Structural basis for catalysis and substrate specificity of *Agrobacterium radiobacter* N-carbamoyl-D-amino-acid amidohydrolase

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**Abbreviations:** D-NCAase, *N*-carbamoyl D-amino acid amidohydrolase; HPG, *N*-carbamoyl-D-*p*-hydroxyphenylglycine; CSHase, *N*-carbamoylsarcosine amidohydrolase; r.m.s.d., root mean square deviation

**Running title:** crystal structure of mutant D-NCAase in free and bound forms

**Keywords:** *N*-carbamoyl-D-amino-acid amidohydrolase; D-NCAase; nitrilase; C172-E47-K127 catalytic triad; *N*-carbamoylsarcosine amidohydrolase; NitFhit; D-enantiomer; substrate specificity
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ABSTRACT

N-carbamoyl-d-amino-acid amidohydrolase is an industrial biocatalyst to hydrolyze
N-carbamoyl D-amino acids for producing valuable D-amino acids. The crystal
structure of N-carbamoyl-D-amino-acid amidohydrolase in the unliganded form exhibits
a α-β-β-α fold. To investigate the roles of C172, N173, R175, and R176 in catalysis,
C172A, C172S, N173A, R175A, R176A, R175K, and R176K mutants were
constructed and expressed respectively. All mutants showed similar CD spectra and
had hardly any detectable activity except for R173A that retained 5% of relative activity.
N173A had a decreased value in $k_{cat}$ or $K_m$, while R175K or R176K showed high $K_m$
and very low $k_{cat}$ values. Crystal structures of C172A and C172S in its free form and in
complex form with a substrate, along with N173A and R175A have been determined.
Analysis of these structures shows that the overall structure maintains its four-layer
architecture and that there is limited conformational change within the binding pocket
except for R175A. In the substrate-bound structure, side chains of E47, K127, and
C172S cluster together toward the carbamoyl moiety of the substrate and those of N173,
R175, and R176 interact with the carboxyl group. These results collectively suggest
that a C172-E47-K127 catalytic triad is involved in the hydrolysis of the carbamoyl
moiety and that R175 and R176 are crucial in binding to the carboxyl moiety, hence
demonstrating substrate specificity. The common Glu/Asp-Lys-Cys triad observed
among N-carbamoyl-D-amino-acid amidohydrolase, NitFhit, and another carbamoylase
Chen et al. suggests a conserved and robust platform during evolution, enabling to catalyze the reactions toward a specific nitrile or amide efficiently.
The enzyme *N*-carbamoyl D-amino acid amidohydrolase (D-NCAase) hydrolyzes *N*-carbamoyl D-amino acids to D-amino acids, carbon dioxide, and ammonia (1). Several microorganisms produce D-NCAase activity including *Agrobacterium* (2-4), *Arthrobacter* (5), *Comamonas* (6), and thermotolerant bacteria such as *Blastobacter* sp. A17p-4 (7) and *Pseudomonas* sp. strain KNK003A (8). Despite low sequence identities among different species, D-NCAases require a strict D-enantiomer of the *N*-carbamoyl amino acid as their substrate (5-7). D-NCAase has been thus utilized as a biocatalyst in pharmaceutical industry to produce valuable D-amino acids due to the high optical specificity. Currently, a two-enzyme reaction process is applied which starts with inexpensive substrate, D,L-5 monosubstituted hydantoins that are synthesized from corresponding aldehydes. The first step is to hydrolyze the substrate using a D-specific hydantoinase to produce a D-carbamoyl derivative. The D-carbamoyl derivative is then converted to the corresponding D-amino acid including D-phenylglycine and D-\(\text{p}\)-hydroxyphenylglycine, the basic building blocks of \(\beta\)-lactam antibiotics by a second enzymatic step catalyzed by D-NCAase (2,9).

Crystal structure of D-NCAase reveals a tetramer with 222 symmetry; each monomer shows a four-layer \(\alpha-\beta-\beta-\alpha\) sandwich fold (10,11). Site-directed mutagenesis of H129, H144 and H215 in D-NCAase suggests strict geometric requirements of these conserved residues to maintain a stable conformation of a putative catalytic cleft. Within this pocket, the presumptive active residue, C172, is
just located at the bottom (12). A C172-E47-K127 triad near the floor of this cavity is thus proposed to participate in catalysis, which is similar to the C177-D51-K144 site of  
$N$-carbamoylsarcosine amidohydrolase (CSHase) (11). Interestingly, the Nit domain of *Caenorhabditis elegans* NitFhit protein (13) shows a similar fold with a presumptive identical C-E-K catalytic triad. Given the structural information and a global sequence analysis, nitrilases, amidases including D-NCAase, N-acyltransferases and presumptive amidases are classified as nitrilase superfamily that all comprises a C-E-K catalytic triad (14). The active cysteine is postulated to attack a carbon in specific nitrile- or amide-hydrolysis or amide-condensation reactions, resulting in synthesis of various natural products. None of the crystal structures of the nitrilase superfamily however had substrates in the active site. The interpretation of the substrate specificity has thus largely relied on modeling (10,11). In D-NCAase, a number of residues nearby C172, particularly N173, R175, and R176 that are located at the same loop of a solvent-accessible pocket are indicated to participate in recognizing a substrate. Here, we report that the crystal structures of the catalytically inactive D-NCAases, C172A or C172S in its free form and in complex with a substrate, $N$-carbamoyl-D-$p$-hydroxyphenylglycine (HPG) are extremely similar and that the mutation of the active C172 did not affect the conformation of the active site. Site-directed mutagenesis studies of N173, R175, and R176 as well as crystal structures of N173A and R175A provide further insight for substrate binding and catalytic
mechanism in D-NCAase and may help in the future rational design of useful biocatalysts.

EXPERIMENTAL PROCEDURES

Site-directed Mutagenesis—Site-directed mutagenesis was carried out using a ‘Transformer™ Site-Directed Mutagenesis’ kit from ClonTech (California, USA) with the pQE-NCA clone as the template according to the manufacture’s protocol. In brief, the selection primer was designed to change the *Xho*I site to *Sma*I site on the DNA target. The mutagenic primer was designed to induce a defined mutation into the DNA target of D-NCAase gene. Plasmid DNA isolated from the recipient strain, *E. coli* BMH 71-18 mutS, was digested with *Xho*I and transformed into chemically-treated competent JM109 *E. coli* cells. Mutant plasmids were subjected to DNA sequencing to confirm the successful mutations.

Expression and Purification of Wild Type and Mutant Enzymes—The recombinant wild type and mutant enzymes expressed in *E. coli* were isolated as previously described (15). The purified protein was analyzed by a SDS-PAGE gel to verify the purity. The protein concentration was assayed according to the Bradford method (16) with bovine serum albumin as a standard.

Enzymatic Assays—The D-NCAase activity was assayed by monitoring the release
of ammonium product which could be colorized using Berthelot reaction to produce blue indophenol (625 nm) (17). The $K_m$ (mM) and $k_{cat}$ (min$^{-1}$) values for wild type and mutant D-NCAase were determined from initial velocity data in reactions containing enzyme, 0.1 M sodium phosphate buffer (pH 7.0, 37 °C), 5 mM EDTA with varying concentration of HPG (1–10-fold $K_m$).

Circular Dichroism of Wild Type and Mutant D-NCAases—Circular dichroism (CD) experiments were performed on an AVIV CD spectropolarimeter (model 62A DS). All scans were performed between 200 and 260 nm (0.1-cm path length) on solutions containing protein (0.5 mg ml$^{-1}$), 10 mM HEPES, pH 7.0, and 1 mM EDTA and were determined as the average of three scans. To access the thermal stability of wild type or mutant D-NCAases, the change of ellipticity at 222 nm was monitored as the protein sample was heated from 20 to 96 °C with a 2 °C increment. Melting temperature ($T_m$) curve was normalized according to the highest CD signal as 1 and the lowest CD signal as 0. $T_m$ value was calculated at the temperature with the CD signal of 0.5.

Crystallization—D-NCAase crystals were obtained by vapor diffusion in hanging drops by mixing the protein solution (~15 mg ml$^{-1}$) with precipitating solution at room temperature as previously described (15). C172A and C172S mutants in the presence or absence of HPG (2 mM) were initially grown as microcrystals with the precipitating condition of 1.20 M lithium sulfate and 0.1 M HEPES buffer at pH 7.0. Microseeding method was then applied to obtain large single crystals (0.5 × 0.4 × 0.1 mm). Crystals
of N173A and R175A were formed directly within one week under 1.02 M and 1.24 mM lithium sulfate in 0.1 M HEPES buffer at pH 7.0, respectively. For R176A and R176K, no crystals were obtained. All crystals belong to space group \( P2_1 \) with comparable cell dimensions (Table I) and 4 molecules per asymmetric unit as that of wild-type D-NCAase (11).

Data Collection and Processing—For data collection, crystals were transferred to mineral oil for a few minutes and then flash-frozen in a liquid nitrogen stream. C172A crystal data were collected at –150 °C using a MSC X-Stream Cryo-system with a double-mirror-focused CuK\( \alpha \) X-ray radiation generated from a Rigaku RU-300 rotating anode generator at Macromolecular X-ray Crystallographic Laboratory of National Tsing Hua University, Hsinchu, Taiwan. C172S, C172A-HPG and C172S-HPG crystal data were collected on beamline 6A at Photon Factory, Japan, using an ADSC Quantum 4R CCD detector. Each data set was processed and scaled with MOSFLM (18) and the CCP4 program suites (19). R175A and N173A crystal data were collected on BL12B2 Taiwan beamline at Spring-8, Japan, using an ADSC Quantum 4R CCD detector. Data were processed with the HKL/HKL2000 suite (20). The statistics of the data collections are given in Table I.

Structure Determination and Refinement—The wild-type crystal model omitting solvent molecules (PDB entry code, 1FO6) was used to calculate a difference Fourier map with the coefficients \( 2F_o - F_c \) and calculated phases for each mutant or
mutant-substrate complex. A tetramer with the α/β fold was seen for each a mutant or mutant-substrate complex. Clearly visible density for the substituted side chain in a mutant or that for the bound substrate was observed. A model was thus readily built for each mutant or mutant-substrate complex using the program O version 8.0 (21).

Structure refinement was carried out with REFMAC5 program (22). The four molecules of the asymmetric unit were refined independently first by restrained refinement procedure using the maximum-likelihood function. Five percent of the reflections were randomly selected and used to compute a free $R$ value ($R_{\text{free}}$) for cross-validation of the model. Sigma A-weighted $2F_o - F_c$ and $F_o - F_c$ electron density maps were generated after each cycle of refinement step. The maps were then inspected to modify the model manually on an interactive graphics workstation with the program O. The progress of the refinement was evaluated by the improvement in the quality of the maps as well as the reduced values for $R$ and $R_{\text{free}}$. Non-crystallographic symmetry restraints as well as geometrical restraints were then applied and gradually relaxed during the refinement. A cis-peptide between Met73 and Pro74 in each mutant and a sulfate molecule with strong density in C172A or C172S were then manually built into the model. In couple with ARP/wARP program (23), water molecules were introduced automatically into the model. TLS refinement (24) prior to individual isotropic B value refinement was used to further reduce the $R$ and $R_{\text{free}}$ values. The stereochemistry of the protein model was assessed using the program
PROCHECK (25). Estimates of the coordinate errors were made using the method of Read (26). A summary of data collection and the refinement statistics is shown in Table I.

Structure comparisons among wild type D-NCAase, mutant D-NCAase, and mutant-substrate complex structures were carried out with the program LSQMAN (27) by superimposing overall Cα atoms of a monomer. For binding-site comparison, Cα atoms or side chain atoms of 12 residues surrounding the binding pocket (E47, K127, H144, E146, C172, N173, R175, R176, N197, T198, H201 and N202) were superimposed. A comparison of D-NCAase with the Nit domain of NitFhit (PDB entry code, 1EMS), or CSHase (PDB entry code, 1NBA) was done by superimposing side chains of three catalytic residues (E47, K127 and C172 in D-NCAase; E54, K127, and C169 in Nit; D51, K144, and C177 in CSHase). The pictures of 3D structure models were prepared with MOLSCRIPT (28) coupled to RASTER3D (29) programs. The figures of electron density map were prepared with PyMOL (30).

RESULTS AND DISCUSSION

Expression and Enzymatic Analysis of D-NCAase Mutants—Based on the D-NCAase-HPG model (11), C172, N173, R175, and R176 located in a short loop near the floor of the binding pocket were chosen for mutational analysis. C172 was
replaced with alanine or serine and expressed in *E. coli*, respectively. After purification by affinity chromatography, a major band of an apparent molecular mass of ~36 kDa was observed on a SDS-PAGE gel for each mutant (Fig. 1). Approximately 10 mg of pure C172A protein and 5 mg of pure C172S protein per liter harvest were obtained, respectively. Enzymatic assay showed greatly reduced activity for both mutants: there was less than 0.1% of relative activity for C172S and no detectable activity for C172A.

N173A, R175A and R176A mutants were then constructed, expressed, and purified, respectively (Fig. 1). Both R175A and R176A showed no detectable activity, while there was less than 5% of relative activity for N173A as compared with that of the wild-type enzyme. We further generated R175K and R176K mutants. For either one, there was less than 0.1% of relative enzymatic activity.

N173A, R175K, and R176K were subjected for kinetic analysis. As shown in Table II, R175K and R176K had ~2.5- and 4-fold higher $K_m$ value, respectively, as compared with that of wild type (Table II). Moreover, the $k_{cat}$ value was significantly reduced for either of two, resulting in an extremely lower $k_{cat}/K_m$ value than that of wild type. The N173A mutant had ~13-fold reduced $k_{cat}$ but 2.5-fold lower $K_m$.

**CD Spectroscopy of Wild Type and Mutant D-NCAases**—CD studies were performed to assess the conformational integrity and thermal stability for wild type, C172S, N173A, R175K, R176A, and R176K. All mutants exhibited far ultraviolet CD spectra nearly identical to that of wild-type D-NCAase (data not shown), indicating...
a similar secondary structure. To compare the stability of the wild type and mutant proteins, the unfolding of the protein was then monitored by the change in ellipticity at 222 nm as the temperature of the sample was increased. All transitions were found to be cooperative and irreversible and had comparable thermal stabilities with $T_m$ of 63 to 71 °C (Table II). These results suggest that each of the created mutants did not affect the secondary structure as well as the thermal stability of the protein.

**Crystal Structures of C172A, C172S, R175A, N173A, C172A-HPG and C172S-HPG**—The crystal structure of C172S was determined to 2.2 Å by molecular replacement method. Residues 3–304 were continuous and defined well in the electron density map. The final model was refined to an $R$ of 18.8% ($R_{free} = 26.7\%$) (Table I). Similarly, the structure of C172A was determined and refined to 2.0 Å resolution, with an $R$ of 17.9% ($R_{free} = 23.5\%$). Crystals of R175A and N173A were obtained under a similar crystallization condition as that for wild-type enzyme. Structures were then determined at 2.0 Å ($R = 19.0\%, R_{free} = 24.6\%$) and 1.95 Å ($R = 15.5\%, R_{free} = 20.9\%$) for R175A and N173A, respectively. Estimated coordinate error values are given in Table I. As shown in Fig. 2, the substituted side-chain electron density in residue 172 was clearly visible for either C172S (Fig. 2A) or C172A (Fig. 2B). Each of these mutant structures shows four subunits (ABCD) with 222 symmetry and is best described as a dimer of dimers like that of the wild-type structure. Moreover, the monomeric subunit of each mutant demonstrates the wild-type $\alpha$-$\beta$-$\beta$-$\alpha$ architecture with modest
deviation in the overall Cα atoms (Table III).

The C172A-HPG and C172S-HPG structures were determined and refined to an R of 17.5% ($R_{free} = 23.3\%$) and 18.6% ($R_{free} = 26.5\%$), respectively (Table I). As seen in Fig. 3A, the $2F_o - F_c$ map unambiguously identified the location and orientation of the substrate in either complex structure. The model consists of four subunits (ABCD) and four substrate molecules bound to the catalytic site of each subunit (Fig. 3B). Like the free-form structure, the monomer has a α/β type structure with two central β sheets and two helices packed on either side. The four substrates are located in a solvent-accessible cleft (Fig. 3C) near the interface of the compact dimers AB and CD, where a long C-terminal fragment extends from a helix to a site near a dyad axis and associates with another monomer. The root mean square deviation in the overall Cα atoms between the superimposed structures with or without substrate is 0.228 Å for C172A and 0.327 Å for C172S, thus indicating limited conformational change in the overall structure upon substrate binding (Table III).

*The Binding Pocket*—The substrate is bound in a pocket surrounded by three large loops (46–61, 127–146 and 197–206) and a short loop (172–178). A number of residues from those loops including E47, K127, H144, E146, A172/S172, N173, R175, R176, N197, T198, H201 and N202 interact with HPG, particularly with the carboxamyl and the carboxyl moieties ($\leq 3.8$ Å) (Fig. 4A). Superposition of the Cα atoms of the binding-site region between the wild type and mutant structures shows virtually
identical conformation (C172A, 0.150 Å; C172S, 0.147 Å), indicating that substitution of cysteine with serine or alanine in residue 172 did not perturb the structure of the binding pocket (Table III). Likewise, the comparison of the free form with the substrate-bound form showed very limited change (Table III and Fig. 4B), suggesting a sturdy site for substrate binding. In the free form of either C172A or C172S structure, a sulfate ion is bound near residue 172 (Fig. 2). Its O2 atom (S172 (Oγ)-sulfate (O2), 2.81 Å) is found in the nearly equivalent position that is occupied by an O atom in the carboxyl group of the HPG molecule (S172 (Oγ)-HPG (O9), 2.86 Å) (Fig. 3A). The O atom from a sulfate ion molecule thus interacts with –OH of S172 in the same manner as the carboxyl group of HPG in the substrate-bound form.

There are 11 interactions (≤ 3.8 Å) between the carboxyl moiety of HPG and the binding pocket (S172, N173, R175, R176, N197 and T198) in the C172S-HPG complex (Fig. 4C). Among these, five hydrogen bonds are found: S172 (Oγ)-HPG (O9), 2.86 Å, N173 (Nδ2)-HPG (O8), 3.16 Å, R175 (Nη1)-HPG (O9), 3.06 Å, R176 (Nε3)-HPG (O8), 2.51 Å, T198 (Oγ1)-HPG (O9), 2.82 Å. The loss of enzymatic activity for R175A or R176A indicates that the interactions with HPG are essential in hydrolyzing HPG. The finding of higher $K_m$ value and very low enzymatic activity for R175K or R176K indeed suggests the crucial role of the guanidinyl group of R175 or R176 in binding to the carboxyl group of HPG. Structural comparison between R175A and wild type enzymes shows little deviation (0.212 Å) in the overall Cα atoms.
There is however significant conformational alteration within the binding pocket (Fig. 5A). The most apparent difference is that the orientation of the side chain of N173 essentially switches to a different direction in R175A (N173 (Nδ)-K127 (Nζ), 2.97 Å in R175A vs. 6.40 Å in wild-type D-NCAase). Other lesser variations such as the Sγ atom of C172 is slightly apart from that in the wild-type structure (0.61 Å) are also observed. These results thus collectively suggest that R175 and R176 are critical in maintaining a proper conformation to fit a substrate with a carboxyl group, hence determining the substrate specificity. We also examine the structure of N173A mutant that did not completely lose its relative activity (5%). As shown in Table III, N173A shares a homologous overall structure. Within the binding pocket, N173A also shows minor conformational alteration (Fig. 5B), indicating that N173 is much less important in maintaining a conformation for substrate binding, unlike that for R175A. In contrast, there is tighter substrate binding affinity upon substitution of N173 with alanine (~2.5-fold lower $K_m$). One possible interpretation is that the much larger side chain of N173 that protrudes outward the pocket may hinder the docking of a substrate into the right orientation toward the presumed reactive Sγ atom of its neighbor C172. It is nevertheless noted that the Sγ atom of C172 points away from the original position (0.37 Å) in N173A, which may explain why it had lower relative activity and $k_{cat}$ (8-fold reduced $k_{cat}$).

For the carbamoyl moiety, there are 14 interactions (E47, K127, H144, E146, S172,
N173, and N197) including 7 hydrogen bonds in the C172S-HPG structure. Among these, the hydroxyl group of S172 extending from the carboxyl end of a β-strand (residues 164 to 170), sits at the very bottom of this pocket and points directly to the C7 atom (2.92 Å) of the carbamoyl moiety of HPG (Fig. 4C). Side chains of two nearby residues, E47 and K127, cluster around that of S172, and face together as a triad (S172-E47-K127) toward the carbamoyl group; the side chain of E47 is situated close the N atom of the carbamoyl moiety (E47 (Oε1)-HPG (N12), 3.05 Å; E47 (Oε2)-HPG (N12), 3.33 Å) while the Nζ atom of K127 sits near the O13 atom of the carbamoyl moiety (2.98 Å) (Fig. 4C). Several interactions are observed among polar groups of E47, K127, and S172 (E47 (Oε1)-S172 (Oγ), 3.39 Å; E47 (Oε2)-S172 (Oγ), 3.99 Å, and K127 (Nζ)-S172 (Oγ), 3.76 Å) which may facilitate to polarize S172 Oγ atom in C172S or C172 Sγ atom of wild-type enzyme. Taken together, these results suggest that the clustered C172-E47-K127 triad forms a robust platform to catalyze an amidohydrolytic reaction when binds to a substrate such as HPG; C172 with a nucleophilic Sγ atom plays a key role in directly attacking the C7 atom of the carbamoyl group, E47 acts as a general base, and K127 stabilizes a tetrahedral transition state. A possible catalytic mechanism that consists of two steps is thereby proposed: (i) an acylation reaction with the carbamoyl moiety of substrate to cleave the susceptible C-N bond and the production of an NH₃ molecule and (ii) deacylation of the acyl-enzyme intermediate to yield a D-amino acid and a CO₂ molecule (10,11).
The carboxyl group of E146 also interacts with the catalytic triad via 5 interactions including a hydrogen bond with K127 (E146 (O€2)–K127 (Nζ2), 2.65 Å). An imidazole ring from H144 sits just above the side chain of E146 (H144 (N€2)-E146 (O€2), 2.58 Å) thus making a hydrogen network and fixing the side-chain geometry of K127, H144, and E146. The finding that H144A had a significant drop in the relative activity (11) and that E146 makes a hydrogen bonding with the carbamoyl group of HPG (E146 (O€2)–HPG (N12), 2.97 Å in C172S-HPG complex) suggest the role of H144 and E146 in maintaining the binding pocket as well as in supporting the docking of a substrate.

Apart from those, F53, P131, N197, P199, H201, and N202 located on three loops (46–61, 127–146 197–206) are also in close proximity to the substrate. The O atom at the main chain of N197 forms a hydrogen bond (2.79 Å) with the N12 atom of the carbamoyl moiety of the substrate, while F53 and P131 make 4 van der Waals contacts (≤ 4.0 Å) with the carbamoyl group. P199, H201, and N202 from a nearby loop (197–206) interacts with the hydroxyphenyl group of HPG that points to the outside space of the binding pocket. It is noted that there is only one strong interaction (N202 (Nδ2)-HPG (O15), 3.13 Å) (Fig. 4C). In the C172A-substrate structure, comparable interactions are also found. The large volume enclosing the hydroxyphenyl group for more van der Waals contacts suggests that D-NCAase favors a substrate with a long/bulky hydrophobic side chain. Indeed, D-NCAase shows broad substrate
specificity toward N-carbamoyl-D-amino acid and hydrolyzes better for larger substrates including D-phenylglycine, D-methionine, and D-leucine (6,8). The finding that D-NCAase had no detectable activity for a small substrate like N-carbamoyl glycine (data not shown) supports this model. In model simulation analysis, an L-enantiomer also bumps onto the 127–146 loop by fitting the carbamoyl group into the active site (data not shown), consistent with its substrate requirement at the D-enantiomeric form (5-7).

**Comparison of the Binding Pocket among Nitrilase, CSHase and D-NCAase**—Although D-NCAase shares low sequence homology with other D-NCAases from other species, the C172-E47-K127 triad is all conserved (11). Another member of the nitrilase superfamily, the Nit domain of *C. elegans* NitFhit protein shows the same four-layer \(\alpha-\beta-\beta-\alpha\) fold with 14.0 Å deviation in the overall C\(\alpha\) atoms (Fig. 6A, left panel), despite lower sequence identity (25%) (13). In support of their related catalytic function, a common C-E-K catalytic triad is seen for D-NCAase and Nit that both belong to the nitrilase superfamily (14) with slight deviation in the polar carboxyl group. It is nevertheless noted that the reactive thiol group of the active cysteine points to different direction (Fig. 6B). Further differences in other regions of the binding pocket are observed. For instance, side chains of R175 and R176 that are responsible to interact with the carboxyl moiety of a substrate in D-NCAase are occupied with those of V172 and R173 in Nit (Fig. 6B), suggesting that
Nit would have its own substrate specificity. We have also compared the D-NCAase structure with that of CSHase, the only other enzyme with available structural coordinates that catalyzes the amidohydrolytic reaction (11,31). Even though CSHase has a distinct structural architecture (three-layer α-β-α fold) and presents the binding pocket in a different way (Fig. 6A, right panel), superposition of the catalytic triad between D-NCAase and CSHase reveals a homologous catalytic triad (C172-E47-K127 in D-NCAase vs. C177-D51-K144 in CSHase) (Fig. 6C), in accord to the related hydrolytic reaction. However, other regions of the binding pocket are essentially different: the residues proposed to bind to the carboxyl moiety in CSHase are from a C-terminal fragment (R202) and that of its neighbor subunit B (K217), respectively, as compared with R175 and R176 from the same loop in D-NCAase. Moreover, a hydrophobic region containing F63, W111, I115, and L120 that may bind to the N-methyl group of the carbamoylsarcosine molecule is only seen in CSHase (Fig. 6C).

In conclusion, we have determined crystal structures of mutant D-NCAases (C172A, C172S, N173, and R175A) as well as substrate-bound complexes (C172A-HPG and C712S-HPG). All structures present the same four-layer sandwich architecture as that of the wild type D-NCAase. The substrate-bound forms reveal that the carbamoyl group of the substrate makes direct contacts with a robust catalytic triad (C172-E47-K127) located at the interior of a solvent-accessible cleft for an amidohydrolytic reaction. R175 and R176 that are situated nearby C172 play crucial
roles in binding to the carboxyl moiety of a substrate as well as maintaining a stable binding platform. The finding that substitution of R175 or R176 with alanine abolished its enzymatic activity further supports this model. For the peripheral portion of the binding pocket, only a substrate that endows a side chain at the D-enantiomeric form can loosely fit into it. A larger side chain can thus make more van der Waals contacts for an enhanced binding. The comparable geometry of C-D/E-K triad seen among D-NCAase, NitFhit, and CSHase suggests a robust and conserved catalytic platform for a related chemical reaction, perhaps being a result of convergent evolution; the increased nucleophilicity of the $\gamma$ atom from the nearby polar groups can therefore attack the C atom of a susceptible C–N bond efficiently. On the other hand, the unique specificity of a particular biocatalyst is acquired from divergence of other regions within the binding pocket as seen from these structures. The elucidation of the structural basis of D-NCAase substrate specificity may thus facilitate the design of mutant enzymes with altered specificity. The catalytic activity and stability of D-NCAase may be also improved by a rational approach.

Acknowledgments—We acknowledge access to Macromolecular X-ray Crystallographic Center of NTHU Instrument Center at Hsinchu, National Tsing Hua University, Taiwan for data collection. We are grateful for the access to the following beamlines for synchrotron data collection: BL-6A at the High Energy Accelerator
Research Organization (KEK), Photon Factory, Japan, BL17B2 beamline at the National Synchrotron Radiation Research Center (NSRRC), Taiwan, and BL12B2 Taiwan beamline at Spring-8, Japan. This work was supported by grants from NSC (NSC91-3112-B-007-011, NSC91-2313-B-007-002, and NSC90-2311-B-007-002), and by Minister of Education (Program for Promoting Academic Excellence of Universities Grant 89-B-FA04-1-4), Taiwan.
REFERENCES

1. Syldatk, C., Läufer, A., Müller, R., and Höke, H. (1990) *Adv. Biochem. Eng. Biotechnol.* **41**, 30–75

2. Olivieri, R., Fascetti, E., Angelini, L., and Degen, L. (1981) *Biotechnol. Bioeng.* **23**, 2173–2183

3. Runser, S., Chinski, N., and Ohleyer, E. (1990) *Appl. Microbiol. Biotechnol.* **33**, 382–388

4. Nanba, H., Ikenaka, Y., Yamada, Y., Yajima, K., Takano, M., and Takahashi, S. (1998) *Biosci. Biotechnol. Biochem.* **62**, 875–881

5. Moller, A., Syldatk, C., Schulze, M., and Wagner, F. (1988) *Enzyme Microb. Technol.* **10**, 618–625

6. Ogawa, J., Shimizu, S., and Yamada, H. (1993) *Eur. J. Biochem.* **212**, 685–691

7. Ogawa, J., Chung, M. C.-M., Hida, S., Yamada, H., and Shimizu, S. (1994) *J. Biotechnol.* **38**, 11–19

8. Ikenaka, Y., Nanba, H., Yamada, Y., Yajima, K., Takano, M., and Takahashi, S. (1998) *Biosci. Biotechnol. Biochem.* **62**, 882–886

9. Takahashi, S., Ohashi, T., Kii, Y., Kumagai, H., and Yamada, H. (1979) *J. Ferment. Technol.* **57**, 328–332

10. Nakai, T., Hasegawa, T., Yamashita, E., Yamamoto, M., Kumasaka, T., Ueki, T., Nanba, H., Ikinaka, Y., Takahashi, S., Sato, M., and Tsukihara, T. (2000) *Structure* **8**, 729–739

11. Wang, W. C., Hsu, W. H., Chien, F. T., and Chen, C. Y. (2001) *J. Mol. Biol.* **306**, 251–261

12. Grifantini, R., Pratesi, C., Galli, G., and Grandi, G. (1996) *J. Biol. Chem.* **271**,
13. Pace, H. C., Hodawadekar, S. C., Draganescu, A., Huang, J., Bieganowski, P., Pekarsky, Y., Croce, C. M., and Brenner, C. (2000) Curr. Biol. 10, 907–917
14. Pace, H. C., and Brenner, C. (2001) Genome Biol 2, REVIEWS 0001.1–0001.9
15. Hsu, W. H., Chien, F. T., Hsu, C. L., Wang, T. C., Yuan, H. S., and Wang, W. C. (1999) Acta Crystallogr. Sect. D Biol. Crystallogr. 55, 694–695
16. Bradford, M. M. (1976) Anal. Biochem. 72, 248–254
17. Gordon, S. A., Fleck, A., and Bell, J. (1978) Ann. Clin. Biochem. 15, 270–275
18. Leslie, A. G. (1999) Acta Crystallogr. Sect. D Biol. Crystallogr. 55 (Pt 10), 1696–1702
19. Collaborative. (1994) Acta Crystallogr. Sect. D Biol. Crystallogr. 50, 760–763
20. Otwinowski, Z., and Minor, W. (1997) Methods Enzymol. 276, 307–326
21. Jones, T. A., Zou, J. Y., Cowan, S. W., and Kjeldgaard, M. (1991) Acta Crystallogr. Sect. A 47, 110–119
22. Murshudov, G. N., Vagin, A. A., and Dodson, E. J. (1997) Acta Crystallogr. Sect. D Biol. Crystallogr. 53, 240–255
23. Perrakis, A., Morris, R., and Lamzin, V. S. (1999) Nat. Struct. Biol. 6, 458–463
24. Winn, M. D., Isupov, M. N., and Murshudov, G. N. (2001) Acta Crystallogr. Sect. D Biol. Crystallogr. 57, 122–133
25. Laskowski, R. A., MacArthur, M. W., Moss, D. S., and Thornton, J. M. (1993) J. Appl. Crystallogr. 26, 283–291
26. Read, R. J. (1986) Acta Crystallogr. Sect. A 42, 140–149
27. Kleywegt, G. J., and Jones, T. A. (1997) Methods Enzymol. 277, 525–545
28. Kraulis, P. J. (1991) J. Appl. Crystallogr. 24, 946–950
29. Merrit, E. A., and Murphy, M. E. P. (1994) Acta Crystallogr. Sect. D Biol.
30. DeLano, W. L. (2002), World Wide Web http://www.pymol.org

31. Romao, M. J., Turk, D., Gomis-Rüth, F.-X., and Huber, R. (1992) *J. Mol. Biol.* **226**, 1111–1130
FIGURE LEGENDS

FIG. 1. **SDS-PAGE analysis of purified D-NCAase mutants.** Lane 1, wild type D-NCAase; lane 2, C172S; lane 3 C172A; lane 4, R175A; lane 5, R175K; lane 6, R176A; lane 7, R176K; lane 7, N173A.

FIG. 2. **The $2F_o - F_c$ electron density map of D-NCAase mutant around residue 172.** A, C172S mutant. B, C172A mutant. Maps are contoured at the 1.5 $\sigma$ level.

FIG. 3. **Crystal structure of the C172S-substrate complex.** A, the $2F_o - F_c$ map of the C172S-HPG complex around HPG, contoured at the 1.5 $\sigma$ level. B, ribbon representation of the homotetrameric structure of the complex, ABCD. The four subunits, A, B, C, and D, are depicted in blue, yellow, red and green, respectively. HPG is drawn as a ball-and-stick model. C, subunit A of C172S with the bound substrate.

FIG. 4. **The binding pocket of the C172S-HPG complex.** A, stereoview of the C172S-HPG binding pocket. HPG is in yellow. Four loops enclosing HPG are colored by blue. E47, K127 and S172 are in red, whereas the other residues binding to substrate are in green. B, superimposed structures between C172S and C172S-HPG complex. The protein backbones of C172S and C172S-HPG are in green and in red, respectively. HPG is in yellow. E47, K127 and S172 residues of C172S (green), and
C172S-HPG (red) are shown by stick structures. The oxygen and nitrogen atoms are colored in red and blue, respectively. C, schematic diagram of HPG bound to C172S. Interactions are shown by dotted lines. The numbering of HPG is in red.

FIG. 5. **Analysis of the active center for R175A and N173A.** A, superposition of active-site residues of wild type (green) with those of R175A (red). B, superposition of active-site residues of wild type (green) with those of N173A (red). Residues are shown by stick structures. The oxygen, nitrogen and sulfur atoms are colored in red, blue, and yellow, respectively.

FIG. 6. **Structural comparison of D-NCAase with Nit or CSHase.** A, comparison of D-NCAase structure with that of Nit (left panel) or CSHase (right panel). The protein backbones of D-NCAase are colored in green, and those of Nit or CSHase are in red. Three catalytic residues (E47, K127 and C172 in D-NCAase; E54, K127, and C169 in Nit; D51, K144, and C177 in CSHase) are shown by stick structures. B, superposition of active-site residues of D-NCAase (green) with those of Nit (red). C, superposition of active-site residues of D-NCAase (green) with those of CSHase (red). Residues essential in the catalysis and substrate binding are shown by stick structures. The oxygen, nitrogen and sulfur atoms are colored in red, blue, and yellow, respectively.
### Table I

**Data collection and refinement statistics**

| Source           | C172A-HPG | C172A | C172S | C172S-HPG | R175A | N173A  |
|------------------|-----------|-------|-------|-----------|-------|--------|
| **Data collection statistics** | KEK BL-6A | CuKα | KEK BL-6A | KEK BL-6A | SPring8 BL-12B | SPring8 BL-12B |
| Cell dimensions  |           |       |       |           |       |        |
| a = 69.50 Å      | 69.24 Å  | 69.42 Å | 69.74 Å | 71.00 Å  | 68.65 Å |
| b = 67.77 Å      | 67.45 Å  | 67.10 Å | 67.97 Å | 67.77 Å  | 67.69 Å |
| c = 138.26 Å     | 137.68 Å | 138.34 Å | 138.36 Å | 136.95 Å | 138.50 Å |
| β = 96.08°       | 96.27°   | 96.12° | 95.93° | 95.96°  |        |
| Resolution (Å)   | 2.00    | 2.20  | 2.00  | 2.40     | 1.95  |
| Highest resolution shell (Å) | 2.07-2.00 | 2.32-2.20 | 2.11-2.00 | 2.53-2.40 | 2.07-2.00 | 2.02-1.95 |
| Completeness (%) | 98.9 (98.9) | 99.8 (99.8) | 95.2 (95.2) | 96.0 (96.0) | 90.7 (98.7) | 99.8 (99.9) |
| Average I/σ(I)   | 5.6     | 5.2   | 7.0   | 5.1      | 7.1   | 7.1    |
| Rmerge (%)       | 7.9     | 12.5  | 8.0   | 13.2     | 9.2   | 10.3   |
| Unique reflections | 81,355  | 63,921 | 78,311 | 45,959   | 79,502 | 92,630 |

| **Refinement statistics** |       |       |       |       |       |       |
|---------------------------|-------|-------|-------|-------|-------|-------|
| Resolution (Å)            | 30.0-2.0 | 30.0-2.2 | 30.0-2.0 | 30.0-2.4 | 25.0-2.0 | 25.0-1.95 |
| Protein atoms             | 9,560 | 9,564 | 9,620 | 9,624 | 9,540 | 9,548 |
| Solvent atoms             | 1,155 | 818  | 769  | 528  | 714  | 1,154 |
| Substrate atoms           |       |       | 60    | 60    |       |       |
| R²                         | 0.179 | 0.188 | 0.175 | 0.186 | 0.190 | 0.155 |
| Rfree                     | 0.235 | 0.267 | 0.233 | 0.265 | 0.246 | 0.209 |
| r.m.s.d. bond length (Å)  | 0.037 | 0.020 | 0.023 | 0.032 | 0.031 | 0.028 |
| r.m.s.d. bond angles (°)  | 2.66  | 1.77  | 1.96  | 2.42  | 2.25  | 2.12  |
| r.m.s.d. torsion angles (°)| 8.07 | 7.56  | 7.67  | 8.54  | 7.77  | 7.30  |
| Estimated coordinate error (Å) | 0.194 | 0.288 | 0.193 | 0.636 | 0.223 | 0.151 |

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*a* Beamline 6A at High Energy Accelerator Research Organization, KEK Photon Factory, Japan.

*b* BL12B2 Taiwan beamline at Japan Synchrotron Radiation Research Institute (JASRI), Spring8, Japan.

*c* Values in parentheses refer to statistics in the highest-resolution shell.

*d* \[ R_{merge} = \frac{\sum |I_o - \langle f \rangle|}{\sum I_o} \]

*e* \[ R = \frac{\sum |F_o - F_c|}{\sum F_o} \]

*f* \[ R_{free} \] was computed using 5% of the data assigned randomly.

*g* r.m.s.d., root mean square deviation.
| D-NCAase | \( k_{cat} \) | \( K_m \) | \( k_{cat}/K_m \) | \( T_m \) |
|----------|-------------|--------|-----------------|------|
| WT       | 5.2×10²     | 1.3    | 4.0×10²         | 63   |
| C172A    | No activity | –      | –               | –    |
| C172S    | Not determined, less than 0.1% activity | –      | –               | 70   |
| N173A    | 3.8×10¹     | 0.52   | 7.3×10¹         | 68   |
| R175A    | No activity | –      | –               | –    |
| R176A    | No activity | –      | –               | 71   |
| R175K    | 2.7×10⁻¹    | 3.2    | 8.5×10⁻²        | 65   |
| R176K    | 6.3         | 5.4    | 1.1             | 65   |

Table II

Kinetic parameters and \( T_m \) values for wild type and mutant D-NCAases.
### Table III

**Comparison of the D-NCAase monomer and binding-site region**

Comparison of root mean square deviations (Å) for the overall Cα atoms in monomer A, and the Cα atoms or all atoms of the binding-site region, between wild type and mutant, wild type and mutant-substrate complex, or the free and the bound structures.

| Comparison                      | Root mean square deviation (Å) |
|---------------------------------|--------------------------------|
|                                 | Cα atoms in monomer A<sup>a</sup> | Cα atoms in binding-site region<sup>b</sup> | All atoms in binding-site region<sup>b</sup> |
| WT<sup>c</sup> vs. C172A        | 0.262                           | 0.150                                    | 0.530                                     |
| WT vs. C172A-HPG                | 0.322                           | 0.202                                    | 0.493                                     |
| C172A vs. C172A-HPG             | 0.228                           | 0.154                                    | 0.359                                     |
| WT vs. C172S                    | 0.202                           | 0.147                                    | 0.252                                     |
| WT vs. C172S-HPG                | 0.299                           | 0.194                                    | 0.662                                     |
| C172S vs. C172S-HPG             | 0.327                           | 0.226                                    | 0.647                                     |
| WT vs. N173A                    | 0.277                           | 0.095                                    | 0.472                                     |
| WT vs. R175A                    | 0.212                           | 0.237                                    | 1.079                                     |

<sup>a</sup> Homologous Cα atoms are compared between superimposed structures.

<sup>b</sup> The residues in binding site for analysis are E47, K127, H144, E146, C172, N173, R175, R176, N197, T198, H201 and N202.

<sup>c</sup> WT, wild-type enzyme.
Chen et al., Fig 1
Chen et al., Fig 2
Chen et al., Fig 3
Chen et al., Fig 4
Chen et al., Fig 6

A

B

C

E47
E54
K127
C172
R175
R176
V172
K127
C169
R173

K127
C172
C169
V172
R173
R176

L120
I115
E47
D51
K127
C177
R175
R176
W111
K144
C172
R175
R176

K127
C172
C177
R175
R176
Structural basis for catalysis and substrate specificity of Agrobacterium radiobacter N-carbamoyl-D-amino-acid amidohydrolase
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J. Biol. Chem. published online April 22, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M302384200

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