Identification of an Active Site-bound Nitrile Hydratase Intermediate through Single Turnover Stopped-flow Spectroscopy

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Background: No direct evidence exists for the direct coordination of nitrile to the Fe$^{3+}$ active site in nitrile hydratases.

Results: The first Fe$^{3+}$-nitrile intermediate species is reported using stopped-flow spectroscopy.

Conclusion: These data establish that the direct ligation of the nitrile substrate occurs during catalytic turnover.

Significance: Understanding the catalytic mechanism of nitrile hydratases is critical to harness their bioremediation and industrial potential.

Stopped-flow kinetic data were obtained for the iron-type nitrile hydratase from Rhodococcus equi TG328-2 (ReNHase) using methacrylonitrile as the substrate. Multiple turnover experiments suggest a three-step kinetic model that allows for the reversible binding of substrate, the presence of an intermediate, and the formation of product. Microscopic rate constants determined from these data are in good agreement with steady state data confirming that the stopped-flow method was appropriate for the reaction. Single turnover stopped-flow experiments were used to identify catalytic intermediates. These data were globally fit confirming a three-step kinetic model. Independent absorption spectra acquired between 0.005 and 0.5 s of the reaction reveal a significant increase in absorbance at 375, 460, and 550 nm along with the hypsochromic shift of an Fe$^{3+}$-S ligand-to-metal charge transfer band from 700 to 650 nm. The observed UV-visible absorption bands for the Fe$^{3+}$-nitrile intermediate species are similar to low spin Fe$^{3+}$-enzyme and model complexes bound by NO or N$_2$. These data provide spectroscopic evidence for the direct coordination of the nitrile substrate to the nitrile hydratase active site low spin Fe$^{3+}$ center.

Nitrile hydratases (NHases)$^2$ catalyze the hydration of nitriles to their corresponding amides under ambient conditions and physiological pH (Scheme 1) (1). NHases have attracted substantial interest as biocatalysts in preparative organic chemistry and are already used in several industrial applications such as the large scale production of acrylamide (1) and nicotinamide (2). For example, Mitsubishi Rayon Co. has developed a microbial process that produces ~95,000 tons of acrylamide annually using the NHase from Rhodococcus rhodo-

chrous J1 (3). More than 3,500 tons of nicotinamide are produced per year via NHase, with yields of >99% and without formation of troublesome byproducts such as acrylic acid (4). NHases have also been employed as bioremediation agents to clean up nitrile-based pesticides, such as bromoxynil (5). Because of their exquisite reaction specificity, the nitrile-hydrolyzing potential of NHase enzymes is becoming increasingly recognized as a truly new type of “green” chemistry.

NHases contain either an Fe$^{3+}$ ion (“iron-type”) or a Co$^{3+}$ ion (“cobalt-type”) in their active sites (6). X-ray crystal structures of both Co-NHase and Fe-NHase reveal that the M$^{3+}$ ion is coordinated by three cysteines, two amide nitrogens, and a water molecule (7). Two of the active site cysteine residues are post-translationally modified to cysteine sulfenic acid (–SOH) and cysteine sulfenic acid (–SOH), yielding an unusual metal coordination geometry, termed a “claw setting.” These Cys oxidation states are essential for NHase activity (8, 9).

The molecular characterization of both iron-type and cobalt-type NHase enzymes has provided some insight into how molecular structure controls enzyme function. Based on these data, and several elegant studies on active site NHase model complexes, four possible reaction mechanisms have been proposed (6, 10). In each, imidate is produced as a reaction intermediate, which then isomerizes to the corresponding amide. The most accepted catalytic mechanism for NHases involves the binding of the nitrogen of the nitrile substrate to the active site metal center; however, no direct evidence has been reported supporting such a mechanism (6, 11). Herein we report the detection of a NHase reaction intermediate, using methacrylonitrile as the substrate that is observed using stopped-flow spectroscopy. These data provide the first direct spectroscopic evidence for nitrile binding to the Fe$^{3+}$ active site in the nitrile hydratase from Rhodococcus equi TG328-2 (ReNHase).

EXPERIMENTAL PROCEDURES

All reagents were purchased commercially and were the highest purity available.

Protein Expression—The ReNHase TG328-2 plasmid was kindly provided by Professor Uwe Bornscheuer (12). The sub-
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SCHEME 1. The hydration of a nitrile to its corresponding amide by nitrile hydratase.

R–C≡N + H₂O → NHase → R–C(O)NH₂

Multiple turnover stopped-flow experiments were initially run at 5 °C using 25 mM methacrylonitrile at pH 7.0 (Fig. 1) to investigate pre-steady state behavior. A noticeable lag is present in the early portion of the reaction, indicating at least two reaction steps. Independent absorption spectra were acquired at 242 nm over the time frame of 0.005–10 s. These data were fit to a double exponential equation providing 〈k₁obs₁ and k₂obs₂〉 of the reaction. k₁obs₁ was designated the fast phase, and k₂obs₂ was designated as the slow phase. Based on these data, a minimal three-step kinetic model is proposed that allows for reversible substrate binding, the presence of an intermediate, and the formation of product (Scheme 2).

The concentration dependence of the reaction rate on methacrylonitrile was examined by plotting the fast and slow phases (k₁obs₁ and k₂obs₂) of the reaction against the substrate concentration (Fig. 2, A and B) to extract the microscopic rate constants of the reaction. The concentration dependence of the fast phase was fit to a linear equation where the slope is the second-order rate constant and the y-intercept is the sum of k₂₁ + k₂ + k₂. This fit provided a k₁ value of 1.0 ± 0.1 mm⁻¹ s⁻¹ and a k₂₁ + k₂ + k₂ value of 12 ± 1 s⁻¹. The nonzero intercept implies that the binding is reversible. The linear fit indicates that binding occurs in a single step (13).

The dependence of k₂obs₂ on substrate was fit to a hyperbolic curve, (k₂obs₂ = kₘₐₓ[S]/(kₕ₂₂ + [S]), where kₘₐₓ is the sum of k₂₁ + k₂ and is equal to the rate at saturated enzyme concentrations. Kₕ₂ is the apparent dissociation constant of an intermediate step following substrate binding, kₘₐₓ was found to be 9 ± 2 s⁻¹, and Kₕ₂ is 1.5 ± 0.8 mm. Subtracting kₘₐₓ(k₂₁ + k₂) from the intercept of the fast phase (k₂₁ + k₂ + k₂) provides k₂₁, which is 3 s⁻¹. The y-intercept of the slow phase provides k₂, which is 1 s⁻¹. Therefore, k₂ is 8 s⁻¹, and Kₕ₄, which is...
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The hyperbolic dependence of the slow phase indicates the presence of a second step following substrate binding, which is independent of substrate concentration.

These data were compared with experimentally determined steady state kinetic data obtained at 5 °C for ReNHase using 0.5–100 mM methacrylonitrile as the substrate at pH 7.0 and 242 nm. Under these conditions, the $V_{\text{max}}$ value is 4.0 ± 0.2 s\(^{-1}\) and the $K_m$ value is 6 ± 1 mM. One assumption under rapid equilibrium, because $k_2$ is greater than $k_{-1}$, is that $K_m$ is greater than $K_d$. Therefore, the steady state $K_m$ value of 6 mM is in good agreement with the $K_d$ value of 3 mM determined from stopped-flow data. However, because the system is not at rapid equilibrium but at the steady state, $K_m = k_2k_3 + k_{-1}k_{-2} + k_{-1}k_3/k_1(k_2 + k_{-2} + k_3)$ for a mechanism with one intermediate before product release (14). That means the experimentally determined $K_m$ value of 6 mM might be different from the $K_d$ value described as $k_{-1}/k_1$. In our case where the values of $k_1$, $k_{-1}$, $k_2$, and $k_{-2}$ were experimentally determined, $K_m$ can only be 0.33 mM (for extremely small $k_3$ values) up to 11 mM (for extremely high $k_3$ values). For this reason, 6 mM is a value for $K_m$ that is compatible with our measurement of the microscopic constants. In fact, values for $k_3$ obtained below in single turnover experiments confirm this, and these values are discussed later.

These data indicate that a three-step reaction mechanism is operative and provide microscopic rate constants. The agreement between theoretical constants calculated from the microscopic rates to steady state data also confirm that stopped-flow experiments using UV detection are valid for the NHase reaction. However, the high concentrations of substrate and subsequent product formation likely obscure potential transient intermediates. To overcome these limitations and to obtain $k_{3\gamma}$, single turnover stopped-flow experiments were performed as only kinetically significant intermediates will be observed directly.

Single turnover stopped-flow experiments are typically not feasible due to the requirement that enzyme concentrations be similar to the $K_m$ value of the substrate. For ReNHase, the $K_m$ value for methacrylonitrile is 190 μM at 25 °C, which is low enough that the enzyme can be kept at a concentration that exceeds the substrate concentration. Additionally, the turnover value determined at the $K_m$ is 5 s\(^{-1}\), placing the reaction well within the limits of the stopped-flow experiment. Therefore, independent absorption spectra were acquired between 350 and 720 nm using 0.33 mM ReNHase and 0.19 mM methacrylonitrile over 0.005–0.5 s of the reaction at 25 °C (Fig. 3). These transient spectra indicate the rapid formation of an Fe$^{3+}$-nitrile species that converts to the resting Fe$^{3+}$ state and product. Independent spectra of enzyme intermediate complexes were extracted after singular value decomposition was applied to the raw data to eliminate noise and to isolate species with significantly different absorption spectra. In total, four species were identified as significantly different, supporting a three-step mechanism. All spectra were then globally fit using the Applied Photophysics Pro-K software to the kinetic model shown in Scheme 2. To verify that Scheme 2 was the best model, simpler and more complicated models were evaluated as well, but a three-step model provided the best global fit. To ensure that a global minimum was reached, the forward rates were varied and then verified by residual analysis, observation of positive fitted spectra, and simulation. The best fit using Scheme 2 as a model provided values for the forward rate constants $k_1$, $k_2$, and $k_3$ of 65 ± 10 mm$^{-1}$s$^{-1}$, 23 ± 3 s$^{-1}$, and 12 ± 4 s$^{-1}$, respectively. The reverse rate constants $k_{-1}$ and $k_{-2}$ were found to be 2.8 ±
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![Graph](image1)

**FIGURE 4.** Concentration profile of two new transient species (red and blue) observed as a function of time under single-turnover assay conditions. The green trace is free enzyme, light blue is substrate, and purple is product. Conditions: 50 mM HEPES, pH 7.0, and 25°C.

![Graph](image2)

**FIGURE 5.** Spectra of the intermediate species (red and blue) generated from singular value decomposition applied to the raw data and the resting enzyme (green) and product complex (purple). Conditions: 50 mM HEPES, pH 7.0, and 25°C.

0.1 and 1.1 ± 0.1 s⁻¹, respectively. Theoretical \( k_{cat} \) and \( K_m \) values were calculated by inserting the microscopic rate constants obtained from single turnover into the following equations derived from the minimal three-step model in Scheme 2.

\[
\begin{align*}
    k_{cat} &= k_2 k_3 / k_2 + k_{-2} + k_3 \\
    K_m &= k_2 k_3 + k_{-1} k_{-2} + k_{-1} k_3 / (k_2 + k_{-2} + k_3)
\end{align*}
\]  

(Eq. 2)

(Eq. 3)

The theoretical \( k_{cat} \) and \( K_m \) values of 8 ± 3 s⁻¹ and 128 ± 50 μM respectivly are in good agreement with steady state values of 5 s⁻¹ and 190 μM, respectively.

These data indicate that the substrate binding step is fast and reversible, corresponding to \( k_1 \), \( k_2 \), is the rate of rearrangement of the enzyme-substrate complex to an enzyme-product complex. Product release is rate-limiting and assigned \( k_3 \). Product release was previously shown to be rate-limiting under steady state conditions for both iron-type and cobalt-type NHase enzymes (15, 16). Concentration profiles for the progress of the reaction confirm a three-step reaction model (Fig. 4) with the observed decrease in free enzyme concentration occurring concomitantly with the formation of an enzyme-substrate complex followed by an enzyme-intermediate complex and the consumption of substrate. The first transient species reaches its maximum concentration at ~0.03 s, after which it begins to disappear, and a second transient species peaks at ~0.1 s.

Singular value decomposition identified four spectrally unique species, with one corresponding to native RenHase enzyme, whereas the second is an Fe³⁺-nitrite intermediate species (Fig. 5). As the reaction proceeds, UV-visible absorbance bands appear at 375, 450, 550, and 650 nm due to an Fe³⁺-nitrite intermediate species. These absorption bands decrease in intensity as the reaction proceeds to product with the band at 550 nm disappearing completely. Extraction of the absorbance data as a function of time at 375 and 550 nm provides curves that are identical to the first and second intermediates in the concentration profile. These data are consistent with the accumulation of an Fe³⁺-nitrite intermediate species that degrades into the resting Fe³⁺ state and product. The origin of this absorption band is likely due to an Fe³⁺→S ligand-to-metal charge transfer (LMCT) band resulting from the strong back donation of the low spin Fe³⁺ center to the nitrile N π* orbitals, similar to NHase-NO and Fe³⁺-N₃ or -NO model complexes (17–19).

Additional evidence for an Fe³⁺-nitrite intermediate species comes from the observed absorption band at ~700 nm. In resting RenHase, this band was assigned to an Fe³⁺→S LMCT band. The observed hypsochromic shift from 700 to 650 nm upon the addition of substrate is indicative of a perturbation at the Fe³⁺ center due to nitrite binding. Blue shifts of similar magnitude have been observed in NHase enzymes and model complexes upon the addition of NO or N₃ and were attributed to an increase in π electron donation from the axial thiolate ligand to the Fe³⁺ ion to compensate for the π-acceptor behavior of the bound ligand (20, 21). Similarly, the absorbance band observed at 450 nm, which has also been assigned as an Fe³⁺→S LMCT band based on resonance Raman data and magnetic circular dichroism model complex data (17, 19), increases in intensity upon substrate binding. Taken together, these data indicate that the observed enzyme-substrate complex is the result of the direct ligation of a nitrite to the active site low spin Fe³⁺ center, which forms an Fe³⁺-nitrite intermediate species.

Direct ligation of a nitrite to the low spin Fe³⁺ center of RenHase is also consistent with the significant increase in absorption observed at 375 nm upon the addition of methacrylonitrile to resting RenHase. In the presence of NO, iron-type NHases show strong absorbance at 370 nm corresponding to an Fe³⁺→S LMCT band that results from the direct coordination of the NO to the Fe³⁺ active site (19, 22). Direct coordination of NO to the low spin Fe³⁺ active site was confirmed by EPR and resonance Raman data, which suggested that NO displaces the axial water molecule, forming an Fe³⁺-NO complex that is inactive. The Fe³⁺→S LMCT band observed at ~700 nm in resting iron-type NHase is not observed in NO-inhibited NHase enzymes but reappears upon light-induced activation (19, 22). However, in the enzyme-substrate intermediate complex, the Fe³⁺→S LMCT band at ~650 nm and a strong absorption at 375 nm are observed. This suggests that upon the addition of nitrite, the absorption band at 375 nm is due to an Fe³⁺→S LMCT transition from nitrite coordination to the Fe³⁺ center in RenHase.

The single turnover data combined with previously reported kinetic and crystallographic data allow a catalytic mechanism to be proposed for iron-type NHase enzymes that involves the...
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direct ligation of the nitrile to the Fe\(^{3+}\) active site (Fig. 6) (6, 16, 22–24). The rate constants provided herein suggest a fast second-order step that involves binding of substrate to the enzyme followed by rearrangement and then product release, which is the rate-limiting step. Displacement of the metal-bound water molecule by a nitrile and coordination to the low spin Fe\(^{3+}\) center activate the CN bond toward nucleophilic attack. Once nucleophilic attack occurs followed by proton transfer, the resulting imidate can tautomerize to form an amide with a sub-sequent proton transfer (15, 16). Finally, the amide product can be displaced by a water molecule and thus provide the regenerated catalyst.

In conclusion, we have identified the first low spin Fe\(^{3+}\)-nitrite intermediate species for an NHase enzyme using single-turnover stopped-flow spectroscopy. The best kinetic model allows for the fast, reversible binding of substrate followed by the formation of an Fe\(^{3+}\)-nitrite intermediate species, a potential rearrangement of ES, and the formation of product. The product release step is rate-limiting, which is consistent with previous steady state kinetic studies for both iron-type and cobalt-type NHase enzymes (15, 16). The observed UV-visible absorption bands for an Fe\(^{3+}\)-nitrite intermediate species at 375, 450, 550, and 650 nm are similar to low spin Fe\(^{3+}\) enzyme and model complexes bound by NO or N\(_3\) and are indicative of strong back donation from the low spin Fe\(^{3+}\) to nitrite \(\pi^*\) orbitals. These data provide spectroscopic evidence for the direct ligation of the nitrite substrate to the low spin Fe\(^{3+}\) active site in NHase.

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FIGURE 6. Proposed catalytic mechanism of nitrile hydratase.
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