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Inorganic Chemistry Article

Theoretical Investigation of the First-Shell Mechanism of Nitrile Hydratase

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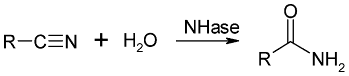
The first-shell mechanism of nitrile hydratase (NHase) is investigated theoretically using density functional theory. NHases catalyze the conversion of nitriles to amides and are classified into two groups, the non-heme Fe(III) NHases and the non-corrinoid Co(III) NHases. The active site of the non-heme iron NHase comprises a low-spin iron ($S = {}^1/_2$) with a remarkable set of ligands, including two deprotonated backbone nitrogens and both cysteine–sulfenic and cysteine–sulfinic acids. A widely proposed reaction mechanism of NHase is the first-shell mechanism in which the nitrile substrate binds directly to the low-spin iron in the sixth coordination site. We have used quantum chemical models of the NHase active site to investigate this mechanism. We present potential energy profiles for the reaction and provide characterization of the intermediates and transition-state structures for the NHase-mediated conversion of acetonitrile. The results indicate that the first-shell ligand Cys114-SO^- could be a possible base in the nitrile hydration mechanism, abstracting a proton from the nucleophilic water molecule. The generally suggested role of the Fe(III) center as a Lewis acid, activating the substrate toward nucleophilic attack, is shown to be unlikely. Instead, the metal is suggested to provide electrostatic stabilization to the anionic imidate intermediate, thereby lowering the reaction barrier.

Introduction

The family of nitrile hydratases (NHases) catalyzes the conversion of nitriles to the corresponding amides (Scheme 1). NHases have received increasing interest during recent years because of their extraordinary properties and their usefulness as biocatalysts in the industrial preparation of acrylamide.[1−3]

The NHase family can be divided into two groups, the non-heme iron-containing NHases and the non-corronoid cobalt-containing NHases. The crystal structures of four NHases have been determined so far, and the results have been intriguing.[4−8] Both the iron and cobalt NHases exhibit a peculiar octahedral coordination sphere around the metal ion, consisting of two *deprotonated* backbone amide nitrogens and three cysteinates (Cys109, Cys112, Cys114; Figure 1; residue numbering as in NHase from *Rhodococcus erythropolis* sp. N-771). Two of the cysteine residues, Cys112 and Cys114, are post-translationally modified to cysteine−sulfinic acid and cysteine−sulfenic acid, respectively.[5,6] This oxidation seems to be essential for the catalytic activity of NHase.[9,10] In the inactivated form of the iron NHase, the sixth coordination site on the metal ion is

Scheme 1. Reaction Catalyzed by NHase.



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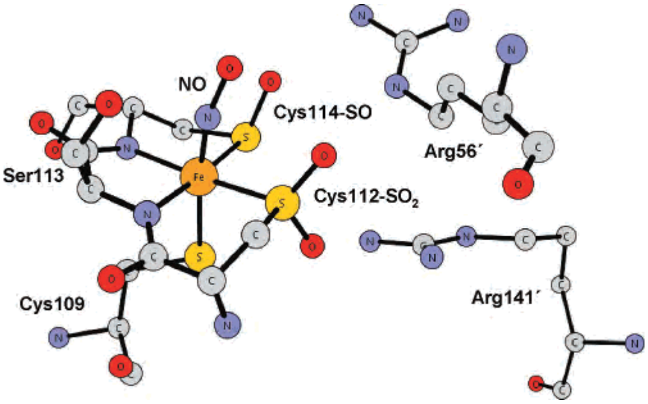

Figure 1. Important residues in the active site of the NO-inactivated iron-containing NHase from *R. erythropolis* sp. N-771 (from X-ray crystal structure 2AHJ[5]).

occupied by a NO molecule. Upon light irradiation, NO dissociates and the activity of NHase is restored.[1,11,12] It is not clear what constitutes the sixth ligand in the active form of NHase; a water or a hydroxide ligand is usually suggested to occupy this position.[4,6−8] This water/hydroxide ligand could possibly be displaced by the nitrile substrate during nitrile hydration.[4,13]

Theoretical calculations have provided significant insight into the unique electronic structure of the NHase active site.[13−19] INDO/S−PUHF (Projected Unrestricted Hartree−Fock) calculations on a series of Fe(III) complexes with different ligands indicated that the deprotonated amide ligands play a role in stabilizing the low-spin Fe(III) ground state of the iron center.[14] Calculations on two low-spin Fe(III) model complexes using either density functional theory (DFT) (BP86VWN) or ROHF−CISD (restricted open-shell Hatree−Fock−configuration interaction including singlet and doublet excitations) showed that the specific orientation of the thiol ligands is crucial for stabilization of the low-spin state.[20] Various inorganic complexes have also been studied using the hybrid density functional method B3LYP, and it was shown that B3LYP successfully reproduces structural properties and correct spin states for Fe(III) complexes aimed at modeling the NHase iron site.[15,16,18,19] B3LYP calculations on models of possible intermediates in the NHase-mediated hydration of acetonitrile indicated that direct coordination of the substrate to the iron center is a possible part of the reaction mechanism.[13]

Synthesis of various Fe(III) and Co(III) complexes with different S/N ligands has also helped to understand the interesting ligand field in the NHase active site.[21−26] However, attempts to synthesize complexes that mimic the catalytic activity of NHase have shown only limited success so far.[26−28] The design of biomimetic complexes of NHase is complicated by the fact that the precise reaction mechanism for nitrile hydration is not known. The very negative reduction potentials of NHase and various Fe(III) model complexes indicate that nitrile hydration occurs through a non-redox reaction mechanism.[22,23,29]

One popular mechanistic proposal for NHase is the first-shell mechanism, in which the substrate coordinates directly to the iron center at the sixth coordination site.[4,27,28] In this mechanism, the Lewis acidity of the iron is suggested to activate the nitrile carbon toward nucleophilic attack.[4,18,28] In general, the presence of an active-site base that abstracts a proton from the attacking water molecule has been invoked, but to our knowledge, no possible candidates for this base have been proposed.

In the present Article, we have investigated the proposed first-shell mechanism with the help of quantum chemical models of the NHase active site. Calculations are performed with the hybrid DFT method B3LYP. DFT, in particular the B3LYP functional, has been used in numerous studies of enzyme active sites containing transition metals, often with great success in explaining reaction mechanisms.[30]

Computational Details

All of the calculations were performed using the hybrid density functional theory (DFT) method B3LYP,[31] as implemented in *Gaussian 03*.[32] For the reference reaction of acetonitrile in water, that is, the nonenzymatic reaction, geometries were optimized in vacuo at the B3LYP/6-31G(d,p) level. Single-point calculations at the B3LYP/6-311+G(2d,2p) level were performed on the optimized geometries to obtain more accurate energies. For the NHase active-site models, geometry optimizations were performed in vacuo with the LANL2DZ basis set, using UB3LYP and a multiplicity of 2 (unless otherwise stated). Single-point calculations using the 6-311+G(2d,2p) basis set were also performed to obtain more accurate energies.

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To approximately account for the effects of the surrounding medium, solvent corrections were calculated as single points at the B3LYP/6-31G(d,p) level using the conductor-like polarizable continuum (CPCM)[33] model. A dielectric constant of $\epsilon = 80$ was used for the reference reaction of acetonitrile in water and $\epsilon = 4$ for the active-site models.

Frequency calculations were performed at the same level of theory as the geometry optimizations to obtain zero-point vibrational energies (ZPVE) and to confirm the nature of the various stationary points. The latter implies no imaginary frequencies for minima and one imaginary frequency for transition states. In model B of the NHase active site, fixing some atoms to their crystallographically observed positions gave rise to a few small negative eigenvalues for the optimized structures. These are very small, on the order of -10 to -20 cm^{-1}, and do not affect the obtained energetic results. One consequence, however, is that the entropic contributions cannot be estimated properly and were thus omitted. These effects are not expected to change the results significantly. The final energies reported below are obtained from the large basis-set calculations, corrected for ZPVE and solvation effects.

Active-Site Models. Two different models of the NHase active site were prepared on the basis of the X-ray crystal structure (2AHJ; Figure 2). Model A is composed of the iron center and parts of the first-shell ligands Cys109, Cys112, Ser113, and Cys114. In the crystal structure, Cys112 is observed as Cys112-sulfinic acid, Cys112-SO$_2$(H), while Cys114 is observed as Cys114-sulfenic acid, Cys114-SO(H). The protonation states of the oxidized cysteines in NHase have not been determined conclusively.[17,34] In model A, both oxidized residues were protonated so that the final overall charge of the model is zero. Model A also contains two water molecules as well as the acetonitrile substrate. During calculations, no atoms were fixed in model A. This minimal model was specifically designed to test the hypothesis of the Lewis acidity of the iron center.

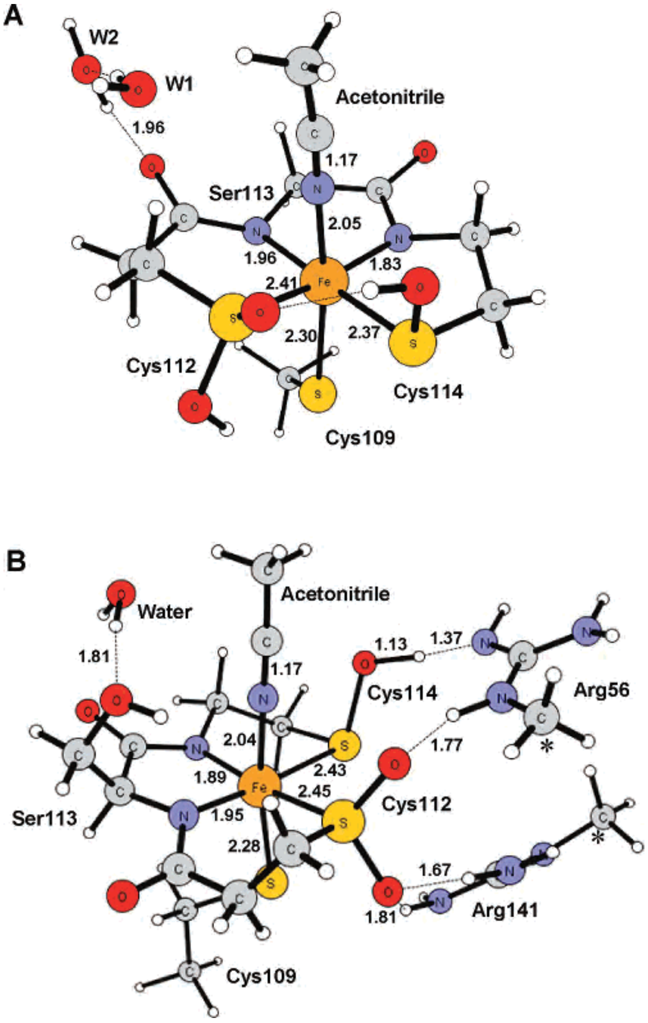

Figure 2. Quantum chemical models of the NHase active site: (A) optimized reactant geometry of model A and (B) optimized reactant geometry of model B. Distances are in angstroms. Asterisks (*) indicate atoms fixed to their crystallographically observed position in calculations.

The somewhat larger model B further includes the entire side chain of Ser113 and models of the two positively charged Arg56 and Arg141 residues in the second shell. Both of these residues form hydrogen bonds to the oxidized cysteines, and Arg56 has been shown to be essential for catalytic activity.[35] It is of interest to examine whether the presence of Arg56 in the active-site model would have an impact on the computed energetics. In the geometry optimizations, both arginine residues in model B were anchored at the truncation carbon to their crystallographically determined positions to preserve the spatial arrangement observed in the crystal structure. These carbons are indicated by asterisks (Figure 2B). Cys112-SO$_2^-$ and Cys114-SO$^-$ were kept in the deprotonated form in model B, which leads to an overall charge of zero.

NHase has a broad substrate specificity and accepts a variety of different aliphatic nitriles, including acetonitrile.[36−38] To model the reaction mechanism of NHase, we used acetonitrile as the substrate,

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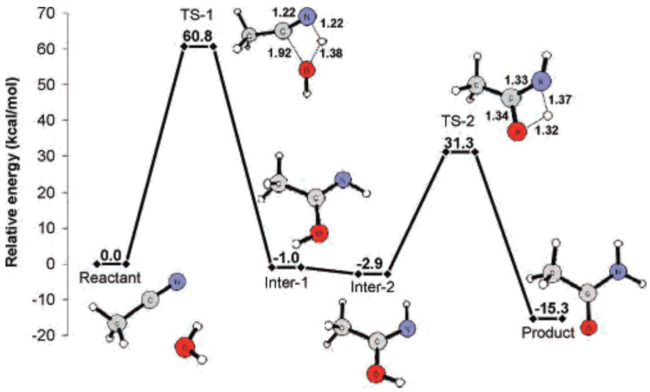

Figure 3. Direct unassisted hydration of acetonitrile to acetamide.

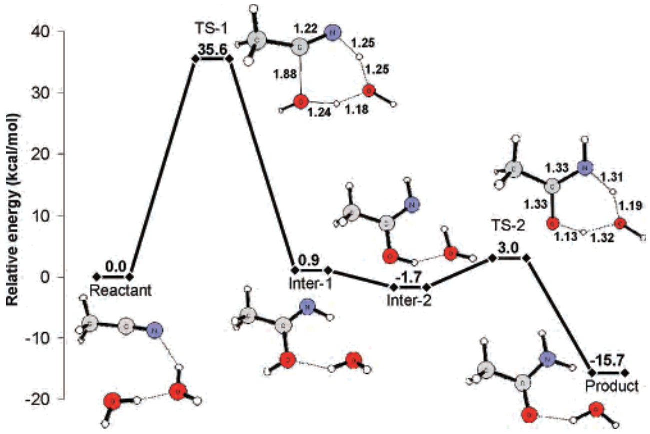

Figure 4. Water-assisted hydration of acetonitrile to acetamide.

which was placed in the sixth coordination position instead of the NO molecule observed in the crystal structure.

Results and Discussion

Before a discussion of the results of the NHase active-site models, it is interesting to study the hydration of acetonitrile in water. The direct unassisted attack of a water molecule on acetonitrile was calculated to have a potential energy curve as displayed in Figure 3. In the first step, the water molecule performs a nucleophilic attack on the nitrile carbon, concerted with the transfer of a proton to the nitrile nitrogen. This step has a calculated barrier as high as 60.8 kcal/mol, and the resulting imidate intermediate (Inter-1) has an energy of −1.0 kcal/mol, making this reaction step almost thermoneutral. The protons on the formed intermediate have to flip to allow for the next step of the reaction, but such flipping can be assumed to occur very fast. The imidate can then tautomerize to give the final amide product in the second step, which has a calculated barrier of 34.2 kcal/mol. The very high barriers are partly due to the large strain at the two transition states, both of which are forced to form highly strained four-membered rings. The strain at the transition state can be alleviated by the introduction of a second water molecule that assists the reaction. Geometries and energies for a water-assisted hydration of acetonitrile are shown in Figure 4. The transition states now form more favorable six-membered rings, and the barriers for both steps are reduced significantly to 35.6 kcal/mol for the first step and only 4.7 kcal/mol for the second step. Introduction of additional water molecules is not expected to lower the barriers any further, and the calculated values of the water-assisted hydration (Figure 4) can thus be considered to represent the barriers for the uncatalyzed nitrile hydration. Experimentally, the NHase-catalyzed nitrile hydration shows barriers on the order of 13−15 kcal/mol.[6,39,40] For the iron-containing *B. imperialis* NHase, an experimental activation energy of 13.8 kcal/mol has been measured for the hydration of acrylonitrile.[39] For the *R. erythropolis* sp. N-771 enzyme, a k_{cat} of 917 s^{-1} has been observed for hydration of methacrylonitrile.[6] Using classical transition-state theory, this can be converted to a barrier of ca. 13 kcal/mol. Also, the cobalt NHases exhibit rate constants on the order of 131−1910 s^{-1} for various nitrile substrates, corresponding to activation barriers of ca. 13−15 kcal/mol.[40] Thus, the calculated barrier of 35.6 kcal/mol for the uncatalyzed hydration of acetonitrile has to be lowered by around 20 kcal/mol by the enzyme to approach the experimentally determined activation energies of NHase. It is not clear how NHase is able to lower the barrier for nitrile hydration, but different general strategies can be envisioned. One possibility is the activation of the substrate to facilitate the attack by water. In the proposed first-shell mechanism, the substrate could be activated through coordination to the iron center. Charge transfer from the substrate to the iron could increase the electrophilicity on the nitrile carbon, thus making the attack here more efficient. Activation of nitriles through coordination to metal ions is a well-known concept in organic chemistry.[41] One example is the Zn(II)-catalyzed tetrazole formation from the addition of azide to organic nitriles.[42,43] Theoretical investigations of the intramolecular [2 + 3] cycloaddition of azide and nitrile showed that coordination of the nitrile group to the Lewis acid Zn(II) reduces the barrier by 5.3 kcal/mol compared to the reaction without zinc.[43] Could the iron cluster of NHase also function as a Lewis acid, activating the substrate toward attack? It has been argued that a low-spin Fe(III) ion in an electron-rich ligand field with five anionic ligands will be a poor Lewis acid.[20,28] However, several factors have been suggested that might enhance the Lewis acidity of the Fe(III) in NHase, including oxidation of Cys112 and Cys114 to sulfenic and sulfinic acids, hydrogen bonding of the cysteine residues to the positively charged arginine residues Arg56 and Arg141, and tautomerization of the deprotonated backbone amides to imidates.[13,28]

Another strategy to lower the barrier for the first reaction step could be through activation of the nucleophile. For example, the nucleophilicity of the water could be increased through abstraction of a proton by an active-site base. Although the involvement of an active-site base has been

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proposed in various mechanisms,[4,28] so far no residue has been identified that can function as such a base. As follows, we have tested whether NHase could lower the barrier for nitrile hydration by employing one of these suggested strategies.

The NHase active-site model A (above) was specifically designed to isolate and investigate the proposed Lewis acidity of the iron atom. This model contains the first-shell ligands to the iron atom with the nitrile coordinated in the sixth position. The preferred spin state of model A was found to be a doublet, in agreement with the experimental observations.[44] The quartet and the high-spin sextet states of model A were higher in energy by 2.6 and 4.7 kcal/mol, respectively. These energies are in line with previous theoretical calculations on a NHase active-site model with direct coordination of acetonitrile to the iron atom.[13] In those calculations, the energy gaps between the doublet state and the quartet and sextet states were found to be 3.0 and 5.0 kcal/mol, respectively, using B3LYP, whereas the BLYP functional yielded gaps of 11.2 and 21.3 kcal/mol, respectively.[13]

In the optimized reactant state of model A (Figure 2A), the nitrile substrate is coordinated to the iron center with a distance of 2.05 Å. This distance changes only slightly to 2.07 Å at the transition state for nucleophilic attack by water on the nitrile carbon (Figure 5A). The distance between the oxygen of the attacking water molecule (W1) and the nitrile carbon is calculated to be 1.69 Å. The second water molecule (W2) facilitates the proton transfer from the nucleophilic water molecule to the substrate nitrogen in the same way as the water-assisted reaction discussed above. The resulting intermediate is shown in Figure 5B. The difference between the uncatalyzed reaction and model A is thus the coordination of the substrate to the iron atom. If this coordination activates the substrate toward attack, this should be reflected in a reduced barrier. Instead, the barrier for nitrile hydration in model A is calculated to be 42.9 kcal/mol (Table 1), which is even higher than the barrier observed for the uncatalyzed reaction in water (35.6 kcal/mol). This demonstrates that coordination to the iron center does not activate the nitrile toward nucleophilic attack.

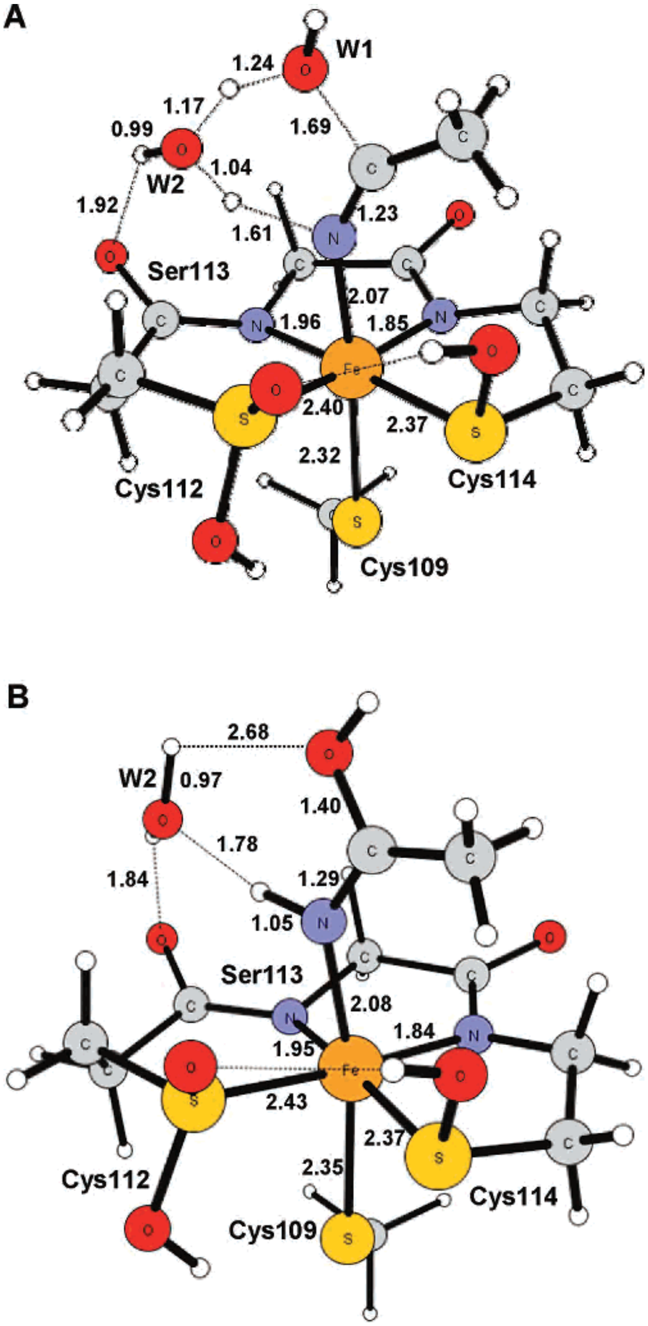

Figure 5. Optimized geometries for model A: (A) transition state for nucleophilic attack by water and (B) intermediate.

Furthermore, we have prepared a larger model of the NHase active site, referred to as model B (Figure 2B). In this model, the side chain of Ser113 is kept because its position indicates that it could assist in shuttling the water proton instead of the second water molecule. Also included in model B are the second shell-residues Arg56 and Arg141, which form hydrogen bonds to Cys112-SO_2^- and Cys114-SO^-.[34] The oxidized cysteine residues Cys112-SO_2^- and Cys114-SO^- were kept in the deprotonated states in model B, leading to an overall charge of zero. During geometry optimization of the reactant species (Figure 2A), one of the protons on Arg56 transferred automatically to the SO^- group of Cys114, rendering both residues uncharged. This proton transfer was also observed in previous quantum chemical calculations on different NHase models[13,17] and is consistent with spectroscopic data that support the presence of a protonated Cys114-SOH moiety in the NHase active site.[17] It is possible, however, that this proton transfer from Arg56 is an artifact of the relative smallness of the employed NHase model and might not occur when more parts are included that can provide proper solvation to the SO^-−Arg^+ ion pair. Cys112-SO_2^- remained unprotonated in our model and is found to form hydrogen bonds to both Arg56 and Arg114. The acetonitrile substrate is coordinated to the iron atom with a distance of 2.04 Å, similar to model A. The critical O−C distance at the transition state for nucleophilic attack is calculated to be 1.68 Å (Figure 6A), also very similar to model A. The Ser113 side chain facilitates proton transfer

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Table 1. Summary of the Computed Energies (kcal/mol) for the First Step of Acetonitrile Hydration, the Nucleophilic Attack of Water on the Nitrile Carbon

	barrier		intermediate	
model	gas phase	with CPCM solvation[a]	gas phase	with CPCM solvation[a]
without catalyst, one water	58.1	60.8	1.1	−1.0
without catalyst, two waters	37.8	35.6	5.4	0.9
model A	45.0	42.9	9.8	3.9
model B	40.2	38.7	4.6	−1.5
model B with Cys114-SO$^-$ as the base	23.6	20.2	22.3,[b] −10.6[c]	16.3,[b] −6.5[c]

[a] $\epsilon = 80$ for models without a catalyst and $\epsilon = 4$ for Models A and B. [b] Unprotonated intermediate. [c] Protonated intermediate.

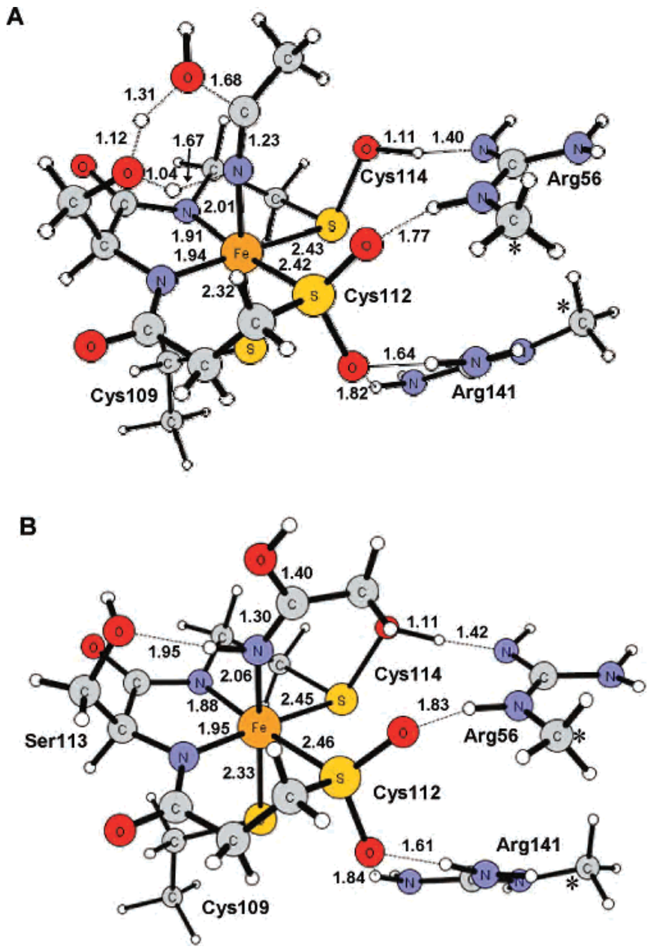


Figure 6. Optimized geometries for model B: (A) transition state for nucleophilic attack by water and (B) intermediate.

from the water molecule to the nitrogen atom of the nitrile substrate. The formed imidate intermediate remains coordinated to the iron atom with a distance of 2.06 Å (Figure 6B). The computed energies for model B are shown in Table 1. The barrier for nucleophilic attack is 38.7 kcal/mol, which is slightly lower than that for model A but still higher than the barrier calculated for the uncatalyzed water-assisted reaction. The resulting intermediate of model B has a calculated energy of −1.5 kcal/mol compared to that of the reactant.

We also optimized the transition-state structures of model B in the quartet and sextet states to investigate whether a change in the spin state might provide a reduction of the barrier. However, for both cases, the observed energies were even higher than that in the doublet state. The barriers were calculated to be 42.1 and 42.6 kcal/mol for the quartet and sextet states, respectively, relative the doublet reactant. A change in the spin state during the reaction is thus not expected (the optimized geometries for the transition state of model B in the quartet and sextet states can be found in the Supporting Information section, Figure S1).

The results of models A and B show thus that the low-spin Fe(III) center is a poor Lewis acid that cannot activate the nitrile toward nucleophilic attack. As noted above, another way to lower the barrier for nitrile hydration could be through activation of the nucleophile, for example by abstraction of a proton from the attacking water molecule by an active-site base. One possible base is the Cys114-sulfenate. We tested this possibility by performing calculations using model B. The transition state for the concerted water attack on the nitrile carbon and proton abstraction by Cys114-SO$^-$ can be seen in Figure 7A (the reactant species of this reaction is shown in the Supporting Information section, Figure S2). At the transition state, the O−C distance is 1.79 Å, and the water proton is located at a distance of 1.25 Å from the water oxygen and 1.16 Å from Cys114-SO$^-$. Because no proton is transferred to the nitrile nitrogen, the resulting imidate is anionic (Figure 7B). Analysis of Mulliken atomic charge populations show that the charge on the substrate nitrogen has changed from +0.11 in the reactant to −0.24 in the intermediate structure. The length of the N−Fe bond has changed from 2.03 Å in the reactant to 1.89 Å in the unprotonated intermediate. Interaction with the Fe(III) ion thus stabilizes the negative charge of the intermediate. The energies computed for this mechanism are shown in Table 1. The barrier for water attack on the nitrile carbon is now significantly reduced to 20.2 kcal/mol, which indicates that activation of water by Cys114-SO$^-$ could be a possible strategy of NHase to lower the barrier for nitrile hydration.

The unprotonated intermediate species has an energy of +16.3 kcal/mol compared to the reactant state (Table 1). In the next step, the proton on Cys114-SOH is transferred to the imidate nitrogen, leading to the protonated imidate intermediate (Figure 7C), which lies 6.5 kcal/mol lower than the reactant species, that is, 22.8 kcal/mol below the unprotonated intermediate (Table 1). This step is thus very fast, and no transition state could be located. Protonation of the nitrogen atom increases the bond distance from imidate to iron to 2.03 Å (Figure 7C). The final step of the reaction is the tautomerization of the intermediate to yield the amide product. This can easily take place either inside or outside the NHase active site, as demonstrated by the calculations of the uncatalyzed reaction (Figure 4).

As noted above, the experimentally determined rate constants of NHase can be translated into activation barriers in the range of 13−15 kcal/mol.[6,39,40] This is somewhat less than the rate-limiting barrier of 20.2 kcal/mol calculated for

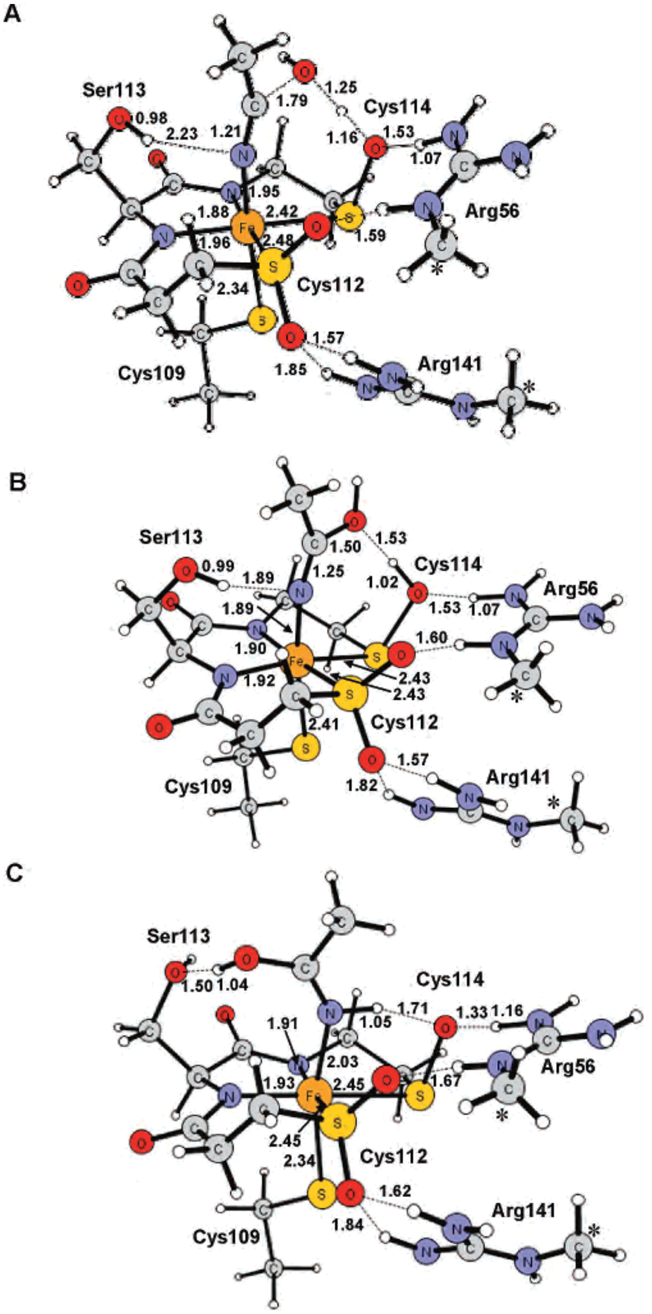
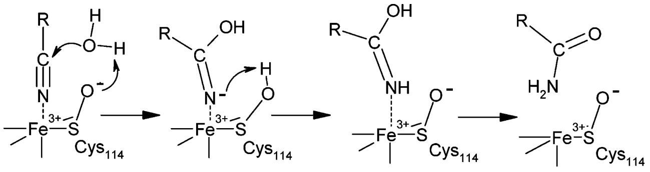


Figure 7. Cys114-SO$^-$ as the catalytic base: (A) concerted transition state for nucleophilic attack and proton abstraction, (B) unprotonated intermediate, and (C) protonated intermediate.

the Cys114-SO$^-$-assisted mechanism (Table 1). It can be envisioned that inclusion of additional elements in the NHase active-site model might reduce the calculated barrier further. For example, mutation of the conserved second-shell residue Tyr68 in the cobalt-containing NHase from *Pseudonocardia thermophila* JCM 3095 has been shown to reduce the rate of acrylonitrile hydration from 1910 to 15.2 s^{-1},[40] corresponding to a barrier increase of roughly 3 kcal/mol. The role of Tyr68 (corresponding to Tyr72 in iron NHase from *R. erythropolis* sp. N-771) is not clear, but a larger model including this residue might establish its role and provide a barrier reduction of several kilocalories per mole.

Scheme 2. A Possible First-Shell Mechanism for NHase-Mediated Nitrile Hydration.



Conclusions

In the present paper, we have investigated the proposed first-shell mechanism of NHase using the DFT method B3LYP. Potential energy curves were calculated for different quantum chemical models of the NHase active site, and the results are summarized in Table 1.

The calculations show that the low-spin Fe(III) center is a poor Lewis acid that does not provide any activation of the nitrile substrate toward nucleophilic attack by water. Instead, the calculations indicate that Cys114-SO$^-$ might function as a catalytic base in the NHase mechanism, abstracting a proton from the attacking water molecule (Scheme 2). A direct involvement of Cys114-SO$^-$ in the reaction mechanism is consistent with experimental data that established Cys114-SO$^-$ as essential for NHase catalytic activity.[9,10] In such a mechanism, the role of the metal ion is to provide electrostatic stabilization to the imidate intermediate, thereby lowering the reaction barrier for the nucleophilic attack.

However, the calculated rate-limiting barrier for this mechanism (20.2 kcal/mol) is 5−7 kcal/mol higher than the experimentally determined barriers for various substrates. Several sources of error can be envisaged here. As always in computational studies, the computational method (in this case the DFT/B3LYP functional and the employed basis sets) cannot be ruled out as a possible source of error. The B3LYP functional is widely used in studies of enzymatic reaction mechanisms, and for systems containing transition metals, it has an estimated error of 3−5 kcal/mol, often overestimating barriers.[45] Another source of error could be the size of the model used in the investigations. Inclusion of other second-sphere residues of the active site might lead to a lower reaction barrier. Finally, it is also possible that this mechanism is not the correct one and that nitrile hydration occurs in a different way in NHase, for example in a second-sphere mechanism. At present, this possibility cannot be ruled out. Further calculations and experiments are necessary to shed more light on these issues. Investigations along these directions are currently underway.

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Supporting Information Available: Optimized geometries for the quartet and sextet states of model B and the reactant structure of model B with the water hydrogen-bonded to Cys114-SO$^-$. This material is available free of charge via the Internet at http://pubs.acs.org.

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