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L-Canavanine Is a Time-Controlled Mechanism-Based Inhibitor of *Pseudomonas aeruginosa* Arginine Deiminase

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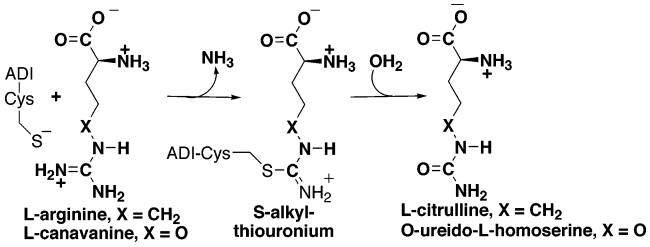
Mechanism-based, irreversible enzyme inhibition has proven to be an effective strategy for the development of therapeutic drugs.[1] Our work in this area focuses on the development of suicide substrate-type inhibitors[2] targeting members of the L-arginine-metabolizing, guanidino-transferring enzyme superfamily.[3] Several members of this family have direct relevance to the treatment of cancer as well as immunological and infectious diseases.[4] A common catalytic strategy, involving active-site cysteine-mediated guanidino-group transfer,[3] is used by these enzymes. This provides the opportunity for the design of inhibitors which are substrate analogues that participate in the first partial reaction to form the covalent enzyme−Cys-alkylthiouronium ion intermediate but fail to or only slowly undergo the ensuing transfer step necessary to regenerate the enzyme catalyst. Here we report the discovery of a novel "slow substrate" inhibitor of the antibiotic target arginine deiminase (ADI), which catalyzes the conversion of L-arginine to L-citrulline and ammonia (Scheme 1). This biological process,

Scheme 1



together with ensuing transformations catalyzed by ornithine transcarbamylase and carbamate kinase, constitutes the L-arginine dihydrolytic degradation pathway,[5] which is essential to cell growth in microbial pathogens[6] but which is notably absent in humans.

L-Canavanine (Scheme 1) is known to be a competitive inhibitor and alternative substrate for most L-arginine-utilizing enzymes,[7] including the ADI from *Streptococcus faecalis*.[8] By using the *Pseudomonas aeruginosa* ADI (PaADI) for inhibitor development,[9−11] we have discovered that L-canavanine is a slow substrate inhibitor. Evidence for this conclusion derives from enzyme inactivation studies. PaADI[9] (10μM) in 20 mM $MgCl_2$ and 50 mM K^+(2-*N*-morpholinoethanesulfonate (pH 5.6 and 25 °C) was preincubated with different concentrations of L-canavanine (0.2, 0.5, 1.0, 4.0, and 10 mM). After various time periods, 10 μL aliquots were mixed with 5 mM L-arginine in 1 mL of 20 mM $MgCl_2$ and 50 mM K^+-(2-*N*-morpholinoethanesulfonate (pH 5.6, 25 °C) and then assayed for ADI activity by measuring L-citrulline production.[12] The rates of inactivation at various L-canavanine concentrations were determined by fitting the enzyme activity data to the single-exponential equation $\ln(v_t/v_0) = -k_{obs}t$, where v_t is the enzyme activity at time t, v_0 is the rate at time zero, and k_{obs} is the inactivation rate (Figure 1A). Data from a plot of first-order rate constant versus L-canavanine concentration (Figure 1B) were fitted to $k_{obs} = (k_{inact}[I])/(K_i + [I])$ to yield the inactivation rate constant of 0.31 ± 0.03 min^{-1} and binding affinity dissociation constant K_i of 1.7 ± 0.5 mM.

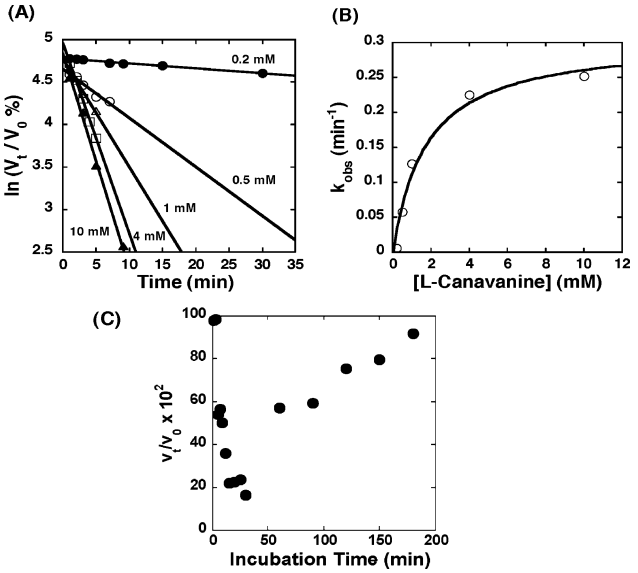


Figure 1. (A) Time-dependent inactivation (ln(% remaining activity in 10 μM PaADI) vs incubation time) as a function of L-canavanine concentration. (B) Concentration dependence of k_{obs} for inactivation of PaADI by L-canavanine. (C) Percent remaining PaADI activity in reaction of 10 μM ADI and 1 mM L-canavanine.

The time course of enzyme activity following incubation of PaADI at subsaturating L-canavanine (1 mM) is shown in Figure 1C. While catalysis by PaADI is inhibited over a ~35 min period, its catalytic activity is regained over longer times. Electrospray ionization mass spectrometric analysis was conducted on HCl-quenched (final concentration 0.1 M) aliquots removed at time intervals during the inactivation−regeneration phases of the reaction of 10 μM ADI and 1 mM L-canavanine. The data show that an enzyme of mass of 46,307 Da, corresponding to the mass of starting recombinant ADI,[9] is present at $t = 0$. Mass spectral analysis of quenched reaction mixtures shows that a labeled protein with a mass 160 Da higher than that of ADI forms and then disappears over the time range of 0−12 h (see Supporting Information). The new protein has a mass expected for an intermediate formed by an ammonia-releasing reaction of PaADI with L-canavanine (Scheme 1).

The close structural similarity between L-arginine and L-canavanine suggests that an analogous pathway is responsible for the inactivation−reactivation cycle. The product formed from L-canavanine by way of a thiouronium intermediate is *O*-ureido-homoserine, which we have identified by mass spectrometric

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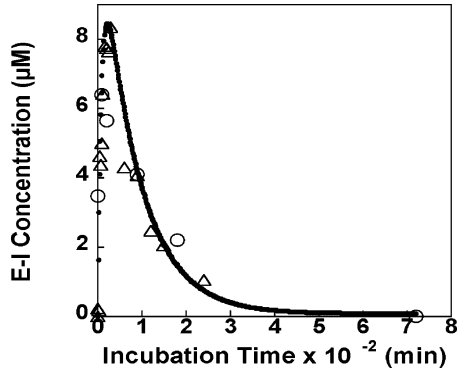

Figure 2. Time course for formation and consumption of *S*-alkylthiouronium intermediate (E−I) calculated from mass spectral (○) and inactivation kinetic (△) data by assuming $[E-I] = [E_{total}]E_{labeled}/(E_{labeled} + E_{unlabeled})$ and $[E-I] = [E_{total}] - [E_{total}](v_t/v_0)$, respectively. The curve was simulated with KINSIM[13] to define the kinetic constants of the kinetic model: $K_1 = 5.88 \times 10^{-4}\ \mu M^{-1}$, $k_2 = 0.12\ min^{-1}$, $k_{-2} = 0.05\ min^{-1}$, $k_3 = 0.01\ min^{-1}$, and $k_{-3} = 8.0 \times 10^{-5}\ min^{-1}$.

analysis (*m*/*z* 178.0825 observed vs *m*/*z* 178.0828 for $C_5H_{11}N_3O_4$), consistent with previous observations.[8] The mass spectral and kinetic data were combined to generate a time course for formation and hydrolysis of the covalent enzyme adduct (E−I) in the reaction with canavanine (Figure 2). The data were fitted to the kinetic model shown in eq 1, by using the simulation program KINSIM[13] to define

$$E + I \underset{}{\overset{K_1}{\rightleftharpoons}} E{\cdot}I \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E{-}I \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} E{\cdot}P \qquad (1)$$

the microscopic rate and kinetic constants derived from Figure 2, to define the microscopic rate constants for the inactivation−reactivation steps. Accordingly, in the canavanine case $k_2 = 0.12\ min^{-1}$ for E−I formation is 12-fold larger than the $k_3 = 0.01\ min^{-1}$ for E−I hydrolysis. The corresponding rate constants for formation and hydrolysis of the Cys-alkylthiouroninum intermediate (Scheme 1, proven by X-ray analysis)[9b] in single-turnover reaction of PaADI with [^{14}C]L-arginine[10] are $k_2 = 13\ s^{-1}$ and $k_3 = 6.5\ s^{-1}$, respectively.[10]

These results show that substitution of an oxygen for the methylene group in arginine reduces the rates of formation and hydrolysis of the covalent enzyme adduct. However, because the change more greatly retards the hydrolysis step, the enzyme is temporarily inactivated. This phenomenon has great potential for the design of new inhibitors to modulate the level of active ADI in the cell in a defined manner. Whereas it is desirable to shut down ADI permanently in human pathogens, this is not the case for the homologous human peptidylarginine deiminase.[4d] Here, it would be desirable to block only the excessive activity that leads to deimination of protein arginine side chains and hence production of arthritis-causing antigens. A time-controlled inhibitor would allow the ADI activity to be attenuated but not eliminated, thus preserving some normal function.

The next step in the development of inhibitors for the guanidino-transferring enzyme family is to understand, and then exploit, the oxygen-substituent effect. The known structures of the ADI−arginine and ADI−thioalkyluronium ion intermediate[9] show that the relative orientation of the reaction center and attacking nucleophile is governed by the guanidine binding residues Asp166 and Asp280. The presence of oxygen in the chain connecting the amino acid and guanidine moieties in L-canavanine and the thiouronium ion intermediate could alter the orientation of the reaction center to impair the hydrolysis step more than the cysteine addition and ammonium ion elimination steps. Alternatively, the electronic effect[14] of oxygen on the electrophilicity of the guanidinium carbon atom might be the controlling factor, amplified in the second partial reaction where water, a weak nucleophile, is involved.

In conclusion, we have shown that L-canavanine is a slow substrate inhibitor of *P. aeruginosa* arginine deiminase that functions by covalent modification of the active-site Cys406 residue in the form of a thiouronium salt. Thus, our earlier proposed[10] strategies for the design of ADI inhibitors that rely on mechanism-based covalent modification of the active-site Cys406 are feasible. The synthesis and evaluation of guanidinium group and chain-modified L-arginine derivatives as slow substrate inhibitors having time-controlled enzyme release properties are underway.

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Supporting Information Available: Mass spectra of mixtures of PaADI and canavanine. This material is available free of charge via the Internet at http://pubs.acs.org.

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