Identification of an active site bound Nitrile Hydratase Intermediate Through Single Turnover Stopped-Flow Spectroscopy†

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Running Title: Identification of an active site bound Nitrile Hydratase Intermediate

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Keyword: Nitrile hydratase, stopped-flow spectroscopy, single turnover, enzyme kinetics

Background: No direct evidence exists for the direct coordination of nitrile to the Fe³⁺ active site in nitrile hydratases.

Results: The first Fe³⁺-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.

Summary

Stopped-flow kinetic data were obtained for the Fe-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 used 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 Fe³⁺-ligand-to-metal charge transfer (LMCT) band from 700 to 650 nm. The observed UV-Vis absorption bands for the Fe³⁺-nitrile intermediate species are similar to low-spin Fe³⁺-enzyme and model complexes bound by NO or N³⁻. These data provide spectroscopic evidence for the direct coordination of the nitrile substrate to the NHase active site low-spin Fe³⁺ center.

Introduction

Nitrile hydratases (NHases) 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
Scheme 1. The hydration of a nitrile to its corresponding amide by nitrile hydratase.

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 rhodochrous* J1(3). More than 3,500 tons of nicotinamide is 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 (‘Fe-type’) or a Co$^{3+}$ ion (‘Co-type’) in their active sites (6). X-ray crystal structures of both Co- 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-sulfinic acid (–SO$_2$H) 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 Fe-type and Co-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 subunit and activator genes were sub-cloned into pET-21a(+) and pET-28a(+), respectively. ReNHase was transformed into NEB Turbo cells (New England Biolabs) for cloning and BL21(DE3) cells (Stratagene) for protein expression. Cells were grown at 37°C in LB media supplemented with kanamycin (50 µg/mL) and ampicillin (100 µg/mL). The cultures were cooled for one hour upon reaching an optical density (OD) of 0.8. Cultures were induced with 0.1 mM Isopropyl-β-D-1-thiogalactopyranoside (IPTG) and 0.25 mM ferrous sulfate and shaken for 16 additional hours at 18°C. Cells were pelleted by centrifugation at 5000 rpm for 5 min.

*Purification of ReNHase.* Cells containing ReNHase were resuspended in 50 mM sodium phosphate buffer at pH 7.5 containing 300 mM NaCl, 40 mM butyric acid, and 10 mM imidazole at a ratio of 3 mL/g of cells and lysed by ultrasonic probe (Misonix Sonicator 3000) in 30 s increments for 4 min. at 21W. The cell lysate was separated from cell debris by centrifugation for 40 min. at 12,500 rpm and purified using immobilized metal affinity chromatography (IMAC) on an AKTA FPLC Chromatographic System (GE) at 4°C. ReNHase was eluted with a linear gradient from 0-100% imidazole buffer (50 mM NaH$_2$PO$_4$ pH 7.5, 300 mM NaCl, 40 mM butyric acid, 500 mM imidazole) at 1 mL/min. followed by buffer exchange to remove butyric acid and imidazole using an Amicon centrifugal filter unit MWCO 30,000 (Millipore). Enzyme purity was assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). A Bradford Assay was performed against bovine serum albumin (BSA) standards to determine protein concentration.
Steady State Kinetic Assay. ReNHase activity was examined using 100 mM methacrylonitrile as the substrate in 50 mM HEPEs buffer, pH 7.0 and 25 °C at 242 nm (ε242 3.2 mM⁻¹cm⁻¹) on a Shimadzu UV-2450 spectrometer. Initial reaction rates were monitored and fit to a modified Hill equation \( y = V_{\text{max}}^e x/(k + x) \) using Origin Pro. One unit of NHase activity was defined as the formation of 1 µmol of amide product formed per minute.

Stopped-Flow Experiments. ReNHase activity towards methacrylonitrile was examined in triplicate using a single mixing Applied Photophysics SX-20 stopped-flow UV-vis spectrophotometer. All data were fit using Pro-Data and Pro-K software by Applied Photophysics. ReNHase activity was monitored at 242 nm by acquiring stopped-flow data from 0.005 to 10 seconds at 5°C using 10 µM enzyme and varying concentrations of methacrylonitrile (0.1 to 25 mM). These data were fit to the double exponential equation:

\[
Y = A_1e^{k_{\text{obs}1}t} + A_2e^{k_{\text{obs}2}t} + C
\]

Single turnover stopped-flow data were obtained using 0.33 mM ReNHase and 0.19 mM methacrylonitrile from 0.005 to 0.5 seconds. Data were reduced by Singular Value Decomposition (SVD) and globally fit to various mechanistic models.

Results and Discussion

Multiple turnover stopped-flow experiments were initially run at 5 °C using 25 mM methacrylonitrile at pH 7.0 (Figure 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 0.005 to 10 s. These data were fit to a double exponential equation providing \( k_{\text{obs}1} \) and \( k_{\text{obs}2} \) for each phase of the reaction. \( k_{\text{obs}1} \) was designated the fast phase and \( k_{\text{obs}2} \) 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).

Scheme 2. Proposed kinetic model for the hydration of methacrylonitrile by ReNHase.

\[
E + S \xrightarrow{k_1} ES \xrightarrow{k_2} EI \xrightarrow{k_3} E + P
\]

The concentration dependence of the reaction rate on methacrylonitrile was examined by plotting the fast and slow phases (\( k_{\text{obs}1} \) and \( k_{\text{obs}2} \)) of the reaction against the substrate concentration (Figures 2A & 2B) in order 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 \( k_1 + k_2 + k_3 \). This fit provided a \( k_1 \) value of 1.0 ± 0.1 mM⁻¹s⁻¹ and a \( k_1 + k_2 + k_3 \) value of 12 ± 1 s⁻¹. The non-zero intercept implies that the binding is reversible. The linear fit indicates that binding occurs in a single step.(13)

The dependency of \( k_{\text{obs}2} \) on substrate was fit to a hyperbolic curve, \( (k_{\text{obs}2} = k_{\text{max}}[S]/(K_{d2} + [S])) \), where \( k_{\text{max}} \) is the sum of \( k_2 + k_3 \) and is equal to the rate at saturated enzyme concentrations. \( K_{d2} \) is the apparent dissociation constant of an intermediate step following substrate binding. \( k_{\text{max}} \) was found to be 9 ± 2 s⁻¹ and \( K_{d2} \) is 1.5 ± 0.8 mM. Subtracting \( k_{\text{max}}(k_2 + k_3) \) from the intercept of the fast phase \( (k_1 + k_2 + k_3) \) provides \( k_3 \), which is 3 s⁻¹. The y-intercept of the slow phase provides \( k_2 \), which is 1 s⁻¹. Therefore, \( k_2 \) is 8 s⁻¹ and \( K_{d1} \), which is \( k_1/k_3 \), is 3 mM. 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 to 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, since \( k_2 \) is greater than \( k_1 \), is that \( K_m \) is greater than \( K_{d1} \). 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, since the system is not at rapid equilibrium but at the steady-state, \( K_m = k_2k_3 + k_1k_2 + k_1k_3/k_1(k_2 + k_3 + k_3) \) for a mechanism with one intermediate before product release (14). That means the experimentally de-
terminated $K_m$ value of 6 mM might be different than the $K_m$ value described as $k_1/k_2$. In our case where the values of $k_1$, $k_2$, $k_3$, and $k_4$ were experimentally determined, $K_m$ can only be 0.33 mM (for extremely small $k_3$ values) up to 11 mM (for extremely high $k_1$ 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 is 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 $k_3$, 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 to 0.5 s of the reaction at 25 °C (Figure 3). These transient spectra indicate the rapid formation of an Fe³⁺-nitrile species that converts to the resting Fe³⁺ state and product. Independent spectra of enzyme intermediate complexes were extracted after singular value decomposition (SVD) was applied to the raw data in order 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 Photo-physics 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 $65 \pm 10$ mM⁻¹ s⁻¹, $23 \pm 3$ s⁻¹, and $12 \pm 4$ s⁻¹, respectively. The reverse rate constants $k_1$ and $k_2$ were found to be $2.8 \pm 0.1$ s⁻¹ and $1.1 \pm 0.1$ 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:

\[ k_{cat} = k_3k_2/k_2+k_2+k_3 \]

\[ K_m = k_2k_3/k_3k_3+k_1k_1k_1(k_2+k_2+k_3) \]

The theoretical $k_{cat}$ and $K_m$ values of $8 \pm 3$ s⁻¹ and $128 \pm 50$ μM, respectively, 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 Fe- and Co-type NHase enzymes (15,16). Concentration profiles for the progress of the reaction confirm a three-step reaction model (Figure 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 seconds.

Singular value decomposition identified four spectrally unique species, with one corresponding to native ReNHase enzyme while the second is an Fe³⁺-nitrile intermediate species (Figure 5). As the reaction proceeds, UV-Vis absorption bands
appear at 375, 450, 550, and 650 nm due to an Fe\(^{3+}\)-nitrile 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 absorption data as a function of time at 375 nm and 550 nm provide curves that are identical to the first and second intermediates in the concentration profile. These data are consistent with the accumulation of an Fe\(^{3+}\)-nitrile intermediate species that degrades into the resting Fe\(^{3+}\) state and product. The origin of this absorption band is likely due to an Fe\(^{3+}\)\(\leftarrow\)S ligand-to-metal charge transfer (LMCT) band resulting from the strong back-donation of the low-spin Fe\(^{3+}\) center to the nitrile N \(\pi^*\) orbitals, similar to NHase-\(\cdot\)NO and Fe\(^{3+}\)-N\(_3\) or -\(\cdot\)NO model complexes (17-19).

Additional evidence for an Fe\(^{3+}\)-nitrile intermediate species comes from the observed absorption band at \(\sim\)700 nm. In resting ReNHase this band was assigned to an Fe\(^{3+}\)\(\leftarrow\)S LMCT band. The observed hypsochroic shift from 700 to 650 nm upon the addition of substrate is indicative of a perturbation at the Fe\(^{3+}\) center due to nitrile binding. Blue shifts of similar magnitude have been observed in NHase enzymes and model complexes upon the addition of NO or N\(_3\) and were attributed to an increase in \(\pi\) electron donation from the axial thiolate ligand to the Fe\(^{3+}\) ion to compensate for the \(\pi\)-accepting behavior of the bound ligand (20,21). Similarly, the absorbance band observed at 450 nm, which has also been assigned as an Fe\(^{3+}\)\(\leftarrow\)S LMCT band based on resonance Raman (RR) data and MCD 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 nitrile to the active site low-spin Fe\(^{3+}\) center, which forms an Fe\(^{3+}\)-nitrile intermediate species.

Direct ligation of a nitrile to the low-spin Fe\(^{3+}\) 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, Fe-type NHases show strong absorbance at 370 nm corresponding to an Fe\(^{3+}\)\(\leftarrow\)S LMCT band that results from the direct coordination of the NO to the Fe\(^{3+}\) active site (19,22). Direct coordination of NO to the low-spin Fe\(^{3+}\) active site was confirmed by EPR and RR data, which suggested that NO displaces the axial water molecule forming an Fe\(^{3+}\)-NO complex that is inactive. The Fe\(^{3+}\)\(\leftarrow\)S LMCT band observed at \(\sim\)700 nm in resting Fe-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 Fe\(^{3+}\)\(\leftarrow\)S LMCT band at \(\sim\)650 nm and a strong absorption at 375 nm are observed. This suggests that upon the addition of nitrile, the absorption band at 375 nm is due to an Fe\(^{3+}\)\(\leftarrow\)S LMCT transition from nitrile coordination to the Fe\(^{3+}\) center in ReNHase.

The single turnover data combined with previously reported kinetic and crystallographic data allow a catalytic mechanism to be proposed for Fe-type NHase enzymes that involves the direct ligation of the nitrile to the Fe\(^{3+}\) active site (Figure 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 activates the CN bond towards nucleophilic attack. Once nucleophilic attack occurs followed by proton transfer, the resulting imidate can tautomerize to form an amide with a subsequent 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\(^{3+}\)-nitrile 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\(^{3+}\)-nitrile 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 Fe- and Co-type NHase enzymes (15,16). The observed UV-Vis absorption bands for an Fe\(^{3+}\)-nitrile 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 nitrile \(\pi^*\) orbitals. These data provide spectroscopic evidence for the direct ligation
of the nitrile substrate to the low-spin Fe$^{3+}$ active site in NHase.

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References

Figure Legends

**Figure 1.** Representative pre-steady state plot of 10 µM *ReNHase* catalyzing the hydration of 250 mM methacrylonitrile in 50 mM HEPES buffer, pH 7.0, and 5 °C at 242 nm over 0.005 to 5 s.

**Figure 2.** Methacrylonitrile concentration dependence on $k_{obs}$. A) The fast phase shows a linear dependence and a non-zero intercept, indicating a fast reversible binding step. B) The slow phase exhibits a hyperbolic dependence with a maximum rate of 8 s$^{-1}$.

**Figure 3.** Absorption spectra collected with mixing times ranging from 0.005 to 0.5 s for the *ReNHase* hydration reaction. The green line indicates resting enzyme while the red line is an intermediate complex. The arrows indicate the direction of major absorption band shifts. Experimental conditions were 0.33 mM *ReNHase* and 0.19 mM methacrylonitrile reacted in 50 mM HEPES, pH 7.0 at 25 °C.

**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.

**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.

**Figure 6.** Proposed catalytic mechanism of nitrile hydratase.
Figure 2.

A.

B.
Figure 3.
Figure 4.
Figure 5.
Figure 6.
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