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Modeling catalytic mechanism of nitrile hydratase by semi-empirical quantum mechanical calculation

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ABSTRACT

Nitrile hydratase (NHase) is an important industrial enzyme capable of converting nitriles to corresponding amides. Utilizing the method of semi-empirical quantum mechanical (QM) calculation by TRITON, the bioconversion process of acrylonitrile to acrylamide catalyzed by NHase was successfully performed on a computer. Crystal structure of a Co-type NHase from *Pseudonocardia thermophila* JCM 3095 (PDB code 1IRE) was selected as the target for acrylonitrile autodock. *In silico* calculations were performed on the NHase–acrylonitrile complex to simulate the enzyme catalysis mechanism by quantitatively comparing energy changes of each reaction pathway. Simulation results showed that active site activation is the first step of NHase catalysis, in which the Co^{2+} coordinated to a water molecule forms a Co–OH complex mediated by the oxidized α-CEA113. Then the oxygen atom in the Co–OH attacks the C atom in the –CN triple bond of acrylonitrile, forming a precursor of acrylamide. Consequently, proton rearrangement happens transforming the precursor into the final product of acrylamide, under the assistance of the hydrogen atom in the –OH group of α-SER112. Gibbs energy changes of three steps corresponding to the active center activation, nucleophilic attack and proton rearrangement are around −31, 23 and −12 kcal/mol, respectively.

1. Introduction

Nitrile hydratase (NHase) [EC 4.2.1.84], an important industrial enzyme involved in microbial hydration of nitriles to corresponding amides, is a metalloenzyme containing either cobalt or iron in its catalytic center [1,2]. Based on this, NHases can be classified into two broad groups: Fe-type NHases and Co-type NHases. The representative Fe-type NHases existing in *Rhodococcus* sp. N-774 and *Pseudomonas chlororaphis* B23 were used for industrial acrylamide production as the first and the second generation catalyst, respectively. These NHases showed unique reactivity to light and nitric oxide (NO) [1,3]. To date, the third generation biocatalyst from *Rhodococcus rhodochrous* J1 has been developed for acrylamide production which contains a cobalt ion at its active center [4].

NHase consists of two subunits, α and β, without homology in amino acid sequence. All NHases contain a highly conserved active center of C–(T/S)–L–C–S–C in the α subunit, in which the Co-type NHases have threonine, whereas the Fe-type NHases have serine residue. Researchers have found that the tertiary structure of the NHase-active center forms an octahedron structure with either Fe or Co ion, mainly composed of four amino acid residues of the α subunit (αCys108, αCys111, αSer112 and αCys113) and two residues of the β subunit (βArg52 and βArg157) [5–7]. Generally, the ligand environments of the metal-ions in both Fe-type and Co-type NHases are similar. In the conserved active center with the common motif of CYS–(THR/SER)–LEU–CSD–SER–CEA, CYS was post-translationally modified into the oxidized forms of CSD, cysteine sulfuric acid (αCys111-SO_2H) and CEA, cysteine sulfenic acid (αCys113-SOH), respectively [8–10]. Site-directed mutagenesis confirmed that these three cysteine residues are essential for active expression of the cobalt-containing H-NHase (high molecular mass-nitrile hydratase) [11].

However, the mechanism of NHase catalysis remains unknown till now. Researchers have suggested three plausible mechanisms of catalysis which are designated as the inner-sphere mechanism, the outer-sphere mechanism and the second outer-sphere mechanism, respectively [3,5]. In the postulated inner-sphere mechanism, the nitrile binds to the metal ion directly and the metal-bound nitrile undergoes hydrolysis by a water molecule. In the outer-sphere mechanism, a hydroxide ion coordinated to the metal ion activates nitriles and attacks on the nitrile substrate. The second outer-sphere mechanism presents that the metal-bound hydroxide will activate another free water molecule from the

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second coordination shell, and this second water attacks the substrate nitrile. Most recently, Peplowski et al. performed auto-docking studies of nitriles and amides into a Co-type NHase [12]. Their analyses of relative positions of crystallographic waters and the best-docked acrylonitrile indicated that the outer-sphere mechanism is more probable [12]. Theoretical investigation by Hopmann et al. showed that based on the first-shell mechanism in which the nitrile substrate binds directly to the low-spin iron in the sixth coordination site, the generally suggested role of the Fe(III) center as Lewis acid, activating the substrate toward nucleophilic attack, is shown to be unlikely [13].

In this work, quantum mechanical calculation was performed to simulate the detailed procedure of nitrile hydratase catalysis by TRITON [14], a graphical software package for modeling enzymatic reactions, analyzing interactions between the active site residues and the substrate, and *in silico* constructing protein mutants. TRITON had been successfully used for the catalytic mechanism study of a haloalkane dehalogenase [15,16]. A novel reasoned catalysis mechanism of NHases was finally constructed including three major steps assigned as the active site activation, nucleophilic attack and proton rearrangement, respectively.

2. Methodology

The crystal structure used for generating the enzyme–substrate complex was obtained from Protein Data Bank (code 1IRE), which is a Co-type NHase from *Pseudonocardia thermophila* JCM 3095 [7].

The tertiary structure of acrylonitrile substrate was generated by software Discovery Studio 2.0 (Accelrys, Inc.). The docking of acrylonitrile into 1IRE was performed by AutoDock3.0.5, the most classical autodock software [17]. Hydrogen atoms were added into the structure file of 1IRE with water molecules remaining. Define the degree of freedom for acrylonitrile as zero, add standard Gasteiger charges as implemented in AutoDock, and generate the calculating grid with size of 80 Å × 80 Å × 80 Å. Distance of the grid node was 0.375 Å. The searching algorithm was LGA (Lamarckian genetic algorithm) with increments of 2.0 Å distance step-size and 10° angle step-size, 50 of searching individuals and 0.02 of mutating rate. Four pre-files, i.e. nitrile hydratase structure, acrylonitrile molecule structure, calculating grid and searching parameters, were used for docking to generate the nitrile hydratase-acrylonitrile complex. A series of binding-energy data were obtained and the conformation with the lowest binding energy was selected as the starting enzyme–substrate complex for following catalysis reaction calculations.

Enzymatic reaction modeling was performed using the TRITON software freely supported by the National Center of Biomolecular Research (http://ncbr.chemi.muni.cz/triton/) consisting of three modules of MOPAC, MODELLER and DRIVER [14]. To include the important Co^{2+} into the calculations, MOPAC7.0 in the original TRITON was renewed as MOPAC2006 [18] to perform the semi-empirical quantum mechanical (QM) calculations. Detailed simulation process was carried out as follows:

Step 1: Load into the structure model of the enzyme–substrate complex. Step 2: Specify the catalysis substrate including the acrylonitrile molecule, water molecule and the cobalt ion. Step 3: Specify functional groups composing the catalysis "cavity" based on the X-ray structure of the enzyme. We divided these active site groups into two types, i.e. the structural amino acids and the reactant amino acids. The structural amino acids assured the correctness of the key residues' conformations, while the reactant amino acids satisfy three constraints as follows: (1) belong to nucleophiles containing –OH group; (2) close to the active site; (3) locating in highly conserved region of NHases. The "cavity" scope was limited as 10 Å distance around the active site. Within this region, the functional groups were finally designated as α-CYS[108], α-CSD[111], α-SER[112], α-CEA[113] and β-TYR[69] which either involving in the octahedron formation of the active site or containing a hydroxyl group. Step 4: Search the reaction pathway of atoms taking part in catalysis. For a single reaction step, we need to specify two interacted atoms, interacting step length and final distance. In a real reaction, substrate will exhibit a trend moving toward the direction with preferable free-energy changing to transform into product, although all atoms are in the state of random thermal movement. For every postulated reaction pathway, there will generate an activation energy result indicating its feasibility. By comparing these energy results, we can revise the reaction pathway approaching to the real mechanism. Step 5: Specify the main chain atoms involving in the calculating "cavity" as immobilized groups to ensure the correct conformation of the catalysis center. Step 6: Determine the QM calculating parameters in MOPAC. The calculation method was chosen as the semi-empirical modified neglect of diatomic overlap (MNDO, the type of Hamiltonian used), the self-consistent field (SCF) equation was selected as conventional mode, the optimization method for the heat of formation minimization was BFGS, the increment step-size was 0.05 Å and the maximum running time was 10,000,000 s. The control parameters were set as GEO-OK, MMOK and PRECISE.

3. Results and discussion

3.1. Active site structure of NHase and docking of acrylonitrile

From the X-ray-observed crystal structure of cobalt-containing nitrile hydratase [7], it can be seen that there is a water molecule involved in the formation of the octahedron structure of the active center, together with amino acids of α-CYS[108], α-CSD[111], α-SER[112] and α-CEA[113], as shown in Fig. 1.

To start the enzyme reaction of NHases, the catalysis substrate (acrylonitrile) needs to be docked into the active center first, adjacent to the metal ion and the octahedron structure as illustrated in Fig. 1. In the docking process of the acrylonitrile molecule into the 3D structure of 1IRE, a suite of binding energy data were obtained ranging from −2.69 to −3.86 kcal/mol which corresponding to 10 distinct conformations of enzyme–substrate complexes. Consequently, the conformation with the lowest binding energy of −3.86 kcal/mol was selected as the starting

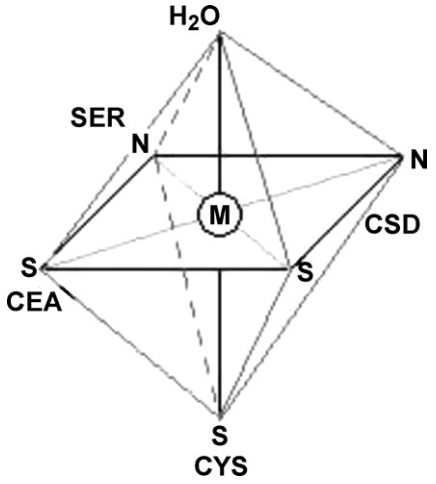

Fig. 1. Schematic octahedron structure of the active site of NHases [7]. The ligands to the cobalt atom (M) include a water oxygen atom, two main chain amide nitrogen atoms (N) (α-SER[112] and α-CEA[113]) and three sulfur atoms (S) of the α-CYS[108], α-CSD[111], and α-CEA[113], where CSD is the post-translationally modified cysteine-sulfunic acid and CEA is the post-translationally modified cysteine sulfenic acid.

structure of the enzyme–substrate complex for modeling the reaction, as shown in Fig. 2. This optimal position exactly locates at the active center of 1IRE close to the cobalt ion. Based on this, detailed reaction steps of NHase catalysis were subsequently simulated by semi-empirical quantum mechanical calculations.

3.2. Step 1 simulation of NHase catalysis designated as active site activation

At first, we specified the reaction pathway in postulation as follows: the oxygen atom in a water molecule (numbering as O2) moves towards the cobalt ion (Co^{2+}), meanwhile a hydrogen atom in the same water moves towards an oxygen atom in CEA, based on 3D structure analyses of the 1IRE-arylontirle active center (as shown in Fig. 3A). The reaction process was simulated by TRITON and its energy (heat of formation) changes of each step were calculated in Fig. 3B. We can see that this postulated activation process consequently exhibits a significant energy decrease accompanying with the distance reduction within O2–Co^{2+} atom, indicating that this is a preferable reaction step for NHase catalysis. Such simulating result supports the first step in three plausible mechanisms of NHases in literature [3,5] that the NHase catalysis was initiated by the metal–OH complex. Furthermore, this active site activation step prepared a new appropriate Co^{2+}–OH nucleophilic attack group for following reactions.

3.3. Step 2 simulation of NHase catalysis designated as nucleophilic attack

In the active center "cavity" we specified to perform the simulation, there are totally three major hydroxyl nucleophilic groups, i.e. the Co^{2+}-bound –OH (generated by the active site activation step), the –OH in α-SER^{112} and the –OH in β-TYR^{69}. By semi-empirical quantum mechanical calculation using TRITON, we simulated each reaction pathway and calculated their energy changes one by one.

At first, the nucleophilic attack performed by the Co^{2+}-bound –OH was simulated. Considering that when the atom O2 attacks C1 in –CN triple bond of AN, the partial charge of N3 in the triple bond would simultaneously change during the reaction progress, the reaction pathway of O2 attacking C1 should include atom N3 and another hydrogen optimized as H4 in the CEA^{111}, as shown in Fig. 4A. Energy changes of this process were summarized in Fig. 4B where totally 25 steps of the reaction were completed. We can see that in the end of this reaction, the heat of formation is higher than that of the initial value, indicating that this process needs to conquer an energy barrier.

Analyzing partial charge changes of atom O2, N3 and C1 by MOPAC, as shown in Fig. 5, it can be seen that at the beginning of the nucleophilic attack, charges on O2, N3 and C1 were around −0.24, −0.14 and 0, respectively. Then the negative charges accumulated in O2 and positive charges enhanced in C1 accompanying with the distance reduction within C1–O2. Meanwhile the negative charge of N3 also significantly increased, enduing it the capability to attract the H4 atom (Fig. 4A). When the atom-distance within C1–O2 reduced to ∼1.56 Å (gradient step 21), the calculated geometry changed into the transition state with charges of −0.38, −0.51 and 0.14 on O2, N3 and C1, respectively. After striding over an activation energy barrier of ∼60 kcal/mol (Fig. 4B), atoms O2–C1 and N3–H4 formed chemical bonds, generating a precursor of acrylamide (AM). In the end, the simulation exhibited another extra step of space displacement functioned by residue α-SER^{112}, by which the distance of N3 to the side chain oxygen atom of α-SER^{112} decreased to 3.4 Å from 4.8 Å, simultaneously the distance of O2–Co^{2+} enhanced to 4.0 Å from 3.3 Å. This means that the precursor prone to be broken away from the active center by nucleophilic attraction of the side chain oxygen atom in α-SER^{112} to the N3-bound hydrogen atom. This is the first step, also a key step for following product generation and release process.

The second assumption for the nucleophilic attack was that the functional group was specified as the –OH in α-SER^{112}. Using the similar simulation method by TRITON, we found that the activation energy barrier for precursor formation in this reaction pathway increased to ∼90 kcal/mol. When changed the nucleophilic attack group to the –OH in β-TYR^{69}, this reaction even cannot proceed due to the absence of a hydrogen atom nearby to form a bond with N3 atom. Collectively, the nucleophilic attack performed by the Co^{2+}-bound –OH exhibits the lowest energy barrier, revealing that this reaction pathway is the most reasonable one.

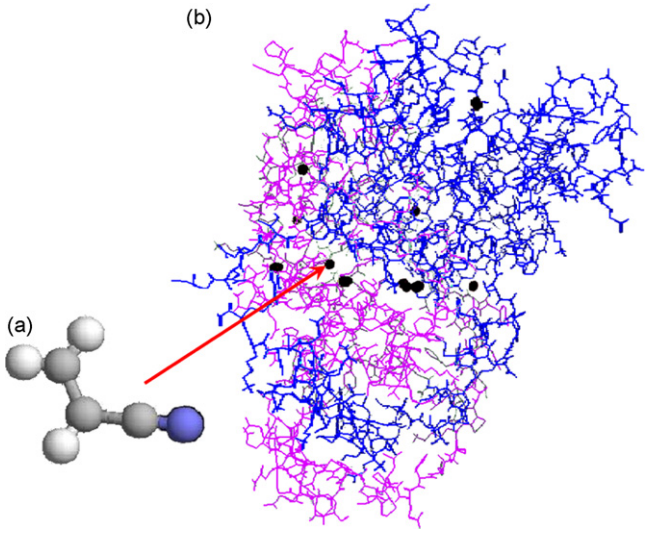

Fig. 2. Autodock of the substrate acylonitrile into the active center of nitrile hydratase 1IRE: (a) the molecule of acrylonitrile; (b) the 3D structure of 1IRE. Ten black dots represent the positions of the autodocked substrates in 1IRE. The arrow points to the optimal docking site of acylonitrile in the active center of 1IRE with the lowest binding energy.

3.4. Step 3 simulation of NHase catalysis designated as proton rearrangement and product generation

To generate a final product of AM based on the Co^{2+}–OH nucleophilic attack pathway simulated above, atom N3 in the AM precursor needs to arrest another –H atom to form the amino group, meanwhile O2 needs to break down with H1 and form a new double bond with C1. Two proton transfer pathways are probable to implement this task. The first is that the H1 atom directly transfers to N3 (H1–N3 pathway, Fig. 6A), and the second is that the H2 atom in the –OH group of α-SER^{112} moves to N3, meanwhile the O2-binding H1 transfers to the original position of H2 forming a new –OH with O4 (H2–N3:H1–O4 pathway, Fig. 6C). Reaction simulations were carried out for both assumptions, and the energy changing results were summarized in Fig. 6B and D, respectively. For the first H1–N3 pathway, the activation energy barrier was ∼94 kcal/mol. For the second H2–N3:H1–O4 pathway, the value reduced to ∼85 kcal/mol although the distance of N3–H2 is longer than that of N3–H1. This indicated that the α-SER^{112}-mediated H2–N3:H1–O4 pathway is more preferable for the step of proton transfer. Consequently, an AM final product was generated at the end of this reaction step.

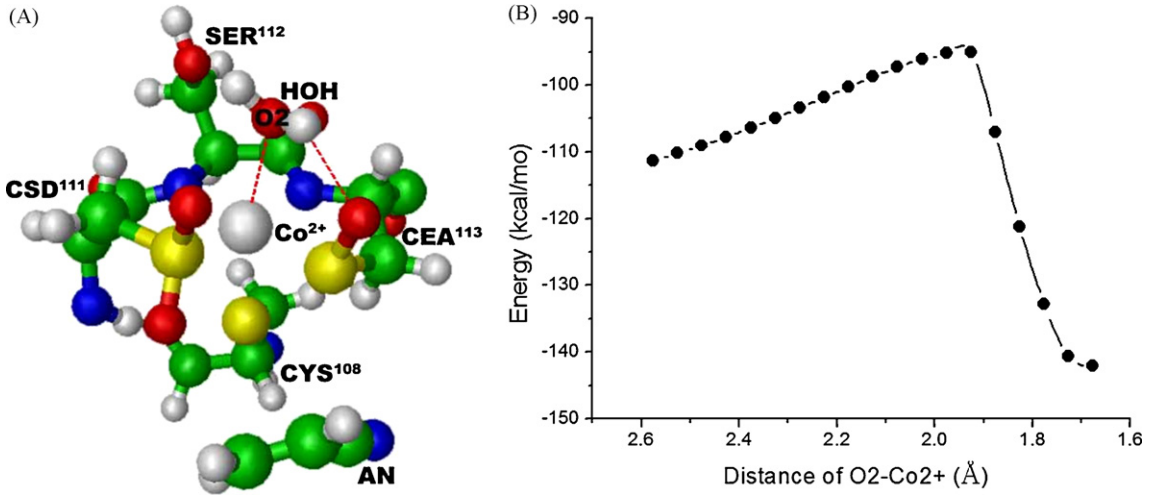

Fig. 3. Pathway sketch map and energy changes in step 1 simulation of NHase catalysis designated as active site activation. (A) Schematic active center structure of the O2–Co^{2+} reaction pathway. Major atoms of O2 (H_2O)–Co^{2+} and O (CEA)–H (H_2O) participating this reaction are linked by dashed line, respectively. (B) Energy (heat of formation) changes of the O2–Co^{2+} reaction pathway. Totally 19 gradient-steps were calculated in this process. AN, acrylonitrile.

3.5. MOPAC calculation test for diverse reaction pathways approaching

Further evaluations on the MOPAC calculation in TRITON showed that reaction postulations for each reaction step can be effectively tested by the pathway processing and its corresponding energy changes. As for cases all with a reasonable reaction pathway, we can calculate and compare their activation energy values, consequently get the optimal specification as described in Fig. 6. However, when a postulated reaction pathway is not reasonable, the gradient reaction step would be terminated during the calculation and we cannot get any results. For example, the postulation specifying the –OH in β-TYR^{69} as a nucleophilic attacker was confirmed infeasible due to the simulating process termination. Another phenomenon for an unreasonable pathway is also probable that the heat of formation of the calculated reaction increases throughout the reaction simulation. For instance, during the nucleophilic attack of Co^{2+}–OH to substrate, we could not get a proper transition state with a rational activation energy change if the reaction pathway merely included the O2–C1 atoms, as shown in Fig. 7. From the diverse approaching tested as above, we can finally obtain the most reasonable and reliable reaction pathway designated as our expected target.

3.6. Construction of the NHase catalysis mechanism

Summarizing the QM-simulation results described above after the substrate docking, we presented that an integrated reaction implemented by NHases converting acrylonitrile (AN) to acylamide (AM) mainly includes three steps as shown in Fig. 8. Step 1 is designated as the active center activation in which a Co^{2+} bound –OH group was formed and acted as the nucleophilic attacker assisted by the oxidized CEA. The Gibbs energy change of this step is about −31 kcal/mol. Step 2 reaction is abbreviated as nucleophilic attack in which the oxygen atom in the Co–OH attacks the C atom in the –CN triple bond and simultaneously the N atom in the triple bond attracts a H atom in CEA^{111}, producing a precursor of the AM product. The Gibbs energy change of this step is around 23 kcal/mol. Step 3 is summarized as the proton rearrangement and product generation where the precursor

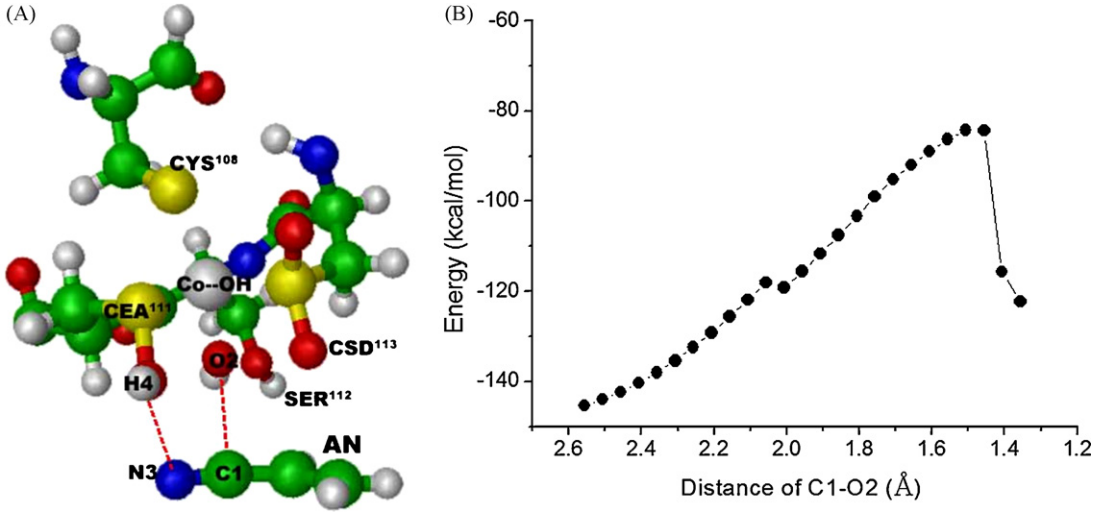

Fig. 4. Pathway sketch map and energy changes in step 2 simulation of NHase catalysis designated as nucleophilic attack performed by Co^{2+}-bound –OH in C1–O2:N3–H4 pathway. (A) Schematic active center structure of the C1–O2:N3–H4 reaction pathway; (B) energy changes of the C1–O2:N3–H4 reaction pathway. There are totally 25 gradient-steps were calculated in this process. Dashed line indicates the major reaction pathway, and the O2 atom was coordinated to metal Co^{2+} (Co–OH). AN, acrylonitrile.

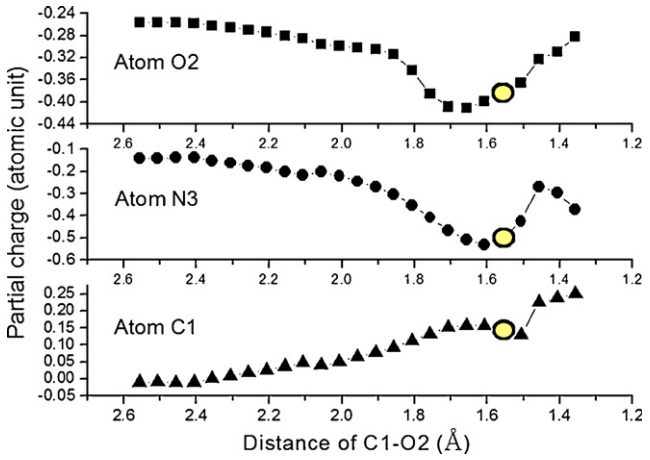

Fig. 5. Changes in partial charges of atom O2, N3 and C1 in the C1–O2:N3–H4 reaction pathway of nucleophilic attack performed by Co^{2+}-bound –OH group. Totally calculating steps are 25. The special circle at step 21 corresponds to the calculated geometry of the transition state.

structure was rearranged to generate the final product of AM under the assistance of the hydrogen atom in the –OH group of α-SER^{112}. The Gibbs energy change of this step is about −12 kcal/mol. Energy comparison of three steps exhibited that the nucleophilic attacking step had the highest energy barrier for completing the entire NHase catalysis.

In this reaction mechanism, the metal-bound –OH is assigned as the nucleophilic attacker, somewhat matching the outer-sphere mechanism in literatures [3,5,12]. Residue α-CEA^{113} plays an important role for activating the active center, which is in consistent with experimental results of Hashimoto et al. that site-directed mutagenesis of this residue would inactivate the enzyme [11]. Residue α-SER^{112} contributes in two aspects, firstly, its side chain –OH can generate a hydrogen bond interacting with the N-bound hydrogen atom in the AM precursor, consequently helping the initial release of the product from the active center; secondly, it participates in the process of proton-rearrangement ensuring a fast and efficient transformation of the intermediate into the final product.

In addition, to effectively promote the release of the final product from the NHase after catalysis, it is highly probable that there is not only one hydrogen bond interaction near the active

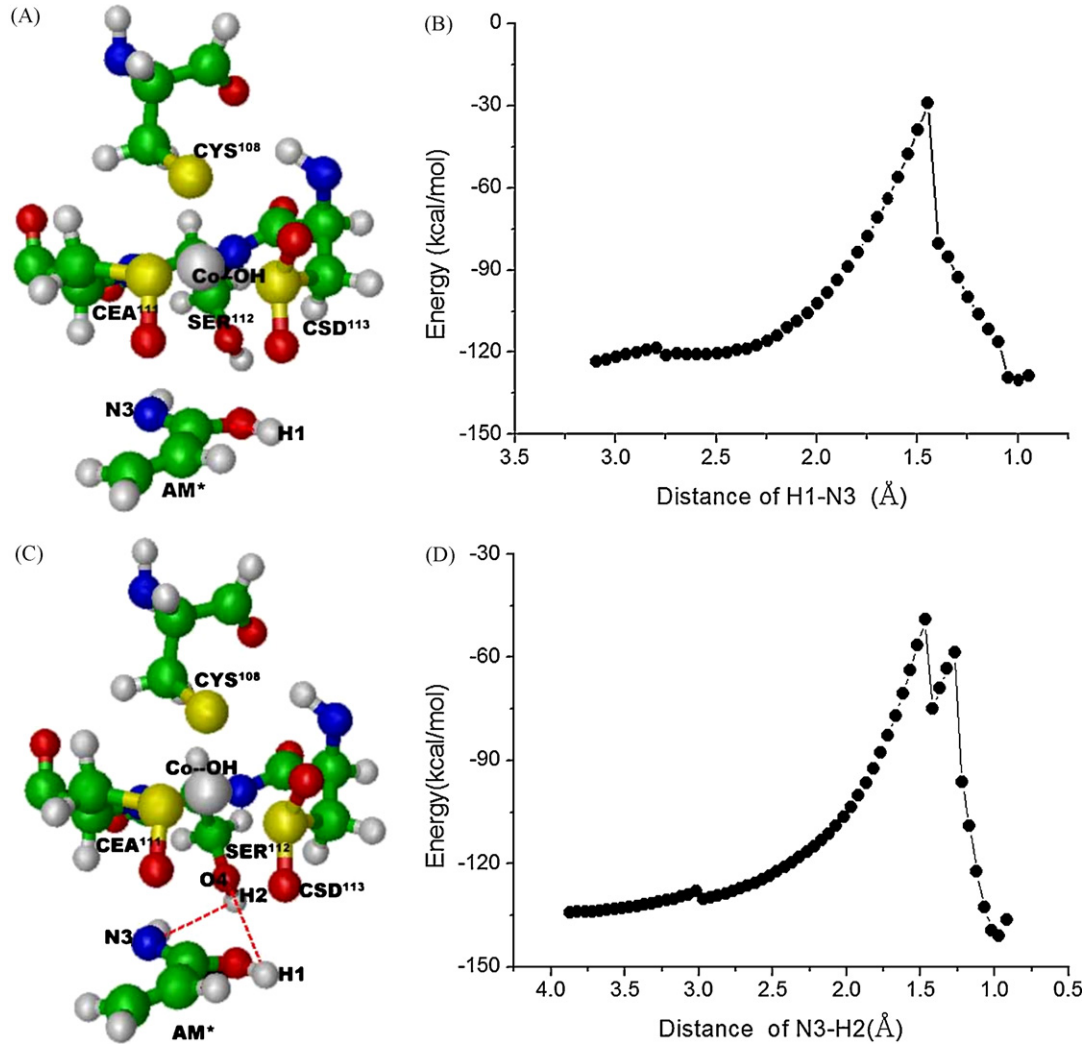

Fig. 6. Pathway sketch map and energy changes in proton transfer/product generation simulation of NHase catalysis. Two pathways, H1–N3 and H2–N3:H1–O4, were simultaneously presented and compared. (A) Schematic structure of the H1–N3 reaction pathway; (B) energy changes of the H1–N3 reaction pathway with totally 44 calculating steps; (C) schematic structure of the H2–N3:H1–O4 reaction pathway; (D) energy changes of the H2–N3:H1–O4 reaction pathway with totally 60 calculating steps. Dashed lines indicate the major reaction pathways, and the metal Co^{2+} coordinated to a new water molecule. AM*, the precursor of acrylamide (AM).

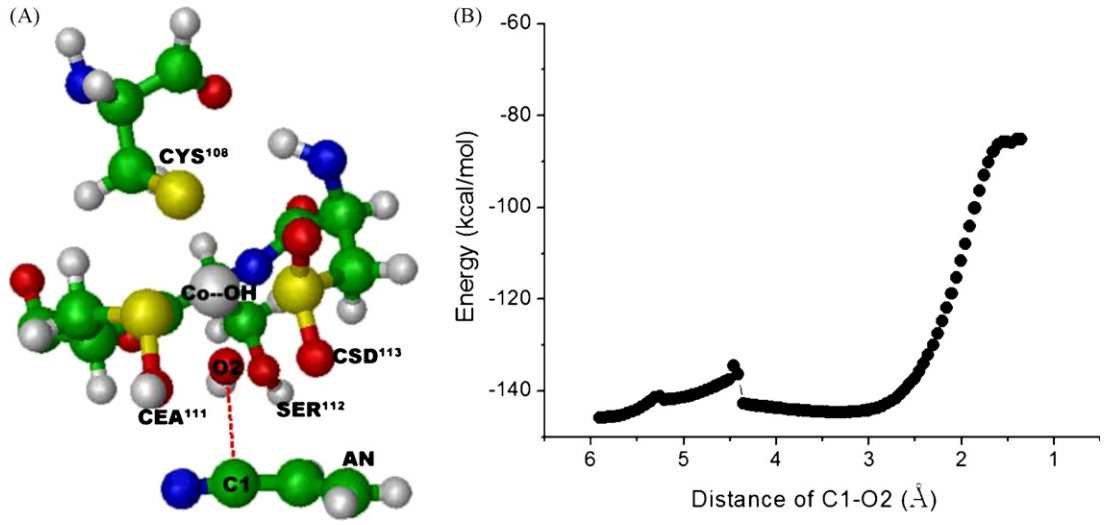

Fig. 7. Pathway sketch map and energy changes in nucleophilic attack simulation assuming that the reaction was performed by merely O2 and C1 atoms. (A) Schematic active center structure of the postulated C1–O2 reaction pathway; (B) energy changes of the postulated C1–O2 reaction pathway. Dashed line links the involving atoms and there are totally 92 gradient-steps were calculated in this process. AN, acrylonitrile.

center working as the deviating "puller". When we spatially searched polar residues with hydroxyl group around the active site, we found that residue α-SER^{122} and β-TYR^{68} located at appropriate positions prone to generate hydrogen bond interaction with the AM molecule, which would "pull" the AM product fast out from the active center, accordingly affect the NHase activity. Our site-directed mutagenesis experiments on α-SER^{122} have revealed that the α-S122C mutation would reduce the NHase activity by ~30%, and the α-S122A mutation would be completely inactive [19]. This confirmed that the nucleophilicity of the side-chain group in α-SER^{122} is important for the activity presence of NHase, in accordance with above postulation. More experiments are in progress to verify the other predictions.

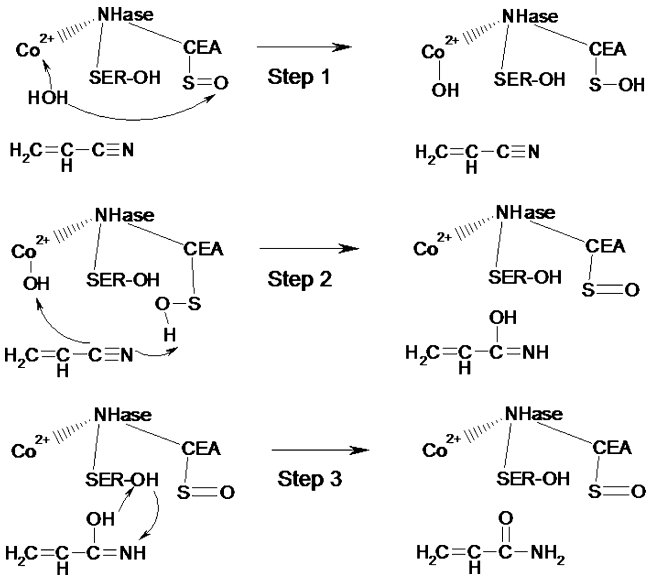

Fig. 8. Schematic catalysis mechanism of NHase converting acrylonitrile to acylamide constructed by semi-empirical quantum mechanical calculation. Step 1, active center activation generating the Co^{2+}-bound –OH group mediated by the CEA^{113} residue. Step 2, nucleophilic attack of Co^{2+}–OH to C atom in substrate, forming the precursor of acrylamide product. Step 3, proton rearrangement and subsequent final product generation mediated by SER^{112} residue.

To date, nitrile hydratases, nitrilases and amidases have been extensively used in biocatalytic transformations to produce various kinds of amides or acids from corresponding nitriles [2,20], which have wide applications in the pharmaceutical industry, chemical industry and environmental protection, therefore is of great interest in research [21–23].

4. Conclusions

We simulated the catalysis process of NHases converting acrylonitrile to acylamide by semi-empirical quantum calculations, and consequently presented a novel but reasoned catalysis mechanism of NHases including three major steps, i.e. active center activation, nucleophilic attack and proton rearrangement/product generation based on the autodock of acrylonitrile into the active center of 1IRE. By quantitative energy change calculations of each reaction step, we revealed the function of the metal ion and some other important residues, such as α-CEA^{113} and α-SER^{112}, in the process of catalysis, and determined the key group implementing the nucleophilic attack as the –OH in the Co^{2+}–OH complex. This catalysis mechanism gives a first detailed description of NHase catalysis supported by quantitative calculations, which is important for further modifications of this enzyme to improve its enzymatic performance and extend its industrial applications.

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