Unraveling the Catalytic Mechanism of Nitrile Hydratases*

Received for publication, May 1, 2006, and in revised form, December 5, 2006. Published, JBC Papers in Press, December 6, 2006, DOI 10.1074/jbc.M604117200

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To elucidate a detailed catalytic mechanism for nitrile hydratases (NHases), the pH and temperature dependence of the kinetic constants $k_{cat}$ and $K_m$ for the cobalt-type NHase from *Pseudonocardia thermophila* JCM 3095 (PtNHase) were examined. PtNHase was found to exhibit a bell-shaped curve for plots of relative activity versus pH at pH 3.2–11 and was found to display maximal activity between pH 7.2 and 7.8. Fits of these data provided $pK_{ES1}$ and $pK_{ES2}$ values of 5.9 ± 0.1 and 9.2 ± 0.1 ($k_{cat}^0 = 130 ± 1$ s$^{-1}$), respectively, and $pK_{ES1}$ and $pK_{ES2}$ values of 5.8 ± 0.1 and 9.1 ± 0.1 ($k_{cat}^0/K_m^0 = (6.5 ± 0.1) × 10^3$ s$^{-1}$ m$^{-1}$), respectively. Proton inventory studies indicated that two protons are transferred in the rate-limiting step of the reaction at pH 7.6. Because PtNHase is stable at 60 °C, an Arrhenius plot was constructed by plotting $ln(k_{cat})$ versus $1/T$, providing $E_a = 23.0 ± 1.2$ kJ/mol. The thermal stability of PtNHase also allowed $\Delta H^0$ ionization values to be determined, thus helping to identify the ionizing groups exhibiting the $pK_{ES1}$ and $pK_{ES2}$ values. Based on $\Delta H^0_{ion}$ data, $pK_{ES1}$ is assigned to $\beta$-Tyr$^{\text{pH}}$, whereas $pK_{ES2}$ is assigned to $\beta$-Arg$^{\text{pH}}$, $\beta$-Arg$^{\text{pH}2}$, or $\alpha$-Ser$^{\text{pH}1}$ (NHases are $\alpha$-betaheterotetramers). A combination of these data with those previously reported for NHases and synthetic model complexes, along with sequence comparisons of both iron- and cobalt-type NHases, allowed a novel catalytic mechanism for NHases to be proposed.

Nitrile hydratase (NHase$^2$; EC 4.2.1.84), one of the enzymes in the nitrile degradation pathway, catalyzes the hydrolysis of nitriles to their corresponding amides in a chemo-, regio-, and/or enatioselective manner at ambient pressures and temperatures at physiological pH (Scheme 1) (1–6). NHases have attracted substantial interest as biocatalysts for industrial applications such as the large-scale production of acrylamide (3, 7–9) and nicotinamide (10). Acrylamide production utilizing the bacterium *Rhodococcus rhodochrous* J1 has increased to >30,000 tons/year (3), whereas >3500 tons of nicotinamide are produced per year (11). Yields of >99% are achieved, and the formation of by-products such as acrylic acid, which plagues traditional methodology, is completely avoided. However, one of the most attractive features of nitrile-metabolizing enzymes is their ability to selectively hydrolyze one cyano group of a dinitrile to its corresponding amine, something that is virtually impossible using conventional chemical methods (12–14). Therefore, the potential use of nitrile-hydrolyzing enzymes for the production of several fine chemicals is increasingly recognized.

NHases are metalloenzymes that contain either a non-heme Fe(III) ion (iron-type) or a non-corrin Co(III) ion (cobalt-type) in their active site and are typically $\alpha$-betaheterotetramers (5, 6, 15, 16). In all known NHases, each $\alpha$-subunit has a highly homologous amino acid sequence (CXYXSCX) that forms the metal-binding site. Cobalt-type NHases contain threonine and tyrosine in the -C(T/S)YSC(Y/T)- sequence of the active center, whereas iron-type NHases contain serine and threonine (6). Recently, both iron- and cobalt-type NHases have been crystallographically characterized (17–22). In all structures published to date, the trivalent metal ion is six-coordinate, with the remaining ligands made up of three cysteines and two amide nitrogens (Fig. 1). Interestingly, two of the active-site cysteine residues are post-translationally modified to cysteinesulfenic acid ($\sim$SO$_2$H) and cysteinesulfenic acid ($\sim$SOH), yielding an unusual metal coordination geometry termed a “claw setting,” and it has been shown that, unless this Cys oxidation process occurs, NHase is inactive (23, 24). In iron-type NHases, nitric oxide (NO) binds in place of a metal-coordinated water/hydroxide molecule, which can be photoactivated, whereas cobalt-type NHases do not bind NO (5). Based on theoretical calculations, the carbon–nitrogen bond in the coordinated amide of both iron- and cobalt-type NHases has significant double bond character, suggesting that it is best represented as an imidometal bond (25).

The structural characterization of both iron- and cobalt-type NHases has provided some insight into how the molecular structure controls the enzyme function. Based on these data and several elegant studies on active-site NHase model complexes (for reviews, see Refs. 5 and 16), four simple reaction mechanisms have been proposed (5, 26). In each reaction, imidate is produced as a reaction intermediate, which then isomerizes to the corresponding amide. Even though a significant amount of structural and synthetic modeling data have been reported for NHases, details of the enzymatic reaction, including which proposed mechanism is operative as well as the nature of the transition state, the identities of groups involved in proton transfers, and the role of the metal ion, remain uncertain. To gain insight into the catalytically important active-site residues and the number of protons that are transferred in the transition state, we have examined the pH and temperature...
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\[
\text{R-CN} + \text{OH}_2 \rightarrow \text{H-N} + \text{OH} \rightarrow \text{R-CH}_2\text{NH}_2
\]

SCHEME 1

**FIGURE 1.** Active site of NHase from *P. thermophila* (Protein Data Bank code 1IRE). The trivalent metal ion is six-coordinate, with three cysteine sulfurs, two amide nitrogens, and one water molecule. Cys\(^{111}\) is post-translationally modified to cysteinesulfenic acid, and Cys\(^{113}\) is modified to cysteinesulfenic acid (20, 21).

dependence of the kinetic parameters and solvent isotope effect of the cobalt-type NHase from *Pseudonocardia thermophila* JCM 3095 (PtNHase). Based on these data, a novel catalytic mechanism is proposed.

**MATERIALS AND METHODS**

**Protein Expression, Purification, and Kinetic Assay**—All chemicals used in this study were purchased from commercial sources and were of the highest quality available. The plasmid encoding the \(\alpha\)- and \(\beta\)-subunits of PtNHase was obtained from the International Patent Organism Depositary (unit.aist.go.jp/ipod/index_e.html) in Japan. The enzyme was purified by a significantly simplified and shortened procedure based on a previously published purification method (19, 20). Briefly, *Escherichia coli* BL21 Star\(^{TM}\) (DE3) cells containing the pUC18-NHase plasmid (20), which includes the genes for the \(\alpha\)- and \(\beta\)-subunits of NHase and an NHase activator protein, were grown at 37 °C in 5 liters of Luria-Bertani broth containing ampicillin (100 mg/liter). When the absorbance reached ~0.8 at 600 nm, the cells were induced by the addition of 0.1 mM isopropyl \(\beta\)-d-thiogalactopyranoside and 0.25 mM Co(II) chloride. The cells were then cultured for an additional 16 h, followed by harvesting by centrifugation. All subsequent manipulations were performed at 4 °C. The cell paste was resuspended in 10 ml of 50 mM Tris-HCl (pH 7.6), sonicated, and clarified by centrifugation. The cell-free extract was fractionated with 40–70% ammonium sulfate and equilibrated with 50 mM phosphate buffer (pH 7.6) containing 1 M (NH\(_4\))\(_2\)SO\(_4\) prior to hydrophobic interaction gradient chromatographic purification using phenyl-Sepharose (1 M (NH\(_4\))\(_2\)SO\(_4\) and 0% isopropyl alcohol \(\rightarrow\) 0 M (NH\(_4\))\(_2\)SO\(_4\) and 0% isopropyl alcohol \(\rightarrow\) 0 M (NH\(_4\))\(_2\)SO\(_4\) and 40% isopropyl alcohol). Active fractions (~15% isopropyl alcohol) were concentrated by ultrafiltration and further purified to >95% (SDS-PAGE) by size exclusion chromatography using Sephacryl S-300.

PtNHase was assayed for catalytic activity using benzonitrile as the substrate (20). In this assay, the hydration of benzonitrile was measured spectrophotometrically by monitoring the formation of benzamide at 242 nm (\(e = 5.5 \text{ mm}^{-1} \text{ cm}^{-1}\)). All assays were performed on a Shimadzu UV-3101PC spectrophotometer equipped with a constant-temperature cell holder and an ISOTEMP 2013D water bath (Fisher). Enzyme concentrations were determined using the Bradford assay with bovine serum albumin as the standard. All assays were performed at 25 ± 0.1 °C in 50 mM potassium phosphate buffer (pH 7.6).

**pH Profiles**—The enzymatic activities of PtNHase at pH 3.2–11.0 were measured using benzonitrile as the substrate. The concentration of each buffer used was 50 mM, and the following buffers were used: borate (pH 8.50–10.50), Tris-HCl (pH 7.00–8.50), phosphate (pH 7.00–7.75), MOPS (pH 6.50–7.00), MES (pH 5.50–6.50), and acetate (pH 3.23–5.50). The kinetic parameters \(k_{cat}/K_m\) were determined using 8–12 different substrate concentrations ranging from 0.2 to 10.0 times the observed \(K_m\) value at each pH studied. Kinetic parameters and fits to the kinetic curves were obtained using IGOR Pro (WaveMetrics, Lake Oswego, OR).

**Solvent Isotope Effect**—All buffers were prepared from a freshly opened bottle of 99.9% \(^2\text{H}_2\text{O}\) (Aldrich). The buffers used in the preparation of all deuterated buffers were in the anhydrous form. The pH of each buffer used was adjusted by the addition of NaOD or DCl (both 99%+ deuterium content; Acros Organics, Geel, Belgium) and corrected for deuteration by adding 0.4 to the reading of the pH electrode (27).

**RESULTS**

**pH Dependence of the Kinetic Parameters**—To examine the reaction mechanism of PtNHase, the kinetic parameters \(k_{cat}, K_m\), and \(k_{cat}/K_m\) were recorded as a function of pH. PtNHase catalyzed the hydration of benzonitrile at pH 7.6 and 25 °C with \(k_{cat} = 123 \pm 1 \text{ s}^{-1}\) and \(K_m = 20.0 \pm 0.1 \text{ M}\) in the absence of \(n\)-butyric acid. These values are indistinguishable from those reported previously (\(k_{cat} = 120 \pm 1 \text{ s}^{-1}\) and \(K_m = 19 \text{ M}\)) (20). PtNHase was found to exhibit a bell-shaped curve for plots of relative activity versus pH at pH 3.2–11 and was found to display maximal activity between pH 7.2 and 7.8. Plots of \(\log(k_{cat})\) and \(\log(k_{cat}/K_m)\) versus pH were prepared for PtNHase and fit to Equations 1 and 2, respectively (Fig. 2) (28, 29).

\[
\log(k_{cat}) = \log(k_{cat}')(1 + [H]/K_{ES1} + K_{ES2}[H]) \quad \text{(Eq. 1)}
\]

\[
\log(k_{cat}/K_m) = \log(k_{cat}'/K_m')(1 + [H]/K_{ES1} + K_{ES2}[H]) \quad \text{Eq. 2)}
\]

where \(k_{cat}'\) is the theoretical maximal velocity; \(k_{cat}'/K_m'\) is the theoretical maximal catalytic efficiency; \(K_{ES1}\) is the ionization constant of the ES complex that affects the acidic side of the pH curve; \(K_{ES2}\) reflects the basic side; and \(K_{\epsilon 1}\) and \(K_{\epsilon 2}\) are the ion-
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**TABLE 1**

| Ionization constants for benzonitrile hydration by PtNHase |  |
|-----------------------------------------------------------|---------|
| $k_{cat}$ vs. pH                                          | 5.9 ± 0.1 |
| $pK_{E1}$                                                 | 9.2 ± 0.1 |
| $pK_{E52}$                                                | 130 ± 1  |

| $k_{cat} / K_m$ vs. pH                                     |  |
|-----------------------------------------------------------|---------|
| $pK_{E1}$                                                 | 5.8 ± 0.1 |
| $pK_{E52}$                                                | 9.1 ± 0.1 |
| $k_{cat} / K_m$ (s$^{-1}$ mm$^{-1}$)                      | (6.5 ± 0.1) × 10$^3$ |

**FIGURE 2.** pH dependence of the kinetic parameters for the hydration of benzonitrile by PtNHase between pH 3.2 and 11. The $K_m$ does not vary with pH. The pH dependence of $k_{cat}$ fit to Equation 1, and the pH dependence of $k_{cat}/K_m$ fit to Equation 2. The kinetic constants obtained from the fits are listed in Table 1. Error bars are smaller than the symbols used.

**FIGURE 3.** Plot of $V_0 / V_n$ versus the atom fraction of deuterium for PtNHase at pH 7.6. $V_0 / V_n$ is defined as (velocity at n atom fraction of deuterium)/(velocity in water). The dashed line represents a linear relationship; the solid line is a direct fit to Equation 4 with fractionation factors $\phi_1 = 0.66$ and $\phi_2 = 0.68$.

Inspection of a plot of log($K_m$) versus pH (Fig. 2) indicated that the $K_m$ does not vary with pH. Therefore, plots of log($k_{cat}$) versus pH and plots of log($k_{cat} / K_m$) versus pH provided similar p$K_a$ values (Table 1).

Examination of a plot of log($k_{cat}$) versus pH revealed a bell-shaped curve that could be fit to Equation 1 (Fig. 2). The slopes of the asymptotes, calculated as described previously (30), for the acidic and basic limbs of log($k_{cat}$) versus pH for PtNHase are 1, indicating that one group is ionized on each limb. A good fit to Equation 1 was obtained, which yielded a $pK_{E1}$ value of 5.9 ± 0.1 and a $pK_{E52}$ value of 9.2 ± 0.1 ($k_{cat} = 130 ± 1$ s$^{-1}$). Similar to plots of log($k_{cat}$) versus pH, fits of log($k_{cat} / K_m$) versus pH to Equation 2 provided a $pK_{E1}$ value of 5.8 ± 0.1 and a $pK_{E52}$ value of 9.1 ± 0.1 ($k_{cat} / K_m = (6.5 ± 0.1) × 10^3$ s$^{-1}$ mm$^{-1}$) (Fig. 2).

**Solvent Isotope Effect**—Solvent isotope effect experiments were carried out on PtNHase using benzonitrile as the substrate at pH 7.6 (p$^3$H = p$^1$H meter reading + 0.4) (27) by substituting hydrogen ($^1$H) with deuterium ($^2$H). $k_{cat}$ values for benzonitrile were measured at several different ratios of D$_2$O and H$_2$O, and the results were plotted as the atom fraction of deuterium versus $V_0 / V_n$, where $V_n$ is the observed velocity at n fraction of deuterium, and $V_0$ is the observed velocity in water (Fig. 3). Proton inventories were obtained by fitting the experimental data to equations derived from the Gross-Butler equation (Equation 3),

$$
V_0 / V_n = \frac{v_T}{v_R} \frac{\prod_j (1 - n + n\phi_j)^j}{\prod_j (1 - n + n\phi_j)^j} \tag{Eq. 3}
$$

where $n$ is the atom fraction of deuterium, $v_T$ is the number of protons transferred in the transition state, $v_R$ is the number of protons transferred in the reactant state, and $\phi$ is the fractionation factor defined as in Equation 4,

$$
\phi = (X^D_i / X^H_i) / (n / (1 - n)) \tag{Eq. 4}
$$

where $X^D_i$ and $X^H_i$ are the mole fractions of deuterons and protons in the i$^{th}$ transition or reactant state (31, 32). At pH 7.6, the data deviate from linearity, and the best fit was obtained for a polynomial, suggesting that at least two protons are transferred in the transition state at this pH (Fig. 3). Because the largest deviation for theoretical proton inventory curves occurs at an...
atom fraction of 0.5 (33, 34), calculation of a midpoint partial solvent isotope effect often helps in determining the number of protons involved in the catalytic reaction. Equations 5–7, derived by Elrod et al. (33), allowed the calculation of midpoint partial solvent isotope effects when the experimental data were obtained at different atom fractions,

\[
\text{One proton: } V_{m}/V_1 = (1 - n_m)(V_d/V_1) + n_m \quad (\text{Eq. 5})
\]

\[
\text{Two protons: } V_{m}/V_1 = ((1 - n_m)(V_d/V_1)^{1/2} + n_m)^2 \quad (\text{Eq. 6})
\]

\[
\text{General solvation: } V_{m}/V_1 = (V_d/V_1)^{-n_m} \quad (\text{Eq. 7})
\]

where \(n_m = 0.49\) (the \(\text{H}_2\text{O}/\text{D}_2\text{O}\) ratio at the midpoint), \(V_{m}/V_1\) equals the midpoint partial solvent isotope effect, and \(V_d/V_1\) represents the total isotope effect (velocity in 100% \(\text{H}_2\text{O}\)/velocity in 100% \(\text{D}_2\text{O}\)). The experimental and calculated midpoint partial isotope effects are presented in Table 2. The presence of \(\text{D}_2\text{O}\) lowered the catalytic activity of PtNHase, resulting in a solvent isotope effect of 2.07 (Fig. 3). The plot of the atom fraction of deuterium versus \(V_{m}/V_1\) (velocity at \(n\) fraction of deuterium/velocity in 100% \(\text{D}_2\text{O}\)) obtained for the reaction of benzonitrile and PtNHase was best fit to Equation 6, suggesting that two protons are transferred during the rate-limiting step at pH 7.6. However, midpoint partial isotope effect calculations do not strongly distinguish between two protons transferred and generalized solvent effects (Table 2). Because the \(K_m\) was found to be independent of pH over the entire pH range studied, the \(\text{D}_2\text{O}/\text{H}_2\text{O}\) for PtNHase is 1.7.

**Temperature Dependence of \(K_m\) and \(k_{cat}\) for PtNHase**—It was reported previously that PtNHase is stable at 60 °C and pH 7.6 (35). These data are very unusual because most enzymes undergo some denaturation at temperatures above 50 °C, resulting in a decrease in \(V_{max}\) (28). This observed thermal stability provides the unique opportunity to probe the thermodynamic properties of the PtNHase-catalyzed hydration of benzonitrile. Initially, the hydration rate of benzonitrile was measured in triplicate between 20 and 60 °C for PtNHase at eight substrate concentrations ranging from 2 to 100 \(\mu\text{M}\). From these data, both \(K_m\) and \(k_{cat}\) values were derived by fitting the experimental data to the Michaelis-Menten equation at each temperature studied. Both the \(k_{cat}\) and \(K_m\) values increased with increasing temperature.

In a simple rapid equilibrium, \(V_{max}/[E] = k_{cat}\), the first-order rate constant. Keeping a constant enzyme concentration, an Arrhenius plot was constructed by plotting \(\ln(k_{cat})\) versus \(1/T\). A linear plot was obtained, indicating that the rate-limiting step does not change as the temperature is increased (28). From the slope of the line, the activation energy \(E_a\) for temperatures between 293 and 333 K was calculated to be 23.0 ± 1.2 kJ/mol (36). Because the slope of an Arrhenius plot is equal to \(-E_a/R\) (where \(R = 8.3145\) J·K\(^{-1}\)·mol\(^{-1}\)), other thermodynamic parameters were calculated by the following relations: \(\Delta G° = -RT\ln(k_{cat}/k_0), \Delta H° = E_a - RT,\) and \(\Delta S° = (\Delta H° - \Delta G°)/T,\) where \(k_0, h, R\), and \(T\) are the Boltzmann, Planck, and gas constants, respectively (Table 3). Assuming that the \(K_m\) is equal to the \(K_m\), a linear plot was obtained for \(\ln(1/K_m)\) versus \(1/T\), which provides \(\Delta H°\) by multiplying the negative slope by \(R\). The following thermodynamic parameters were calculated by the relations: \(\Delta G° = -RT\ln(1/K_m)\) and \(\Delta S° = (\Delta H° - \Delta G°)/T\) (Table 3).

Given the thermal stability of PtNHase, the pH dependence of \(k_{cat}\) at saturating substrate concentrations (100 \(\mu\text{M}\)) at several pH values between 3.2 and 10.5 were also examined at three different temperatures to determine the identity of the ionizing groups exhibiting the \(pK_a\) values of 5.9 and 9.2, respectively. These data were fit to Equation 1, providing three ionization constants, one for each temperature. A plot of ionization constants versus inverse absolute temperature yields the slope (Equation 8),

\[
\text{Slope} = \Delta H_{ion}/2.303 \cdot R
\]

where \(R\) is the gas constant (Fig. 4, A and B) (37). The enthalpies of ionization calculated from these data are 7.6 ± 0.3 and 12.0 ± 0.3 kcal/mol, respectively. The enthalpy of ionization for \(pK_{ES1}\) is in the range for tyrosine or histidine residues (6.0–7.5 kcal/mol), suggesting that the \(pK_{ES1}\) is due to \(\beta\text{Tyr}^{68}\) because no active-site histidine residues exist (34, 38). The enthalpy of ionization for \(pK_{ES2}\) is in the range for serine or arginine residues (12–13 kcal/mol), so it can be assigned to one of the following residues: \(\alpha\text{Arg}^{52}, \alpha\text{Arg}^{137},\) or \(\alpha\text{Ser}^{112}\) (34, 38).

**DISCUSSION**

Simple catalytic mechanisms for NHases have been proposed based on x-ray crystal structures, theoretical modeling studies, synthetic models, and limited kinetic and spectroscopic studies (5, 16, 26, 39). In the most widely accepted mechanism of NHases, the nitrile moiety of the substrate binds directly to the metal center, displacing the active-site water/hydroxide group and allowing the metal ion to act as a Lewis acid, activating the coordinated nitrile toward nucleophilic attack (26). In this mechanism, the nucleophilic water/hydroxide is likely provided/activated by an active-site base. This mechanism was proposed based on observed changes in electronic absorption and EPR spectra upon the addition of nitriles (39). In addition, synthetic model studies have revealed that nitriles can readily exchange with low spin Fe(III) and Co(III) centers (40, 41).
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Examination of a plot of $\log(k_{cat})$ versus pH revealed a bell-shaped curve that yielded a $pK_{ES1}$ value of $5.9 \pm 0.1$ and a $pK_{ES2}$ value of $9.2 \pm 0.1$ ($k_{cat} = 130 \pm 1.0 \text{ s}^{-1}$). The slopes of the asymptotes, calculated as described previously (30), of the acidic and basic limbs of $\log(k_{cat})$ versus pH for PtNHase are 1, indicating that one group is ionized on each limb. These data indicate that one ionizable group ($pK_{ES1}$) must be deprotonated and that a second ionizable group must be in the protonated form ($pK_{ES2}$) in the ES complex for catalysis to occur. Assignment of the observed $pK_{ES}$ values is difficult, but likely candidates for $pK_{ES1}$ are the deprotonation of the metal-coordinated sulfinic acid (putative $pK_a$ in NHase of 7.6) (24) and the protonation of the leaving group or an active-site residue such as $\beta$Tyr$^{68}$. Based on the temperature dependence of the ionization constant, the most likely assignment for $pK_{ES1}$ is $\beta$Tyr$^{68}$. The observed $pK_{ES1}$ value cannot be the metal-bound water molecule because ENDOR data recorded in both $^1$H$_2$O and $^2$H$_2$O as well as in $^{17}$O-labeled water on the iron-type NHase from *Brevibacterium* sp. strain R312 indicate that a water molecule is bound to the metal center at pH 7.5 (45). The observed $pK_{ES2}$ value may be due to the deprotonation of a conserved active-site arginine residue ($\beta$Arg$^{52}$ or $\beta$Arg$^{157}$) that forms a hydrogen bond with both the sulfenic and sulfonic acid ligands of the active site (20). Alternatively, the $pK_{ES2}$ value may be due to the deprotonation of $\alpha$Ser$^{112}$ or the metal-bound water molecule depending on which proposed mechanism is active. Based on the temperature dependence of the ionization constant, the second ionization constant $pK_{ES2}$ is best assigned to either an active-site arginine residue or $\alpha$Ser$^{112}$.

Enzyme-centered ionizable groups were gleaned from plots of $\log(k_{cat}/K_m)$ versus pH because it is possible to determine $pK$ values centered on the free enzyme and free substrate (46). Similar to the plots of $\log(k_{cat}/K_m)$ versus pH, fits of $\log(k_{cat}/K_m)$ versus pH provided a $pK_{ES1}$ value of $5.8 \pm 0.1$ and a $pK_{ES2}$ value of $9.1 \pm 0.1$ ($k_{cat}/K_m = (6.5 \pm 0.1) \times 10^3 \text{ s}^{-1} \text{ mm}^{-1}$). Both asymptotes have slopes of 1, indicating that a single ionizable group exists at both high and low pH values. Similar to $pK_{ES1}$, the $pK_{ES2}$ value is most likely due to $\beta$Tyr$^{68}$, but could also be due to the deprotonation of the metal-coordinated sulfinic acid. Moreover, similar to $pK_{ES2}$, the enzyme-centered $pK_{ES2}$ value is most likely due to $\alpha$Ser$^{112}$, but may also be due to $\beta$Arg$^{52}$ or $\beta$Arg$^{157}$, all of which are strictly conserved.

A very fundamental aspect of the catalytic mechanism of NHases that has not been addressed to date is the chemical identity of the rate-limiting step. Kinetic isotope effect studies are an excellent way to gain an understanding of the nature of the rate-limiting step as well as to probe the transition state of catalytic reactions (47). Primary isotope effects are observed if a bond to the labeled atom is made or broken during the reaction, whereas secondary isotope effects describe processes at other positions. Therefore, we examined the solvent isotope effect of PtNHase using benzonitrile as the substrate at pH 7.6 ($p^2$H/H 1.0 m meter reading + 0.4) (27) by substituting hydrogen ($^1$H) with deuterium ($^2$H). The intrinsic primary isotope effect ($k_{H} / k_{D}$) is related to the symmetry of the transition state for that step (i.e. the larger the isotope effect, the more symmetrical the transition state), with the theoretical limit being 9 at 37 °C in the absence of tunneling effects. For the simplest case, in which...
a single proton produces the solvent isotope effect, a plot of the atom fraction of deuterium versus $V_n/V_D$ would be linear, where $V_n$ is the $k_{cat}$ at a particular fraction of deuterium and $V_D$ is the $k_{cat}$ in buffer containing 100% deuterium oxide (33). The presence of D$_2$O lowers the catalytic activity of PtNHase, resulting in a solvent isotope effect of 2.07. This normal isotope effect suggests that an oxygen–hydrogen bond is broken in the rate-limiting step. For PtNHase, $V_n/V_D$ deviates from linearity, and the best fit indicates that two protons are transferred during catalysis with similar fractionation factors (0.66 and 0.68). Analysis of the midpoint solvent isotope effect also supports involvement of two protons in the reaction. These data may reflect the transfer of a proton from an active-site water molecule to an active-site base to form a more nucleophilic hydroxide and the transfer of a proton from the hydroxyl group of the imine intermediate to form the amide. Conversely, they may represent the transfer of a proton from the hydroxyl group of the imine intermediate to an active-site base, followed by the transfer of a proton from a base to the imine to form the amide. The first fractionation factor ($\phi_f = 0.66$) is characteristic of a proton–oxygen bond (neutral oxygen, 0.8–1.2) with a conventional isotope effect equal to 1 (32). It is likely that, at pH 7.6, the protonation state of $\beta$Trp$^{68}$ (p$K_{a}$ in the enzyme-substrate complex of 5.9 ± 0.1) results in the transfer of a proton to $\alpha$Ser$^{112}$, which eventually donates that proton to the leaving group.

The rate-limiting step in the catalytic reaction is important in understanding the hydration of benzonitrile by PtNHase. Because PtNHase is stable at 60 °C, PtNHase provides the unique opportunity to determine the activation parameters of the ES$^g$ complex over a wide temperature range. Construction of an Arrhenius plot for the hydration of benzonitrile by PtNHase indicates that the rate-limiting step does not change as a function of temperature (38). The $E_a$ for the activated ES$^g$ complex is 23.0 ± 0.12 kJ/mol for PtNHase, which is 60% of the value reported for the iron-type NHase from Brevibacterium imperialis CBS 489-74 (38.4 kJ/mol), suggesting that transition state formation is more viable for the Co(III) enzyme (48). The enthalpy of activation calculated over the temperature range 20–60 °C is 18.0 ± 0.9 kJ/mol, whereas for PtNHase at 25 °C is $-146.0 \pm 0.7$ J/mol. The positive enthalpy is indicative of a conformation change upon substrate binding, likely due to the energy of bond formation and breaking during nucleophilic attack on the scissile carbon–nitrogen triple bond of the substrate. On the other hand, the negative entropy suggests that some of the molecular motions are lost upon ES$^g$ complex formation, possibly due to hydrogen bond formation between catalytically important amino acids, water molecules, and the substrate. This is consistent with the proton inventory data obtained at pH 7.6, where multiple proton exchanges take place. All of these factors contribute to the positive free energy of activation. Because the $K_m$ also increases with temperature, the thermodynamic parameters for the formation of the Michaelis complex at 25 °C were also determined. The observed negative $\Delta G^o$ value indicates that the formation of the ES complex is thermodynamically favorable and that the reaction catalyzed by PtNHase is slightly exothermic. However, $\Delta S^o$ was found to be negative, suggesting the ES complex is highly
process is likely the observed $pK_a$ value of 5.9. That $\beta$Ty68 plays an important catalytic role is consistent with the observed 17-fold decrease in $k_{cat}$ when $\beta$Ty68 is substituted with Phe (20). The fact that $K_m$ also increases by >10-fold upon substitution of $\beta$Ty68 with Phe indicates that $\beta$Ty68 also functions to bind and position substrate, likely stabilizing the transition state. Because the observed 17-fold decrease in $k_{cat}$ when $\beta$Ty68 is substituted with Phe is not that expected for a general base, the strictly conserved residue $\alpha$Ser112, which is also part of the proposed catalytic triad, likely functions as the general base by deprotonating $\beta$Ty68. Displacement of the metal-bound water molecule by a nitrile will activate the carbon–nitrogen bond toward nucleophilic attack and likely place the water molecule in the proper orientation, with regard to the catalytic triad and the carbon–nitrogen bond, for the addition of an oxygen–hydrogen bond across the carbon–nitrogen bond. Based on our preliminary isotope studies, two protons are transferred in the transition state, which we propose is due to a water proton being transferred to the nitrile nitrogen atom and the second to $\beta$Ty68, consistent with our observed normal isotope effect. Once proton transfer occurs, the resulting imidate can tautomerize to form an amide with a subsequent proton transfer from $\alpha$Ser112, which functions to shuttle protons from $\beta$Ty68. Finally, the amide product can be displaced by a water molecule, providing the regenerated catalyst.

Acknowledgment—We thank Dr. Alvan C. Hengge for helpful discussions.

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