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Evidence That Histidine-163 Is Critical for Catalytic Activity, but Not for Substrate Binding to *Escherichia coli* Agmatinase

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Agmatinase (agmatine ureohydrolase, EC 3.5.3.11) from *Escherichia coli* was inactivated by diethyl pyrocarbonate (DEPC) and illumination in the presence of Rose bengal. Protection against photoinactivation was afforded by the product putrescine, and the dissociation constant of the enzyme–protector complex (12 mM) was essentially equal to the K_i value for this compound acting as a competitive inhibitor of agmatine hydrolysis. Upon mutation of His163 by phenylalanine, the agmatinase activity was reduced to 3–5% of wild-type activity, without any change in K_m for agmatine or K_i for putrescine inhibition. The mutant was insensitive to DEPC and dye-sensitized inactivations. We conclude that His163 plays an important role in the catalytic function of agmatinase, but it is not directly involved in substrate binding. © 1999 Academic Press

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Agmatinase (agmatine ureohydrolase, EC 3.5.3.11) catalyses the hydrolysis of agmatine to putrescine and urea (1). Although agmatinase activity is found mainly in bacteria (1–7), its presence in mammalian tissues has been recently reported (8, 9). Agmatine, which results from decarboxylation of arginine by arginine decarboxylase (10), is a metabolic intermediate in the biosynthesis of putrescine and higher polyamines (1) and may have important roles in mammals, regulating several neurotransmitter-related functions (11, 12).

The enzyme from *Escherichia coli* and putative agmatinases from *Scynechocytis*, *Schizosaccharomyces pombe* and *Bacillus subtilis*, has been cloned and the deduced amino acid sequences indicates their homology to all sequenced arginases (13–15), which also catalyses an hydrolytic reaction with production of urea. Considering this and the close structural relationships between their substrates, the question arises as to whether a similar or identical mechanism is involved in catalysis by these enzymes. At present, there is considerable information with respect to the molecular and kinetic properties of arginases from several species and tissues (16–23). Moreover, the crystal three-dimensional structures of the enzymes from rat liver (21) and *Bacillus caldovelox* (24) are available, and mechanisms of arginine hydrolysis have been proposed (21, 23, 24). The currently accepted mechanisms involves the activation of a metal-bound water molecule for nucleophilic attack on the guanidino carbon of arginine (21, 23, 24). It is also known that fully activated rat liver arginase contains a binuclear metal center (21), which is thought to polarize a bound solvent molecule better than one metal ion (25). In addition, a role for a critical histidine, corresponding to His-141 in the sequence of rat liver arginase, has been indicated by chemical modification (22), site directed mutagenesis (19) and X-ray crystallographic studies (21, 24). In contrast with arginase, information with respect to agmatinase is still scarce. It is, however, known that *E. coli* agmatinase also exhibits an absolute requirement of Mn^{2+} for catalytic activity (26). Moreover, a binuclear metal center for this enzyme is suggested by the following facts: (a) active enzyme species which contains 1 Mn^{2+}/active site may be further activated by the metal ion; (b) all ligands for a bimanganese center in arginase, are strictly conserved residues in the sequence of agmatinase (14, 15).

Since the rat liver His141 is also conserved among all sequenced arginases and agmatinases (14, 15), a critical role for one equivalent residue in agmatinase (His163 in *E. coli*), may be reasonably expected. However, there has been no chemical modification or site-

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directed mutagenesis to support this expectation. Hence, we considered it worthwhile to investigate this aspect, which is central not only for delineating the catalytic mechanism of agmatine hydrolysis, but also for a better understanding of the similarities and differences between two enzymes which apparently evolved from a single primordial protein (14, 15). As a first result of our efforts, we now report site directed and chemical modification studies that substantiates the importance of His163 in catalysis by *E. coli* agmatinase.

MATERIALS AND METHODS

Materials. All chemicals were of the highest quality commercially available (most from Sigma Chemical Co.) and were used without further purification. The plasmid pKA5, bearing the *spe*B gene of *E. coli* agmatinase, was kindly supplied by Dr. Stephen Boyle (Virginia Polytechnic Institute and State University). *E. coli* UCC SI was obtained from the Department of Microbiology (University of Concepción). Restriction enzymes, as well as enzymes and reagents for PCR were obtained from Promega. Synthetic nucleotide primers were obtained from Oligopeptido (Universidad de Chile) and [α-^{35}S]dATP from Amersham. The pQE60 *E. coli* expression vector and the Ni–NTA resin were obtained from Quiagen.

Enzyme and protein assays. Enzyme activities were determined by measuring the formation of urea from agmatine in 50 mM Tris–HCl (pH 7.5). In agreement with a previous report (25), results described here were the same, regardless the presence or absence of added Mn^{2+} during the assays for activity. Urea was determined by a colorimetric method with α-isonitrosopropiophenone (27), and protein by means of the standard Bio-Rad protein assay (Bio-Rad, CA), with bovine serum albumin as a standard.

Enzyme preparations. Bacteria were grown with shaking at 37°C in Luria broth in the presence of ampicillin (100 μg/ml). The JM109 strain of *E. coli* was transformed with the plasmid pKA5 carrying the wild-type and mutant agmatinase cDNAs and the expression of the proteins was induced with 1 mM isopropyl β-D-thiogalactopyranoside (IPTG). The wild-type and mutant agmatinase cDNAs were also directionally cloned into the histidine tagged pQE60 *E. coli* expression vector and the histidine-tagged enzymes were expressed in *E. coli* strain JM109, following induction with 1 mM IPTG. All the agmatinase genes were sequenced to confirm that no mistakes resulted from PCR. Histidine-tagged enzymes were purified to homogeneity by metal chelate chromatography over Ni–NTA resin, according to the instructions of the manufacturer. Native agmatinase, extracted from *E. coli* UCC SI, and untagged recombinant enzymes were purified by the procedure described by Satischandran and Boyle (1) The purity of the enzymes was evaluated by SDS–PAGE.

Site-directed mutagenesis. The H163F mutant form of *E. coli* agmatinase was obtained by a two-step PCR (28), using the plasmid pKA5 containing the *spe*B gene of *E. coli* agmatinase as a template. A first PCR product was obtained using the 5′ sense primer 5′-AGTCCATCCATGGGCACCTTAG-3′ and a 3′ complementary primer corresponding to nucleotides 476–498 of agmatinase with a AC → AA substitution at nucleotides 487–488 (sequence: 5′-CATAGTGCCGAAGTCAAATTCAC-3′). Similarly, the second PCR product was obtained using the 5′ sense primer corresponding to nucleotides 476–498 of agmatinase with a CA → TT substitution at nucleotides 487–488 (sequence: 5′-GTGAATTTGACTTCGGCACT-ATG-3′) and the 3″ complementary primer 5′-ATTAATGGCAT-GCTTTACCCGT-3′. Using the PCR products of agmatinase with the CA → TT and TG → AA substitutions in the coding and noncoding strands, respectively, and using the 5′ and 3′ primers mentioned above, the full length agmatinase cDNA coding for the H163F mutant was generated by a second round of PCR. The mutation of agmatinase at nucleotides 487–488 was confirmed by the dideoxynucleotides sequencing method.

Reactions with chemical modifiers. For Rose bengal-sensitized photoinactivation, reaction mixtures containing the enzyme in 50 mM potassium phosphate (pH 7.5) and varying dye concentrations (0–40 μg/ml), were illuminated at 25°C with a 100-W lamp at 20 cm. At intervals, aliquots were withdrawn and assayed for agmatinase activity. Rose bengal without illumination, or irradiation in the absence of Rose bengal had no effect on agmatinase activity.

Reactions with diethyl pyrocarbonate (DEPC) were carried out at 25°C in 50 mM potassium phosphate buffer (pH 7.0), and quenched by addition of 5 mM imidazole. Solutions of DEPC, freshly prepared in ice-cold ethanol, were evaluated spectrophotometrically (29).

Data analysis. Data were computer fitted to the appropriate equations. The pseudo first-order rate constants (k) of inactivation were calculated from the slope of the plots of log (residual activity) versus time of reaction. The dissociation constant of the enzyme-protector complex was determined from the x-intercept of a plot $1/k$ versus the concentration of the protector compound. Kinetic data of competitive inhibition were analyzed by double reciprocal plots, and the corresponding K_i value was determined from a replot of slopes versus inhibitor concentration.

RESULTS AND DISCUSSION

Chemical modification. Initially, attempts were made to use chemical modification with DEPC and its reversal by hydroxylamine, as a probe to evaluate the importance of histidine residues in *E. coli* agmatinase. It is known that of the residues that can potentially react with DEPC, histidine residues can be regenerated with hydroxylamine (29). However, the use of this approach was complicated by the inhibition of agmatinase by hydroxylamine, and also by the ethanol used as a solvent for DEPC. Although the inhibitory effect of ethanol was reversed by dialysis, and this allowed us to demonstrate that the chemical modifier effectively inactivates the enzyme, it was not possible to relate the inactivation to the ethoxycarbonylation of histidine residues, since all the attempts for reversal of hydroxylamine inhibition by dialysis or dilution of the inhibited enzyme solutions, were unsuccessful. Moreover, significant inhibition was produced by hydroxylamine concentrations considerably lower than those commonly used for reversal of DEPC inactivation of enzymes (29). As an example, at 30 mM agmatine, 50% inhibition of enzyme activity was produced by about 5 mM hydroxylamine. Although the mechanism of hydroxylamine inhibition of agmatinase was beyond the scope of this study, one plausible explanation would be inner-sphere coordination of hydroxylamine and Mn^{2+} in the active site of agmatinase.

As an alternative to DEPC, we decided to use illumination in the presence of Rose bengal, which exhibits a high degree of specificity for histidine residues at neutral pH (30–32). As shown in Fig. 1A, illumination of agmatinase led to inactivation which was dependent both upon Rose bengal concentration and the duration of light exposure and followed pseudo first order kinet-

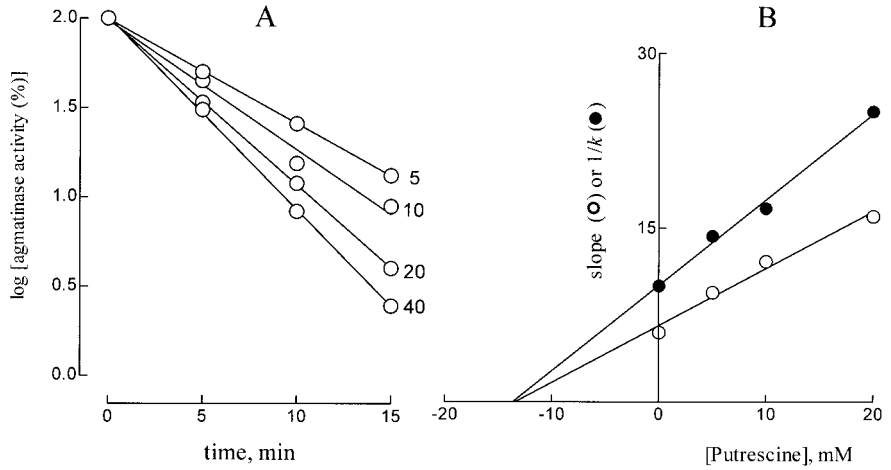

FIG. 1. (A) Pseudo-first-order inactivation by illumination of *E. coli* agmatinase in the presence of the indicated μM concentrations of Rose bengal. (B) Determination of the dissociation constants of the enzyme-inhibitor and enzyme–protector complexes; the concentration of Rose bengal was 40 μM.

ics at all dye concentrations. The semi logarithmic plots of the percentage activity remaining as a function of time of irradiation were linear up to about 90% inactivation and even after prolonged illumination in the presence of Rose bengal, about 5% activity was retained by the enzyme. The photoinactivation of agmatinase was partially quenched by 10 mM N_3^- but remained essentially unaltered in the presencie of 0.5 mM Fe $(CN)_6^{3-}$, indicating the operation of a mechanism in which energy is transferred from the excited triplet sensitizer to molecular oxygen to form a singlet oxygen ($^1O_2$), which, in turn, react with the acceptor (30).

Protection against inactivation was afforded by putrescine, a product and competitive inhibitor of agmatine hydrolysis. Moreover, essentially the same value of 12 mM was calculated for the dissociation constants of the enzyme-inhibitor and enzyme-protector complexes (Fig. 1B). It seems, therefore, safe to assume that putrescine protect by binding to the active site and forming a complex which is very similar, if not identical, to the enzyme–inhibitor complex. The linearity of the plot $1/k_{obs}$ versus putrescine concentration indicates that putrescine totally protected against the inactivation and, therefore, that Rose bengal cannot inactivate the enzyme-inhibitor complex. On the other hand, the specificity of protection was indicated by the fact that ornithine, which is structurally related to putrescine, had no inhibitory effect on agmatine hydrolysis and afforded no protection against photoinactivation of the enzyme. Thus, results obtained favors the conclusion that photoinactivation involves residue(s) located at or near the active site. Guanidinium chloride was also a competitive inhibitor of agmatine hydrolysis by agmatinase (K_i = 100 ± 10 mM), but it was unable to protect against photoinactivation of the enzyme. Considering guanidinium chloride as an analogue of urea, the lack of protection would be interpreted as indicating that the guanidinium group of the substrate do not interact with the photosensitive residue, or it is not in such a close proximity as to prevent its inactivation.

Although results described in the legend to Fig. 1 were those specifically obtained from studies using native species of *E. coli* agmatinase, these were essentially the same for wild-type histidine-tagged and untagged recombinant species of the enzyme. On the other hand, results to be described below, corresponding to untagged species, were not altered by the presence of the extra six histidine residues.

Site-directed mutagenesis. That the conserved His-163 is the target for DEPC and dye-sensitized inactivation, was indicated by the properties of enzyme species in which this residue was replaced by phenylalanine by site directed mutagenesis. Upon mutation, agmatinase activity was reduced to about 1–3%

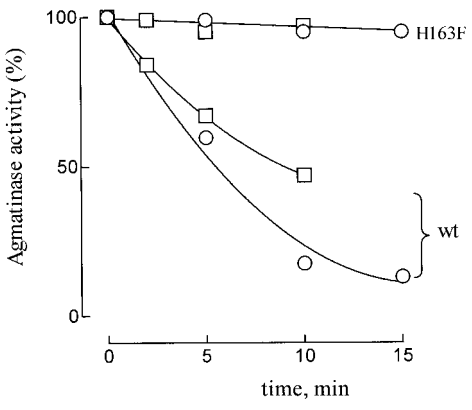

FIG. 2. Effect of incubation with 2 mM DEPC or illumination of wild-type and H163F mutant *E. coli* agmatinases in the presence of 20 μM Rose bengal. DEPC (□), Rose bengal (○).

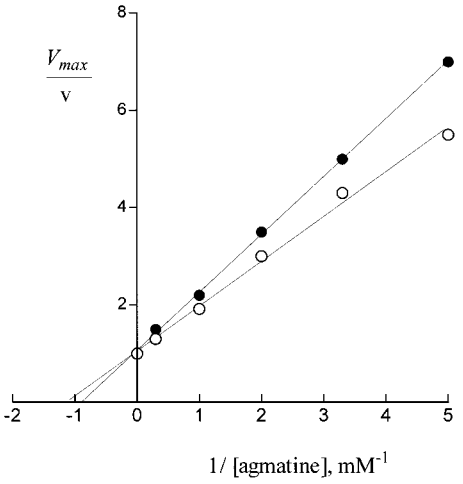

FIG. 3. K_m values of wild-type (○) and H163F (●) mutant agmatinases. Values of V_{max} were determined from double reciprocal plots of initial velocities versus substrate concentrations.

of wild type activity, and this activity was not altered by illumination in the presence of Rose bengal or incubation of the mutant enzyme with DEPC (Fig. 2).

The K_m value for agmatine remained practically unaltered as a consequence of replacement of His163 with phenylalanine. From the slopes of the lines in Fig. 3, calculated values were 0.92 and 1.12 mM, respectively. The K_i for competitive inhibition by putrescine (12 ± 2 mM at pH 7.5) was also essentially the same for wild-type and mutant forms of the enzyme. We may, therefore, conclude that the imidazole ring of His163 is critical for catalytic activity, but not for substrate binding, although a close proximity between this residue and putrescine in the enzyme-putrescine complex is indicated by the ability of the product to protect against dye-sensitized inactivation of native and wild type recombinant species. Although a subtle structural change cannot be totally excluded, the replacement of the side chain of His163 with phenylalanine do not seem to induce a gross conformational change, as indicated by the unaltered stability of the protein to trypsin inactivation. In fact, the activities of wild type and mutant forms remained unaltered after incubation with trypsin (0.1 mg/ml) for 20 min at 25°C.

In conclusion, our present findings substantiates the importance of His-163 for catalytic activity of *E. coli* agmatinase. Further work is, evidently, required to clarify the exact role played by His163 in catalysis by agmatinase and studies addressed to this aspect are currently underway in our laboratory. Any mechanism needs to incorporate the essentially equal K_m and K_i values for wild-type and H163F mutant enzymes, and the fact that agmatinase activity is severely reduced, but not totally abolished by chemical modification or replacement of His139 with phenylalanine, which is roughly similar in size to histidine, but lacks its acid/base and hydrogen bonding capabilities. Among the possibilities to be considered, His163 would act in transition state stabilization by hydrogen bonding, which is expected to increase the rate of reaction of bound substrate, without a direct effect on binding of the substrate in the ground state (33). On this basis, destabilization of the transition state would explain the considerably lower catalytic activity of the H163F mutant species of agmatinase. Another interesting alternative emerges if one considers the explanation given by Kanyo *et al.* (21) for the residual activity of the mutant H141N of rat liver arginase. According to these researchers, His141 would act as a proton shuttle from bulk solvent to the ε-amino group of ornithine before product dissociation and facilitating the ionization of a metal-bound water by mediating proton transfer to bulk solvent, and the residual activity of the mutant may result from direct proton transfer from bulk solvent. A similar role would be assigned to His163 in *E. coli* agmatinase.

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