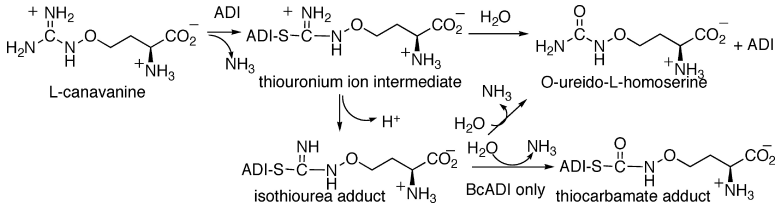
Article

Inactivation of Microbial Arginine Deiminases by l-Canavanine

Ling Li, Zhimin Li, Danqi Chen, Xuefeng Lu, Xiaohua Feng, Elizabeth C. Wright, Nathan O. Solberg, Debra Dunaway-Mariano, Patrick S. Mariano, Andrey Galkin, Liudmila Kulakova, Osnat Herzberg, Kari B. Green-Church, and Liwen Zhang

J. Am. Chem. Soc., **2008**, 130 (6), 1918-1931 • DOI: 10.1021/ja0760877



More About This Article

Additional resources and features associated with this article are available within the HTML version:

- Supporting Information
- Links to the 1 articles that cite this article, as of the time of this article download
- Access to high resolution figures
- Links to articles and content related to this article
- Copyright permission to reproduce figures and/or text from this article

View the Full Text HTML

J|A|C|S
ARTICLES
Published on Web 01/19/2008

Inactivation of Microbial Arginine Deiminases by L-Canavanine

Ling Li,[†] Zhimin Li,[†] Danqi Chen,[†] Xuefeng Lu,[†] Xiaohua Feng,[†] Elizabeth C. Wright,[†] Nathan O. Solberg,[†] Debra Dunaway-Mariano,*[,†] Patrick S. Mariano,*[,†] Andrey Galkin,[‡] Liudmila Kulakova,[‡] Osnat Herzberg,[‡] Kari B. Green-Church,[§] and Liwen Zhang[§]

Department of Chemistry and Chemical Biology, University of New Mexico, Albuquerque, New Mexico 87131, Center for Advanced Research in Biotechnology, University of Maryland Biotechnology Institute, Rockville, Maryland 20850, and Mass Spectrometry and Proteomics Facility, Campus Chemical Instrument Center, The Ohio State University, Columbus, Ohio 43210

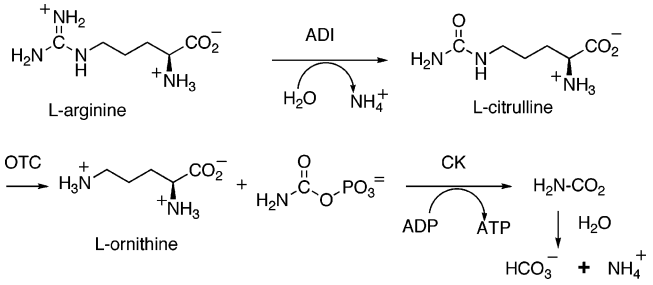
Received August 13, 2007; E-mail: mariano@unm.edu

Abstract: Arginine deiminase (ADI) catalyzes the hydrolytic conversion of L-arginine to ammonia and L-citrulline as part of the energy-producing L-arginine degradation pathway. The chemical mechanism for ADI catalysis involves initial formation and subsequent hydrolysis of a Cys-alkylthiouronium ion intermediate. The structure of the *Pseudomonas aeruginosa* ADI-(L-arginine) complex guided the design of arginine analogs that might react with the ADIs to form inactive covalent adducts during catalytic turnover. One such candidate is L-canavanine, in which an N−methylene of L-arginine is replaced by an N−O. This substance was shown to be a slow substrate-producing O-ureido-L-homoserine. An in depth kinetic and mass spectrometric analysis of *P. aeruginosa* ADI inhibition by L-canavanine showed that two competing pathways are followed that branch at the Cys-alkylthiouronium ion intermediate. One pathway leads to direct formation of O-ureido-L-homoserine via a reactive thiouronium intermediate. The other pathway leads to an inactive form of the enzyme, which was shown by chemical model and mass spectrometric studies to be a Cys-alkylisothiourea adduct. This adduct undergoes slow hydrolysis to form O-ureido-L-homoserine and regenerated enzyme. In contrast, kinetic and mass spectrometric investigations demonstrate that the Cys-alkylthiouronium ion intermediate formed in the reaction of L-canavanine with *Bacillus cereus* ADI partitions between the product forming pathway (O-ureido-L-homoserine and free enzyme) and an inactivation pathway that leads to a stable Cys-alkylthiocarbamate adduct. The ADIs from *Escherichia coli*, *Burkholderia mallei*, and *Giardia intestinalis* were examined in order to demonstrate the generality of the L-canavanine slow substrate inhibition and to distinguish the kinetic behavior that defines the irreversible inhibition observed with the *B. cereus* ADI from the time controlled inhibition observed with the *P. aeruginosa*, *E. coli*, *B. mallei*, and *G. intestinalis* ADIs.

Introduction

L-Arginine deiminase (ADI) catalyzes the hydrolysis of L-arginine to form citrulline and ammonia. This reaction is the first step in the L-arginine degradation pathway (Scheme 1)[1−4] that is present in a wide range of bacterial species [5−9] and in parasitic protists such as *Giardia intestinalis*,[10] *Trichomonas vaginalis*,[11] *Trichomonas fetus*,[12] and *Hexamita inflate*.[13] For pathogenic bacteria, the pathway is required for virulence and for energy production under anaerobic conditions.[14−17]

Scheme 1



[†] University of New Mexico.
[‡] University of Maryland.
[§] The Ohio State University.

(1) Abdelal, A. T. *Annu. Rev. Microbiol.* **1979**, *33*, 139−168.
(2) Cunin, R.; Glansdorff, N.; Pierard, A.; Stalon, V. *Microbiol. Rev.* **1986**, *50*, 314−352.
(3) Zuniga, M.; Perez, G.; Gonzalez-Candelas, F. *Mol. Phylogenet. Evol.* **2002**, *25*, 429−444.
(4) (a) Dong, Y.; Chen, Y. Y.; Snyder, J. A.; Burne, R. A. *Appl. Environ. Microbiol.* **2002**, *68*, 5549−5553. (b) Knodler, L. A.; Sekyere, E. O.; Stewart, T. S.; Schofield, P. J.; Edwards, M. R. *J. Biol. Chem.* **1998**, *273*, 4470−4477.
(5) Baur, H.; Luethi, E.; Stalon, V.; Mercenier, A.; Haas, D. *Eur. J. Biochem.* **1989**, *179*, 53−60.
(6) Shibatani, T.; Kakimoto, T.; Chibata, I. *J. Biol. Chem.* **1975**, *250*, 4580−4583.
(7) Kondo, K.; Sone, H.; Yoshida, H.; Toida, T.; Kanatani, K.; Hong, Y. M.; Nishino, N.; Tanaka, J. I. *Mol. Gen. Genet.* **1990**, *221*, 81−86.
(8) Weickmann, J. L.; Fahrney, D. E. *J. Biol. Chem.* **1977**, *252*, 2615−2620.

10.1021/ja0760877 CCC: $40.75

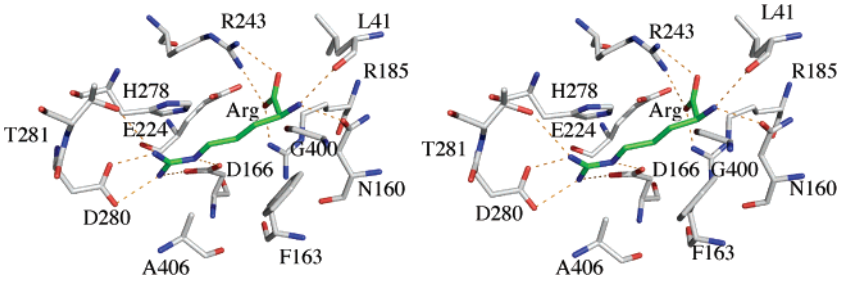


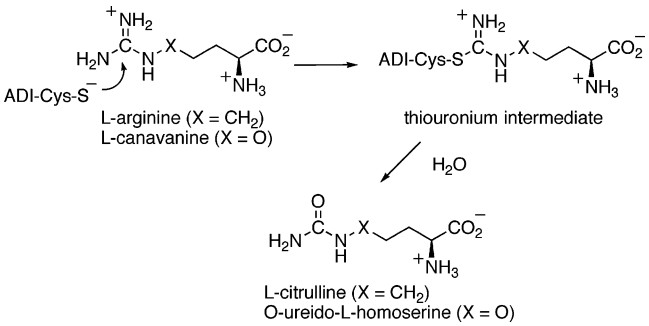
Figure 1. Stereoscopic representation of the C406A mutant of PaADI active site bound with L-arginine prepared using the graphics program Pymol[45] and the X-ray crystallographic coordinates for the structure of the C406A PaADI (L-arginine) complex (PDB ID 2A9G).[19]

For the human parasite *G. intestinalis*, which lacks mitochondria, the L-arginine degradation and the glycolysis pathways serve as the primary source of energy. Flux studies have shown that the L-arginine degradation sequence is dominant in the production of ATP.[10] Furthermore, gene silencing experiments carried out with *G. intestinalis* indicate that this pathway is required for survival of the trophozites in culture (Kulakova, unpublished result). In light of the fact that the pathway is not present in humans, the enzymes involved represent significant targets for the development of drugs to treat giardiasis.

The investigation described below focuses on the development of inhibitors for L-arginine deiminase (ADI), the first enzyme in the L-arginine degradation pathway. The initial phase of our studies of the structure and catalytic mechanism of this enzyme concentrated on the *Pseudomonas areuginosa* ADI (PaADI). The structure determination of PaADI set the stage for work on inhibitor design. Specifically, structural investigations showed that the active site of PaADI is located in a narrow crevice formed by a network of charged residues (Figure 1).[18,19] The results of site-directed mutagenesis and substrate analog investigations demonstrated that ligand binding energy is derived from ion pair and hydrogen bonding interactions between active site residues and the guanidinium, carboxylate and ammonium groups of the substrate.[19,20] Thus, it is clear that high affinity inhibitors of PaADI must possess the functional groups of L-arginine.

The discovery that PaADI employs an active site Cys in nucleophilic catalysis[21] provided the foundation for mechanism-based strategies for inhibitor design for ADI[22] and its close relatives, protein arginine deiminase (PAD4)[23,24] and dimethylarginine dimethylaminohydrolase (DDAH).[25,26] Specifically, the chemical pathway for catalysis by ADI involves addition of the active site Cys-thiolate[27] to the guanidinium carbon of L-arginine followed by elimination of ammonia to generate the Cys-alkylthiouronium intermediate (Scheme 2). In the second partial reaction in the catalytic pathway, nucleophilic addition of a His activated water molecule to the Cys-alkylthiouronium carbon takes place with ensuing elimination of the Cys-thiolate and formation of the product L-citrulline.[19,20]

Scheme 2



One strategy we have formulated[22] for the design of effective ADI inhibitors is founded on the assumption that differences in the electronic and steric demands of the two partial reactions involved in the catalytic process could be used advantageously. Accordingly, the approach would employ L-arginine analogs that are capable of reacting with the ADI Cys-thiolate anion to form the thiouronium intermediate but, by appropriate design, these intermediates would either not, or only slowly, undergo the hydrolysis partial reaction required to regenerate the active form of the enzyme. Inhibitors adhering to these design features should have a high potential for effective *in vivo* ADI inhibition owing to their high water solubility, specific targeting of ADI, and efficient transport into the pathogen via the L-arginine transporter.[28]

The L-arginine analog selected initially to test this strategy is L-canavanine (Scheme 2), a compound that differs from the

(9) Schimke, R. T.; Berlin, C. M.; Sweeney, E. W.; Carroll, W. R. *J. Biol. Chem.* **1966**, *241*, 2228−2236.
(10) Schofield, P. J.; Edwards, M. R.; Matthews, J.; Wilson, J. R. *Mol. Biochem. Parasitol.* **1992**, *51*, 29−36.
(11) Linstead, D.; Cranshaw, M. A. *Mol. Biochem. Parasitol.* **1983**, *8*, 241−252.
(12) Yarlett, N.; Lindmark, D. G.; Goldberg, B.; Moharrami, M. A.; Bacchi, C. J. *J. Eukaryotic Microbiol.* **1994**, *41*, 554−559.
(13) Biagini, G. A.; Yarlett, N.; Ball, G. E.; Billetz, A. C.; Lindmark, D. G.; Martinez, M. P.; Lloyd, D.; Edwards, M. R. *Mol. Biochem. Parasitol.* **2003**, *128*, 11−19.
(14) Silva. L. M.; Baums, C. G.; Rehm, T.; Wisselink; H. J.; Goethe, R.; Valentin- Weigand, P. *Vet Microbiol* **2006** *115*, 117−27.
(15) Gruening, P.; Fulde, M.; Valentin-Weigand, P.; Goethe, R. *J. Bacteriol.* **2006** *188*, 361−9.
(16) Makhlin, J.; Kofman, T.; Borovok, I.; Kohler, C.; Engelmann, S.; Cohen, G.; Aharonowitz, Y. *J. Bacteriol.* **2007** *189*, 5976−86.
(17) Seggewiss, J.; Becker, K.; Kotte, O.; Eisenacher, M.; Yazdi, M. R.; Fischer, A.; McNamara, P.; Al Laham, N.; Proctor, R.; Peters, G.; Heinemann, M.; von Eiff, C. *J. Bacteriol*. **2006**, *188*, 7765−77.
(18) Galkin, A.; Kulakova, L.; Sarikaya, E.; Lim, K.; Howard, A.; Herzberg, O. *J. Biol. Chem.* **2004**, *279*, 14001−14008.
(19) Galkin, A.; Lu, X. F.; Dunaway-Mariano, D.; Herzberg, O. *J. Biol. Chem.* **2005**, *280*, 34080−34087.
(20) Lu, X. F.; Li, L.; Wu, R.; Mariano, P. S.; Dunaway-Mariano, D. *Biochemistry* **2006**, *45*, 1162−1172.
(21) Lu, X.; Galkin, A.; Herzberg, O.; Dunaway-Mariano, D. *J. Am. Chem. Soc.* **2004**, *126*, 5374−5375.
(22) Lu, X. F.; Li, L.; Feng, X. H.; Dunaway-Mariano, D.; Engen, D.; Mariano, P. S. *J. Am. Chem. Soc.* **2005**, *127*, 16412−16413.
(23) Luo, Y.; Arita, K.; Bhatia, M.; Knuckley, B.; Lee, Y. H.; Stallcup, M. R.; Sato, M.; Thompson P. R. *Biochemistry* **2006**, *45*, 11727−36.
(24) Luo, Y.; Knuckley, B.; Lee, Y. H.; Stallcup, M. R.; Thompson, P. R. *J. Am. Chem. Soc.* **2006**, *128*, 1092−3.
(25) Stone, E. M.; Schaller, T. H.; Bianchi, H.; Person, M. D.; Fast, W. *Biochemistry* **2005**, *44*, 13744−52.
(26) Hong, L; Fast, W, *J. Biol. Chem.* **2007**, *282*, 34684−34692.
(27) Li, L.; Li, Z.; Wang, C. H.; Xu, D.; Mariano, P. S.; Dunaway-Mariano, D.; Guo, H. *Biochemistry* submitted for review.
(28) Knodler, L. A.; Schofield, P. J.; Edwards, M. R. *Microbiology* **1995**, *141*, 2063−2070.

Table 1. Kinetic Rate Constants for ADI-Catalyzed Conversion of L-Canavanine (Can) to O-Ureido-L-homoserine Plus Ammonia and L-Arginine (Arg) to L-Citrulline Plus Ammonia

ADI	pH	substrate	k_{cat} (s^{-1})	K_M (mM)	k_{cat}/K_M ($M^{-1}s^{-1}$)
PaADI	5.6	Can	0.62 ± 0.04	0.7 ± 0.1	8.9×10^2
		Arg[a]	6.3 ± 0.1	0.14 ± 0.01	4.5×10^4
BcADI	7.0	Can	0.46 ± 0.04	2.3 ± 0.4	2.0×10^2
		Arg[b]	4.4 ± 0.1	0.09 ± 0.01	4.9×10^4
EcADI	6.0	Can	0.42 ± 0.03	2.1 ± 0.3	2.0×10^2
		Arg[c]	3.2 ± 0.1	0.32 ± 0.2	1.0×10^4
BmADI	5.6	Can	0.09 ± 0.01	1.2 ± 0.1	7.5×10^1
		Arg[d]	1.3 ± 0.1	0.09 ± 0.01	1.4×10^4
GiADI	7.5	Can	0.32 ± 0.04	1.9 ± 0.2	1.7×10^2
		Arg[e]	2.6 ± 0.1	0.16 ± 0.01	1.6×10^4

[a] From ref 20. Reaction solutions initially contained 0.1 μM PaADI, 0.1−2.0 mM L-arginine, and 20 mM $MgCl_2$ in pH 5.6, 50 mM K^+MES buffer at 25 °C. [b] Reaction solutions initially contained 0.03 μM BcADI, L-arginine (0.5−5 K_m), 10 mM ketoglutarate, 0.2 mM NADH, 0.9 mg of glutamate dehydrogenase (Sigma, type II) in pH 7.0, 50 mM K^+HEPES buffer at 25 °C. [c] Reaction solutions initially contained 1.0 μM EcADI, 0.1−5.0 mM L-arginine, and 20 mM $MgCl_2$ in pH 6.0, 50 mM Bis-Tris-HCl buffer at 25 °C. [d] Reaction solutions initially contained 1.0 μM BmADI, 0.1−1.0 mM L-arginine, and 20 mM $MgCl_2$ in pH 5.6, 50 mM K^+MES buffer at 25 °C. [e] Reaction solutions initially contained 1.0 μM GiADI and 0.1−2.0 mM L-arginine in pH 7.5, 50 mM K^+HEPES buffer at 25 °C.

native substrate by replacement of an N−methylene group by a N−oxygen moiety. We believed that this modification would not significantly impair binding to the active site nor would it prevent reaction with the Cys-thiolate anion. On the other hand, the N−O for N−CH_2 replacement might alter the hydrolysis step by (1) introducing an α-effect,[29] (2) inducing proton loss from the thiouronium ion intermediate, and/or (3) causing misalignment of the electrophilic carbon with respect to the His-bound water nucleophile.

The results of an in depth kinetic and mass spectral study of the L-canavanine reaction catalyzed by ADIs derived from *Pseudomonas aeruginosa* (PaADI), *Bacillus cereus* (BcADI), *Escherichia coli* (EcADI), *Burkholderia mallei* (BmADI), and *G. intestinalis* (GiADI) are described below. In this investigation, L-canavanine was shown to be a substrate for all of the ADIs, which promote its conversion to O-ureido-L-homoserine by using the same pathway employed for transformation of L-arginine to L-citrulline (Scheme 2). However, in each case the Cys-alkylthiouronium intermediate derived from L-canavanine partitions between product formation and generation (by proton loss) of the Cys-alkyl-isothiourea adduct, from which the regeneration of a catalytically active enzyme is slow, yet finite. Among the ADIs examined, BcADI is unique because the Cys-alkylthiourea adduct undergoes irreversible formation of a Cys-alkylthiocarbamate adduct.

Results and Discussion

ADI Catalyzed Hydrolysis of L-Canavanine to Form Ammonia and O-Ureido-L-homoserine. Earlier, Kihara and Snell[30] observed that L-canavanine is a slow substrate for catalysis by the ADI derived from *Streptococcoccus faecalis*. Since that time, it has been shown that this L-arginine analog is a slow substrate for other ADIs (e.g., from *Mycoplasma arthritidis*,[31] *Giardia intestinalis*[4b]) as well as a low affinity competitive inhibitor of other types of L-arginine-utilizing enzymes.[32]

Scheme 3

$$\text{ADI} + \text{L-canavanine} \underset{}{\overset{1/K_m}{\rightleftharpoons}} \text{ADI•L-canavanine} \xrightarrow{k_{cat}} \text{ADI} + \text{O-ureido-L-homoserine} + NH_3$$

The slow turnover of L-canavanine by ADI is suggestive of an α-effect[29] promoted by the N−O moiety. To determine which step(s) in the chemical pathway (Scheme 2) is most affected and to determine the generality of the effect, kinetic analyses were carried out using ADIs obtained from distantly related microbes. The initial velocities of PaADI, BcADI, BmADI, EcADI, and GiADI catalyzed transformations of L-canavanine to O-ureido-L-homoserine and ammonia were determined as a function of L-canavanine concentration. The initial velocities (v_o) were found to increase with increasing L-canavanine concentration until saturation of the ADI occurred. The data obtained from these experiments were fitted to $v_o = (k_{cat}[S]/(K_m+[S])$ (see Experimental section in Supporting Information) to yield k_{cat} and K_m values, defined in the kinetic sequence given in Scheme 3. Because L-canavanine is a slowly reacting substrate, the binding steps in these processes can reach thermodynamic equilibrium, in which case the K_m values are equivalent to dissociation constants of the ADI(L-canavanine) complexes.

The k_{cat} and k_{cat}/K_m values for the ADI-catalyzed L-canavanine and L-arginine hydrolysis reactions, measured at the optimal pH for each ADI, are given in Table 1. The data show that for all ADIs the respective k_{cat}/K_m and k_{cat} values for the L-canavanine reaction are ca. 100-fold and ca. 10-fold smaller than those for L-arginine hydrolysis. Thus, replacement of the N−methylene group in the natural substrate by the N−O group in L-canavanine reduces the binding affinities to all ADIs as well as the rates of catalytic turnover.

Formation of the Cys-S-alkylthiouronium Intermediate During Single Turnover Reaction between BcADI and L-Arginine. By carrying out a single turnover reaction of [^{14}C]-L-arginine with excess BcADI, in which rapid acid quench is applied at various time points, we have demonstrated that the BcADI-catalyzed hydrolysis of L-arginine proceeds via a Cys-S-alkylthiouronium intermediate (Figure 2A). The time course data for [^{14}C]-L-arginine consumption and [^{14}C]-L-citrulline formation as well as for the formation and disappearance of radiolabeled BcADI were fitted to the kinetic mechanism shown in Scheme 4 using the kinetic simulation program KINSIM[33] to define rate constants for formation of the intermediate from the BcADI(L-arginine) complex (k_2) and for hydrolysis of the intermediate to form the BcADI(citrulline) complex (k_3). For the reaction of 104 μM BcADI with 49 μM [^{14}C]-L-arginine at pH 7 and 25 °C, k_2 is 50 s^{-1} and k_3 is 9 s^{-1}. The amount of the Cys-S-alkylthiouronium intermediate reaches a maximum value of 6% of the BcADI(L-arginine) complex at 30 ms, after which it decreases (Figure 2A).

Because [^{14}C]-L-canavanine is not readily available, a mass spectrometric based analysis procedure was used to monitor the

(29) (a) Hudson, R. F. *Angew. Chem., Int. Ed.* **1973**, *12*, 36−56. (b) Gerstein, J.; Jencks, W. P. *J. Am. Chem. Soc.* **1964**, *86*, 4655−4663. (c) This effect is proposed to be responsible for cysteine protease inhibition by azapeptide analogs (ref 29d). (d) Magrath, J.; Abeles, R. H. *J. Med. Chem.* **1992**, *35*, 4279−4283.
(30) Kihara, H.; Snell, E. E. *J. Biol. Chem.* **1957**, *226*, 485−495.
(31) Smith, D. W.; Ganaway, R. L.; Fahrney, D. E. *J. Biol. Chem.* **1978**, *253*, 6016−6020.
(32) Rosenthal, G. A. *Q. Rev. Biol.* **1977**, *52*, 155−178.
(33) Barshop, B. A.; Wrenn, R. F.; Frieden, C. *Anal. Biochem.* **1983**, *130*, 134−145.

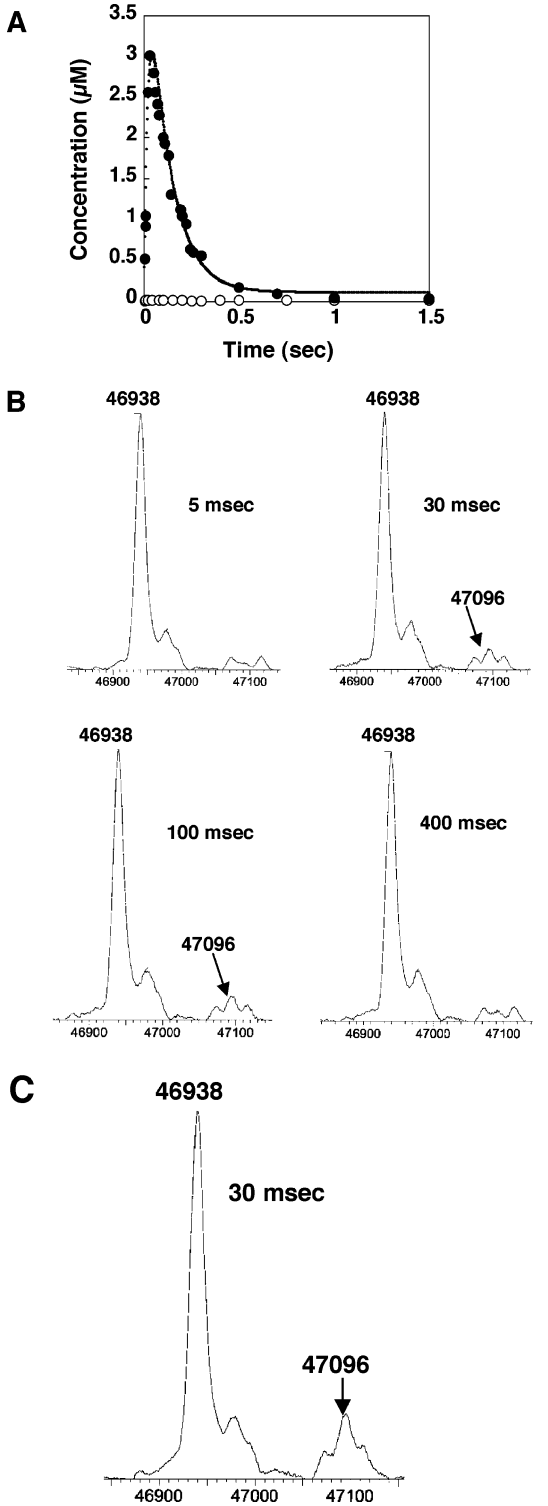


Figure 2. (A) Time courses for the formation and decay of the ^{14}C-labeled enzyme from the reaction of wild-type BcADI (●) and Cys400Ser mutant (○). The dotted curve was obtained by fitting the data points to the kinetic model shown in Scheme 4 by using the program KINSIM[33] and $k_2 = 50$ s^{-1}, $k_{-2} = 0.1$ s^{-1}, $k_3 = 9$ s^{-1}, and $k_{-3} = 0.1$ s^{-1}. (B) Electrospray mass spectra of BcADI thiouronium intermediate from the reaction of 207 μM BcADI and 96 μM L-arginine in 50 mM K^+HEPES at pH 7.0 and 25 °C at 5, 30, 100, and 400 ms. The peak at 46 938 Da is from unmodified BcADI whereas the peak at 47 096 Da is from the thiouronium intermediate formed from BcADI and L-arginine. (C) Electrospray mass spectra of BcADI thiouronium intermediate from the reaction of 207 μM BcADI and 9.6 mM L-arginine in 50 mM K^+HEPES at pH 7.0 and 25 °C at 30 ms. The peak at 46 938 Da is from unmodified BcADI whereas the peak at 47 096 Da is from the thiouronium intermediate formed from BcADI and L-arginine.

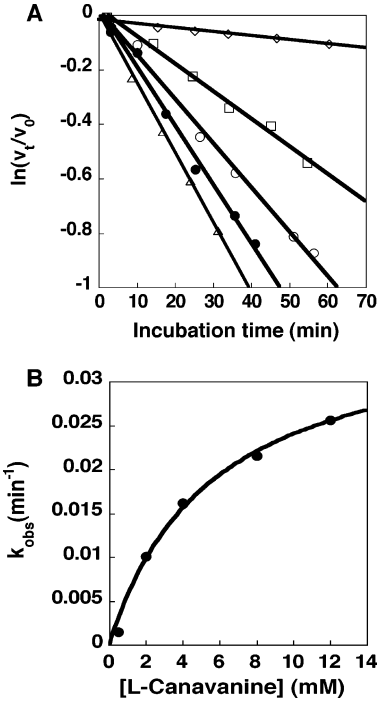


Figure 3. (A) Time and concentration dependence of the inactivation of BcADI by L-canavanine in 50 mM K^+HEPES at pH 7.0 and 25 °C (0.5 mM (◇), 2 mM (□), 4 mM (○), 8 mM (●), and 12 mM (△)). (B) Plot of k_{obs} vs L-canavanine concentration with data fitting to the equation $k_{obs} = (k_{inact}[\text{L-canavanine}]/(K_I + [\text{L-canavanine}])$ to obtain K_I and k_{inact} values.

Scheme 4

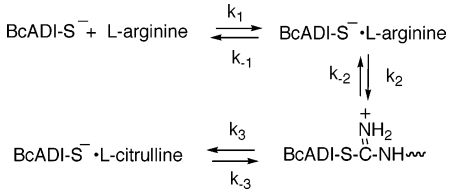


Table 2. Kinetic Rate Constants k_{inact} and K_I for Inactivation of ADI by L-Canavanine and the Ratio of k_{cat} for ADI Catalyzed L-Canavanine Hydrolysis and the k_{inact} for L-Canavanine Induced ADI Inactivation[a]

ADI	pH	k_{inact} (s^{-1})	K_I (mM)	k_{cat}/k_{inact}
PaADI	5.6	$5.2 \pm 0.5 \times 10^{-3}$	1.7 ± 0.5	120
BcADI	7.0	$0.63 \pm 0.01 \times 10^{-3}$	5.0 ± 1.0	730
EcADI	6.0	$1.15 \pm 0.03 \times 10^{-3}$	5.8 ± 0.7	370
BmADI	5.6	$1.32 \pm 0.03 \times 10^{-3}$	2.2 ± 0.2	70
GiADI	7.5	$1.63 \pm 0.06 \times 10^{-3}$	2.2 ± 0.2	200

[a] Kinetic data are reported in Figure 3 (text) and Figure S3 (Supporting Information).

time course for formation and consumption of the Cys-S-alkylthiouronium intermediate during a single turnover reaction of the BcADI(L-canavanine) complex (see below). Prior to initiating these experiments, this method was tested on the BcADI(L-arginine) single turnover reaction. Accordingly, reaction of 207 μM BcADI with 96 μM L-arginine at pH 7 and 25 °C was carried out in a rapid quench apparatus. The acid-quenched samples, collected at reaction times of 5, 30, 100, and 400 ms, were analyzed by using mass spectrometry. As can be seen in Figure 2B, only BcADI (46 938 Da) and not the BcADI thiouronium intermediate (47 096 Da) was observed for mixtures quenched after 5 and 400 ms reaction times. In contrast,

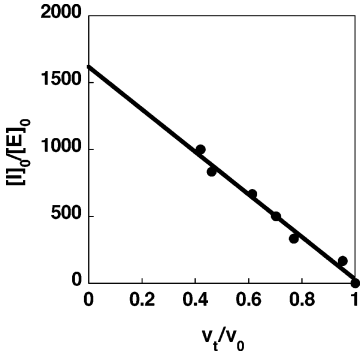


Figure 4. Determination of the ratio of rate constants for BcADI turnover and inactivation by L-canavanine. A plot of the ratio of [L-canavanine]$_0$/[BcADI]$_0$ vs the fraction of BcADI activity remaining, where [BcADI]$_0$ = 6 μM, reactions at pH 7.0 and 25 °C, and 1 h incubation times.

the 47 096 Da BcADI thiouronium intermediate was observed at a level of 8% of the total BcADI and 17% of the BcADI-(L-arginine) complex in mass spectra of reaction mixtures quenched after 30 and 100 ms. The reliability of these measurements was verified by carrying out duplicate experiments.

Importantly, the mass spectrometry-based time course for formation of the thiouronium intermediate and that for the overall BcADI catalyzed L-arginine hydrolysis reaction compare favorably to those determined by employing the radioisotope technique (Figure 2A). The mass spectrum of the quenched sample from reaction of 207 μM BcADI with 9.6 mM L-arginine (pH 7 and 25 °C) terminated at 30 ms shows that ca. 16% of the BcADI has been converted to the thiouronium intermediate (Figure 2C). Unlike the single turnover reaction, in this process BcADI is saturated with L-arginine and therefore a larger fraction of the BcADI accumulates as the thiouronium intermediate.

Inactivation of BcADI by L-Canavanine. A striking observation made during the steady-state kinetic studies with L-canavanine is that BcADI is irreversibly inactivated during the course of multiple turnovers to product. This phenomenon was further investigated by incubating BcADI with L-canavanine and monitoring the time- and concentration-dependent loss of enzyme activity (Figure 3A). A plot of the pseudo-first-order rate constants for inactivation (k_{obs}, calculated from the data reported in Figure 3A) vs L-canavanine concentration is shown in Figure 3B. From inspection of this plot it is clear that k_{obs} increases with increasing L-canavanine concentration until BcADI becomes saturated. The data were fitted to the kinetic equation $k_{obs} = (k_{inact}[I]/(K_I+[I])$ to obtain the K_I and k_{inact} values (Table 2). The ratio $k_{cat}/k_{inact} = 730$ defines the relative efficiencies of the product-forming and the enzyme-inactivating pathways. This partition ratio was determined independently by using the titration method.[34] Inactivation reactions were carried out in which the [canavanine]/[enzyme] ratio ([I]$_0$/[E]$_0$) was varied between 0 and 1000 (Figure 4). The plot of the [I]$_0$/[E]$_0$ vs v_t/v_0 yielded a partition ratio of product formation to enzyme inactivation of 1617 at pH 7.0 and 25 °C.

Comparison of the Reaction Pathways Leading to O-Ureido-L-homoserine and Ammonia and to Inactived BcADI. The structure of the covalent adduct present in the L-canavanine inactivated BcADI was investigated by using mass spectrometric analysis of acid-quenched reaction mixtures of BcADI (10 μM) and L-canavanine (12 mM) (pH 7, 25 °C). With increasing reaction time, the intensity of the mass peak corresponding to native BcADI (46 938 Da) decreases and that associated with the covalent adduct (47 098 Da) increases (see Supporting Information Figure S1). The difference between the masses of native and inactivated BcADI was found to be 160 Da (Figure 5A and Supporting Information Figure S1).

In Figure 5B is shown an overlay of progress curves for (1) the formation of the O-ureido-L-homoserine, (2) the fraction of native BcADI remaining (peak height at 46 938 Da/(peak height at 47 098 Da + peak height at 46 938 Da)), and (3) the fraction

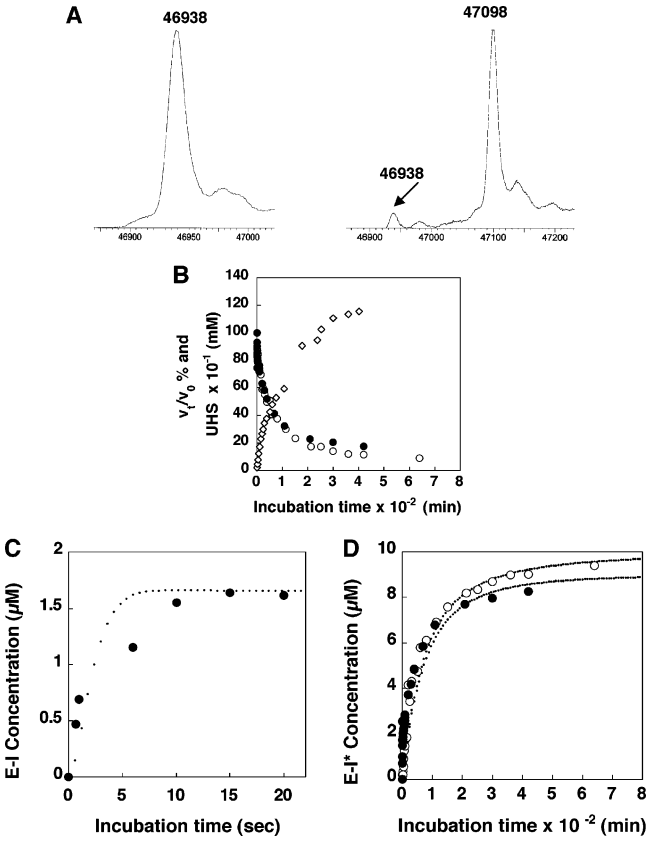


Figure 5. (A) Electrospray mass spectra of BcADI present in aliquots taken from an incubation mixture initially containing 10 μM BcADI and 12 mM L-canavanine in 50 mM K^+HEPES at pH 7.0 and 25 °C. Aliquots were withdrawn at time 0 s and 23 h, quenched with HCl, and then passed through a 10-KD filter to remove unreacted L-canavanine. The peak at 46 938 Da is from unmodified BcADI whereas the peak at 47 098 Da is from the covalent-adduct formed from BcADI and L-canavanine. (B) A plot of the percent remaining catalytic activity and product O-ureido-homoserine formation in BcADI incubated with L-canavanine vs the time period of the incubation. The incubation mixture initially contained 10 μM BcADI and 12 mM L-canavanine in 50 mM K^+HEPES at pH 7.0 and 25 °C. The fraction of native BcADI remaining is calculated from kinetic (○) and mass spectrometric (●) data, respectively. The formed product O-ureido-homoserine (◇) was measured using the fixed-time, colorimetric assay described in the Experimental section (Supporting Information). (C) Time course for formation of the BcADI S-alkylthiouronium intermediate (E-I), calculated from mass spectrometric data (●) by assuming [E-I] = [E_{total}]-$E_{labeled}$/($E_{labeled}$ + $E_{unlabeled}$) (Scheme 4). The curve was simulated with KinTek. (D) Data obtained by analysis of aliquots of reaction mixtures initially containing 10 μM BcADI and 12 mM L-canavanine in 50 mM K^+HEPES at pH 7.0 and 25 °C. Time course for formation of BcADI S-alkylthiocarbamate adduct (E-I*) calculated from mass spectrometric data (○) and kinetics (○) by assuming [E-I*] = [E_{total}]$E_{labeled}$/($E_{labeled}$ + $E_{unlabeled}$) and [E-I*] = [E_{total}] − [E_{total}](v_t/v_0) (Scheme 7). The curve was simulated with KinTek.

(34) Silverman, R. *Mechanism-based Enzymes Inactivators: Chemistry and Enzymology*; Academic Press: Boca Raton, FL, 1988.

of BcADI activity remaining. The parallel nature of the latter two profiles demonstrates that BcADI inactivation correlates with the formation of the 47 098 Da enzyme adduct. The progress curve for product formation shows that the L-canavanine is consumed within the first 6 h of the reaction. The mass spectrum measured at 23 h (Figure 5A) reveals that 95% of the BcADI is present as the 47 098 Da adduct. The reversion of inactivated BcADI to the native enzyme is estimated to occur at a rate $<6 \times 10^{-5}$ min^{-1}.

Taken together, the data demonstrate that L-canavanine inactivation of BcADI is associated with irreversible formation of an enzyme adduct with a mass of 47 098 Da that takes place at a rate that is ca. 1000-fold slower than the conversion of L-canavanine to O-ureido-L-homoserine. The inactivated BcADI has the same molecular mass as that of the BcADI Cys-S-alkylthiouronium species (Scheme 2), the reactive intermediate in the catalytic pathway. However, the inactive adduct must differ from the thiouronium intermediate because its rate of formation is ca. 1000-fold slower than that of product formation.

By using the mass spectrometry-based methodology employed to monitor formation of the Cys-S-alkylthiouronium intermediate in the L-arginine catalyzed reaction, the short time period (0−20 s) time course of the appearance of the Cys-S-alkylthiouronium intermediate in reaction of BcADI with L-canavanine was determined (Figure 5C) along with the longer period (0−800 min) time course associated with the BcADI inactivation reaction (Figure 5D). The observed burst, associated with formation of the Cys-S-alkylthiouronium intermediate of mass 47 098 Da, rises to a level of 16% (Figure 5C) at a rate of 37 min^{-1}. This burst is followed by the accumulation of the 47 098 Da inactivated enzyme and a concurrent depletion of the native enzyme (Figure 5D) at a rate of 4.7×10^{-2} min^{-1}.

Structure of the L-Canavanine Inactivated BcADI. The C400S BcADI mutant was used to show that the active site Cys400 is required for formation of the L-canavanine inactivated enzyme. As anticipated, kinetic assays for formation of L-citrulline or O-ureido-L-homoserine from L-arginine or L-canavanine, respectively, reveal that the BcADI C400S mutant does not catalyze either deimination reaction. Specifically, 50 μM BcADI C400S, incubated with 10 mM L-arginine or 10 mM L-canavanine at pH 7 and 25 °C for 23 h does not produce detectable levels ($k_{cat} < 1 \times 10^{-6}$ s^{-1}) of either product. Likewise, mass spectrometric monitoring of the reaction of 50 μM BcADI C400S with 12 mM L-canavanine at pH 7 and 25 °C for 6 h shows that an enzyme adduct of 160 Da higher mass is not produced.

One possible explanation of the formation of inactivated BcADI in the reaction with L-canavanine is that the Cys400-S-alkylthiouronium intermediate in the catalytic pathway undergoes rearrangement by migration of the thiouronium group to a neighboring nucleophilic residue. This possible scenario was ruled out by using MALDI-TOF and ESI-MS/MS mass spectrometry to unambiguously assign the site at which modification takes place in the L-canavanine inactivation reaction. L-Canavanine inactivated BcADI was subjected to tryptic digestion and the resulting peptides were analyzed and sequenced by using ESI MS/MS. Individual peptides were identified by comparing their observed and theoretical masses.

Table 3. MALDI-MS Peaks of Proteolytic Cleavage of BcADI with Trypsin Endoproteinase[a]

fragment	observed mass (Da)	calculated mass (Da)
394−399	599.29	559.3378
246−250	636.30	636.3469
175−180	759.34	759.3936
175−181	915.45	915.4941
400−407	936.45	936.4464
127−134	948.52	948.5002
193−199	976.47	976.4999
400−407[b]	1096.47	936.4464
82−89	1063.51	1063.5531
400−408	1064.48	1064.5413
175−182	1071.53	1071.5952
257−266	1141.65	1141.6767
200−208	1149.58	1149.6164
183−192	1272.58	1272.6544
51−60	1279.55	1279.6071
400−410	1292.66	1292.6529
61−71	1319.75	1319.7323
61−71[c]	1335.62	1319.7323
82−92	1417.76	1417.8167
239−250	1417.76	1417.8274
400−410[b]	1452.66	1292.6529
181−192	1584.82	1584.8572
378−393	1796.93	1796.9394
235−250	1805.04	1805.0397
183−196	1825.95	1825.9311
181−196	2138.18	2138.1333
61−80	2230.22	2230.2447
183−199	2230.22	2230.1371
1−20	2273.33	2273.2691
181−199	2542.43	2542.3393
294−315	2561.40	2561.3034
1−24	2711.64	2711.5395
267−293	3121.46	3122.5484
267−293[c]	3138.48	3122.5484
200−234	3978.02	3980.0558

[a] See Experimental section in Supporting Information for details. [b] Peptides generated from L-canavanine inactivated BcADI. [c] Difference caused by oxidation of the methionine residue, a common occurrence with this technique. During the MALDI analysis, partially oxidized methionines undergo facile loss of the methyl sulfoxide to generate a triplet peaks in which the adjacent pairs are separated by 16 and 48 Da. Three peptides, M175-R180, N294-R315 and A267-K293, formed by cleavage of inactivated BcADI undergo Met oxidation. The peaks at 759.3936, 2561.3034, and 3122.5484 Da correspond to the three unmodified peptides whereas the peaks at 1319.7323, 2577.2983 and 3138.5433 Da correspond to the same peptides transformed to the corresponding methionine sulfoxides.

As shown in Figure S2 (Supporting Information) and Table 3, protonated molecular ions ($M+H^+$) for 33 peptides were detected by using MALDI-TOF analysis of the tryptic digest, achieving an overall protein sequence coverage of 65%. Based initially on the X-ray crystal structure of the published PaADI structure[18,19] and now on the recently determined structure of the BcADI C265S mutant (Galkin and Herzberg, unpublished data), we know that the BcADI residues Ser402, His270, Asp272, Glu215, Asp218, and Glu219 are potential nucleophilic residues located in close proximity to Cys400. The peptides containing these six residues are included in the 33 tryptic digest peptides, and the mass spectrometric data show that none of them are modified.

Major peptides containing the native and modified forms of the active site Cys400 BcADI are observed by mass spectrometric analysis of the tryptic digest of L-canavanine inactivated BcADI (Table 3). Included in this group are C^{400}-MSMPIVRKDI^{410} (mass of unmodified 1292 Da and modified 1452 Da), C^{400}MSMPIVR^{407} (mass of unmodified 936 Da and

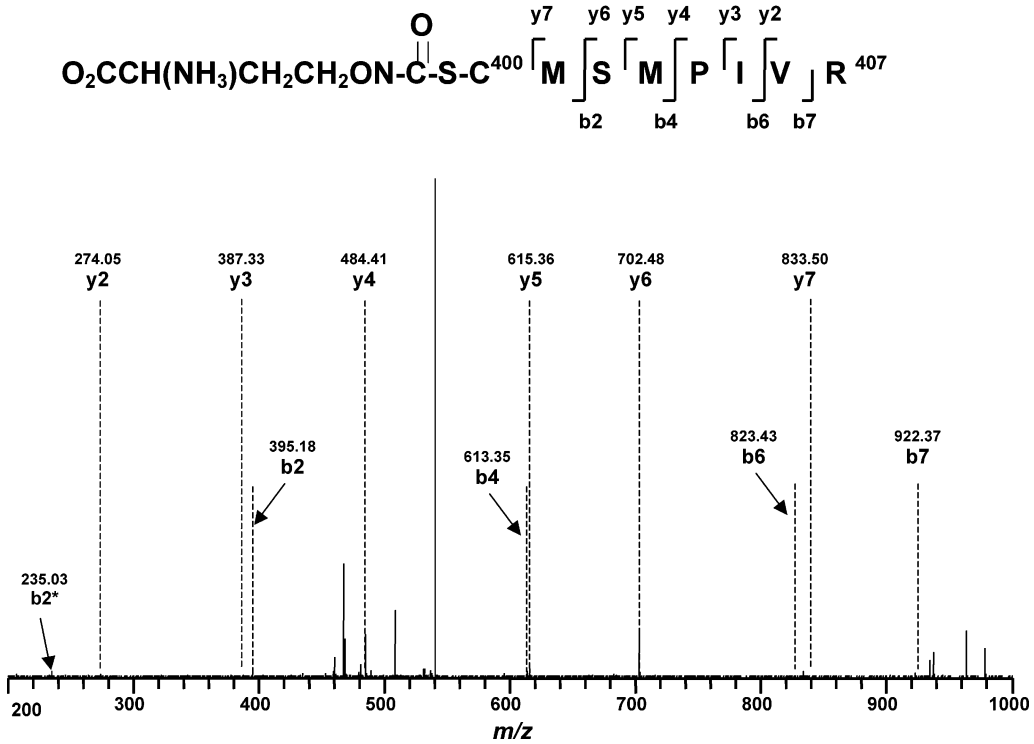


Figure 6. ESI-MS/MS spectrum of modified peptide $C^{400}MSMPIVR^{407}$obtained after tryptic digestion of L-canavanine inactivated BcADI containing several b-ions, which gain 160 Da mass units due to the covalent adduct formation at Cys400.

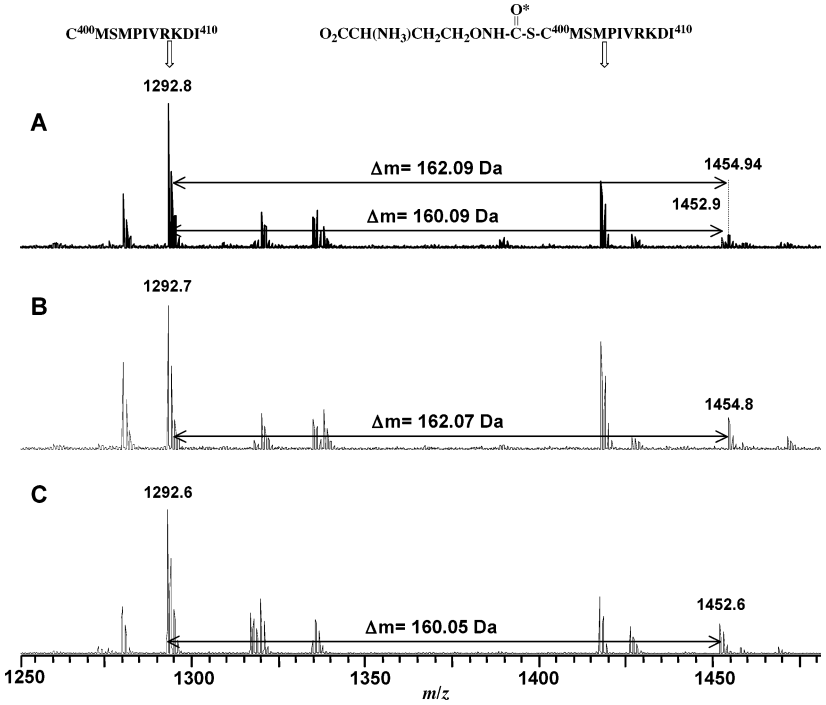
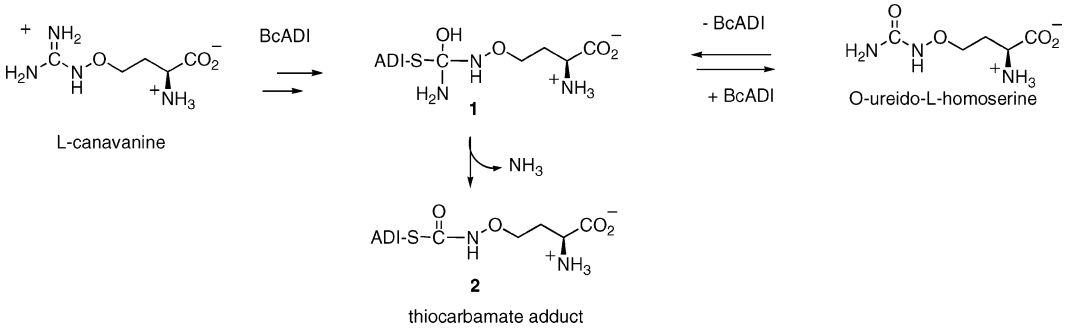


Figure 7. MALDI-TOF mass spectra of peptides $C^{400}MSMPIVRKDI^{410}$ (mass of unmodified 1293 Da and modified 1453 or 1455 Da) obtained from tryptic digestion of inactivated enzyme generated by treatment of BcADI with L-canavanine in (A) 1:1 $H_2{}^{18}O$ and $H_2{}^{16}O$, (B) $H_2{}^{18}O$, and (C) $H_2{}^{16}O$. The respective 300 μL solutions of 100 μM BcADI in 50 mM K^+HEPES (pH 7.0, 1 mM DTT) and 120 mM L-canavanine in 50 mM K^+HEPES (pH 7.0) were first lyophilized to remove all water. Then 300 μL of $H_2{}^{18}O$ were added to each solution prior to mixing and incubating for 7 h. Parallel reactions were run in an identical fashion in $H_2{}^{16}O$ and a 1:1 mixture of $H_2{}^{18}O$ and $H_2{}^{16}O$. The enzymes obtained in each case were subjected to tryptic digestion followed by MALDI-TOF analysis of the Cys400 containing peptide $C^{400}MSMPIVRKDI^{410}$.

modified 1096 Da). The observation of Cys400 containing peptide fragments with a 160 Da higher mass is consistent with Cys400 being the site of L-canavanine promoted modification. Quantitative analysis of the peaks associated with the unmodified and modified peptides indicates that *ca.* 16% of the protein is modified by L-canavanine. This observation suggests that the modification is partially reversed during the tryptic digestion process.

Scheme 5



Collision induced dissociation (CID) MS/MS analysis[35,36] was carried out on the C^{400}MSMPIVR^{407} peptide in order to demonstrate unambiguously that Cys400 is the site of adduct formation in L-canavanine inactivated BcADI (Figure 6). The daughter ions most commonly seen by using this technique are produced by cleavage of peptide amide bonds with charge retention at the N-terminal (b ions) and C-terminal (y ions) positions.[37] The mass difference between b or y ions in a given series corresponds to the mass of the amino acid residue. The set of all LC/MS/MS data collected on L-canavanine inactivated BcADI was processed by using the Mascot Distiller software with standard data processing parameters and searched by MASCOT (Matrix Science, Boston, MA) and PEAKS (Bioinformatics Solutions Inc., Waterloo, ON, Canada). Results from the best match for the peptide C^{400}MSMPIVR^{407}, which were manually verified, suggest that canavanine modification occurs at Cys400. Several y-type ions are observed, including *m*/*z* 274.05, 387.33, 484.41, 615.36, 702.48, and 833.50 (y_2-y_7, respectively). Five b-type ions, including *m*/*z* 235.03, 395.18, 613.35, 823.43, and 922.37 (b_2, b_2*, b_4, b_6, b_7, respectively) are also observed with a mass difference of 160 Da, which corresponds to the attachment of the thiouronium group of canavanine. The remaining b-type ions are also shifted to 160 Da higher mass. These observations demonstrate that Cys400 is the only site in BcADI at which covalent adduct formation takes place upon treatment with L-canavanine.

The results of the ESI-MS/MS studies described above give solid support for the conclusion that the covalent modification present in inactivated BcADI is at the same Cys residue that functions as the nucleophile in the catalytic conversion of L-canavanine to O-ureido-L-homoserine. In order to gain additional information for characterization of the inactivated enzyme, mass spectrometric analysis was carried out on the peptide C^{400}MSMPIVRKDI^{410} (mass of unmodified peptide is 1292 Da; mass of modified peptide is 1452 Da) obtained from tryptic digestion of inactivated enzyme generated by treatment of BcADI with L-canavanine in $H_2{}^{18}O$, in $H_2{}^{16}O$ or in a 1:1 mixture of $H_2{}^{18}O$ and $H_2{}^{16}O$. The resulting protein mixtures were subjected to tryptic digestion followed by MALDI-TOF analysis of the Cys400 containing peptide C^{400}MSMPIVRKDI.^{410} In Figure 7 are displayed mass regions for C^{400}MSMPIVRKDI^{410} generated from native and L-canavanine inactivated BcADI. As can be seen by viewing these spectra, L-canavanine modification conducted in $H_2{}^{18}O$ results in the production of inactivated

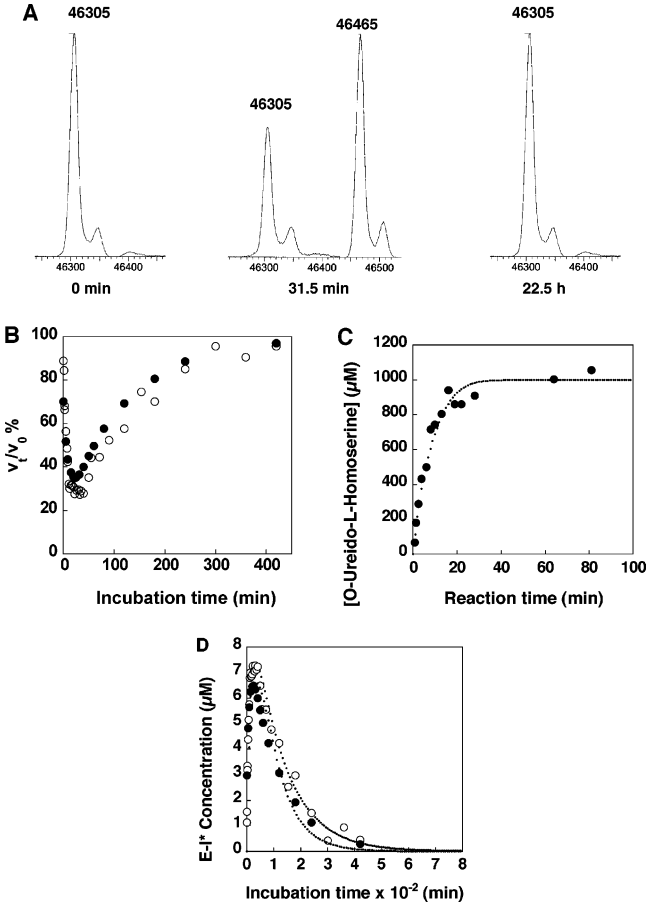


Figure 8. (A) Electrospray mass spectra of PaADI present in aliquots taken from an incubation mixture initially containing 10 μM PaADI and 1 mM L-canavanine in 50 mM K^+MES at pH 5.6 and 25 °C. Aliquots were withdrawn at time 0 s, 31.5 min, and 22.5 h and quenched with HCl then passed through a 10-KD filter to remove unreacted L-canavanine. The peak at 46 305 Da is due to native PaADI and the peak at 46 465 Da is associated with the covalent adduct formed between PaADI and L-canavanine (Scheme 7). (B) Percent remaining PaADI activity in reaction of 10 μM PaADI and 1 mM L-canavanine at pH 5.6 and 25 °C. The fraction of native PaADI remaining is calculated from kinetic (○) and mass spectrometric (●) data. (C) Product O-ureido-homoserine formation in PaADI incubated with L-canavanine vs the time period of the incubation. The incubation mixture initially contained 10 μM PaADI and 1 mM L-canavanine in 50 mM K^+-MES at H 5.6 and 25 °C. O-Ureido-homoserine was measured using the fixed-time, colorimetric assay described in the Experimental section (Supporting Information). (D) Time course for formation and consumption of the inactive enzyme calculated from inactivation kinetic (○) and mass spectrometric (●) data by assuming $[E\text{-}I^*] = [E_{total}] - [E_{total}](v_i/v_0)$ and $[E\text{-}I^*] = [E_{total}]E_{labeled}/(E_{labeled} + E_{unlabeled})$. The curve was simulated with KinTek.

(35) Raymackers, J.; Daniels, A.; De Brabandere, V.; Missiaen, C.; Dauwe, M.; Verhaert, P.; Vanmechelen, E.; Meheus, L. *Electrophoresis* **2000**, *21*, 2266−2283.
(36) Gustafsson, E.; Thoren, K.; Larsson, T.; Davidsson, P.; Karlsson, K. A.; Nilsson, C. L. *Rapid Commun. Mass Spectrom.* **2001**, *15*, 428−432.
(37) Biemann, K. Appendix 5. Nomenclature for peptide fragment ions (positive ions). *Methods Enzymol.* **1990**, *193*, 886−887.

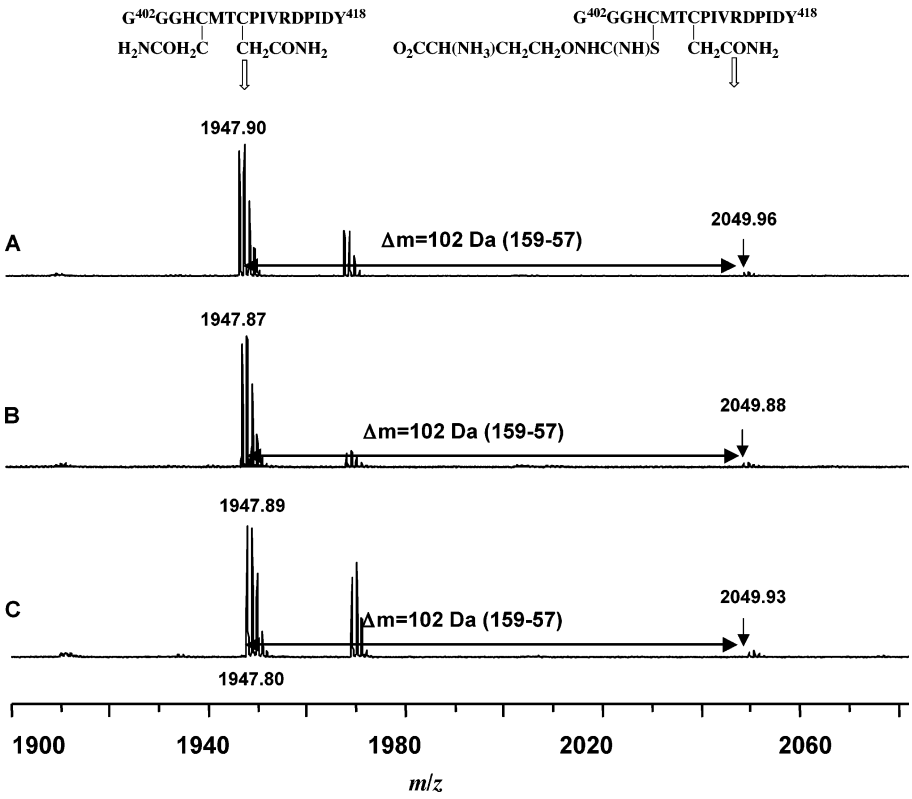


Figure 9. MALDI-TOF mass spectra of peptides GGGHC^{406}MTCPIVRDPIDY^{418} obtained by tryptic digestion of PaADI, partially inactivated by reaction with L-canavanine in (A) 1:1 $H_2{}^{18}O$ and $H_2{}^{16}O$, (B) $H_2{}^{18}O$, and (C) $H_2{}^{16}O$, followed by alkylation of unmodified cysteines with iodoacetamide. Respective 450 μL solutions of 100 μM PaADI in 50 mM MES (pH 5.6, 20 mM $MgCl_2$) and 20 mM L-canavanine in 50 mM MES (pH 5.6, 20 mM $MgCl_2$) were first lyophilized to remove all water. Then 450 μL of $H_2{}^{18}O$ were added to each solution prior to mixing and incubating for 7 min. Parallel reactions were run in an identical fashion in $H_2{}^{16}O$ and a 1:1 mixture of $H_2{}^{18}O$ and $H_2{}^{16}O$. The protein mixtures obtained were subjected to tryptic digestion followed by MALDI-TOF analysis of the Cys406 containing peptide GGGHC^{406}MTC^{409}PIVRDPIDY^{418} in which Cys409 contains an S-acetamido group (mass 57).

BcADI that yields a modified peptide, which is 2 mass units higher than that generated by tryptic digestion of inactivated BcADI formed in $H_2{}^{16}O$.

Taking into account the molecular mass of inactivated BcADI, the requirement for participation of Cys400 in its formation, and the fact that a water derived oxygen is incorporated into the adduct, we assign the structure of the inactivated enzyme the BcADICys400-alkylthiocarbamate (**2**, Scheme 5). We envision the formation of the thiocarbamate to occur from the tetrahedral adduct (**1**, Scheme 5) upon elimination of ammonia. The tetrahedral adduct **1** might be formed through a pathway that branches from the chemical pathway leading from the substrate L-canavanine to the product O-ureido-L-homoserine or alternatively, it might be formed through a pathway that originates with the product O-ureido-L-homoserine. Either route would lead to formation of a Cys400-S-alkyl thiocarbamate derivative **2** of BcADI, which has a mass of 47 098 Da which is 160 (from reaction in $H_2{}^{16}O$) or 162 (from reaction in $H_2{}^{18}O$) mass units higher than that of the native enzyme. To distinguish between these two possibilities, conditions for the occurrence of the back reactions were simulated by incubating BcADI with O-ureido-L-homoserine and ammonia for 1 h under the same conditions used for inactivation of BcADI by L-canavanine (Figure 3A). The observation that the activity of BcADI remains unchanged (data not shown) under these conditions leads to the conclusion that inactivated BcADI is not formed by the reaction of the BcADI with the O-ureido-L-homoserine, and therefore that it is formed via a minor pathway which branches from the pathway leading from L-canavanine to O-ureido-L-homoserine.

PaADI, EcADI, BmADI, and GiADI Reactions with L-Canavanine. To test the generality of L-canavanine inactivation of ADI, inactivation reactions of PaADI, EcADI, BmADI, and GiADI were monitored at the pH optimum for catalysis of L-arginine hydrolysis to determine the time and concentration dependent loss of enzyme activity (Supporting Information Figure S3). The plots of the pseudo-first-order rate constants of inactivation (k_{obs}) as a function of L-canavanine concentration are hyperbolic, showing that the rate of inactivation is slower than the rate of formation of the ADI(L-canavanine) complex (Figure S3 Supporting Information). The data were used to obtain the K_I and k_{inact} values provided in Table 2. Although some variation can be seen in the K_I and k_{inact} values for the five ADIs, no correlation with either structure divergence or pH optima appears to exist. The k_{inact} value for each ADI is considerably smaller than the value of k_{cat} for product formation (Tables 1 and 2).

The most remarkable finding arising from these studies is that, in contrast with BcADI where inactivation by L-canavanine is essentially irreversible, inactivation of PaADI, EcADI, BmADI, and GiADI is reversible. A thorough study of the PaADI catalyzed reaction of L-canavanine was carried out to define the kinetics of the inactivation and reactivation processes. The results, summarized in Figure 8, were obtained for the reaction of PaADI (10 μM) with a sub-saturating concentration (1 mM) of L-canavanine at pH 5.6 and 25 °C. In Figure 8A are shown selected mass spectrometric data measured for reaction mixtures quenched at various times (the complete data set is shown in Supporting Information Figure S4). The changes,

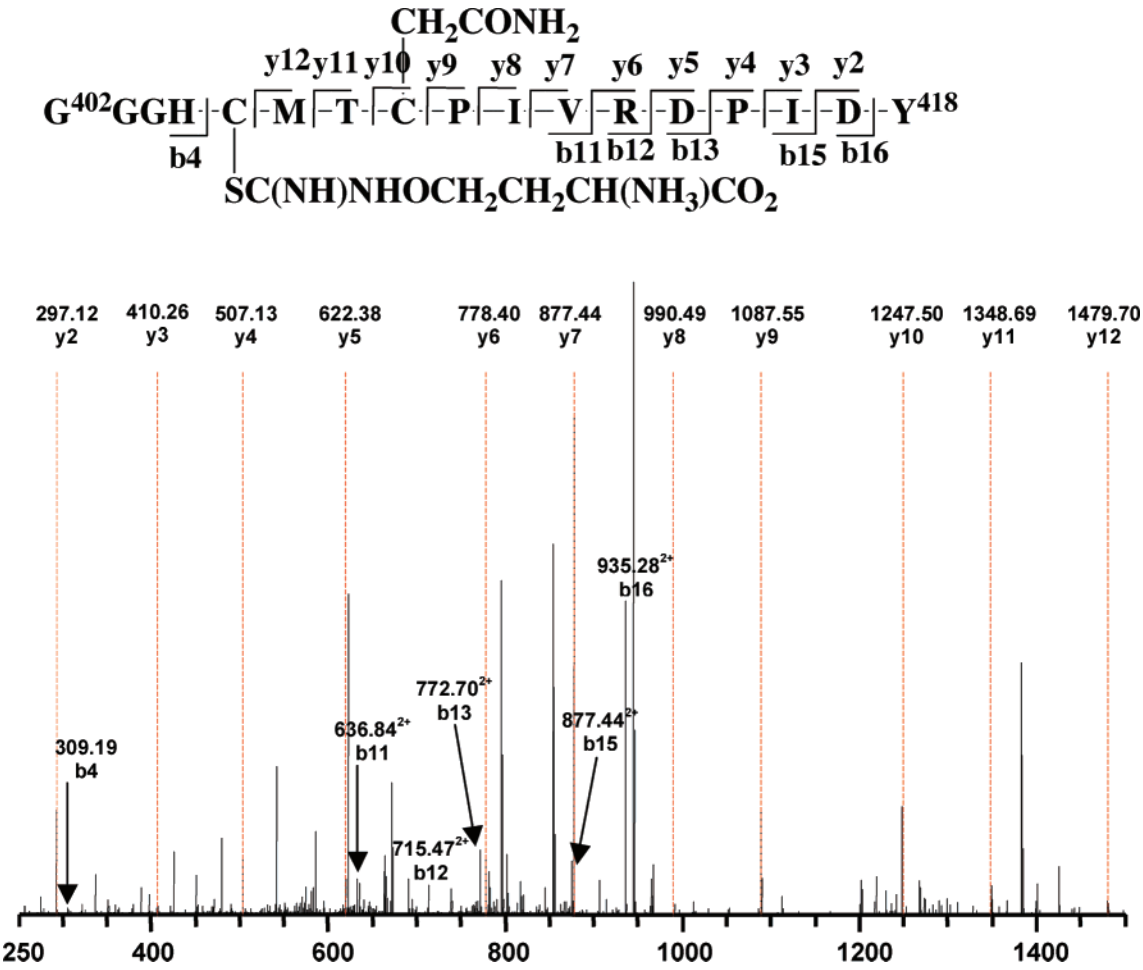


Figure 10. ESI-MS/MS spectrum of the peptide $G^{402}GGHC^{406}MTC^{409}PIVRDPIDY^{418}$ after L-canavanine inactivation of PaADI, Cys409 alkylation with iodoacetamide, and tryptic digestion.

taking place over the 0−23 h time course of the reaction, reveal the formation of an enzyme adduct of mass 46 465 Da, which corresponds to the calculated mass of a PaADI Cys-alkylthiourionium intermediate (Scheme 2). This adduct accumulates to *ca.* 70% maximally and then slowly reverts to native PaADI.

Shown in Figure 8B is a plot of the fraction of the remaining activity of PaADI vs reaction time. Enzyme activity is first lost and then regained. Also shown in Figure 8B is the time profile of the fraction of native PaADI (46 305 Da) remaining as determined by mass spectrometric analysis of acid quenched aliquots. The activity loss and build-up of the 46 465 Da mass species are closely correlated as are the regeneration of activity and the conversion of the 46 465 Da enzyme adduct back to the native PaADI.

The progress curve for formation of the O-ureido-L-homoserine product reaches saturation at 30 min (Figure 8C), suggesting that the level of inactive enzyme is determined by (1) the prevailing concentration of canavanine, (2) the rates at which the thiouronium intermediate partitions to product and to inactive enzyme, and (3) the rate at which the inactive enzyme returns to the thiouronium ion intermediate and/or undergoes hydrolysis to regenerate active enzyme. The time course for the formation and consumption of the inactive enzyme species, calculated from the mass spectrometric and the kinetic data (Figure 8B), are shown in Figure 8D. The time course data (Figures 8C and 8D) were fitted using the simulation program KinTek (KinTek Corp; Austin, Texas) and the calculated the k_{cat}, k_{inact}, K_m, and K_I values (Tables 1 and 2) to set limits on the individual rate constants. This treatment gave rate constants for the conversion of the PaADI(L-canavanine) complex to the ADI-thiouronium intermediate ($k_2 \approx 40$ min^{-1}) and for conversion of the ADI-thiouronium intermediate to product ($k_3 \approx 40$ min^{-1}). Because we do not have a direct measurement of the formation of the ADI-thiouronium intermediate, the values of these rate constants are not well constrained.

The rate constant, derived from the data of Figure 8, for the conversion of the PaADI(L-canavanine) complex to the inactive enzyme is defined as 4.0×10^{-1} min^{-1}, whereas the rate constant for conversion of the inactive enzyme to active enzyme is the product complex is 8.5×10^{-3} min^{-1}. The difference between these rate constants and between the corresponding rate constants measured for the BcADI reaction (4.7×10^{-2} min^{-1} and $<6 \times 10^{-5}$ min^{-1}, respectively) nicely accounts for the observed L-canavanine time-controlled inhibition of PaADI and the irreversible inactivation of BcADI.

In order to characterize the structure of the covalent adduct, mass spectrometric analysis was carried out on the peptides $GGGHC^{406}MTCPIVRDPIDY^{418}$, obtained by tryptic digestion of PaADIs that are partially inactivated by reaction with L-canavanine in $H_2{}^{18}O$, in $H_2{}^{16}O$ or in a 1:1 mixture of $H_2{}^{18}O$ and $H_2{}^{16}O$. The protein mixtures were treated with iodoacetamide to alkylate the unmodified cysteine residues, before subjecting them to tryptic digestion and MALDI-TOF analysis of the Cys406 containing peptide $GGGHC^{406}MTC^{409}$-

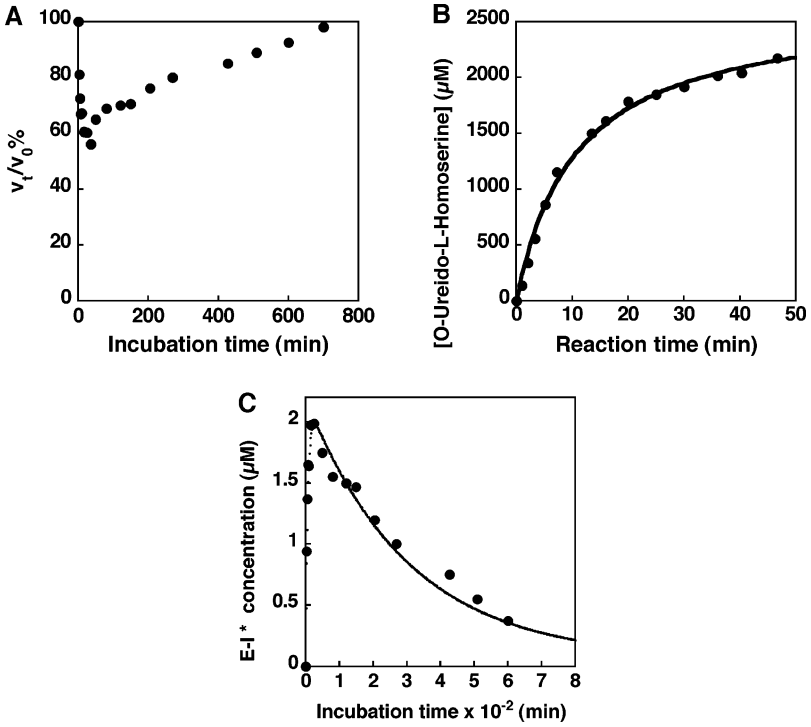


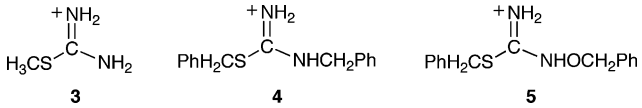
Figure 11. (A) Percent remaining GiADI activity in reaction of 5 μM GiADI and 2 mM L-canavanine at pH 7.5 and 25 °C. (B) Product O-ureido-homoserine formation catalyzed by GiADI incubated with L-canavanine vs the time period of the incubation. The incubation mixture initially contained 5 μM GiADI and 2 mM L-canavanine at pH 7.5 and 25 °C. O-Ureido-homoserine (●) was measured using the fixed-time, colorimetric assay described in the Experimental section (Supporting Information). (C) Time course for formation and consumption of the inactive enzyme calculated from inactivation kinetic data by assuming $[E\text{-}I^*] = [E_{total}] - [E_{total}](v_t/v_0)$. The curve was simulated with KinTek.

Table 4. Simulated Rate Constants for the Formation of the Inactive ADI Adduct ($^*k_{inact}$) and for Its Conversion to Product (k_{react})[a]

ADI	$^*k_{inact}$ (min^{-1})	k_{react} (min^{-1})
PaADI	0.4	0.0085
BcADI	0.047	$<5 \times 10^{-5}$
EcADI	0.073	0.05
BmADI	0.08	0.02
GiADI	0.1	0.0032

[a] Fitted kinetic data are shown in Figures 5, 8, and 11 of the text and Figure S3 of the Supporting Information.

Scheme 6



PIVRDPIDY[418]. This peptide contains the acetamido group derived from iodoacetamide (mass 57) and the urea or carbamate group derived from L-canavanine. As can be seen by viewing the spectra of the peptides displayed in Figure 9, the peptides derived from PaADI reacted with L-canavanine in $H_2{}^{18}O$ vs $H_2{}^{16}O$ have the same mass. Therefore, the covalent adduct does not contain a solvent derived oxygen atom.

For the purpose of demonstrating that the inactivation reaction of L-canavanine with PaADI is not due to translocation of the thiouronium group from its initial Cys 406 position to Cys409 or another nucleophilic site, collision induced dissociation (CID) MS/MS analysis[35,36] was carried out on the $G^{402}GGHC^{406}$-MTC^{409}PIVRDPIDY[418] peptide. Results from the best match for the peptide (Figure 10), which were manually verified in a manner described above, show that the L-canavanine modification occurs at the catalytic Cys406. The low pK_a of a L-canavanine thiouronium model described in the following section, suggests that the modified PaADI is the Cys406-S-alkylthiourea adduct formed by proton loss from the Cys406-S-alkylthiouronium ion intermediate.

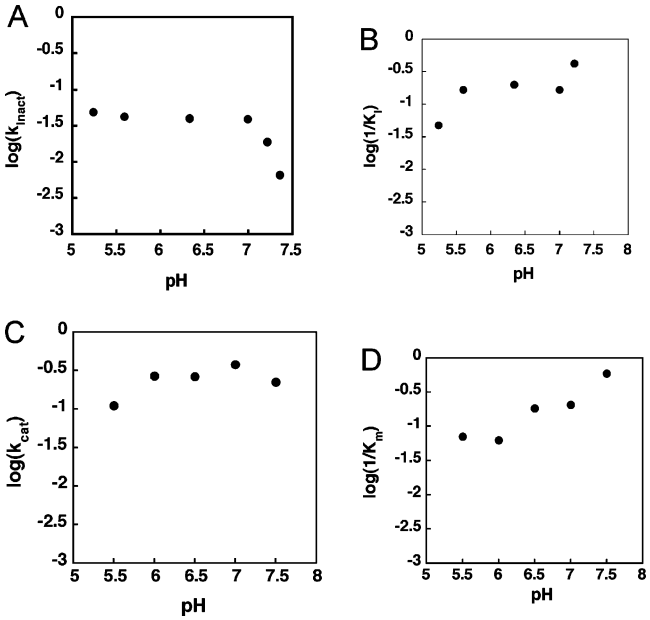


Figure 12. pH profiles for k_{inact} (A) and K_I (B) of L-canavanine induced BcADI inactivation and for k_{cat} (C) and K_m (D) of BcADI catalyzed L-canavanine hydrolysis to O-ureido-homoserine and ammonia. For details see the Experimental section (Supporting Information).

The kinetics of the L-canavanine inactivation process operative in the other ADIs were briefly examined. GiADI is of particular interest because of its sequence divergence from the bacterial ADIs, and its optimal activity pH range from 5 to 7.5,

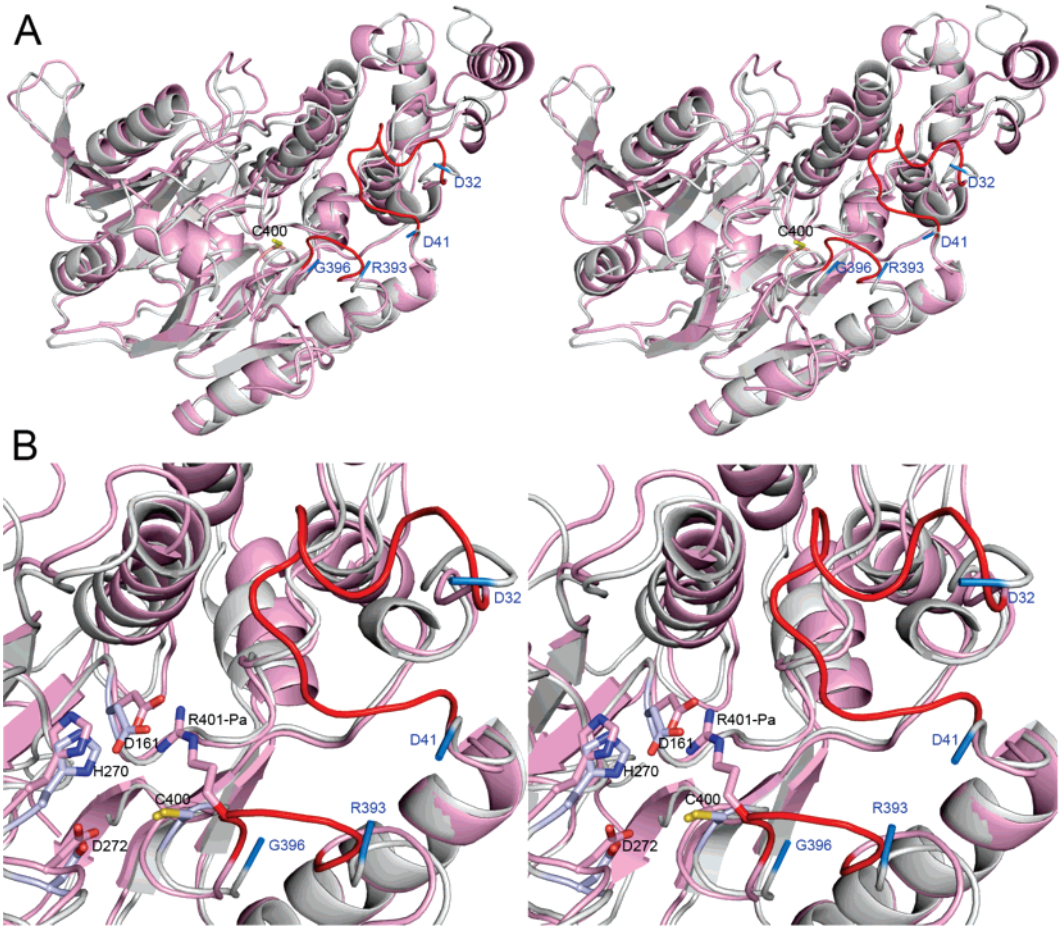
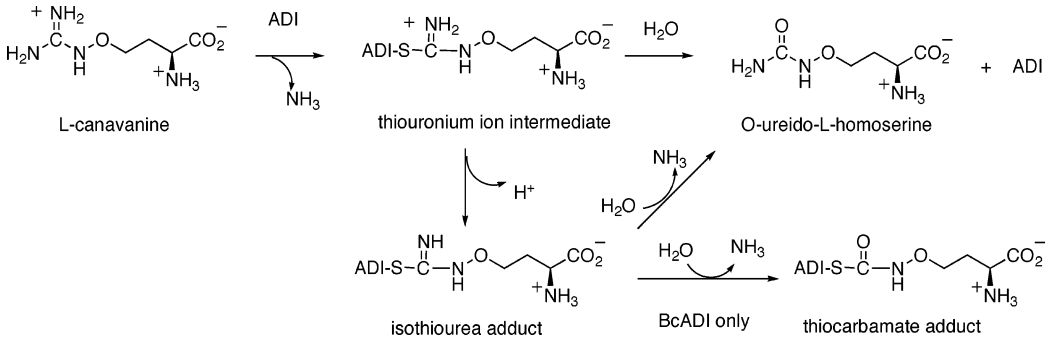


Figure 13. Superpositions of BcADI and PaADI structures. (A) Stereoscopic view of a ribbon representation of the superposed PaADI and BcADI monomers. PaADI is depicted in pink color and BcADI is depicted in gray color. The regions exhibiting the largest difference associated with the active site correspond to two loops. For PaADI, these loops are highlighted in red. For BcADI, the loops are largely disordered and the ordered terminal residues are labeled and highlighted in blue. Residue numbering corresponds to BcADI. The catalytic cysteine residue is shown in stick model. (B) Stereoscopic view of the superposed PaADI and BcADI active site region using the same color scheme as in (A). Atomic colors are as follows: oxygen, red; nitrogen, blue; sulfur, yellow; carbon, light blue for PaADI and pink for BcADI. Residue numbers correspond to BcADI, except that Arg401 of PaADI is labeled R401-Pa (the corresponding Arg397 in BcADI is disordered). The figures were generated usin the X-ray crystallographic coordinates for the structure of the C406A PaADI (L-arginine) complex (PDB ID 2A9G)[19] and the apo BcADI C265S mutant (Galkin and Herzberg, unpublished results).

Scheme 7



which distinguishes both GiADI and BcADI from PaADI, BmADI, and EcADI (optimal activity pH range <6).[20,27] The fact that BcADI and PaADI have pH optima of 7.0 and 5.6, respectively require that measurements of their inactivation kinetics be carried out under different pH conditions. Consequently, studies with GiADI provide information about whether differences observed between L-canavanine inactivation of BcADI and PaADI is a result of differences in reaction pH. In Figure 11A is shown the time course for the loss and regain of GiADI (5 μM) activity in reaction with 2 mM L-canavanine in 50 mM K^+HEPES buffer (pH 7.5) at 25 °C. The time course for the L-canavanine to O-ureido-L-homoserine conversion (Figure 11B) shows that the activity of GiADI decreases by ca. 45% with incubation time and following the depletion of L-canavanine (ca. 40 min) 100% of the original enzyme activity is slowly regained. The time course for formation and consumption of the inactive enzyme (shown in Figure 11C), was fitted using KinTek to define the rates of GiADI thiouronium ion intermediate formation and conversion to O-ureido-L-homoserine equivalent at ~61 min^{-1}, the rate of GiADI inactivation at 1 ×

10^{-1} min^{-1}, and the rate of reactivation at 3.2×10^{-3} min^{-1}. Thus, like PaADI, GiADI undergoes L-canavanine promoted time controlled inhibition, not irreversible inactivation, supporting the conclusion that the solution pH is not the factor determining the unique behavior of BcADI. The same conclusion is evident from the pH profile for L-canvanine inactivation which shows efficient irreversible inactivation at pH 5.6 (see below).

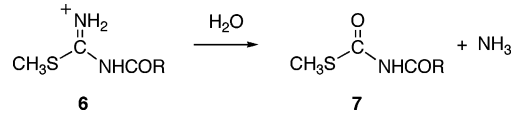
Similar kinetic characteristics were observed for BmADI (reaction mixtures of 20 μM BmADI and 5 mM L-canavanine at pH 5.6) and EcADI (20 μM EcADI with 10 mM L-canavanine at pH 6.0) (Figure S5, Supporting Materials). Like with PaADI and GiADI, in both cases L-canavanine inactivation is reversible. The individual rate constants for inactivation and reactivation for BmADI and EcADI are listed in Table 4 along with those for the other ADIs. The ratios of these rate constants define the efficiency of L-canavanine inhibition of each ADI.

Chemical Models of the L-Arginine and L-Canavanine Derived Thiouronium Intermediates. The ADI catalyzed deimination reaction pathway shown in Scheme 2 contains a hydrolysis step that involves attack of a bound water molecule on a thiouronium ion intermediate.[19,20,38] Because the pK_a of the model conjugate acid of S-methylisothiourea (**3**, Scheme 6) is 9.8,[39] it is reasonable to expect that the L-arginine derived ADI Cys-alkylthiouronium ion intermediate will be protonated at the pH optimum for ADI catalysis (pH 5−7.5).[20,27] Corroboration for this hypothesis was sought by examining the model compounds S-benzyl-*N*-benzylthiouronium bromide (**4**) and its N-benzyloxy analog **5** (Scheme 6). The syntheses of these substances are described in the Supporting Information. The thiouronium pK_a values of **4** and **5** were determined by measuring ^{13}C NMR chemical shifts for the corresponding thiouronium carbon atoms as a function of solution pH (pH profiles shown in Figure S6A and B). The L-arginine thiouronium model **4** was shown to have a pK_a value of 8.5 ± 0.2 which is close to the value determined for the conjugate acid of S-methylisothiourea. In contrast, **5**, a model of the L-canavanine thiouronium salt, has a remarkably lower pK_a value of 3.0 ± 0.1. Thus, the replacement of the N−CH_2 by an N−O moiety has a pronounced effect on the pK_a of the thiouronium group. Although inexplicably large, this change is consistent with differences between the pK_a of L-arginine (12.5) and L-canavanine (6.6).[40] By using the ^{13}C NMR monitored pH titration method (Figure S6C), the L-canavanine pK_a was determined to be 7.8 ± 0.1, a value not too different from the one measured earlier using a potentiometric based method.[37]

pH Profiles for L-Canavanine Inactivation of BcADI. The pH profiles for k_{inact}, K_I, k_{cat}, and K_m of the L-canavanine reaction with BcADI are not easily interpreted (Figure 12). To the extent that K_I and K_m measure L-canavanine-BcADI binding affinities, the pH profiles reflect contributions made by ionization of the uncomplexed L-canavanine guanidinium group and ionizable groups in the active site of the enzyme. Also, the k_{inact} and k_{cat} pH profiles are composites of ionization associated with the L-canavanine guanidinium group, the active site ionizable groups in the BcADI(L-canavanine) complex, the Cys-alkylthiouronium group, and the active site His in the BcADI thiouronium ion intermediate. Consequently, the pH profiles cannot be used to define the role played by proton loss from the L-canavanine derived thiouronium intermediate in the inactivation process.

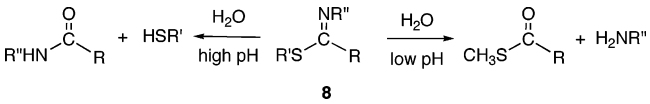
In any event, assuming that the thiouronium ion formed in the ADI active site by reaction with L-canavanine has a pK_a that is similar to that of the model **5**, it is likely that deprotonation of the intermediate to form a corresponding isothiourea would be facile.

Scheme 8



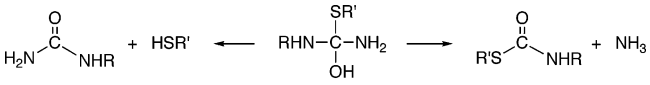
6 **7**

Scheme 9



8

Scheme 10



9

Conclusion

A unified model which accounts for the reversible L-canavanine covalent modification of PaADI, EcADI, BmADI, and GiADI (which leads to time controlled inhibition) and the irreversible L-canavinine covalent modification of BcADI, (which leads to permanent inhibition) is presented in Scheme 7. Based on an analysis of the acid dissociation constants of the chemical models (Scheme 6), it is likely that the Cys-alkylthiouronium ion formed in the reaction of L-canavanine will undergo ready deprotonation in competition with its reaction with His-activated water to form the Cys-alkyl-isothiourea adduct. We expect that this less electrophilic species will react more slowly with water than the Cys-alkylthiouronium intermediate to from O-ureido-L-homoserine and the active form of the enzyme.

In the reaction of BcADI with L-canavanine, an alternative pathway is available to the isothiourea generated by deprotonation of the initially formed Cys-alkyl-thiouronium intermediate. This process leads to generation of a thiocarbamate adduct (Scheme 7). Although unique among the L-canavanine + ADI reactions explored in this investigation, hydrolytic reactions of this type have precedent in the chemistry of thiouronium salts and closely related thioimidate esters. For example, in contrast to the typical reactions of thiouronium salts in basic solution, which form thiol and urea products, Klayman and his coworkers[41] observed that treatment of the N-electron withdrawing group substituted S-methyl analogs (**6** in Scheme 8) with water results in formation of the corresponding thiocarbamates **7**. In a related fashion, studies[42] of the pH dependent hydrolysis of thioimidates (**8**, Scheme 9) have shown that thioesters and amines are produced predominantly at low pH and that amides

(38) Das, K.; Butler, G. H.; Kwiatkowski, V.; Clark, A. D., Jr.; Yadav, P.; Arnold, E. *Structure* **2004**, *12*, 657.
(39) Albert, A.; Goldacre, R.; Phillips, J. *J. Chem. Soc.* **1948**, 2240.
(40) Tomiyama, T. I. *J. Biol. Chem.* **1935**, *111*, 45; Boyar, A.; Marsh, R. E. *J. Am. Chem. Soc.* **1982**, *104*, 1995−1923.
(41) Klayman, D. L.; Shine, R. J.; Bower, J. D. *J. Org. Chem.* **1972**, *37*, 1532−1537.
(42) Chaturvedi, R. K.; MacMahon, A. E.; Schmir, G. L. *J. Am. Chem. Soc.* **1967**, *89*, 6984−6993.

and mercaptans are the major products generated at high pH. These findings suggest that tetrahedral intermediates (**9**, Scheme 10) produced by water addition to thiouronium salts/isothioureas can undergo loss of either thiols and amines, depending on the nature of the environment or substituents.

Perhaps more relevant to the observations made in our studies with BcADI are the results of investigations of active site Cys thiolate mediated nitrile hydrolysis catalyzed by papain and by asparagine synthetase B. These studies have shown that the tetrahedral intermediate formed by attack of water on protonated thioimidate intermediates can eliminate either ammonia or the Cys thiolate, depending on the particular involvement of active site residues in wild type and mutant enzymes.[43,44] These findings prompted us to carry out a comparison of the active site of PaADI[18,19] with that of BcADI, the latter of which has only recently become available (Galkin and Herzberg, unpublished data) (Figure 13). As expected, the catalytic and substrate binding residues present in the active site of PaADI are conserved in the active site of BcADI. However, the active site of BcADI exhibits two disordered loop regions that span residues 394−395 and residues 33−40. In contrast, these regions are ordered in the structure of PaADI. Assuming that the crystal structures reflect the mobility of a loop that is in solution, the observations indicate that the active site of BcADI is more accessible to bulk solvent than that of PaADI. This difference might underlie the irreversible partitioning of the L-canavanine + BcADI derived isothiourea intermediate to the inert thiocarbamate adduct. Clearly, more work is required to clarify this issue.

Acknowledgment. Support for this study by NIH (DDM, OH, PSM: AI-059733; KBG-C: CA-16058) is gratefully acknowledged. We also thank Ken Johnson for helpful suggestions and for use of the pilot KinTek simulation program.

Supporting Information Available: Experimental section, Figures S1−6, and NMR spectra of **4** and **5**. This material is available free of charge via the Internet at http://pubs.acs.org.

JA0760877

(43) Dufour, E.; Storer, A. C.; Menard, R. *Biochemistry* **1995**, *34*, 16382−16388.
(44) Boehlein, S. K.; Rosa-Rodriguez, J. G.; Schuster, S. M.; Richards, N. G. *J. Am. Chem. Soc.* **1997**, *119*, 5785−5791.
(45) http://pymol.sourceforge.net, DeLano Scientific LLC, San Carlos, California.