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

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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. Although ICH is similar to human DJ-1 (1.6 Å Cα root mean square deviation), structural differences in the vicinity of Cys101 disfavor the facile oxidation of this residue that is functionally important in human DJ-1 but would be detrimental to ICH activity. The ICH active site region also exhibits surprising conformational plasticity and samples two distinct conformations in the crystal. ICH represents a previously uncharacterized clade of the DJ-1 superfamily that possesses a novel enzymatic activity, demonstrating that the DJ-1 core fold can evolve diverse functions by subtle modulation of the environment of a conserved, reactive cysteine residue.

Isocyanides (also called isonitriles) are a class of organic compounds that contain a triply bonded carbon-nitrogen functional group (R-N≡C). Isocyanides differ from nitriles in that the nitrogen atom is bonded to the organic moiety in the isocyanides. In one resonance structure, an isocyanide has a formal positive charge on the nitrogen and a formal negative charge on the carbon atom. In another resonance form, the carbon atom has substantial carbenoid character and can act as an electrophile in its singlet state (Fig. 1A). Structural and spectroscopic analyses of methyl isocyanide indicate that the linear triple-bonded species is the dominant resonance structure (1, 2), although the nature of the R group substituent may influence the relative contribution of these two resonance forms (3). As a consequence of these resonance forms, isocyanides are versatile organic compounds that possess both nucleophilic and electrophilic character at the carbon atom (3). Isocyanides are notoriously pungent and typically toxic, and several biologically produced isocyanides with broad antimicrobial activities have been characterized (4), including brassicicolin A (5), xanthochelin (4), welwitindolinone (6), and darulcin A and B (7).

Despite the natural occurrence of several cytotoxic isocyanide compounds, only one enzyme has been characterized to date that specifically degrades organic isocyanides. This enzyme, originally named isonitrile hydratase (EC 4.2.1.103), was isolated from Pseudomonas putida strain N19-2 in a screen of soil-dwelling bacteria that could survive acclimatization to medium containing 0.02% (v/v) (~1.6 mM) cyclohexyl isocyanide (8). The enzyme has subsequently been renamed cyclohexylisocyanide hydratase (ICH)5 and is a 48-kDa homodimeric protein that catalyzes the addition of water to the isocyanano group of various organic isocyanides to yield the corresponding N-formamide (Fig. 1B). Previous enzymological studies of ICH have shown that catalysis requires a conserved cysteine residue (Cys101) in the active site, the enzyme is highly selective for 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

5 The abbreviations used are: ICH, isocyanide hydratase; ADP, atomic displacement parameter; ASU, asymmetric unit; CHES, N-cyclohexyl-2-aminoethane sulfonic acid; ESU, estimated standard uncertainty; NF, N-(2-naphthyl)formamide; NIC, 2-naphthyl isocyanide; r.m.s., root mean square.
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 pK$_a$ 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 pK$_a$ 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 peptidases 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 (Glu$^{18}$ 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 (Cys$^{101}$) makes key hydrogen bonds with a protonated aspartic acid (Asp$^{17}$) and a nearby ordered water molecule that are required for significant ICH activity. Structural changes in the environment of Cys$^{101}$ disfavor the formation of cysteine-sulfinate (Cys-SO$_2$)$^-$ that is observed in human DJ-1, although Cys$^{101}$ 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 Cys$^{101}$, 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’ Ndel restriction site and a 3’ Xhol restriction site. The ICH gene was cloned between the Ndel and Xhol 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 three vector-derived amino acids at the N terminus (GSH), resulting in a 231-amino acid protein with 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 (GE Healthcare). The final protein ran as a single band of 28 kDa on overloaded Coomassie Blue-stained SDS-PAGE. Purified ICH was concentrated to 20 mg/ml using a centrifugal concentrator (Amicon, Billerica, MA), supplemented with 2 mM DTT, and stored at −80 °C.

**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 (GE Healthcare). The final protein ran as a single band of 28 kDa on overloaded Coomassie Blue-stained SDS-PAGE. Purified ICH was concentrated to 20 mg/ml using a centrifugal concentrator (Amicon, Billerica, MA), supplemented with 2 mM DTT, and stored at −80 °C.

**Crystal Growth and Data Collection—**All crystals of ICH were grown at room temperature using the hanging drop vapor diffusion method. For wild-type, C101S, and C101A ICH, drops containing 2 μl of ICH at 20 mg/ml and 2 μl of reservoir solution (24–26% PEG 3350, 200–250 mM magnesium chloride, 100 mM CHES, pH 9.3, or 100 mM Tris-HCl, pH 8.6) were equilibrated against 500 μl of reservoir solution. The choice of buffer is significant because inspection of the final electron density maps shows that ICH crystals grown from CHES-buffered solutions contain a molecule of CHES bound in the active site (C101S ICH in this study). In contrast, crystals grown from Tris-buffered solutions (such as wild-type and C101A ICH) have similar morphology and diffraction properties but no bound buffer in the active site. Plate-shaped crystals in space group P2₁, measuring ~50 × 300 × 300 μm formed in 1–3 days. D17E and T102S ICH formed prismatic rod-shaped crystals in space group I2₁2₁2₁ with approximate dimensions 200 × 200 × 700 μm. For D17E ICH, 2 μl of 24 mg/ml protein was mixed with 2 μl of reservoir solution (12% PEG 4000, 240 mM ammonium acetate, 100 mM sodium acetate, pH 4.6). For T102S ICH, 2 μl of 17 mg/ml protein was mixed with 2 μl of reservoir solution (19–22% PEG 4000, 140–160 mM sodium citrate, pH 5.6). In both cases, crystals appeared after 2–3 days at room temperature. All crystals were cryoprotected by passage through six solutions of incrementally increasing amounts of ethylene glycol to a final concentration of 30% (v/v) in the reservoir solution. The cryoprotected crystal was rapidly cooled by immersion into liquid nitrogen.

For T102S ICH, x-ray diffraction data were collected at the University of Nebraska Macromolecular Structural Core Facility from a single crystal maintained at 110 K using a MicroMAX-007 rotating copper anode x-ray generator (Rigaku/MSC, The Woodlands, TX) operating at 40 kV and 20 mA, Osmic Blue confocal optics, and a Raxis IV++ image plate detector. For crystals of wild-type, C101S, C101A, and D17E ICH, diffraction data were collected from single crystals at 110 K at BioCARS beamline 14-BMC at the Advanced Photon Source (Argonne, IL) using 0.9-Å incident radiation and an ADSC Q315 CCD detector. In situ annealing (35) of all crystals was performed by blocking the cold nitrogen stream for 7 s and typically resulted in lowered mosaicity and improved data scaling statistics. Due to the high resolution diffraction of crystals of wild-type, C101S, C101A, and D17E ICH (dmin ~ 1.0 Å), separate high and low resolution passes were used to record the total dynamic range of the data. All data were indexed, scaled, and merged using HKL2000 (36) with final data statistics in Table 1.

**Structure Solution and Refinement—**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 SHELX97 (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, Oy1 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-liberation-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

|                  | Wild type | C101A | C101S | T102S | D17E |
|------------------|-----------|-------|-------|-------|------|
| Data collection  |           |       |       |       |      |
| X-ray source     | APS 14BMC | APS 14BMC | APS 14BMC | Rotating Cu anode | APS 14BMC |
| X-ray wavelength (Å) | 0.9 | 0.9 | 0.9 | 1.54 | 0.9 |
| Space group      | P2₁       | P2₁   | P2₁   | I₂,2,₁,₁ | I₂,2,₁,₁ |
| Cell dimensions a, b, c (Å) | 56.58, 56.47, 68.23 | 56.66, 40.39, 83.83 | 56.31, 56.40, 68.30 | 38.60, 102.72, 121.11 | 38.70, 102.57, 121.10 |
| β (degrees)      | 112.49    | 104.52 | 112.416 | 112.416 | 112.416 |
| Resolution (Å)   | 37-1.05   | 20-1.03 | 37-1.00 | 37-1.00 | 37-1.00 |
| Rmerge (%)       | 0.07 (0.70) | 0.06 (0.57) | 0.05 (0.56) | 0.09 (0.56) | 0.08 (0.87) |
| Completeness (%) | 97.1 (94.2) | 99.0 (94.9) | 98.4 (96.4) | 99.5 (97.2) | 99.2 (98.0) |
| Redundancy (%)   | 6.0 (4.6) | 4.0 (3.0) | 3.8 (3.4) | 7.4 (5.9) | 8.6 (5.5) |

Refinement

|                  | Wild type | C101A | C101S | T102S | D17E |
|------------------|-----------|-------|-------|-------|------|
| PDB code         | 3NON      | 3NOO  | 3NOQ  | 3NOR  | 3NOV |
| Program          | SHELX97   | SHELX97 | SHELX97 | Refmac5²  | SHELX97 |
| Resolution (Å)   | 37-1.05   | 20-1.03 | 37-1.00 | 78-1.90 | 37-1.05 |
| Rmerge (%)       | 13.9      | 12.9   | 12.4   | 15.5   | 14.3 |
| Rfree (%)        | 16.9      | 16.6   | 14.6   | 20.2   | 17.4 |
| Rwork (%)        | 14.0      | 13.0   | 12.5   | 15.8   | 14.2 |
| No. of protein residues | 456 | 455 | 455 | 231 | 230 |
| No. of water atoms | 496 | 444 | 445 | 240 | 330 |
| Rmerge (Å²)      | 12.4      | 15.2   | 12.2   | 12.0   | 14.4 |
| Rfree (Å²)       | 22.4      | 24.7   | 23.4   | 20.3   | 31.2 |
| Bond lengths (Å) | 0.006     | 0.007  | 0.007  | 0.010  | 0.007 |
| Bond angles (Å)  | 0.020     | 0.021  | 0.021  | 0.021  | 0.021 |
| Bond angles (degrees) | 1.1 |       |       |       |      |

* Values in parentheses are for highest resolution shell.
* Rmerge is calculated according to Equation 1,

\[
R_{\text{merge}} = \sum_{hkl} \frac{\| \sum_i F_{hkl} - \langle F_{hkl} \rangle \|}{\sum_i F_{hkl}}
\]  

where \( i \) is the \( i \)th observation of a reflection with index \( h, k, l \), and angle brackets indicate the average over all \( i \) observations.

* Rwork is calculated according to Equation 2,

\[
R_{\text{work}} = \frac{\sum_{hkl} F_{hkl} - F_{\text{calc}}}{\sum_{hkl} F_{hkl}}
\]  

where \( F_{\text{calc}} \) is the calculated structure factor amplitude with index \( h, k, l \), and \( F_{hkl} \) is the observed structure factor amplitude with index \( h, k, l \).

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 POVscopt+ (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 LIV-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, \( N-(2\text{-naphthyl}) \) 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 \( \epsilon_{251} = 39,330 \text{ M}^{-1} \text{ cm}^{-1} \) 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 μM 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 $K_m$ and $V_{max}$ values using Prism (GraphPad Software, San Diego, CA). Reported errors in $k_{cat}$, $K_m$, and $V_{max}$ 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–I) and 10 β-stands (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 (I) 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 αI 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 Å² 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 P2₁ 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 Å² versus 2700 Å²). 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-

**FIGURE 2. Structure of P. fluorescens ICH and comparison with human DJ-1.** A, the ICH protomer is represented as a ribbon diagram, with lettered α-helices colored yellow and numbered β-strands colored blue. The long C-terminal helix J is specific to ICH and its close homologues. B, a ribbon diagram of the ICH homodimer viewed down the molecular 2-fold axis, with one protomer colored green and the other colored blue. ICH crystallizes as a dimer in the ASU. The conserved and reactive cysteine residue (Cys101) is located at the bottom of a solvent-exposed pocket in the ICH dimer that is partially occluded by helix J. Primes are used to distinguish between the residues belonging to the two protomers in the dimer. C, anisotropic ADPs for the Cα atoms of the ICH dimer are shown as 90% thermal ellipsoids in a divergent eye stereo view. The largest displacements are colored red (20 Å²), and the smallest are colored blue (1 Å²). Note the elevated mobility of helix J and the asymmetry in the anisotropic ADPs in the two molecules of the dimer, reflecting the fact that the two molecules in the ASU are dynamically distinct. D, ICH (green and blue ribbons) and human DJ-1 (orange and gold ribbons) are superimposed, showing the high degree of structural similarity. The presence of the long C-terminal helix J in ICH is the most prominent structural difference between these two proteins.
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. The electron density contoured at 1.5σ is shown in blue; 2mFo − DFc, electron density contoured at 5.5σ, is shown in purple, and mFo − DFc, difference electron density contoured at 4σ, is shown in green. Hydrogen bonds are shown as dashed lines with distances given in Å. (B) The active site of ICH, centered on the conserved and catalytically essential cysteine residue. The electron density contoured at 1.5σ, 2mFo − DFc, electron density contoured at 5.5σ, and mFo − DFc, difference electron density contoured at 4σ, are shown in color. Hydrogen bonds are shown as dashed lines with distances given in Å. The thiol sulfur atom of reduced Cys101 is within 3.4 Å of the oxygen atom of Cys101-SO−2, indicating that Cys101 is partially oxidized. In the 1.00 Å resolution crystal structure of ICH 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.

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 value 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 different 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 ~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

TABLE 2

| Protein | $k_{cat}$ | $K_m$ | $K_i$ | $k_{cat}/K_m$ |
|---------|---------|-------|-------|-------------|
| Wild type | 0.216 ± 0.003 | 52.2 ± 2 | 52.2 ± 2 | 4154 |
| C101S | ND | ND | ND | ND |
| C101A | ND | ND | ND | ND |
| T102S | 0.234 ± 0.003 | 30.7 ± 1 | 30.7 ± 1 | 7622 |
| T102V | 0.092 ± 0.010 | 20.3 ± 5 | 20.3 ± 5 | 4532 |
| D17E | neg | neg | neg | neg |
| D17N | neg | neg | neg | neg |
| D17V | neg | neg | neg | neg |

* Reported only for T102V, which exhibits substrate inhibition.
* ND, no detectable activity.
* neg, negligible activity. The activity of these mutants was reproducibly higher than background but could not be reliably quantified using this assay.

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
helix in the C101A ICH structure (Fig. 5C). In some places, particularly Thr153, 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 Ile152, 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 Asp17 Residue Is Critical for ICH Catalysis—Because the likely protonated side chain of Asp17 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 Asp17. Site-directed mutation of Asp17 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 Asp17 is critical to ICH function. Although D17V and D17N can be crystallized, crystal structures that refined to Rfree 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 Glu17 side chain occludes the water binding site near residue 17 and causes a change in the conformation of Cys101 (Fig. 6A). The loss of enzymatic activity for D17E may be due either to displacement of Cys101, 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 Cys101. Although it is difficult to determine which change results in loss of enzymatic activity, we note that Cys101 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 Asp17, 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.

Thr102 Is Important but Not Essential for ICH Catalysis—Thr102 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 Thr102 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 $k_{cat} = 0.234 \pm 0.003 \text{s}^{-1}$, $K_m = 31 \pm 1 \mu M$. The crystal structure of T102S ICH shows unexpected disorder in the active site, with the neighboring Ser102 and Cys101 side chains exhibiting spatially correlated alternate side chain conformations (Fig. 6B). The disorder at Cys101 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 Cys101 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 Ser102 closely correspond to the native Thr102 side chain, and the nearby water molecule is conserved (Fig. 6B). The T102V mutant, from this study, to play a significant role in substrate binding but is not an essential residue for ICH catalysis. Thr102 may also indirectly contribute to ICH catalysis by influencing the pK_a value of the Cys101 thiol through hydrogen bonding, although testing this will require additional study.

Arg214 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 cationic side chain of Arg214 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 carbendoxone resonance form of the isocyanidic 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 carbendoxone 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 work that initially characterized ICH (9), and the strict requirement for Cys$^{101}$ supports a mechanism that involves nucleophilic attack by a thiolate to give this intermediate. The water molecule observed between Asp$^{17}$ and Cys$^{101}$ is proposed to play a key role in hydrating the isocyanide. In this mechanism, Asp$^{17}$ activates water for proton donation and then for

**DISCUSSION**

The structure of ICH provides the first detailed view of a new class of DJ-1 superfamily enzymes. ICH, like other members of the DJ-1 superfamily, requires a conserved cysteine residue for function. Other characterized members of the DJ-1 superfamily use this cysteine residue either for redox sensing (13, 24) or, in the peptidases, as a catalytic nucleophile (28, 30, 32, 52). 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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