycoside C, 1260 Da.

Miscellaneous Compounds. Dimyristoyl-*sn*-glycerophosphocholine, bovine brain phosphatidylserine (acyl residues 18:0, 54%; 18:1, 41%; higher homologues, 5%), and egg phosphatidylcholine and phosphatidic acid (acyl residues 16:0,

* Author to whom correspondence should be addressed.

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