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Theoretical Investigation of the Second-Shell Mechanism of Nitrile Hydratase

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Nitrile hydratases (NHases) are biocatalytically important enzymes that are utilized in the industrial production of acrylamide and nicotinamide. There are two different classes of NHases, harbouring either a low-spin Fe^{III} or a low-spin Co^{III} ion in the active site, in each case with the same peculiar set of ligands, involving deprotonated backbone amides and oxidized cysteine residues. The detailed reaction mechanism of NHase has not been established yet, but different proposals have been put forward. Depending on the binding site of the substrate, these can be divided into first-shell and second-shell mechanisms, respectively. Recently, we have investigated different first-shell mechanisms using quantum-chemical active-site models based on the iron-dependent NHase (*Inorg. Chem.* **2007**, *46*, 4850). Here we continue our investigation of the NHase reaction by exploring two different variations of the second-shell mechanism of the iron-dependent NHase. In the first, a metal-bound hydroxide ion performs a nucleophilic attack on the nitrile substrate, while in the second investigated mechanism, the oxidized cysteine, Cys114-SO^-, acts as the nucleophile. We report energies, optimized transition state, and intermediate geometries for both investigated mechanisms. The calculated barriers are similar to the previously reported first-shell mechanism involving Cys114-SO^- as catalytic base.
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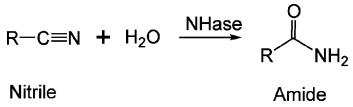
Introduction

Nitrile hydratases (NHases, EC 4.2.1.84) are bacterial enzymes that catalyze the conversion of nitriles to their corresponding amides (Scheme 1). They are of particular importance for the industrial production of acrylamide and nicotinamide.[1–3] Two different classes of NHases are recognized, the non-heme Fe^{III}-containing NHases and the non-corrin Co^{III}-containing NHases.[4,5] Despite the different metal preferences, the two classes exhibit very large similarities in primary and secondary structures, and are generally assumed to utilize the same reaction mechanism.[5,6]



Scheme 1. Nitrile hydration reaction catalyzed by NHases.

The X-ray crystal structures of several cobalt and iron NHases from different bacterial sources have been determined.[6–11] The metal ion was found to have an octahedral coordination with a peculiar set of ligands, involving two deprotonated backbone amides and three cysteine residues, two of which are post-translationally modified to cysteinesulfenic (Cys-SOH) and cysteinesulfinic (Cys-SOOH) acids (Figure 1).[8] The asymmetric oxidation of the cysteines seems to be essential for the catalytic activity of NHase,[12–15] because reconstitution of NHase under anaerobic conditions (resulting in lack of cysteine oxidation),[12] or oxidation of *both* cysteines to cysteinesulfinic acid, abolishes the catalytic activity of NHase.[14] The identity of the sixth metal ligand is not clear. In the inactive form of the iron-containing NHase, the sixth ligand is a nitric oxide molecule (Figure 1).[8] However, in the active form of NHase, the sixth ligand might be a water molecule or a hydroxide ion, or possibly the nitrile substrate, as will be discussed in more detail below.

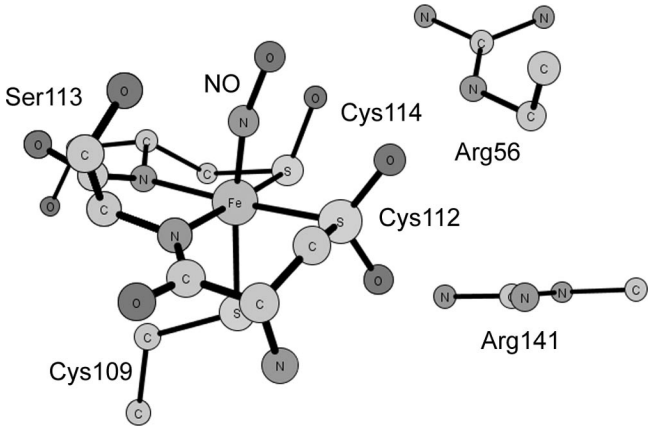


Figure 1. Active site in the inactive form of the NHase from *R. erythropolis*. N-771 (PDB 2AHJ).[8]

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The first-shell ligand sphere in NHase is absolutely conserved, and so are several second-sphere residues. These include two arginine residues, which are forming hydrogen bonds to the oxidized cysteines (Figure 1). Site-directed mutagenesis of one of these arginines, Arg56 (numbering as in NHase from *R. erythropolis* N-771), leads to lack of catalytic activity.[16] One possible explanation is that the arginine residues are essential for proper orientation of the oxidized cysteine residues.

NHase and model complexes aimed at mimicking the active site of NHase have been studied intensively both experimentally and theoretically.[17–54] However, the precise reaction mechanism for nitrile hydration remains elusive. Two classes of reaction mechanisms have been considered repeatedly in the literature.[3,7,17,50] These will here be referred to as the first-shell and second-shell mechanisms, respectively (Scheme 2). In the first-shell mechanism, the nitrile coordinates directly to the metal ion, which possibly acts as a Lewis acid, activating the substrate towards nucleophilic attack by water (Scheme 2, A). Several variants of the first-shell mechanism exist, which mainly differ in regard to the proposed identity of catalytic base that activates the water molecule.[51,54]

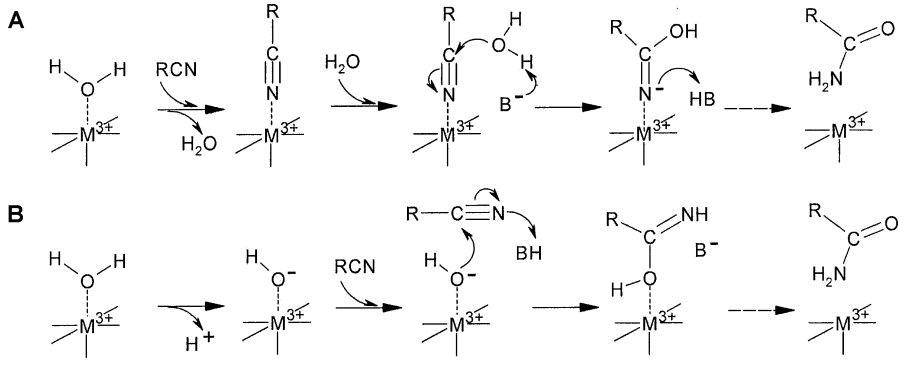
In the second-shell mechanism, the substrate does not interact with the metal atom. The sixth metal ligand is instead a hydroxide ion, which performs a nucleophilic attack on the nitrile (Scheme 2, B).[3,7,17] In an alternative variant of the second-shell mechanism, the nucleophile is not a hydroxide molecule but instead the oxidized Cys114-SO$^-$ residue, as discussed in more detail below.[17,55]

We have recently investigated the first-shell mechanism of NHase using density functional theory.[54] Calculations were performed using a quantum-chemical active-site model based on the iron-dependent NHase from *R. erythropolis*. N-771 (PDB 2AHJ).[54] It was shown that coordination of the substrate to the iron atom does not lower the barrier for nitrile hydration, indicating that the low-spin Fe^{III} center is a poor Lewis acid. The computed barrier for water attack on the metal-coordinated substrate was 38.7 kcal/mol, making this mechanism unlikely.[54] However, we showed that if Cys114-sulfenic acid activates the attacking water molecule, the barrier for the first-shell mechanism can be reduced dramatically to only 20.2 kcal/mol (Scheme 3).[54] The calculated barrier is somewhat higher than what different experimentally determined reaction rates indicate (employing classical transition-state theory, the experimental rates correspond to barriers of 13–15 kcal/mol),[9,52,56] and it therefore remains possible that this proposed NHase mechanism is not the correct one.

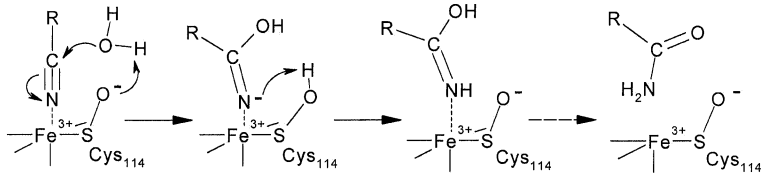
In this paper, we extend our studies of the iron-containing NHase by investigating two different variations of the second-shell mechanism. In the first variant, a metal-bound hydroxide ion is generated, which attacks the nitrile substrate. In the second mechanism investigated here, the attacking nucleophile is instead the sulfenate side chain of Cys114-sulfenic acid. Optimized geometries and energetics for both mechanisms are presented, and the likelihood of the studied mechanisms is discussed.

Computational Details

All calculations were performed using the hybrid density functional theory method B3LYP,[57] as implemented in Gaussian03.[58] Geometry optimizations were done in vacuo at the UB3LYP/LANL2DZ level, employing a multiplicity of 2. To avoid large artificial movements of the second-shell arginine residues in the model, the point of trun-



Scheme 2. Two possible reaction mechanisms of NHase. A: first-shell mechanism, involving direct metal coordination of the substrate; B: second-shell mechanism, in which a metal-coordinated hydroxide ion attacks the substrate in the second shell.



Scheme 3. Recently suggested variant of the first-shell mechanism of NHase, based on DFT calculations.[54]

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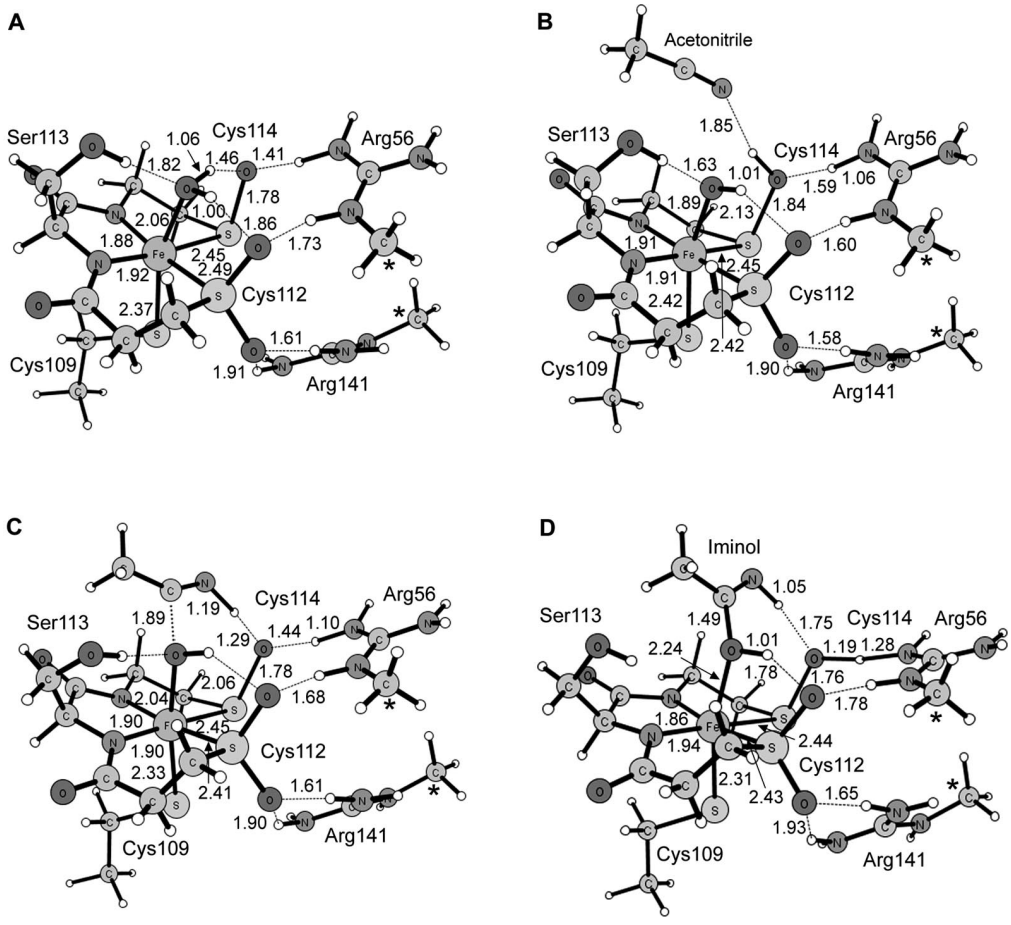

Figure 2. Optimized geometries for NHase-mediated hydration of acetonitrile using a second-shell mechanism. Distances are in Å. Asterisks mark atoms kept fixed to their crystallographically observed positions during geometry optimizations. A: reactant with metal-bound water; B: reactant complex; C: concerted transition state; D: iminol intermediate.

cation of each arginine residue was kept fixed to its crystallographically observed position during geometry optimizations (see Figure 2).

A homogeneous polarizable medium was employed to model the effect of the missing protein environment on the energetics of the reaction mechanism. Single-point calculations were performed on the optimized geometries using the conductor-like polarizable continuum model (CPCM)[59] with a dielectric constant of $\varepsilon = 4$. Zero-point vibrational energies were calculated by performing frequency calculations on all optimized geometries at the same level of theory as the geometry optimizations. In the frequency calculations, several stationary points exhibited one small negative eigenvalue (in the order of –10 to –20 cm^{-1}), which is due to the freezing scheme employed in geometry optimizations. However, this does not affect the obtained energetics and can thus be accepted.

The 6-311+G(2d,2p) basis set was employed to perform single-point calculations on the optimized geometries in order to obtain more accurate energies. The final energies reported here are the large-basis-set energies, corrected for CPCM and ZPVE effects.

Active-Site Models

The active-site model employed in this study is based on the X-ray crystal structure of NHase from *Rhodococcus erythropolis* N-771 (PDB 2AHJ).[8] The model is composed of the active-site iron atom, the first-shell ligands Cys109, Cys112, Ser113, and Cys114, and the second-shell ligands Arg56′ and Arg141′. The included residues were truncated as shown in Figure 2. The points of truncation of the second-shell arginine residues were kept fixed during geometry optimizations. A water molecule was manually placed in the sixth coordination site of the metal atom. The two oxidized cysteine residues, Cys114-sulfenic acid and Cys112-sulfinic acid, were modeled in their ionic forms. The protonation state of these cysteines in NHase has not been determined conclusively, but they are proposed to exist either both in their ionic state,[21] or as Cys112-SO_2^- and Cys114-SOH.[47]

In the calculations of the active-site model it was assumed that the iron is in the Fe^{III} state and that the overall multiplicity of the complex is 2, as demonstrated from experimental and theoretical results.[4,17,17,40,45,60]

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NHase has a broad substrate specificity, catalyzing the conversion of a variety of different aliphatic nitriles.[61–63] In the present investigation, the second-shell mechanism of NHase was studied with acetonitrile as substrate, as in our previous study of the first-shell mechanism.[54]

Results and Discussion

The active-site model of the iron–NHase was optimized with a water molecule placed in the sixth coordination site of the metal ion (Figure 2, A). In the optimized model, the two second-shell arginines are hydrogen-bonded to the oxidized cysteines. Arg141 forms two hydrogen bonds to Cys112-SO_2^- of lengths 2.59 Å and 2.77 Å, respectively (here hydrogen-bond length refers to distance between donor and acceptor atoms). An additional hydrogen bond is formed from the secondary amino group of Arg56 to Cys112-SO_2^- (2.70 Å), while one primary amino group of Arg56 is hydrogen-bonded to Cys114-SO^- (2.52 Å). The water molecule forms hydrogen bonds to the Ser113 hydroxy group and to both Cys112-SO_2^- and Cys114-SO^-. The lengths of these bonds are 2.81 Å, 2.72 Å, and 2.47 Å, respectively. The second-shell mechanism in Scheme 2, B proposes that prior to nitrile hydration, the metal-bound water molecule is converted to a hydroxide ion by removal of a proton. To the best of our knowledge, no suggestion has been put forward on how the formation of the hydroxide ion occurs. The optimized geometry indicates that Cys114-SO^- would be a good candidate to act as the base that deprotonates the metal-bound water. In the optimized active-site model, the distance from the sulfenate oxygen atom to the water proton is only 1.46 Å. The water O–H bond is elongated and has a length of 1.06 Å (Figure 2, A). However, every attempt to transfer a proton from the water molecule to the sulfenate side chain resulted in back-transfer of the proton to the water molecule. Only addition of the acetonitrile substrate to the active-site model enabled optimization of a structure, in which the proton had been transferred to the sulfenate side chain (Figure 2, B). In this structure, the former water proton is hydrogen-bonded to the nitrile nitrogen atom. Interestingly, the energy of this complex is very close to the combined energies of the separated nitrile and the active-site complex with water bound to the iron atom. The difference is only 0.3 kcal/mol, when solvation is considered (Figure 3).

In the next step of the proposed mechanism (Scheme 2, B), the metal-bound hydroxide ion attacks the nitrile substrate. The negative charge evolving on the nitrogen atom is suggested to be stabilized through proton transfer to the substrate (Scheme 2). The reactant complex (Figure 2, B) indicates that the protonated cysteinesulfenic acid could function as the proton donor. We optimized the concerted transition state for nucleophilic attack of the metal-bound hydroxide ion on the nitrile and the simultaneous proton transfer from sulfenic acid to the substrate (Figure 2, C). The barrier for this step (calculated with respect to the separated reactants) is 22.7 kcal/mol (Figure 3). At the transition state, the hydroxide ion is less tightly bound to the iron atom, with an oxygen–iron bond length of 2.04 Å compared to 1.89 Å in the reactant complex. The critical O–C bond length is 1.89 Å, while the proton originating from Cys114-sulfenic acid is 1.19 Å from the nitrile nitrogen atom.

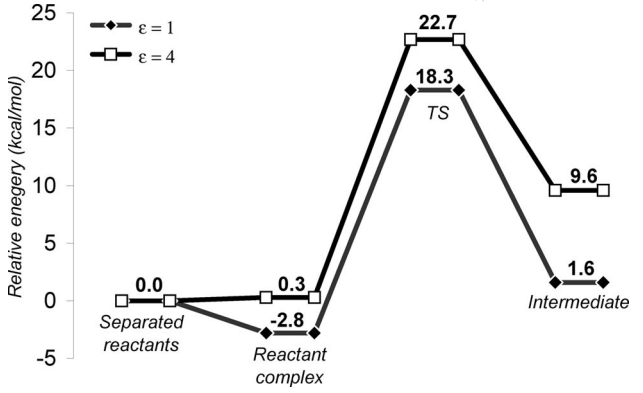
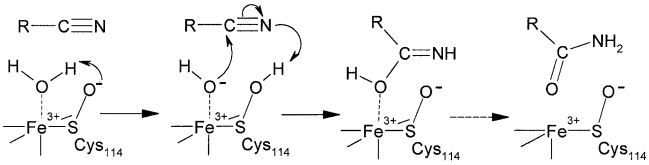


Figure 3. Computed energies for NHase-mediated hydration of acetonitrile through a metal-bound hydroxide ion.

The iminol intermediate formed upon the nucleophilic attack remains coordinated to the metal ion (Figure 2, D). However, the oxygen–iron bond is elongated from 1.89 Å in the reactant complex to 2.24 Å in the intermediate. The hydrogen bond from Ser113 to the hydroxide ion is broken. The computed energy of the intermediate is +9.6 kcal/mol compared to the separated reactants (Figure 3). The subsequent tautomerization of the iminol to the amide product was not studied with the NHase active-site model here. It can be envisioned that the iminol molecule dissociates from the metal ion and spontaneously tautomerizes to the amide product. Previous calculations have shown that this tautomerization reaction easily can proceed in water, with a barrier of only 4.7 kcal/mol.[54]

Scheme 4 summarizes the mechanism discussed above, while the energetic profile is shown in Figure 3. The calculated barrier for attack of the metal-bound hydroxide ion on the nitrile substrate (22.7 kcal/mol) is similar to the calculated barrier of the recently investigated first-shell mechanism of NHase (20.2 kcal/mol; Scheme 3).[54] Both mechanisms must thus be considered equally likely or unlikely at this point.



Scheme 4. Second-shell mechanism, involving attack of a metal-bound hydroxide ion on the nitrile, as investigated in this paper.

Another alternative second-shell mechanism suggests that the sulfenate side chain of Cys114-SO^- is the nucleophile that attacks the nitrile substrate. A mechanism of this kind has previously been suggested for a cobalt-containing active-site mimic of NHase, and could also be a possibility for the enzymatic reaction.[17,55] Here, we have investigated

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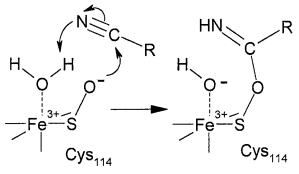
the first step of this mechanism, the attack of the sulfenate side-chain of Cys114-SO$^-$ on the nitrile substrate (Scheme 5). The same active site as above was employed, with water bound in the sixth coordination site of the iron complex (Figure 2, A). As above, the nucleophilic attack on the nitrile triple bond will lead to formation of a negative charge on the nitrogen atom, which requires stabilization through hydrogen bonding or proton transfer. In this case, an obvious proton donor would be the metal-bound water molecule. It can be assumed that the pK_a of this water molecule is lowered significantly through metal binding,[35] so that it can function as an acid here.



Scheme 5. Alternative second-shell mechanism involving sulfenic acid (Cys114-SO$^-$) as nucleophile.

We have optimized a concerted transition state, involving attack of the sulfenate oxygen atom on the nitrile carbon atom, combined with proton transfer from the water ligand to the substrate nitrogen atom (Figure 4, A; Scheme 5). The computed barrier is 22.2 kcal/mol (Figure 5), which is very similar to the barrier for the second-shell mechanism involving nucleophilic attack by the metal-bound hydroxide ion (22.7 kcal/mol; Figure 3). At the transition state, the distance from the attacking oxygen atom to the nitrile carbon atom is 1.77 Å. The Ser113 side-chain is hydrogen-bonded to the water molecule, which facilitates proton transfer to the substrate.

The resulting intermediate formed following the sulfenate attack was also optimized (Figure 4, B) and was calculated to have a relative energy of +9.9 kcal/mol compared to the reactant species (Figure 5). At the intermediate, the sulfur–oxygen bond is elongated to 2.03 Å (Figure 4, B), compared to 1.78 Å in the reactant (Figure 2, A). The following steps of this reaction mechanism must involve dissociation of the sulfur–oxygen bond in order to liberate the

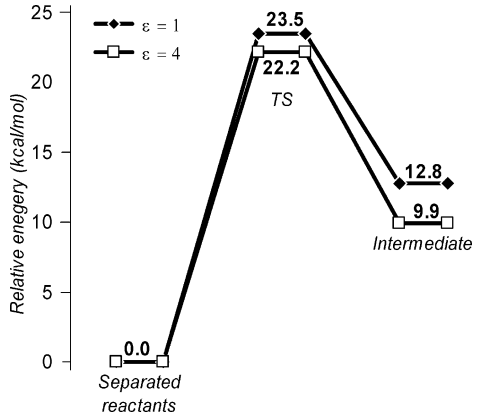


Figure 5. Computed energies with sulfenic acid (Cys114-SO$^-$) as nucleophile.

amide product. We have not considered this step explicitly in our calculations, but it can be envisioned that a hydroxide ion might attack the sulfur atom, thereby releasing the product.[16] This would at the same time result in the regeneration of the cysteinesulfenic acid. The metal-bound hydroxide ion could fulfil this role, either itself or through activation of another water molecule.

Conclusions

In this paper, density functional theory was employed to investigate the proposed second-shell mechanism of NHase, involving attack of a metal-bound hydroxide ion on the nitrile substrate. Our calculations indicate that a metal-bound water ligand could be transformed into a hydroxide ion through proton transfer to Cys114-SO$^-$. Nucleophilic attack of the metal-bound hydroxide ion on the substrate could then occur concertedly with proton transfer from Cys114-SOH to the nitrile nitrogen atom (Scheme 4). We optimized the concerted transition state for this mechanism, which has a computed barrier of 22.7 kcal/mol.

Another variant of the second-shell mechanism, in which Cys114-SO$^-$ acts as the nucleophile, was also investigated in

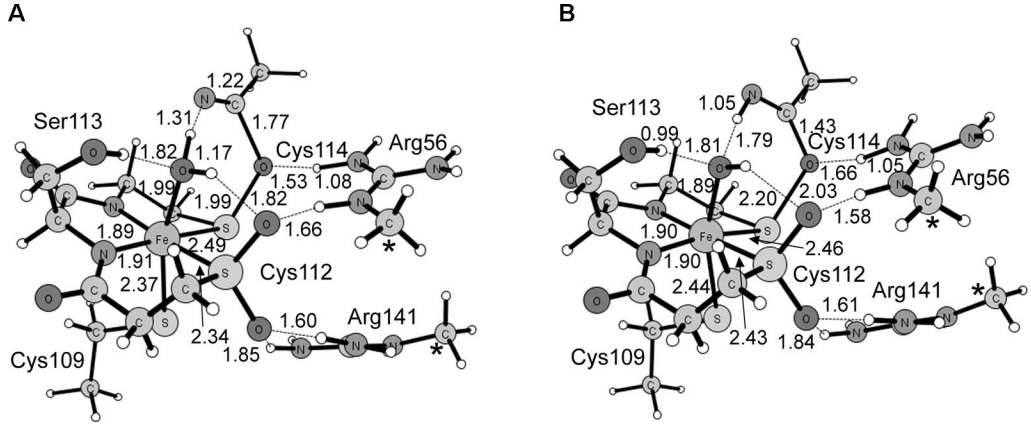


Figure 4. Optimized NHase active-site geometries with sulfenic acid (Cys114-SO$^-$) as nucleophile. A: concerted transition state; B: sulfur-linked intermediate.

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this paper (Scheme 5). In our calculations, the metal-bound water molecule functions as the acid which protonates the nitrile substrate. The computed barrier for the concerted transition state is 22.2 kcal/mol, which is very similar to the second-shell mechanism utilizing the metal-bound hydroxide ion. Thus, both investigated mechanisms exhibit barriers that are similar to the recently investigated first-shell mechanism, in which Cys114-SO$^-$ activates a water molecule for nucleophilic attack on the metal-bound substrate (computed barrier of 20.2 kcal/mol; Scheme 3).[54] At this stage it is not possible to single out any of these three mechanisms as the correct one. Different experimentally determined rates for NHase indicate barriers of 13–15 kcal/mol,[9,52,56] which is 5–9 kcal/mol below the barriers computed here. It is possible that the discrepancy is due to the inherent errors of the employed B3LYP functional. These errors have been estimated to 3–5 kcal/mol for relative energies of systems containing transition metals. However, it is also possible that the correct NHase mechanism is neither of the considered reaction pathways. In this context it is noteworthy that Cys114-SO$^-$ acts as either base or nucleophile in all three mechanisms that in our calculations have close to feasible barriers. Although the exact role of Cys114-SO$^-$ cannot be pinpointed yet, it seems likely that it is one of the key players in NHase-mediated nitrile hydration. More calculations are currently underway in our laboratory to investigate other mechanistic possibilities.

Acknowledgments

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