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Arginine Deiminase Uses an Active-Site Cysteine in Nucleophilic Catalysis of L-Arginine Hydrolysis

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Arginine deiminase (EC 3.5.3.6) catalyzes the hydrolysis of L-arginine to citrulline and ammonium ion; the first step of the L-arginine degradation pathway used by specialized microorganisms as an essential pathway for energy production[1] (Scheme 1). The deiminase has attracted much attention because it can be used to reduce nitric oxide levels[2] and therefore serve as a cancer chemotheraputic agent targeting angiogenesis and tumor growth.[3] With the recent discovery of the arginine degradation pathway in microbial pathogens, arginine deiminase has emerged as a novel drug target. It is within this context that investigations of the structure[4] and the mechanism of catalysis of arginine deiminase from *Pseudomonas aeruginosa* were initiated in our laboratories.

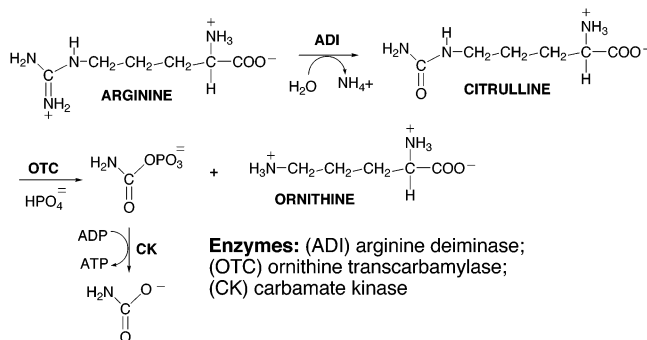
Arginine deiminase shares structural homology with peptidyl-arginine deiminase,[5] amidinotransferase,[6] and *N*(G),*N*(G)-dimethyl-arginine deiminase.[7] Mechanistic studies of this important enzyme family have not been reported. Each of these enzymes possesses a "Cys-His-Glu" motif in their active sites. The conserved cysteine (Cys) residue may mediate group transfer from the guanidinium centers of the respective substrates, as is illustrated with arginine deiminase in Scheme 2.[8] The use of an enzyme active-site Cys residue in nucleophilic catalysis of group-transfer reactions is well precedented.[9]

The possibility of Cys nucleophilic catalysis has important implications for the development of arginine deiminase inhibitors, where design strategies may center on the use of mechanism-based, irreversible inhibitors. In this communication, we report the results from transient-state kinetic experiments, which show that arginine deiminase catalysis proceeds via a covalent adduct formed by active-site Cys406 displacement of ammonia from the guanidinium carbon of L-arginine.

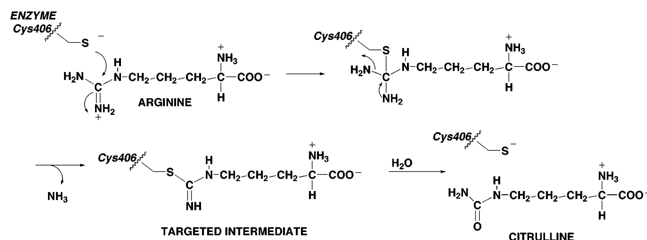
The enzyme was obtained for kinetic study by overexpression of the cloned gene in *Escherichia coli*.[4] Two site-directed mutants, in which the active-site Cys406 was replaced with serine (Ser) (OH replaces SH functionality) or with alanine (Ala) (CH_3 replaces CH_2-SH side chain), were prepared by PCR to serve as controls in the kinetic experiments. The initial velocity of citrulline formation in reaction solutions initially containing 0.1 μM wild-type or 10 μM mutant arginine deiminase and 0.1−5.0 mM L-arginine in 50 mM K^+(2-*N*-morpholino-ethane-sulfonate)/20 mM $MgCl_2$ (pH 5.6, 25 °C) was measured using the published assay procedure.[10] The steady-state kinetic constants of the wild-type enzyme, determined under these optimal conditions, are $k_{cat} = 6.3 \pm 0.2\ s^{-1}$ and $K_m = 140 \pm 20\ \mu M$. The observed inactivity of the two mutants ($k_{cat} < 1 \times 10^{-4}\ s^{-1}$), supports the proposed role of the Cys406 in nucleophilic catalysis.

The next step was to demonstrate the intermediacy of a covalent adduct formed between Cys406 and the substrate. The "thiourea"

Scheme 1



Scheme 2



functionality of the targeted intermediate shown in Scheme 2 is known to be chemically stable in aqueous solution.[11] It was therefore feasible to test the proposed chemical pathway via intermediate trapping using radiolabeled substrate in combination with rapid-quench techniques. The single-turnover time courses for the consumption of [^{14}C-1]L-arginine and formation of [^{14}C-1]L-citrulline were measured (using HPLC separation[12] and scintillation counting), as was the time course for the formation and consumption of [^{14}C]-labeled enzyme (isolated by CCl_4-induced precipitation) (Figure 1). The reaction was carried out at 25 °C and pH 5.6 with enzyme in ~50-fold excess to substrate. If the K_m is used as an estimate of the dissociation constant for the enzyme−substrate complex, ~80% of the [^{14}C-1]L-arginine is predicted to be associated with enzyme upon mixing. The citrulline formation and arginine decay curves, shown in Figure 1A, were fitted to a first-order rate equation to yield apparent rate constants $k = 3.6 \pm 0.1\ s^{-1}$ and $k = 4.2 \pm 0.1\ s^{-1}$, respectively. The time courses for the formation and decay of ^{14}C-labeled enzyme (Figure 1B), were fitted using the simulation program KINSIM and the kinetic model shown in Figure 1B to define the rate constant of intermediate formation as 13 s^{-1} and the rate constant for intermediate consumption as 6.5 s^{-1}. At 120 ms, the intermediate reaches a maximal level of 7% of the estimated level of bound substrate. The ^{14}C-labeled enzyme is judged to be a kinetically competent intermediate, because it is formed faster than is product, and it is consumed at a rate consistent with that of product formation. The level of

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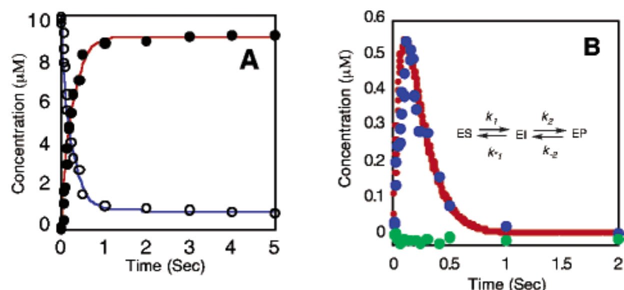

Figure 1. Time courses for the single-turnover reaction of 520 μM arginine deiminase (wild-type or Cys406Ser mutant) and 10 μM [^{14}C-1]L-arginine in 50 mM K^+Mes/20 mM $MgCl_2$ (pH 5.6) at 25 °C. Reactions were carried out in a KinTek rapid-quench instrument using 0.6 M HCl as the quench, CCl_4 to precipitate the enzyme, and reversed-phase HPLC to separate the [^{14}C-1]L-arginine and [^{14}C-1]L-citrulline: (A) (○) [^{14}C-1]L-arginine and (●) [^{14}C-1]L-citrulline time course data generated with the wild-type enzyme were fitted with the first-order equations $[S]_t = [S]_{max} - ([P]_{max}(1 - e^{-kt}))$ and $[P]_t = [P]_{max}(1 - e^{-kt})$ where "k" is the first-order rate constant; $[S]_t$ and $[P]_t$ are the substrate and product concentrations at time "t". (B) Time courses for the formation and decay of the ^{14}C-labeled enzyme from the reaction of wild-type arginine deiminase (blue ●) and Cys406Ser arginine deiminase (green ●). The red curve was obtained by fitting the data points to the kinetic model shown by using the program KINSIM and $k_1 = 13$ s^{-1}, $k_{-1} = 0.05$ s^{-1}, $k_2 = 6.5$ s^{-1}, and $k_{-2} = 0.02$ s^{-1}.

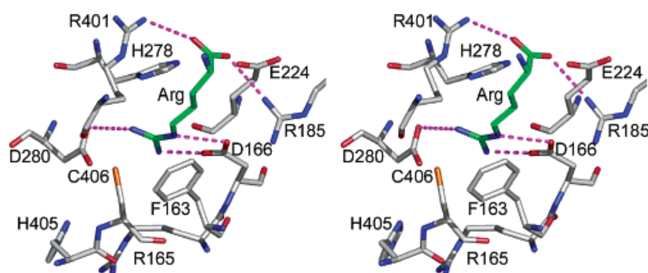

Figure 2. Stereoscopic view of a modeled L-arginine (green), docked in the active site of adenosine deiminase (figure adapted from reference 4). The Cys406 is oriented for nucleophilic attack at the guanidinium carbon. Electrostatic interactions are indicated by dotted lines (magenta) (sulfur: orange; oxygen: red; nitrogen: blue).

maximum accumulation of the intermediate is small (typical of what is observed for the accumulation of covalent enzyme intermediates[13]), thus indicating that its rate of formation is not significantly faster than its rate of reaction. This is also apparent from the substrate decay curve, which largely mirrors the product formation curve.

The next step was to show that the Cys406 residue plays an essential role in the formation of the reaction intermediate, as is prescribed by the proposed chemical pathway (Scheme 2). For this purpose the Cys406Ala and Cys406Ser mutants were employed. As reported earlier, the two mutants do not catalyze product formation. The chemical pathway also predicts that neither mutant will catalyze the formation of ^{14}C-labeled enzyme. Reaction of the mutant enzymes with limiting [^{14}C-1]L-arginine under the same conditions used for the wild-type enzyme (Figure 1B) failed to produce ^{14}C -labeled enzyme. The results obtained from studies of the Cys406Ser mutant (shown in Figure 1B) were matched by those observed for the Cys406Ala (data not shown). The conclusion drawn from these observations is that the Cys406 thiol group is essential for the formation of the reaction intermediate.

The X-ray structure of the arginine deiminase active site (see Figure 2) suggests that replacement of Cys406 with a Ser residue will not perturb the active-site residues.[14] Thus, failure of the Cys406Ser mutant to form the reaction intermediate can be attributed to the removal of the active-site nucleophile. Moreover, the X-ray structure and modeled arginine show that the Cys406 thiol group is perfectly oriented for attack at the guanidinium carbon of the L-arginine as it is held in place by H-bonds formed with several active-site residues. His278 is the probable base.

In summary, the close proximity of the Cys406 thiol to the guanidinium carbon of the L-arginine substrate, coupled with the essential role that it plays in the formation of the reaction intermediate, serves as strong evidence to support the proposed role of Cys406 in nucleophilic catalysis.[15] Nucleophilic catalysis in arginine deiminase has important implications for the mechanisms of catalysis in other members of the same enzyme family, which also conserve the active-site Cys. In addition, the results suggest that arginine deiminase inhibitor design strategies may be based on mechanism-based, covalent modification of the active-site Cys406.

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(14) Following the submission of this manuscript we have solved the structure of the Cys406Ala mutant complexed with L-arginine. The new structure confirms the published model[4] and demonstrates that replacement of Cys406 with Ala does not alter the configuration of active-site residues.

(15) Note in added proof: the X-ray coordinates of the *Mycoplasma arginini* arginine deiminase in complex with the product citrulline have been released at the PDB ID: 1LXY by authors Das, K.; Buttler, G. H.; Kwiztkowski, V.; Yadav, P.; Arnold, E. This structure reveals a covalent adduct formed between the active-site Cys and the citrulline carbonyl carbon, consistent with nucleophilic addition of the Cys at the electrophilic center.

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