Evolution of New Enzymatic Function by Structural Modulation of Cysteine Reactivity in *Pseudomonas fluorescens* Isocyanide Hydratase

Mahadevan Lakshminarasimhan  
*University of Nebraska - Lincoln*

Peter Madzelan  
*University of Nebraska Lincoln, pmadzelan3@unl.edu*

Ruth Nan  
*Washington University in St. Louis*

Nicole Marie Milkovic  
*University of Nebraska - Lincoln*

Mark A. Wilson  
*University of Nebraska - Lincoln, mwilson13@unl.edu*

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Evolution of New Enzymatic Function by Structural Modulation of Cysteine Reactivity in *Pseudomonas fluorescens* Isocyanide Hydratase*

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Mahadevan Lakshminarasimhan 1,2, Peter Madzelen 1, Ruth Nan 5, Nicole M. Milkovic, and Mark A. Wilson 1

From the Department of Biochemistry and the Redox Biology Center, University of Nebraska, Lincoln, Nebraska 68588-0664

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Isocyanide (formerly isonitrile) hydratase (EC 4.2.1.103) is an enzyme of the DJ-1 superfamily that hydrates isocyanides to yield the corresponding N-formamide. In order to understand the structural basis for isocyanide hydratase (ICH) catalysis, we determined the crystal structures of wild-type and several site-directed mutants of *Pseudomonas fluorescens* ICH at resolutions ranging from 1.0 to 1.9 Å. We also developed a simple UV-visible spectrophotometric assay for ICH activity using 2-naphthyl isocyanide as a substrate. ICH contains a highly conserved cysteine residue (Cys101) that is required for catalysis and interacts with Asp17, Thr102, and an ordered water molecule in the active site. Asp17 has carboxylic acid bond lengths that are consistent with protonation, and we propose that it activates the ordered water molecule to hydrate organic isocyanides. In contrast to Cys101 and Asp17, Thr102 is tolerant of mutagenesis, and the T102V mutation results in a substrate-inhibited enzyme.

Isocyanides differ from nitriles in that the nitrogen atom is bonded to the organic moiety in the isocyanide compounds, only one enzyme has been characterized to catalyze the hydration of isocyanides and will not catalyze the hydrolysis of the corresponding nitrile compounds, and the enzyme has a broad substrate tolerance for organic isocyanides (8, 9). ICH is interesting because, in addition to catalyzing the hydration of an unusual class of compounds, it is also a new and poorly characterized member of the large DJ-1 superfamily.

The DJ-1 superfamily comprises several phylogenetic clades with distinct structural features, oligomerization, and putative
active sites (10–12). Examples of functionally validated chaperones, proteases, peptidases, transcription factors, and stress response proteins are all found in the DJ-1 superfamily. Although the cellular roles of many of these proteins remain unknown, the majority of functionally characterized DJ-1 superfamily proteins are implicated in the stress response. This group includes the human protein DJ-1, which confers protection against oxidative stress (13, 14) via several different proposed activities (15) and is involved in multiple diseases, including Parkinsonism (16), various cancers (17), and ischemic injury (18, 19).

A common feature of proteins in the DJ-1 superfamily is that they contain a highly conserved cysteine residue that is essential for the functions of many of these proteins (10–12). This cysteine typically adopts an energetically strained set of backbone torsion angles and is located at a sharp turn between an N-proximal β-strand and a C-proximal α-helix called the nucleophile elbow (20–22). In human DJ-1, this cysteine residue has a thiol pKₐ value of ~5 and therefore exists as a reactive cysteine thiolate at physiological pH (23). Cys¹⁰⁶ in DJ-1 is particularly susceptible to oxidation and forms cysteine-sulfenic and -sulfonic acids during oxidative stress (13, 24). Recent studies have shown that Cys¹⁰⁶-sulfenic acid is an important post-translational modification of human DJ-1 and is favored over other types of cysteine modification by the local hydrogen bonding environment around the thiolate (25). In particular, Cys¹⁰⁶-sulfenic acid is stabilized through hydrogen bonding with a highly conserved and protonated glutamic acid (Glu¹⁸), and this interaction also contributes to the low Cys¹⁰⁶ thiol pKₐ value in the reduced form of DJ-1 (23, 25). Further emphasizing the importance of this interaction, structural and computational studies (12) show that the Cys-Glu hydrogen-bonded interaction and oxidation of the conserved cysteine are observed in several other DJ-1 superfamily proteins from diverse organisms, including Drosophila melanogaster (26), Escherichia coli (21), Saccharomyces cerevisiae (22, 27), Pyrococcus horikoshii (28), and Deinococcus radiodurans (29).

Because cysteine thiols are excellent nucleophiles, some of the uncharacterized DJ-1 superfamily members may be enzymes of unknown function that use this conserved cysteine for catalysis. Currently, the only members of the DJ-1 superfamily with experimentally verified and physiologically relevant enzymatic activities are a subset of proteases and hydrolases (Pfpl (30), PH1704 (28), and E. coli Hsp31 (31, 32)) that employ a papain-like Cys-His-Asp/Glu catalytic triad that includes the conserved cysteine residue. ICH does not contain this catalytic triad and thus represents a new activity and type of active site in the DJ-1 superfamily. ICH belongs to a previously uncharacterized clade of the superfamily with several close homologues, as well as possessing weaker homology to transcription factors that are fusions of an AraC-like helix-turn-helix DNA binding domain and a DJ-1-like domain (33). In addition, ICH and its close homologues are distinguished from most other members of the DJ-1 superfamily by the replacement of the highly conserved protonated glutamic acid (Glu¹⁸ in human DJ-1; see above) with an aspartic acid. This substitution suggests the hypothesis that functional diversity has evolved in the DJ-1 superfamily through a subtle modulation of the active site environment of the conserved reactive cysteine in order to achieve different chemical outcomes. This hypothesis, which has precedent in other protein families (34), would help explain the varied functions of characterized DJ-1 superfamily proteins and also provides an example of natural selection capitalizing on the unique reactivity of the cysteine thiol(ate) to achieve functional diversity while maintaining structural parsimony.

We have conducted a combined structural and enzyme kinetics study of Pseudomonas fluorescens ICH to investigate the role of the structural environment of the active site cysteine residue in the function of this unusual enzyme. ICH is a homodimer in the crystal and in solution and is structurally similar to human DJ-1 and closely related proteins, and the enzyme active site lies near the dimerization interface. A critical and conserved cysteine residue (Cys¹⁰¹) makes key hydrogen bonds with a protonated aspartic acid (Asp¹⁷) and a nearby ordered water molecule that are required for significant ICH activity. Structural changes in the environment of Cys¹⁰¹ favor the formation of cysteine-sulfinate (Cys-SO₂⁻) that is observed in human DJ-1, although Cys¹⁰¹ is partially oxidized to a cystine-sulfenic acid in the ICH crystal, possibly due to x-ray irradiation. The structure supports and expands upon a previously proposed mechanism for ICH that involves nucleophilic attack by Cys¹⁰¹, followed by the addition of water to the enzyme-linked thioimidate species and release of the N-formamide, although the details of this proposed mechanism require additional study.

**EXPERIMENTAL PROCEDURES**

**Cloning and Mutagenesis of P. fluorescens Pf-5 ICH**—The coding sequence for ICH was PCR-amplified from the genomic DNA of P. fluorescens Migula strain Pf-5 (American Type Culture Collection number BAA-477D) using primers that incorporated a 5’ NdeI restriction site and a 3’ XhoI restriction site. The ICH gene was cloned between the NdeI and XhoI restriction sites of the bacterial expression vector pET15b (Novagen, Darmstadt, Germany) such that the expressed protein carries an N-terminal, thrombin-cleavable hexahistidine tag. After thrombin cleavage, the final recombinant ICH protein has a calculated molecular mass of 24,158 Da. ICH sequence numbering throughout this paper is for the untagged native protein amino acid sequence. All point mutations (D17V, D17N, D17E, C101S,
C101A, T102S, and T102V) were generated by site-directed mutagenesis using Pfu Turbo DNA Polymerase (Stratagene, La Jolla, CA) and appropriate mutagenic primers. All ICH constructs were verified by DNA sequencing (Eurofins MWG Operon and UNL DNA sequencing core facility).

**Protein Expression and Purification—*E. coli* strain BL21(DE3) (Novagen, Darmstadt, Germany) bearing the ICH-pET15b construct was grown in LB broth supplemented with 100 μg/ml ampicillin at 37 °C with shaking at 250 rpm until the A600 reached 0.5–0.7. Overexpression of ICH was induced by the addition of 0.5 mM isopropyl β-d-thiogalactopyranoside, followed by 5 h of incubation at 37 °C. Cells were harvested by centrifugation, frozen in liquid nitrogen, and stored at −80 °C until needed.

Frozen cells were thawed on ice and resuspended in lysis buffer (50 mM HEPEs, pH 7.5, 300 mM KCl, 10 mM imidazole). The suspended cells were lysed by the addition of hen egg white lysozyme to a final concentration of 1 mg/ml at 4 °C for 30 min, followed by sonication and centrifugation to remove cell debris. The cleared supernatant was applied to a His-select Ni2+ metal affinity resin to remove any uncleaved protein that retained the histidine tag. The recombinant hexahistidine tagged ICH was purified using Ni2+ metal affinity chromatography according to standard procedures, using 200 mM imidazole to elute bound protein. The eluted protein was dialyzed overnight at 4 °C against storage buffer (25 mM HEPES, pH 7.5, 100 mM KCl), and protein concentration was determined using a calculated extinction coefficient of ϵ280 = 15,600 M⁻¹ cm⁻¹ based on the amino acid composition of *P. fluorescens* ICH. The histidine tag used for protein purification was removed by thrombin cleavage (2 units of thrombin/mg of ICH) at room temperature for 4 h, followed by passage over Ni2+ metal affinity resin to remove any uncleaved protein that retained the histidine tag. Thrombin was removed by passage over benzamidine-Sepharose resin (GE Healthcare). The final protein ran as a single peak into liquid nitrogen.

**Crystal Structure of Isocyanide Hydratase**

Crystal Structure of Isocyanide Hydratase—The crystal structure of wild-type ICH was determined by molecular replacement using the structure of human DJ-1 (Protein Data Bank code 1P5F (20), 28% sequence identity) as a search model in the program Phaser (37) in the CCP4 suite. The automated rotation and translation functions found a clear top solution with a final Z-score of 8.6 for the ICH dimer in the ASU. The initial molecular replacement model was improved using automated rebuilding in ARP/wARP (38) as implemented in the CCP4 program suite. ARP/wARP constructed and refined a nearly complete initial model with an Rfree (39) of 21% for data collected on a rotating anode x-ray source to 1.7 Å resolution. This initial model was subjected to manual editing and then was further refined against the 1.05 Å resolution synchrotron data set in SHELX-97 (40) using conjugate gradient minimization against an intensity-based least squares target function with geometric and atomic displacement parameter (ADP) restraints. All refinements used a bulk solvent correction to allow inclusion of the low resolution data. The final models for all atomic resolution data sets (wild-type, C101S, C101A, and D17E ICH) contain anisotropic ADPs and were refined with riding hydrogens on all atoms except the O of serine, O of tyrosine, O of threonine, and Nδ1 of histidine due to the ambiguous hydrogen placement for these atoms. The model for T102S ICH was refined in Refmac5 (41) in the CCP4 suite against a maximum likelihood amplitude-based target function with geometric and isotropic ADP restraints. Translation-libration-screw refinement of ADPs in Refmac5 was used once the structural model for T102S ICH reached convergence (42, 43). Final model statistics are provided in Table 1.
Crystal Structure of Isocyanide Hydratase

TABLE 1
Data collection and refinement statistics

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Refinement

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Structure Analysis—All refined crystal structures were subjected to geometric, packing, and electron density validation using COOT (44) and MolProbity (45). Over 98% of the residues are in the favored regions of the MolProbity Ramachandran plot, and the only outlier is Ile152. This residue is located in an area that is conformationally polymorphic in ICH (see “Results”). Superposition of crystal structures was performed using the secondary structure matching algorithm (46) as implemented in COOT. Solvent-accessible surface areas were calculated using the refined coordinates in the PISA web server (47) at the European Bioinformatics Institute. Structural figures were made with Povscript+ (48).

Bond length analysis of Asp17 in ICH was performed in SHELX-97 by eliminating all geometric restraints (DFIX, DANG, and CHIV) on this residue, followed by 10 cycles of conjugate gradient refinement. The model was then subjected to a single cycle of unrestrained full matrix least squares refinement followed by matrix inversion in order to determine the estimated standard uncertainties (ESUs) on refined parameters. No coordinate shifts were applied to the model, and the coordinates deposited with the Protein Data Bank were refined with restraints.

Kinetic Characterization of ICH Using UV-visible Spectrophotometry—A UV spectrophotometric assay was developed using 2-naphthyl isocyanide (NIC) as a substrate to measure the kinetics of ICH catalysis. This method allows the evolution of the product, N-(2-naphthyl) formamide (NF), to be monitored continuously at 251 nm, where NF absorbs strongly but NIC has a negligible absorbance at the micromolar concentrations used in the assay (supplemental Fig. 1). The absorbance measurements for NF formation were converted to concentrations using an extinction coefficient of ε251 = 39,330 M^-1 cm^-1 for NF measured in the reaction buffer (100 mM KH2PO4/K2HPO4, pH 7.4, 50 mM KCl, 20% DMSO). Although the naphthyl moiety of the substrate allows for spectrophotometric detection of product formation, it also renders the substrate poorly soluble in aqueous solution. Consequently, 20% DMSO is used as a co-solvent. DMSO has strong UV absorption below ~230 nm; therefore, the region of the spectrum below this wavelength is not usable, although this may be ameliorated with an alternate choice of cosolvent. The assay was validated by comparing the UV absorption spectrum of the reaction product with authentic NF (supplemental Fig. 1), comparing the HPLC profiles of the reaction product with NF, and by using electrospray ioniza-
tion-MS to confirm that NF is the product of the ICH-catalyzed reaction. Both NIC and NF were purchased from Sigma-Aldrich at 97% purity.

The assay was performed using NIC prepared as a 1 M stock in 100% DMSO and diluted to the required concentrations in reaction buffer. Approximately 150 μl of the reaction buffer and substrate were mixed and pre-equilibrated at 25 °C for 1 min in a quartz cuvette held in a Peltier temperature controller, followed by the addition of ICH (1 μM final enzyme concentration) in order to initiate the reaction. Initial rates of reaction were measured by monitoring absorbance at 251 nm during the first minute using a Cary50 UV-visible spectrophotometer (Varian, Palo Alto, CA). The reaction exhibited saturation kinetics with respect to NIC concentration, and all reactions were performed in triplicate. The averaged data and S.D. values were plotted and fit using nonlinear regression to the Michaelis-Menten model in order to determine $K_m$, $V_{max}$, and $k_{cat}$ values using Prism (GraphPad Software, San Diego, CA). Reported errors in $k_{cat}$, $K_m$, and $K_s$ values are from the fit procedure. Increasing the amount of DMSO to 30% (v/v) did not change the kinetic parameters, indicating that the enzymatic properties of ICH are not significantly altered by the DMSO cosolvent in this concentration range. The activities of multiple batches of ICH were compared both when freshly purified and after rapid freezing in liquid nitrogen and storage at −80 °C, with no loss of activity detected after a single freeze-thaw cycle.

**Sedimentation Equilibrium Ultracentrifugation**—Sedimentation equilibrium ultracentrifugation was performed at 20 °C using a Beckman Coulter XL-I analytical ultracentrifuge, an An Ti-50 rotor and absorbance optics. ICH samples were thawed on ice and dialyzed for 3 h at 4 °C against 25 mM HEPES, pH 7.5, 100 mM KCl, and 1 mM DTT. After dialysis, the samples and buffer were centrifuged to remove particulates and diluted to the required concentrations (0.25, 0.50, and 1.00 mg/ml) in the dialysis buffer. Samples (110 μl) and buffer (125 μl) were loaded in a six-sector carbon-filled Epon sample cell fitted with quartz windows. Sedimentation equilibrium ultracentrifugation was performed at 1.7 × 10^4, 2.0 × 10^4, and 2.4 × 10^4 rpm, and the absorbance of each sample was monitored at 275 nm as a function of radius. Scans at each speed were collected after 20 and 22 h of centrifugation and compared to ensure that equilibrium had been reached. The partial specific volume of the protein samples and the solvent density were calculated using the program SedNTerp (49), using the partial specific volume of Tris as a substitute for the HEPES buffer that was used in this experiment. All nine data sets (three protein concentrations at three different speeds) were globally fit using the analytical ultracentrifugation data analysis routine provided by Beckman-Coulter in Origin 6.0.

**RESULTS**

**Description of the ICH Structure**—The 1.05 Å resolution crystal structure of *P. fluorescens* ICH contains 10 α-helices (A–J) and 10 β-strands (β1–β10) arranged in the core α-β flavodoxin-like fold that is characteristic of members of the DJ-1 superfamily (Fig. 2A). ICH is distinguished from previously characterized members of the DJ-1 superfamily by the presence of an additional long, nine-turn C-terminal α-helix (J) that makes contacts along the length of helices D and G. Helix J wraps around the “top” of the ICH protomer and partially occludes the conserved cysteine residue (Cys101) in the active site. As a consequence, Cys101 lies at the bottom of a narrow and deep pocket in ICH. In addition, helix αJ contains multiple residues that are near the active site, including Arg124, whose guanidinium group is within ~8 Å of Cys101. Helix J has elevated anisotropic ADPs, and the electron density exhibits clear evidence of conformational disorder, indicating elevated flexibility in this part of the molecule.

ICH is a homodimer in the crystal (Fig. 2B), burying 4800 Å^2 of total surface area at the dimer interface, as calculated by the European Bioinformatics Institute PISA server (47). The asymmetric unit of ICH in space group P2_1 contains both protomers of the ICH dimer, which superimpose with a Cα r.m.s. deviation of 0.38 Å. The small differences between the two noncrystallographic symmetry-related molecules reside primarily in residues 54–64 and the C-terminal α-helix (residues 192–227); these are flexible regions of the proteins that also display elevated ADPs that differ in magnitude among the equivalent Cα atoms in the two protomers of the ICH dimer (Fig. 2C). The DJ-1 superfamily is notable for the prevalence and diversity of oligomerization exhibited by its members (21), and dimerization appears to be the most common form of self-association in the superfamily. The ICH dimer is structurally analogous to that formed by both human DJ-1 (Protein Data Bank entry 2OR3 (23); Cα r.m.s. deviation = 1.6 Å) and *E. coli* YajL (Protein Data Bank entry 2AB0 (21); Cα r.m.s. deviation = 1.7 Å). However, the presence of 45 additional residues at the C terminus of ICH contributes two structural elements that are not present in either DJ-1 or its close homologues: an irregularly structured strand that lies between a hydrophobic groove created by helices H and I, followed by a kinked nine-turn C-terminal α-helix J (Fig. 2D). These additional structural elements result in a substantially larger amount of total buried surface area in ICH compared with human DJ-1 (4800 Å^2 versus 2700 Å^2). A sedimentation equilibrium ultracentrifugation experiment conducted at multiple rotor speeds and ICH concentrations (see “Experimental Procedures”) demonstrates that ICH is an obligate homodimer in solution with a measured molecular mass of 43.7 kDa, which agrees reasonably well with the expected dimeric mass of 48 kDa (supplemental Fig. 2).

Although ICH is clearly related to previously characterized DJ-1 homologues, the closest structural match for ICH in a DALI search of the Protein Data Bank is 3EWN, a protein of unknown function from *Pseudomonas syringae* that was solved by the New York SGX Research Center for Structural Genomics after the structure for ICH was determined. The high degree of sequence and structural similarity of these two proteins (45% sequence identity; Cα r.m.s. deviation = 1.0 Å), a similar array of active site residues, and their presence in closely related prokaryotes suggest that they might share similar functions. It would be interesting to experimentally test whether 3EWN contains ICH activity because this could be a useful means to determine if ICH activity is adventitious or conserved. This unresolved question is important because there is currently no clearly established biological role for an ICH activity, although
homologues of this enzyme appear to be abundant in the pseudomonads and related prokaryotes (see “Discussion”).

The Active Site Environment of the Conserved Cysteine in ICH Is Distinct from DJ-1—The active site of ICH is centered on a conserved cysteine residue (Cys101) located at the bend between β-stand 6 and α-helix F called the “nucleophile elbow” (Fig. 3A). The cysteine residue at this location in other DJ-1 superfamily members typically has strained backbone torsion angles in the marginal or unfavorable region of Ramachandran space (20, 28, 29, 31, 50–53). In ICH, Cys101 has marginal backbone torsion values of $\phi = 60$, $\psi = -141$ that are more favorable than other DJ-1 superfamily members (approximately $\phi = 75$, $\psi = -110$). This difference is due primarily to subtle structural changes at residue 120 (ICH numbering) that favor different backbone torsion angles due to van der Waals contact with the backbone carbonyl oxygen of residue 101.

Similar to other DJ-1 superfamily proteins, ICH requires Cys101 for enzymatic activity (9). The active site environment of Cys101 is dominated by hydrogen bonding to residues Asp17 and Thr102 (Fig. 3A). In contrast to human DJ-1, where the cysteine residue is followed by an alanine, the bulkier side chain of Thr102 in ICH occludes the “amide pocket” region comprising the amide nitrogen atoms of Gly71 and Thr102 (Fig. 3B). In human DJ-1, the amide pocket stabilizes one of the two oxygen atoms in the cysteine-sulfinic acid form of Cys106 (13). In ICH, this pocket is distorted by steric conflicts with the long, discretely disordered side chain of Arg214 present in ICH but not in DJ-1 (Fig. 3B). As a consequence of the occlusion and distortion of the amide pocket and the altered hydrogen bonding environment created by the shorter side chain of Asp17, Cys101 in ICH cannot readily oxidize to form cysteine-sulfinic acid in a way that is structurally analogous to the well studied case of human DJ-1. This is advantageous for ICH function because Cys101 is very likely to function as the key active site residue in ICH only in its reduced form.

Despite having a diminished capacity for oxidation to cysteine-sulfinic acid, Cys101 in crystalline wild-type ICH has a feature in the $2mF_o - DF_c$ electron density map that is consis-
Crystal Structure of Isocyanide Hydratase

The active site region of ICH and comparison with human DJ-1. A, the active site of ICH, centered on the conserved and catalytically essential cysteine residue. 2mFo−DFe, electron density contoured at 1.5σ is shown in blue; 2mFo−DFe, electron density contoured at 5.5σ is shown in purple, and mFo−DFe, difference electron density contoured at +4σ is shown in green. Hydrogen bonds are shown as dashed lines with distances given in Å. Cys101 is partially oxidized to the sulfenic acid by x-ray-induced photochemistry involving an ordered water molecule near Cys101. The 2mFo−DFe electron density at 5.5σ (purple) shows that the carboxylic acid of Asp17 is protonated, with bonding density for the carbon- oxygen double bond present for only one of the two oxygen atoms. Bond length analysis using the 1.05 Å resolution diffraction data for wild-type ICH confirms that Asp17 is protonated, B, a divergent eye stereo view of the ICH active site (darker) superimposed on the corresponding region of human DJ-1 (Protein Data Bank code 1P5F) (lighter). Despite broad similarities, the environment around Cys101 in ICH is altered and cannot form the stabilizing hydrogen bonds (dashed lines) that favor functionally important cysteine oxidation in human DJ-1. Particularly important are the structural changes at Asp17 and Gly98 that modulate the reactivity of Cys101 and favor enzymatic activity in ICH.

The thiol sulfur atom of reduced Cys101 is within 3.4 Å of Asp17 in the active site. Asp17 is noteworthy because in all previously characterized members of the DJ-1 superfamily, this residue is a functionally important glutamic acid. Inspection of the 2mFo−DFe electron density for Asp17 (Fig. 3A) shows that one carbon-oxygen bond has shared electron density for a double bond between the two atoms, whereas the other carbon-oxygen single bond lacks this electron density between the atoms, suggesting that the Asp17 carboxylic acid side chain is protonated. This is supported by atomic resolution bond length analysis of Asp17 (see “Experimental Procedures”), which gives carboxylic acid oxygen–carbon bond lengths of 1.198 ± 0.014 and 1.323 ± 0.014 Å (averages of the two molecules in the ASU). These bond lengths are consistent with protonation of the carboxylic acid side chain of Asp17, although we cannot directly observe the associated hydrogen atom in the electron density maps. However, we note that a deprotonated carboxylic acid with unequal carbon-oxygen bond lengths would lack resonance stabilization and thus would be expected to have a pKa value near that of an alcohol (i.e. pKa = 14−16). Therefore, such a species should be protonated at physiological pH.

We propose that the protonated carboxylic acid side chain of Asp17 donates a hydrogen bond to the cysteine thiol(ate), similar to the structurally equivalent glutamic acid in human DJ-1 (23). Due to the shorter length of the aspartic acid side chain, however, a water molecule can be accommodated between the thiol of Cys101 and Asp17, which may play an important functional role in the enzyme’s mechanism, as described below.

Kinetic and Structural Characterization of ICH Active Site Mutations—Previous work has shown that ICH is a promiscuous enzyme that can accept a wide range of organic isocyanides as substrates (8). Consequently, although the native substrate of the enzyme is unknown, commercially available isocyanides can be used to study ICH catalysis. Capitalizing on the broad substrate tolerance of ICH, we developed a spectrophotometric assay for ICH activity that uses NIC as substrate (see “Experimental Procedures”). P. fluorescens ICH is a modest enzyme in these assay conditions, converting NIC to NF with a kcat of 0.216 ± 0.003 s−1 and a Km of 52 ± 2 μM at 25 °C and pH 7.4 (Fig. 4 and Table 2). These catalytic values are differ substantially from those previously measured for P. putida ICH using an HPLC-based assay with cyclohexyl isocyanide as substrate (Km = 16.2 mM, kcat = 16 s−1) (8). These differences are possibly due to minor differences in the enzymes (cloned from P. fluorescens versus P. putida) or the bulky and more conformationally restricted aromatic naphthyl moiety in NIC as compared with cyclohexyl isocyanide. Mutation of Cys101 to serine completely eliminates ICH enzymatic activity (Table 2), supporting previous observations that C101A is an inactive mutant using an HPLC-based assay (9). Therefore, the thiolate of Cys101 is proposed to be a critical nucleophile that initiates the hydration of isocyanides (see “Discussion”). Notably, human DJ-1 has negligible detectable ICH activity in this assay.

Cys101 Mutants and Structural Polymorphism in the ICH Active Site—Despite being similarly inactive enzymes, the crystal structures of C101S and C101A ICH are surprisingly different in the active site region. The C101S mutation is highly conservative, and the only notable change compared with the wild-type enzyme is the stronger electron density for a water molecule between Asp17 and residue 101 (Fig. 5A). Due to its proximity to the putative Cys101 nucleophile, this water mole-
cule is a good candidate for the water molecule that is added to the isocyanide during catalysis. In wild-type ICH, this water molecule is present but subject to photochemically driven oxidation of Cys101 to the sulfenic acid (see above). Consequently, the electron density for this water is more prominent in C101S ICH because it is not reacting with the cysteine thiol during x-ray irradiation.

In contrast to the conservative C101S mutant, the C101A mutation results in correlated displacements of \( \approx 2 \) Å for the nearby residues 151–165 due to the loss of a hydrogen bond between the thiol(ate) of Cys101 and the amide hydrogen of Ile152. Interestingly, Ile152 is also the only Ramachandran plot outlier in wild-type ICH. This leads to substantial changes near the active site and the introduction of two water molecules into the area around C101A that are absent in other ICH structures (Fig. 5B). The structural change accompanying the C101A mutation helps explain the puzzling difference electron density around residues 151–171 that was observed for the wild-type protein (Fig. 5C). This residual electron density is difficult to model but agrees reasonably well with the displacement of this

![Figure 4](image_url)

**FIGURE 4.** Michaelis-Menten plot of ICH kinetics. A, hyperbolic representation of the kinetics of catalysis by wild-type ICH and certain active site point mutants. The background uncatalyzed rate of NF formation is also shown. Mutations at Thr102 have variable impact on ICH catalysis, with the T102S mutant improving catalytic power, whereas the T102V mutant results in substrate inhibition of the enzyme. All data were measured in triplicate, and the average values are shown as symbols with associated S.D. values as error bars. B, a double reciprocal (Lineweaver-Burk) plot of the data in A. Data were plotted and fit using Prism (GraphPad Software, La Jolla, CA).

**TABLE 2**

<table>
<thead>
<tr>
<th>Protein</th>
<th>( k_{cat} ) (s(^{-1}))</th>
<th>( K_m ) (µM)</th>
<th>( k_{cat}/K_m ) (s(^{-1}) M(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>0.216 ± 0.003</td>
<td>52.2 ± 2</td>
<td>4154</td>
</tr>
<tr>
<td>C101S</td>
<td>ND (^a)</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>C101A</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>T102S</td>
<td>0.234 ± 0.003</td>
<td>30.7 ± 1</td>
<td>7622</td>
</tr>
<tr>
<td>T102V</td>
<td>0.092 ± 0.010</td>
<td>20.3 ± 5</td>
<td>254 ± 61</td>
</tr>
<tr>
<td>D17E</td>
<td>neg. (^c)</td>
<td>neg.</td>
<td>neg.</td>
</tr>
<tr>
<td>D17N</td>
<td>neg.</td>
<td>neg.</td>
<td>neg.</td>
</tr>
<tr>
<td>D17V</td>
<td>neg.</td>
<td>neg.</td>
<td>neg.</td>
</tr>
</tbody>
</table>

\(^a\) Reported only for T102V, which exhibits substrate inhibition.

\(^b\) ND, no detectable activity.

\(^c\) neg., negligible activity. The activity of these mutants was reproducibly higher than background but could not be reliably quantified using this assay.
helix in the C101A ICH structure (Fig. 5C). In some places, particularly Thr\textsuperscript{153}, the difference electron density suggests even larger displacements than those observed in C101A ICH. Therefore, the entire stretch of residues from 151 to 171 samples at least two conformations in the wild type protein. This structural polymorphism suggests that, by eliminating the hydrogen bond with the amide of Ile\textsuperscript{152}, the C101A mutation alters the protein energy landscape so as to strongly favor the “shifted” conformation that is less populated but still sampled to some extent in wild-type ICH.

The Protonated Asp\textsuperscript{17} Residue Is Critical for ICH Catalysis—Because the likely protonated side chain of Asp\textsuperscript{17} hydrogen bonds to a bound water molecule in the wild-type protein (Fig. 3A), this residue was mutated to structurally similar amino acids that retained or eliminated the hydrogen bonding ability of Asp\textsuperscript{17}. Site-directed mutation of Asp\textsuperscript{17} to asparagine (D17N), valine (D17V), and glutamic acid (D17E) all have a profoundly negative impact on the enzyme (Table 2 and Fig. 4). The observation that all of these mutations are similarly detrimental to ICH activity suggests that hydrogen bond donation by Asp\textsuperscript{17} is critical to ICH function. Although D17V and D17N can be crystallized, crystal structures that refined to \( R_{free} \) values below 30% could not be obtained for these proteins due to extensive disorder. However, the D17E mutant forms large crystals that diffract well. The 1.05 Å resolution crystal structure of D17E ICH shows that the Glu\textsuperscript{17} side chain occludes the water binding site near residue 17 and causes a change in the conformation of Cys\textsuperscript{101} (Fig. 6A). The loss of enzymatic activity for D17E may be due either to displacement of Cys\textsuperscript{101}, to the loss of a direct hydrogen bond between the carboxylic acid and the thiol(ate), or to the loss of the bound water that resides between residue 17 and Cys\textsuperscript{101}. Although it is difficult to determine which change results in loss of enzymatic activity, we note that Cys\textsuperscript{101} samples a similarly displaced conformation in the catalytically active T102S mutation (see below), suggesting that this is not responsible for the loss of enzymatic activity. Instead, we propose that it is the loss of the water molecule near Asp\textsuperscript{17}, which is a candidate for the added water in isocyanide hydration, that is the more likely contributor to the poor activity of D17E ICH. More speculatively, this may also be true for D17V and D17N ICH.

Thr\textsuperscript{102} Is Important but Not Essential for ICH Catalysis—Thr\textsuperscript{102} was previously proposed to be a catalytically essential residue based on the inactivity of the T102A mutation (9). In this study, more conservative mutations at Thr\textsuperscript{102} have a varied impact on ICH activity, and this residue is not absolutely required for ICH catalysis. The T102V mutation, which is incapable of hydrogen bonding, results in the appearance of substrate inhibition with significant residual ICH activity (Fig. 4 and Table 2). In contrast, the more conservative T102S mutation increases enzyme activity to 0.234 ± 0.003 \( s^{-1} \), \( K_m = 31 ± 1 \) \( \mu M \). The crystal structure of T102S ICH shows unexpected disorder in the active site, with the neighboring Ser\textsuperscript{102} and Cys\textsuperscript{101} side chains exhibiting spatially correlated alternate side chain conformations (Fig. 6B). The disorder at Cys\textsuperscript{101} results in a change in the conformationally plastic region comprising residues 151–171, similar to that observed for C101A ICH (Fig. 5B). Remarkably, this disorder at the putative nucleophilic Cys\textsuperscript{101} residue appears to have no negative effect on ICH activity, which is slightly enhanced in this mutant enzyme. Otherwise, the T102S mutant is structurally similar to the wild-type protein; the two rotameric states of Ser\textsuperscript{102} closely correspond to the native Thr\textsuperscript{102} side chain, and the nearby water molecule is conserved (Fig. 6B). Thr\textsuperscript{102} appears, from this study, to play a significant role in substrate binding but is not an essential residue for ICH catalysis. Thr\textsuperscript{102} may also indirectly contribute to ICH catalysis by influencing the pK_a value of the Cys\textsuperscript{101} thiol through hydrogen bonding, although testing this will require additional study.

Arg\textsuperscript{214} Facilitates Anion Binding near the Active Site—Attempts to crystallize the catalytically impaired C101S and D17V mutants of ICH bound to NIC or NF were unsuccessful. Although there is currently no direct structural information about how ICH interacts with its substrate, the electron density from the five structures determined in this study suggests that the catalytic side chain of Arg\textsuperscript{214} preferentially interacts with anions in the various crystallization conditions used (Fig. 7). In wild-type ICH, this area is populated by bound ethylene glycol from the cryoprotection solution, which suggests an affinity for neutral polar molecules as well. The significance of these adventitious interactions is unclear, although the persistent accumulation of anionic molecules from different crystallization conditions in the ICH active site provides circumstantial support to the hypothesis that the native substrate(s) for ICH possesses an anionic moiety.
nucleophilic attack of the resulting hydroxide to resolve the thioimidate (Fig. 8C). Based on our analysis of crystal structures of ICH at pH values up to 9.3, we propose that Asp$^{17}$ is constitutively protonated, although we cannot rule out a role for this residue as a general acid/general base. We note that the bond lengths of Asp$^{17}$ are consistent with protonation of the carboxylic acid side chain; however, we cannot directly observe the associated proton using x-ray diffraction. NMR experiments may provide an alternative way to evaluate the protonation state and pK$_a$ value of Asp$^{17}$. A correlated proton transfer event in Fig. 8D gives the N-formamide, followed by release of the product and entrance of another water molecule that restores the enzyme to its resting state in Fig. 8A.

ICH was originally isolated from bacteria that were acclimated to grow on media containing 0.02% cyclohexyl isocyanide, which is toxic to most microorganisms (8). Therefore, this enzyme is capable of detoxifying isocyanides in vivo. However, it is unclear if ICH activity is the primary biological function of these proteins. Natural isocyanides exist (4), and thus there is potential evolutionary value to this activity, but it is also possible that this protein’s ICH activity is robust enough to be subject to positive selection even if this activity is adventitious. Circumstantial evidence supporting a primary role for ICH activity is that homologues of this protein exist in many pseudomonads but appear to be rare or absent in higher eukaryotes. This is significant, because pseudomonad bacteria (including genera Pseudomonas, Burkholderia, and Ralstonia) are known for their remarkable catabolic malleability, including the ability to degrade xenobiotic compounds, such as trinitrotoluene (55), nylon oligomers (56), and polychlorinated biphenyls (57). Therefore, it would not be surprising for these bacteria to either natively possess the ability to degrade isocyanides or to repurpose existing enzymes to acquire this activity under positive selective pressure. However, a BLAST search identifies many putative ICH homologues that retain all of the catalytically important residues and are also fused to a helix-turn-helix AraC-like DNA binding domain (33). This indicates a role for these proteins in transcription that is not obviously connected to isocyanide degradation. Therefore, it is possible that the function of this protein and its close homologues is significantly more complex than currently appreciated.

The combined structural and enzymological data in this study allow a tentative reaction mechanism to be proposed for ICH (Fig. 8). In this mechanism, ICH uses a catalytic Cys$^{101}$ nucleophile to attack organic isocyanides at the carbon atom (Fig. 8A). Because the carbendoxon resonance form of the isocyanide group is the more electrophilic, it is used to illustrate ICH catalysis. We note, however, that the linear structure of most isocyanides indicates that the carbendoxon resonance form is a minor contributor (1–3), and this may contribute to the relatively slow rate of ICH catalysis. A thioimidate intermediate is produced by the attack of Cys$^{101}$ on the isocyanide carbon atom and proton abstraction from the nearby ordered water (Fig. 8B). This is consistent with our results, but there are currently no data that establish the existence of the thioimidate. The formation of a thioimidate intermediate was first proposed in previous study allow a tentative reaction mechanism to be proposed for ICH. The proposed ICH mechanism has some similarities with the cysteine protease mechanism; however, the Pfpl/DJ-1 group of proteases contain a standard catalytic triad with an active site configuration and protein oligomerization state different from those found in ICH (28). We find that the ICH active site is also different from DJ-1 and that these differences disfavor facile cysteine oxidation in ICH that would inhibit its activity. In contrast, the environment of the conserved cysteine residue in human DJ-1 strongly favors the formation of a cysteine-sulfenic acid, which allows DJ-1 to respond to perturbations in cellular redox homeostasis (13, 24, 25). Therefore, ICH represents a
distinct group of DJ-1 superfamily proteins whose unusual activity is an evolutionary consequence of changes in the environment of a conserved, reactive cysteine residue. More generally, the DJ-1 superfamily exemplifies the functional versatility of cysteine in the context of a conserved protein structural architecture. With further study, the DJ-1 superfamily may provide a showcase of the full range of cysteine chemistry.

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