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Full Reaction Mechanism of Nitrile Hydratase: A Cyclic Intermediate and an Unexpected Disulfide Switch

Kathrin H. Hopmann*

Centre for Theoretical and Computational Chemistry, Department of Chemistry, University of Tromsø, N-9037 Tromsø, Norway

Supporting Information

ABSTRACT: The full reaction mechanism of nitrile hydratase has remained elusive, despite extensive theoretical and experimental studies. A novel reaction mechanism for nitrile hydratase is proposed here, with remarkable features and very feasible barriers. Our results, obtained on the basis of large quantum-mechanical active site models, identify Cys-SO$^-$ as the nucleophile, performing a direct nucleophilic attack on the metal-coordinated nitrile. This implies the formation of an intriguing cyclic intermediate, which subsequently is cleaved through attack of the axial cysteine on the sulfenate, thereby forming a disulfide bond. In this mechanism, nitrile hydration occurs without directly involving a water molecule. Subsequent water-mediated disulfide cleavage regenerates the active site. This is the first example of a disulfide switch directly implicated in an enzymatic reaction mechanism.

Nitrile hydratases (NHases, EC 4.2.1.84) are bacterial enzymes, which have been utilized in various industrial processes to catalyze the hydration of nitriles to amides.[1] Two NHase types are known, the nonheme iron and the noncorrinoid cobalt NHases. The NHase active site contains a low-spin metal ion ($S = {}^1/_2$ Fe^{III} or $S = 0$ Co^{III}) with a conserved coordination sphere involving two deprotonated backbone amides and three cysteines.[2] Two of the cysteines are post-translationally modified to cysteine sulfinic and cysteine sulfenic acid, respectively.[3] Additionally, conserved serine, tyrosine, and arginine residues are present in the active site (Figure 1).

Despite extensive efforts aimed at unraveling the mechanistic details of NHase,[2b,c,4−11] the full reaction pathway of this enzyme has remained elusive. Our quantum-chemical studies of a variety of inner- and outer-sphere mechanisms did not allow for identification of a preferred pathway.[10] In the course of our work, we were the first to provide evidence that Cys$_{114}$-SO$^-$ (numbering as in PDB 2AHJ) could function as a base that activates a nucleophilic water molecule.[10a] Experimental findings by Hashimoto et al. on NHase-mediated conversion of *tert*-butylisonitrile supported this mechanism.[7d] However, the nucleophilic properties of Cys$_{114}$-SO$^-$ could also point to a mechanistic alternative: direct attack of the sulfenate onto the substrate. Experimental results by Heinrich et al. on an NHase biomimetic complex supported an *outer-sphere* mechanism involving sulfenate as the nucleophile.[4] In our calculations, however, this pathway was not more feasible than alternative NHase mechanisms.[10b] Subsequently, we found that if nucleophilic attack by the sulfenate occurs through an *inner-sphere* mechanism,[10d] this provides a facile first reaction step, resulting in the formation of an unparalleled cyclic intermediate (Scheme 1). Interestingly, very recent experimental results provide additional support for the role of cysteine sulfenate as the nucleophile.[11b] However, neither the recent study[11b] nor the earlier theoretical work[10d] have provided a satisfactory explanation on how such a cyclic intermediate can be converted into the amide product.

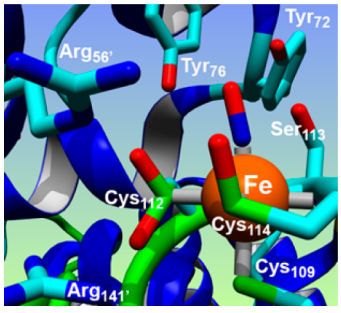
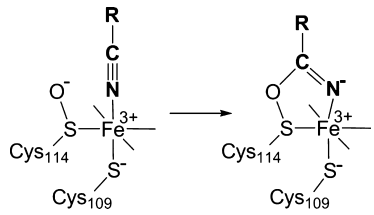


Figure 1. NHase active site showing the Fe^{III} coordination sphere and some conserved residues (PDB 2AHJ[3a]).

Scheme 1. Putative Cyclic Intermediate[a]



[a]Proposed by us on the basis of theoretical results[10d] and very recently supported by experimental results by Holz and coworkers.[11b]

Here we put forward a full reaction mechanism for NHase, involving the cyclic intermediate (Scheme 1), which is cleaved through an unexpected disulfide formation with the axial cysteine, without the involvement of a water molecule. Subsequent water-mediated cleavage of the disulfide regenerates the active site.

The full reaction pathway (Scheme 2) was studied with a large active site model of Fe^{III}-NHase [see the Supporting Information and Figure S1 for computational and model details]. In the first

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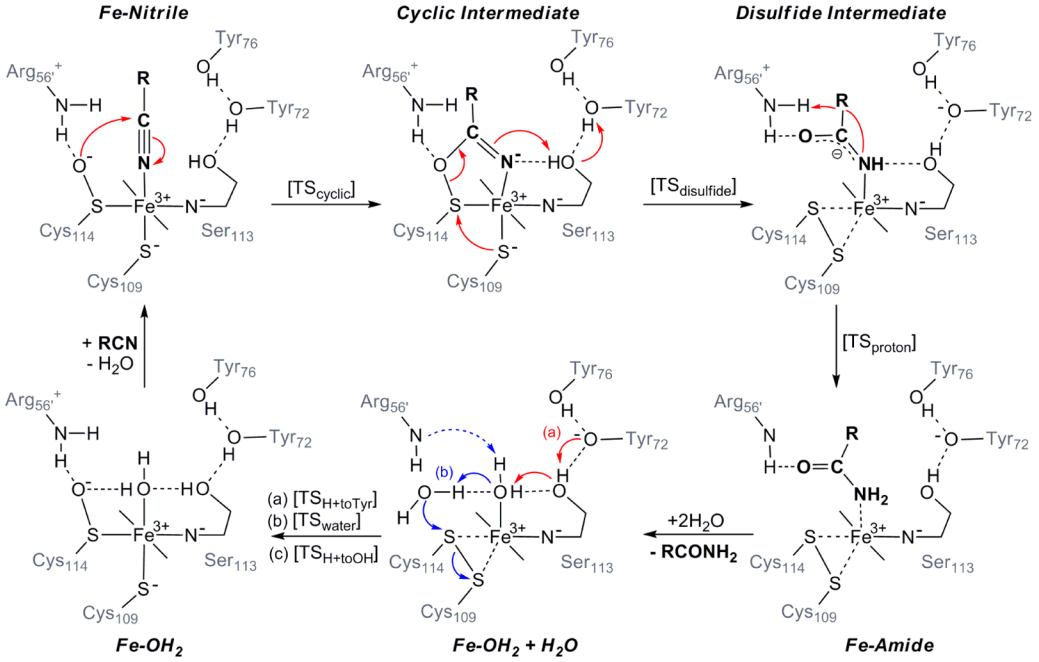
Scheme 2. Full Reaction Mechanism of NHase As Proposed in This Work[a]



[a]The attack of Cys_{114}-SO^- on the coordinated nitrile forms a cyclic intermediate. Cys_{109}–S–S–Cys_{114} disulfide formation promotes cleavage of the latter to give the amide. Active-site regeneration occurs through attack of water on the disulfide (see Scheme S2 in the SI for details).

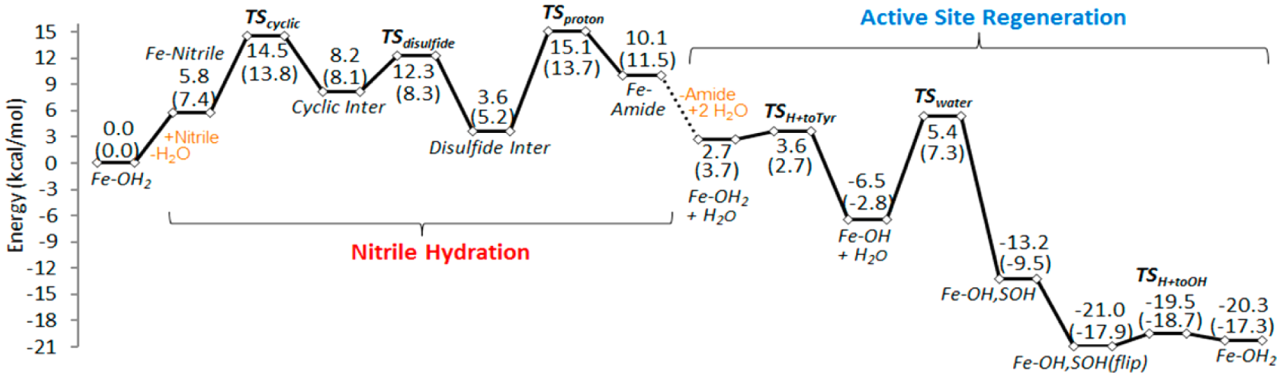


Figure 2. Computed electronic energies (enthalpies in parentheses)[13] for nitrile hydration and active-site regeneration.

step, Cys_{114}-SO^- attacks the coordinated nitrile to form a cyclic intermediate (TS_{cyclic}; Figure 3A). The enthalpy barrier for this step is only 6.4 kcal/mol relative to the Fe-Nitrile species (Figure 2). If it is assumed that the metal initially coordinates a water molecule (Fe-OH_2) and the cost of exchanging water for nitrile is added, the barrier becomes 13.8 kcal/mol (see Scheme S1 in the SI for the ligand-exchange calculation).

Different pathways can be envisioned for conversion of the cyclic intermediate to the amide (Figure S2 in the SI): (i) attack of water on the carbon atom; (ii) attack of water on the sulfur atom; (iii) cleavage of the intermediate in the absence of water. The first alternative has a high barrier (38 kcal/mol[10d]), whereas the second possibility could not be achieved computationally due to the lack of an appropriate residue for activation of the water molecule. Instead, we propose that cleavage of the cyclic intermediate occurs through attack of Cys_{109} on the sulfur atom (Scheme 2, $TS_{disulfide}$), with a barrier of only 8.3 kcal/mol relative to the Fe-OH_2 species (Figure 2). This results in the formation of a Cys_{109}–Cys_{114} disulfide bond, concomitant with cleavage of the Cys_{114}–substrate bond and proton transfer from Tyr_{72} via Ser_{113} to the substrate (Figure 3B). The oxygen atom of Cys_{114} is incorporated into the amide product. Note that the disulfide is formed from a thiolate and a sulfenate, implying that the overall oxidation state of the sulfur centers does not change (in contrast to disulfide formation from two thiols, which formally requires oxidation). Although there have been proposals for disulfide switches as modulators of protein function,[12] this appears to be the first example of a reversible disulfide switch implicated in an enzymatic reaction mechanism.

Following formation of the disulfide intermediate, the substrate nitrogen abstracts a proton from $Arg_{56'}$ (TS_{proton}, Scheme 2 and Figure 3C), which has a barrier of 13.7 kcal/mol (Figure 2). Protonation of the substrate oxygen, forming an iminol, is energetically less preferable. Following amide displacement, the active site is regenerated through sequential steps of proton transfer to Tyr_{72}, attack of water on the disulfide, and proton transfer to $Arg_{56'}$ (Schemes 2 and S2 in the SI). The enthalpic proton-transfer barriers appear underestimated; this was also observed earlier for low-barrier proton-transfer steps.[14] The regenerated active site (Fe-OH_2) has a relative energy of

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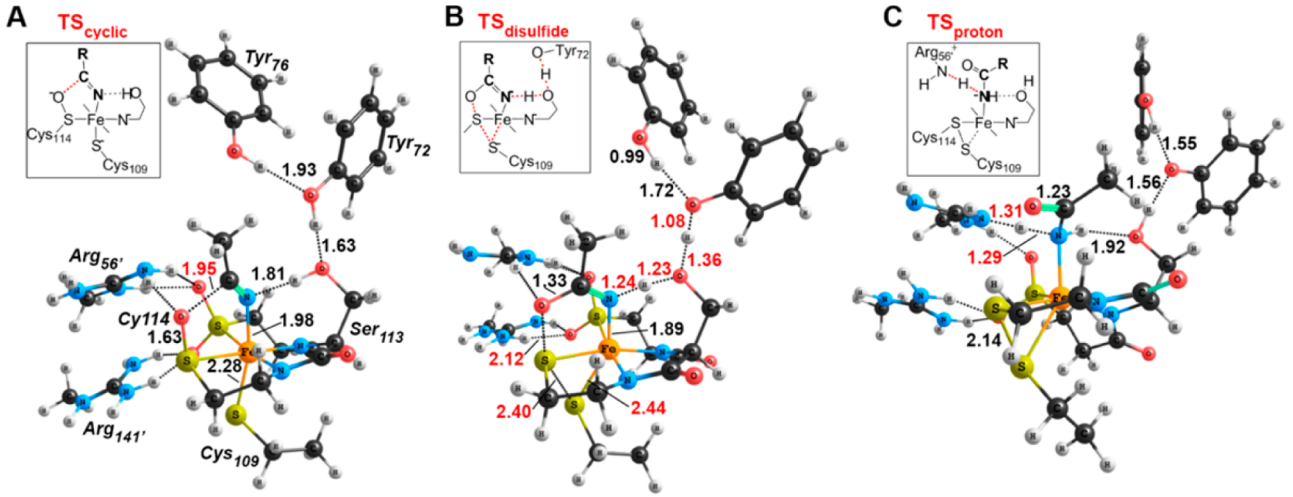

Figure 3. Optimized transition states for NHase-mediated nitrile hydration. (A) Nucleophilic attack of Cys_{114}-SO^- on the nitrile carbon. (B) Attack of Cys_{109}-S^- on the sulfur atom of Cys_{114}, cleavage of the cyclic intermediate, and proton transfer from Tyr_{72}. (C) Proton transfer from $Arg_{56'}$. The insets show bonds that break or form (dashed red lines); distances are given in angstroms (in red for forming/breaking bonds).

−17.3 kcal/mol, corresponding to the enthalpy change for converting acetonitrile to acetamide. Interestingly, the final two structures, involving Cys_{114}-sulfenic acid with a metal-bound hydroxide [Fe-OH,SOH(flip)] and Cys_{114}-sulfenate with a metal-bound water (Fe-OH_2), are quasi equienergetic. This might explain differing assignments for the protonation state of Cys_{114}.[15] The overall barrier of this novel mechanism is 13.8 kcal/mol, in excellent agreement with experimental rates (translating to barriers of ∼13−15 kcal/mol) and lower than any previous proposal.[10] Experimental results, showing the importance of Cys_{114}, $Arg_{56'}$, and Tyr_{72} for catalysis,[7a,b,e] further support this proposal. Future studies are relevant to confirm the here-proposed role of Cys_{109} in nitrile hydration.

■ ASSOCIATED CONTENT

Supporting Information

Computational details, ligand-exchange reactions, proposals for cleavage of the cyclic intermediate, active-site regeneration, and coordinates of all optimized structures. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: kathrin.hopmann@uit.no.

Notes

The authors declare no competing financial interest.

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