Catalytic Mechanism of Nitrile Hydratase Proposed by Time-resolved X-ray Crystallography Using a Novel Substrate, tert-Butylisonitrile

Nitrile hydratases (NHases) catalyze the hydration of nitriles to the corresponding amides and are used as catalysts in the production of acrylamide, making them one of the most important industrial enzymes (1, 2). NHases contain a non-heme Fe(3+) or non-corrin Co(3+) catalytic center. Iron-type NHases show unique photoactivity; the enzyme is inactivated by nitrosylation in the dark and immediately reactivated by photo-induced denitrosylation (3–5). The protein structure is highly conserved among all known NHases (6–9) as well as a related enzyme, thiocyanate hydrolase (10). The metal site is also conserved, with a distorted octahedral geometry. All ligand residues are involved in a strictly conserved motif of the α subunit, Cys1-Xaa-Leu-Cys2-Ser-Cys3, where two amide nitrogens of Ser and Cys3 and three Cys sulfurs are coordinated to the metal (6). Cys2 and Cys3 are post-translationally modified to cysteine-sulfenic acid and cysteine-sulfenic acid, respectively (7), which probably take deprotonated forms at the metal site (11). The sixth ligand site is occupied by a solvent molecule (8) or by a NO molecule in nitrosylated iron-type NHase (7).

Several reaction mechanisms have been proposed based on the protein structures (1, 6). First, nitriles directly bind to the metal to facilitate the nucleophilic attack of a water molecule on the nitrile carbon. In the other mechanisms, a water molecule activated by the metal directly or indirectly attacks nitriles trapped near the metal. In all cases, the metal is suspected to function as a Lewis acid. By reconstituting iron-type NHase from recombinant unmodified subunits, we demonstrated that the post-translational modifications of its cysteine ligands are essential for its catalytic activity (12). We also found that specific oxidation of the cysteine sulfenic acid ligand to cysteine sulfenic acid resulted in irreversible inactivation (13). Kovacs and co-workers (14) studied the ligand exchange reaction in the low spin Co(3+)–containing NHase model complexes and concluded that the trans-thiolate sulfur played an important role in promoting the ligand exchange at the sixth site. Later, by using a sulfenate-ligated iron complex, they showed that protonation/deprotonation states of the sulfenate oxygen were modulated by the unmodified Cys thiolate ligand (15).

The atomic coordinates and structure factors (code 2ZPB, 2ZPE, 2ZPF, 2ZPG, and 2ZPI) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).}

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†The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1 and S2.

‡The abbreviations used are: NHase, nitrile hydratase; RNHase, nitrile hydratase from R. erythropolis N771; tBuNC, tert-butylicisonitrile; tBuNH2, tert-butylicamine; ATR-FTIR, attenuated total reflectance-Fourier transform infrared.
### EXPERIMENTAL PROCEDURES

**Materials**—Nitrile hydratase from *R. erythropolis* N771 (ReNHase) was inactivated by endogenous NO molecules in living cells in the dark (4, 23). ReNHase was purified in the nitrosylated form in the dark as described previously (23). The purified nitrosylated ReNHase was stored in 50 mM Tris-HCl, pH 7.5, at −80 °C in the dark at a concentration of 20 mg/ml. The concentration of the nitrosylated NHase was determined by measuring the absorbance at 280 nm (22). Although the other product derived from the isonitrile carbon was not identified, the kinetic analyses revealed that the $K_m$ for tBuNC was comparable with that for methacrylonitrile, whereas $k_{cat}(1.8 \times 10^{-2} \text{ s}^{-1})$ was $1.8 \times 10^5$ times smaller. In this study, taking advantage of the slow reactivity of tBuNC as well as the photo-reactivity of nitrosylated inactive ReNHase (3, 4), we obtained structural evidence on the reaction mechanism by studying the time course of the tBuNC catalysis with X-ray crystallography. Based on the results, we propose a reaction mechanism in which the sulfenate group of αCys$^{114}$-SO$^-_2$ plays a key role in the catalysis.

**ATR-FTIR Measurements**—The nitrosylated ReNHase (70 mg/ml) in 50 mM sodium phosphate, pH 7.5, was loaded on a silicon ATR prism, and an NHase reaction with tBuNC was photoactivated by white light illumination for 1 min. FTIR spectra with 10-s scans were recorded at 0, 20, 30, and 60 min after illumination. Light-induced difference spectra were calculated by subtracting the dark spectra from each spectrum after illumination. The base-line distortion was corrected by subtracting the corresponding spectra measured in the same manner but without illumination.

For CO detection, 6 µl of hemoglobin (50 mg/ml) in 50 mM Tris-HCl, pH 7.5, was lightly dried on a silicon ATR prism, and 6 µl of the nitrosylated ReNHase sample (70 mg/ml) in Tris-HCl, pH 7.5, was placed beside the hemoglobin. The sample space was sealed with a CaF$_2$ plate, to be supplied to the ReNHase solution as a vapor. The sample was stabilized at room temperature in the dark for 4 h.

FTIR spectra were measured on a Bruker IFS-66/S spectrophotometer equipped with an MCT detector (D313-L). All of the spectra were recorded at 4 cm$^{-1}$ resolution. A single-beam spectrum was recorded for 100 s before illumination, and ten spectra (100 s scans) were successively recorded after 10 s of illumination by continuous white light from a halogen lamp (Hoya-Schott HL150; 60 milliwatt cm$^{-2}$ at the sample).
glycol 8000, 0.10 M Tris-HCl, pH 7.5, 0.30 M MgCl₂) and equilibrated against 0.40 ml of precipitant solution. Crystals with dimensions of approximately 0.4 × 0.3 × 0.3 mm³ grew within a day in the dark at 20 °C. When crystals of the nitrosylated NHase were dissolved in 50 mM Tris-HCl, pH 7.5, the enzyme solution exhibited trace amounts of methacrylonitrile hydratation activity in the dark, but it had a specific activity of 7.3 × 10² units/mg after light-induced denitrosylation (10,000 lx) with a cold light illumination system (LG-PS2; Olympus, Tokyo, Japan) for 15 min.

Preparation of the ReNHase Crystals without or with tBuNC—Crystals of the nitrosylated ReNHase were first vapor-soaked with cryoprotectant solution (30% polyethylene glycol 8000, 0.10 M Tris-HCl, pH 7.5, 0.60 M MgCl₂) for 1 day by being swapped in mother liquor. They were then vapor-soaked for a day with mother liquor solution containing tBuNC at a final concentration of 0.10 M. After being mounted, ReNHases in the crystals were activated by light-induced denitrosylation (10,000 lx) with a cold light illumination system (LG-PS2; Olympus), and the reaction proceeded for 18, 120, 340, and 440 min at 20 °C. At each elapsed time, the reaction was terminated by flash cooling with N₂ gas at 95 K.

### X-ray Data Collections, Structure Determinations, and Refinements

Diffraction data were collected using a Quantum 315 CCD detector (Area Detector Systems Corporation, Poway, CA) at the beamline BL-5A (λ = 1.000 Å) of the Photon Factory (Tsukuba, Japan) at 95 K. Each data set was indexed, merged, and scaled with the HKL2000 program suite (24). The

![Absorbance(a.u.)](image)

**FIGURE 3.** The CO stretching region of the ATR-FTIR spectra of hemoglobin. Hemoglobin was loaded on an Si ATR prism, and NHase in an H₂¹⁶O (A) or H₂¹⁸O (B) buffer and tBuNC were separately placed in a sealed space (Fig. 2). The spectra at 0 (blue), 20 (cyan), 30 (green), and 60 (red) min after photoactivation of NHase were recorded.

### TABLE 1

Data collection and refinement statistics

| Nitrosylated NHase* | 0 min*,a | 18 min* | 120 min* | 340 min* | 440 min* |
|---------------------|----------|---------|----------|----------|----------|
| **Data collection**  |          |         |          |          |          |
| Space group         | C2       | C2      | C2       | C2       | C2       |
| Cell dimensions     |          |         |          |          |          |
| a (Å)               | 114.7    | 114.0   | 114.0    | 114.1    | 113.9    |
| b (Å)               | 60.5     | 60.0    | 60.0     | 60.1     | 60.2     |
| c (Å)               | 81.9     | 81.7    | 81.7     | 81.7     | 81.4     |
| α, β, γ (°)         | 125.0    | 125.1   | 125.1    | 125.1    | 125.1    |
| Wavelength (Å)      | 1.00000  | 1.00000 | 1.00000  | 1.00000  | 1.00000  |
| Resolution (Å)      | 50.0-1.30| 50.0-1.48| 50.0-1.48| 50.0-1.39| 50.0-1.59|
| Resolution of highest resolution shell (Å) | 1.35-1.30 | 1.53-1.48 | 1.53-1.48 | 1.44-1.39 | 1.65-1.59 |
| Rmerge             | 0.051    | 0.036   | 0.038    | 0.038    | 0.036    |
| Rmerge of highest resolution shell | 0.293    | 0.118   | 0.108    | 0.271    | 0.208    |
| I/σ(I)             | 24.4     | 26.8    | 26.1     | 24.1     | 27.9     |
| I/σ(I) of highest resolution shell | 24.4   | 26.8    | 26.1     | 24.1     | 27.9     |
| Completeness (%)    | 98.8     | 98.3    | 97.0     | 98.0     | 97.3     |
| Completeness of highest resolution shell (%) | 97.2     | 93.6    | 93.1     | 90.2     | 85.3     |
| Redundancy          | 3.7      | 2.1     | 2.0      | 1.8      | 1.8      |

| **Refinement**      |          |         |          |          |          |
| Resolution (Å)      | 7.96-1.30| 8.00-1.48| 8.00-1.48| 7.98-1.39| 7.99-1.59|
| No. reflections     | 105,634  | 69,876  | 68,831   | 84,855   | 55,536   |
| Rmerge/Rfree       | 16.9/18.7| 16.8/19.4| 16.8/19.0| 17.7/20.1| 15.8/18.5|
| No. atoms          |          |         |          |          |          |
| Protein             | 3,288    | 3,252   | 3,255    | 3,222    | 3,185    |
| Ligand/ion         | 4        | 5       | 5        | 5        | 12       |
| Water              | 756      | 663     | 643      | 601      | 538      |
| B-factors          |          |         |          |          |          |
| Protein            | 11.7     | 13.4    | 14.1     | 14.5     | 11.2     |
| Ligand/ion         | 18.7     | 16.6    | 20.9     | 29.8     | 18.6     |
| Water              | 28.8     | 28.3    | 29.1     | 20.7     | 27.2     |
| Root mean square deviations |          |         |          |          |          |
| Bond length (Å)    | 0.007    | 0.008   | 0.008    | 0.007    | 0.009    |
| Bond angles (°)    | 1.151    | 1.198   | 1.188    | 1.184    | 1.184    |
| Ramachandran plot  | Most favored (%) | 98.5    | 98.0    | 98.2    | 98.5    |
| Also allowed region (%) | 1.5      | 2.0     | 1.8      | 1.5      | 1.8      |

*a One crystal was used to collect the data of each complex.

*b 0 min* represents nitrosylated NHase soaked with tBuNC.
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RESULTS AND DISCUSSION

Identification of the Product from the Isonitrile Carbon by ATR-FTIR Measurements—To identify all products except for the amine, the reaction was monitored using ATR-FTIR. tBuNC was added as a vapor to nitrosylated NHase, and the enzyme was activated by light-induced denitrosylation. Several prominent peaks, all arising from tert-butylamine (tBuNH₂), were observed in the spectrum at 2950 cm⁻¹, 2870 cm⁻¹, and 2837 cm⁻¹, respectively. These peaks were attenuated by the addition of tBuNC, indicating the consumption of amine.

Time-resolved X-ray Crystallography of the Reaction of RenHase with tBuNC—Crystals of nitrosylated RenHase were soaked with tBuNC, and the reaction was started by light-induced denitrosylation at 25°C. At 18, 120, 340, and 440 min, the sample was collected and flash-cooled at 95 K, at which point the crystal structure was determined. Details of data collection and refinement statistics are summarized in Table 1. Unfortunatley, we could not collect data from the crystals that were incubated longer because those crystals were damaged. The overall structure at each elapsed time was essentially unchanged except for the pocket above the Fe(III) center (Fig. 4). αCys₁₁²-SO₂⁻ (αCSD₁₁₂) and αCys₁₁⁴-SO⁻ (αCSO₁₁₄) modifications were clearly observed in all of the structures determined.

Before soaking with tBuNC, an NO molecule was observed at a distance of 2.1 Å from the Fe(III) center (Fig. 4A). The Fe-N(NO) distance is 0.6 Å longer than observed in the previous structure (Protein Data Bank code 2ahj). In the previous structure, NO was likely to be pushed toward Fe(III) by 1,4-dioxane, the co-precipitant used (7). After soaking with tBuNC, the electron density of tBuNC was clearly observed in the pocket (Fig. 4B) with the tert-butyl group facing the NO molecule coordinated to the Fe(III). Because of the limited space in the hydrophobic pocket, the bulky tert-butyl group must face the iron in its nitrosylated state. In addition to the original conformation (conformer A), Sy of βMet₄₀ took another conformation (conformer B) with occupancies of A:B = 0.25:0.75. Movement of Sy of βMet₄₀ to conformer B is likely due to the occupation of the hydrophobic pocket by tBuNC. We hypothesize that conformer B is less stable because of steric hindrance between Sy and the amide oxygen of βMet₄₀ (Fig. 5).

At 18 min, electron densities of NO and tBuNC, especially that of the isonitrile group, were attenuated (Fig. 4C). Sy of βMet₄₀ remained disordered, but the occupancy of conformer A increased to 0.55. At 120 min, the NO disappeared, and a tBuNC molecule was coordinated to Fe(III) with an Fe-C(-NC) bond length of 2.4 Å (Fig. 5).
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FIGURE 6. Proposed catalytic mechanisms of NHase. A, isonitrile hydrolysis. B, nitrile hydration.

length of 2.1 Å (Fig. 4D). βMet40 took conformer A again. The rotation of the tBuNC molecule could be driven by the recovery of βMet40 to conformer A.

The $F_o - F_e$ electron density at 340 (supplemental Fig. S1) and 440 min (Fig. 4E) were very similar to one another but distinct from those observed at 120 min. In both structures, the $F_o - F_e$ electron density corresponding to the tert-butyl group was moved ~1.0 Å away from the iron, and an extra electron density was observed near the isonitrile carbon as well as the sulfenate oxygen of αCys114. When the products, tBuNH2 and CO, were included in the calculation of the electron density at 440 min, the refined model of tBuNH2 was well fit on the $2F_o - F_e$ electron density, but that of CO was not (supplemental Fig. S2). In addition, two positive electron densities were observed near the CO molecule in the $F_o - F_e$ electron density. Alternatively, we calculated the electron density at 440 min by assuming the presence of only tBuNH2. As shown in Fig. 4F, tBuNH2 was well fit on the $2F_o - F_e$ electron density, and two positive electron densities were observed above the iron ion and near Oδ of the sulfenate group, in the $F_o - F_e$ electron density. We assigned the positive densities as the carbon of the isonitrile and the solvent water molecule (named as H2Oa), respectively (Fig. 4F). All distances of Fe-C(-NC), C(-NC)-N(-NC), C(-NC)-O(H2Oa), N(-NC)-O(H2Oa), and O(H2Oa)-O(-SO) converged at less than 2.2 Å (Table 2). The O(H2Oa)-O(-SO) distance cannot be explained. These atoms may be disordered because the occupancies of H2Oa and Oδ of αCys114-SO− converged on 0.50. Interestingly, a positive difference density was observed below $S_y$ of αCys114-SO− in the $2F_o - F_e$ electron density map after coordination of tBuNC (Fig. 4, D–F). The distance between the density and $S_y$ of αCys114-SO− is 1.4 Å, and the angle Oδ(αCys114-SO−) − $S_y$(αCys114-SO−) − the density was 133°. The positive density may represent an alternative position of Oδ of αCys114-SO−.

Proposed Catalytic Mechanisms of NHase—Based on the results, we propose the following catalytic mechanism: the tBuNC substrate binds the metal directly, and then a water molecule, activated by Oδ of αCys114-SO−, makes a nucleophilic attack on the isonitrile carbon to produce tBuNH2 and CO (Fig. 6A). Considering the similarity between isonitriles and nitriles, nitrile hydration is likely to proceed in a similar manner (Fig. 6B). When a nitrile coordinates to the metal, the nitrile carbon is attacked by a water molecule, activated by Oδ of αCys114-SO−. The low $k_{cat}$ value for isonitrile may be due to limited accessibility of the activated water molecule because of steric hindrance by Oδ of αCys114-SO−. Nitrile coordination to the Fe3+ was suggested by electron spin resonance measurements (30).

Involvement of αCys114-SO− in the catalytic reaction had been suggested by our previous studies using the inhibitor, 2-cyano-2-propyl hydroperoxide (13), specifically oxidizing αCys114-SO− to Cys-SO2−, and the site-directed mutant NHases (31). Yano et al. (32) have extensively studied the N2S2(tBuNC)2-type Co3+ model complexes with different sulfur oxidation states and concluded that sulfur oxidations promoted the Lewis acidity of the Co3+ center and that the sulfinyl oxygen exhibited a nucleophilic character. Theoretical calculation studies have indicated that Oδ of αCys114-SO− could be a catalytic base when nitrile coordination was assumed (19). These antecedent studies support the mechanism of the substrate coordinated to the iron being attacked by water activated by αCys114-SO−. Recently, involvement of the Ser ligand (αSer112, corresponding to αSer113 of RenHase) and of the vicinal Tyr and Trp residues (βTyr68 and βTrp72; corresponding to βTyr72 and βTyr76 of RenHase) in the catalytic mechanism was suggested by temperature- and pH-dependent kinetic studies of the Co-type NHase from Pseudomonas aeruginosa (33). However, the corresponding residues of RenHase were unchanged during our investigations (Fig. 4).

Our findings represent the first structural evidence of reaction intermediates in NHase catalysis. The present results demonstrate a reaction mechanism in which the sulfinyl group of αCys114-SO− plays a key role in the catalysis. Cysteine oxidation has been found to play important roles in various proteins (34). The present work reveals a novel role of cysteine sulfenic acid as a catalytic base.

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