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CATALYSIS BY ARGININE DEIMINASE: EVIDENCE FOR A COVALENT INTERMEDIATE

Douglas W. Smith and David E. Fahrney

Department of Biochemistry, Colorado State University
Fort Collins, Colorado 80523

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SUMMARY

Arginine deiminase (EC 3.5.3.6) catalyzes the hydrolysis of arginine to ammonia and citrulline. This reaction is postulated to occur in three steps: (1) formation of the Michaelis complex, (2) the formation of an amidino-enzyme intermediate and liberation of ammonia, and (3) the rate-determining step, hydrolysis of the amidino-enzyme. The enzymic reaction is accelerated 5-fold by 0.2 M imidazole. This striking effect is expected for the amidino-enzyme mechanism but otherwise is difficult to explain. The putative amidino-enzyme intermediate can be demonstrated by quenching the [^{14}C]arginine-arginine deiminase reaction at low pH. Under these conditions, 0.5 equivalents of ^{14}C label per mol enzyme dimer were covalently bound.

Arginine deiminase from Mycoplasma arthritidis is a dimer composed of apparently identical subunits (1). The enzyme catalyzes the hydrolysis of a C-N bond in the guanidino group of arginine and related guanidine derivatives. The products are ammonia and the corresponding ureido analog of the substrate. N^{α}-Methylarginine, D-arginine, homoarginine, and argininic acid undergo enzymic hydrolysis 200-3000 times more slowly than L-arginine (2). The K_m value of arginine is about 4 μM, whereas the nonspecific substrates have K_m values ranging from 1.2 to 16 mM. Our tentative interpretation is that K_m for a nonspecific substrate may approximate the substrate dissociation constant K_S, since competitive inhibition constants of nonreactive analogs are of the same magnitude.

The low K_m value for arginine may have mechanistic implications. For a number of hydrolases, the mechanism involves attack by a nucleophilic group of the enzyme to form a covalent intermediate in contrast to direct attack by water. Scheme 1 depicts a similar stepwise mechanism for arginine deiminase.

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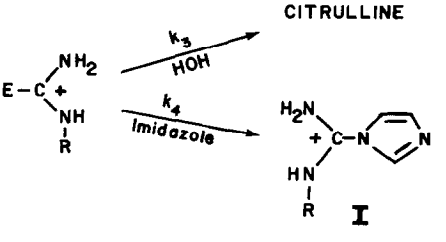
In accordance with the assumption that the pathway involves an amidino-enzyme intermediate, the steady-state rate equations yield $K_m = K_S k_3/(k_2+k_3)$ and $k_{cat} = k_2 k_3/(k_2+k_3)$. The assumption that the k_3 step, hydrolysis of the amidino-enzyme, is rate determining would explain the low K_m value for arginine. If $k_3 << k_2$, then $k_{cat} = k_3$ and $K_m = K_S\ (k_3/k_2)$. Thus K_m is not a dissociation constant and is considerably smaller than K_S.

In view of the hypothesis that the arginine deiminase reaction involves an intermediate, it is natural to explore the effect of added nucleophiles on this reaction. Perhaps the most convincing evidence for covalent catalysis would come from a "substrate labeling" experiment. Results from both approaches are discussed below.

RESULTS

Kinetic evidence for a covalent intermediate — Imidazole accelerates the rate of ammonia formation, an effect in accord with the formation of an amidino-enzyme intermediate. Analysis of the kinetics involving the reaction of an added nucleophile with this intermediate predicts a constant value for K_m/V. As Fig. 1 shows, this effect is observed. Also the rate of ammonia formation could be increased so greatly by imidazole only if hydrolysis of the amidino-enzyme (k_3) were the rate-determining step. A plot of k_{cat} against imidazole concentration is linear; accordingly, k_{cat} does not approach k_2 at the highest imidazole concentration tested. This in turn suggests that k_2 is larger than k_3.

It is important to know the products in any mechanistic study. Unfortunately, attempts to demonstrate the formation of an amidino-imidazole (I)



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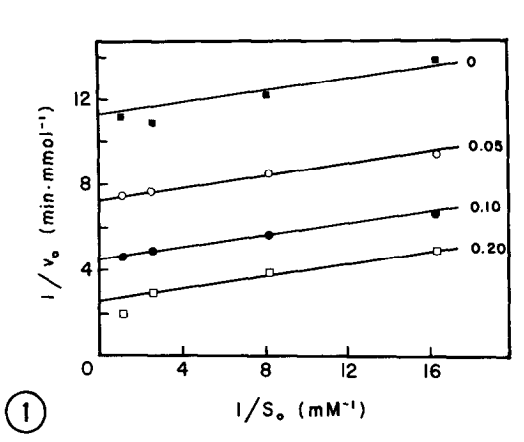


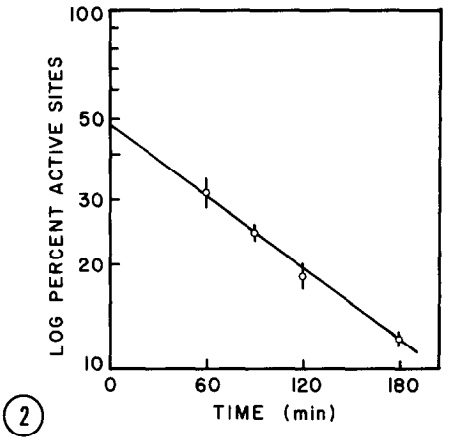


Fig. 1. Effect of imidazole on the hydrolysis of arginine with arginine deiminase from Mycoplasma arthritidis at pH 7.2, 25°. The release of ammonia was measured in the presence of imidazole at the concentrations (M) indicated. The ionic strength was held at 0.2 M by NaCl.

Fig. 2. Time course of loss of ^{14}C label from arginine deiminase at pH 7.0, 0-4°. The enzyme was labeled by quenching the steady state enzymic hydrolysis of [guanidino-^{14}C]arginine in 1 M HCl. Unbound label was removed from the labeled enzyme by continuous ultrafiltration at pH 7 for the time period indicated after the reaction mixture was quenched. The amount of enzyme-bound label is expressed on a semilogarithmic plot as percent of available catalytic sites, assuming two catalytic sites per enzyme molecule. The ^{14}C label is removed from the "quenched" enzyme with a rate constant of 10^{-4} sec^{-1}, compared with 29 sec^{-1} for the enzymic hydrolysis (k_{cat}) of arginine at pH 7.

during rate acceleration by imidazole were unsuccessful. The literature on the hydrolysis of amidines leaves little doubt that I should be completely hydrolyzed in seconds (3).

Direct evidence for a covalent intermediate — If k_3 is rate determining, then high concentrations of arginine would drive all the enzyme to the amidino-enzyme form. Were this the case, quenching of the steady state reaction mixture by acid might be a suitable method for isolating the covalent intermediate.

The enzymic hydrolysis of L-[guanidino-^{14}C]arginine was quenched in 1 M HCl. After quenching the reaction, the pH was adjusted to 7.0 and aliquots were rapidly dialyzed in Dow Hollow-Fiber ultrafiltration units. The dialyzed aliquots were then assayed for protein concentration and ^{14}C, with the results shown in Fig. 2. To our dismay the "[^{14}C]enzyme" loses ^{14}C. The loss follows

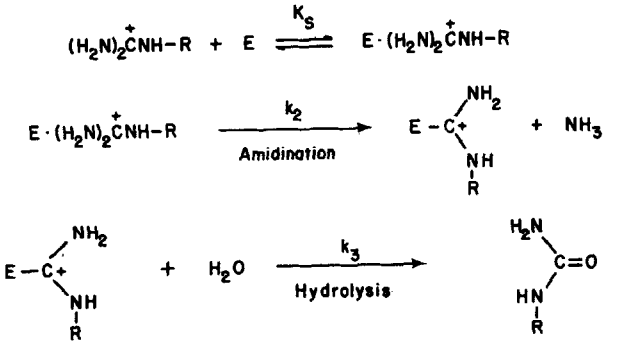
first order kinetics, with a half life of 100 min at pH 7.0, 0-4°.

The question immediately arises whether any ^{14}C is covalently bound to the enzyme, since the relatively short dialysis time periods may not permit complete removal of free [^{14}C]arginine and [^{14}C]citrulline from the enzyme solution. Two experiments designed to answer this question involved determining the amount of ^{14}C-label incorporated into the enzyme when the active site is protected. In the first experiment lysine, a competitive inhibitor (K_i = 15 mM), was present at a concentration of 0.1 M before addition of [^{14}C]arginine. The amount of "[^{14}C]enzyme" measured after 2 and 3 hours rapid dialysis was 25-30% of that determined in the absence of lysine. In the second experiment, the active site was protected by formamidinium ion, an active-site-directed irreversible inhibitor discussed in the following communication (1). In the presence of 10 mM formamidine, nominal amounts of ^{14}C were detected in the dialyzed enzyme solutions after 1.5 hours of dialysis. After 3 hours the amount of ^{14}C corresponded to less than 0.1% of the total number of catalytic sites, compared to 14% observed with only [^{14}C]arginine in the reaction mixture. These results indicate that the dialysis technique removed unbound ^{14}C rapidly enough to allow detection of enzyme-bound label. The absence of enzyme-bound label when formamidine was included in the reaction medium, together with reduced labeling in the presence of lysine, provides direct evidence for covalent substitution of the enzyme during catalysis.

We alluded earlier to the expectation that the amidino-enzyme would be the predominant form of the enzyme under steady state conditions. However, extrapolating back to zero time in Fig. 2, we see that only half of the available sites are labeled. The interpretation of this result is the subject of the next communication in this series, in which evidence for half-of-the-sites reactivity is discussed (1).

DISCUSSION

Tentatively, we propose the hypothesis that catalysis by arginine deiminase may involve a covalent intermediate as the substrate moves along the



SCHEME 1

reaction coordinate (Scheme 1). The stepwise hydrolytic mechanism postulated in Scheme 1 has a precedent. L-Arginine:glycine amidinotransferase from hog liver has been found to catalyze the transfer of an amidino group from arginine to the enzyme (4):

$$\text{L-Arginine} + \text{enzyme-SH} \rightleftharpoons \text{L-ornithine} + \text{enzyme-S-}\overset{+}{C}(NH_2)_2$$

$$\text{Enzyme-S-}\overset{+}{C}(NH_2)_2 + \text{glycine} \rightleftharpoons \text{enzyme-SH} + \text{guanidinoacetate}$$

If the amidino-enzyme intermediate is isolated, it hydrolyzes at neutral pH to urea and free enzyme. Thus the amidinotransferase mechanism is an example of the stepwise mechanism presented in Scheme 1.

Whether or not catalysis by arginine deiminase involves a covalent intermediate, we still have much to learn about the mechanism of this enzyme.

METHODS

Arginine deiminase was prepared from M. arthritidis as described previously (5). The concentration of enzyme was determined from the absorbance at 278 nm using $\varepsilon = 9.52 \times 10^4\ M^{-1}\ cm^{-1}$ (6). The rate of ammonia formation during the arginine deiminase-catalyzed hydrolysis of L-arginine was monitored spectrophotometrically by a coupled reaction with glutamate dehydrogenase (5). Only enzyme preparations from log-phase cultures having a specific activity of at least 18 units/mg were utilized in this investigation. One unit of activity is defined as that amount of enzyme which hydrolyzes 1 μmol of L-arginine in 1 min at 25°, pH 7.2. The concentration of active sites was calculated using the relation

$$[\text{active sites}] = 2 \times [E_T] \times \frac{\text{specific activity}}{20.4\ \text{units/mg}}$$

to allow for small differences in specific activity of different enzyme prepa-

rations. For homogeneous arginine deiminase having a specific activity of 20.4 units/mg, the value of k_{cat} is 29 s^{-1}.

Kinetic studies of rate acceleration by imidazole were performed in 50 mM Tes, pH 7.2, 25°. The ionic strength was held at 0.2 M by NaCl.

Substrate labeling of the enzyme was performed by injecting 0.1 ml aqueous [guanidino-^{14}C]arginine (0.1 μmol; 210 mCi/mmol) into 0.9 ml 0.01 M sodium phosphate (pH 7.0) containing 17 nanomol arginine deiminase agitated by a vortex mixer. Approximately 1 sec after addition of substrate, the reaction was quenched by injecting 0.1 ml concentrated HCl. The quenched reaction mixture was then diluted with 2.4 ml 0.2 M phosphate buffer (pH 7.0) to which 0.5 ml 2 M NaOH had been added. Aliquots were dialyzed against 0.1 M phosphate buffer (pH 7.0) in Dow Hollow-Fiber ultrafiltration units (cellulose acetate fibers, 50,000-dalton exclusion limit) at 0-4°. After dialysis for 0.5-3 hours, the absorbance at 278 nm was determined. One ml of dialyzed enzyme solution was added to 10 ml Aquasol for ^{14}C determination in a Packard 3310 liquid scintillation counter.

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