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Insights into catalytic activity of industrial enzyme Co-nitrile hydratase. Docking studies of nitriles and amides

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Abstract Nitrile hydratase (NHase) is an enzyme containing non-corrin Co^{3+} in the non-standard active site. NHases from *Pseudonocardia thermophila* JCM 3095 catalyse hydration of nitriles to corresponding amides. The efficiency of the enzyme is 100 times higher for aliphatic nitriles then aromatic ones. In order to understand better this selectivity dockings of a series of aliphatic and aromatic nitriles and related amides into a model protein based on an X-ray structure were performed. Substantial differences in binding modes were observed, showing better conformational freedom of aliphatic compounds. Distinct interactions with postranslationally modified cysteines present in the active site of the enzyme were observed. Modeling shows that water molecule activated by a metal ion may easily directly attack the docked acrylonitrile to transform this molecule into acryloamide. Thus docking studies provide support for one of the reaction mechanisms discussed in the literature.

Keywords Amides · Biotechnology · Docking · Nitrile hydratase · Nitriles

Introduction

Nitrile hydratase (NHase) is an enzyme containing non-corrin Co^{3+} or non-heme Fe^{3+} metal ion in the non-standard active site [1–3]. NHases from *Pseudonocardia thermophila* catalyse reaction of nitriles hydration to corresponding amides (Fig. 1) and are working agents in white biotechnol-

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ogy [4]. Amides are used as flocculants, components of synthetic fibers, vitamins (PP) or soil conditioners. The biggest NHase plants are located in Japan (Mitsubishi Rayon Co., Ltd), France (SNF Floerger) and China (Lonza Guangzhou Fine Chemicals) [5]. The molecular details of the catalytic activity of NHases are not known. Known is the fact of NHases substrate selectivity. Some types of enzymes prefer aliphatic nitriles and others catalyse aromatic ones better [6]. Recently very promising stereoselective NHases have been reported [7, 8]. In our opinion NHase is a promising protein for rational design of biocatalysts.

The enzyme is composed of two subunits - α (23 kDa) and β (26 kDa). In all known NHases the active site exhibits the common motif: CYS-X-Y-CSD-SER-CEA, where CSD and CEA are postranslationally modified cysteine residues: CSD is cysteine-sulfinic acid (αCys111-SO$_2$H) and CEA is cysteine sulfenic acid (αCys113-SOH, see Fig. 2) [1–3].

Till 2006 14 X-ray structures have been solved [1, 9–11] and intensive research have been done [12–16], but the catalytic activity is not explained yet. There are suggested three possible catalytic mechanisms [9]. Obviously, the initial step is nitrile (and at least one water molecule) entry into the vicinity of the active site. In all the mechanisms the metal ion acts as a Lewis acid. In the first mechanism (M1) the nitrile binds to the metal ion. This leads to increased electrophilicity of the nitrile carbon and facilitates water molecule attack on the substrate. Another postulate (M2) is that rather a hydroxide ion coordinated to the Lewis acid activates nitriles, thereby acts as a nucleophile attacking the carbon bound to nitrile nitrogen. The third mechanism (M3) assumes that the hydroxide ion coordinated to the metal ion activates a water molecule from the second coordination shell, and this activated water attacks the carbon atom of nitrile. After hydration an amide is ready to leave the enzyme.
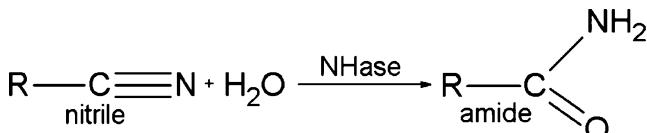
Fig. 1 Reaction catalysed by nitrile hydratase

Fig. 3 Substrates (*left*) and products (*right*) used in docking calculations. For abbreviations see the text

None of the solved 14 structures of NHases contains nitrile nor amide molecule. Known ligands are nitric oxide that reversibly binds to Fe-NHases (dissociation follows after light exposure) and inhibitors: 1,4-dioxane, *n*-butyric acid and cyclohexyl isocyanide [9, 10]. Since preferential catalytic efficiency for aromatic or aliphatic substrates may be related to orientations of these ligands in NHases interior, information on relative binding modes is highly desirable.

This paper for the first time presents hypothetical binding places of substrates (acrylonitrile, ACN; methacrylonitrile, MAN; benzonitrile, BNT; nicotinonitrile, NCN) and products (acryloamide, ACA; methacryloamide, MAA; benzoamide, BAM; nicotinamide, NCA), see Fig. 3, of cobaltous nitrile hydratase (Co-NHase).

Since the active site model contained a water molecule bound with Co^{3+} ion the first shell, presented data may give support either to the mechanism M2 or M3 only. Present docking studies provide a framework for detailed QM/MM calculations necessary for the complete explanation of the Co-NHase enzymatic activity.

Materials and methods

The docking of ligands into deprotonated *Pseudonocardia thermophila* JCM 3095 nitrile hydratase was performed using AutoDock 3.0.5 [17], the most cited docking software [18]. The crystal structure (pdb code 1IRE) was used as the reference protein [10]. Charges on the non-standard active site were taken from Gaussian 98 [19] Hartree-Fock
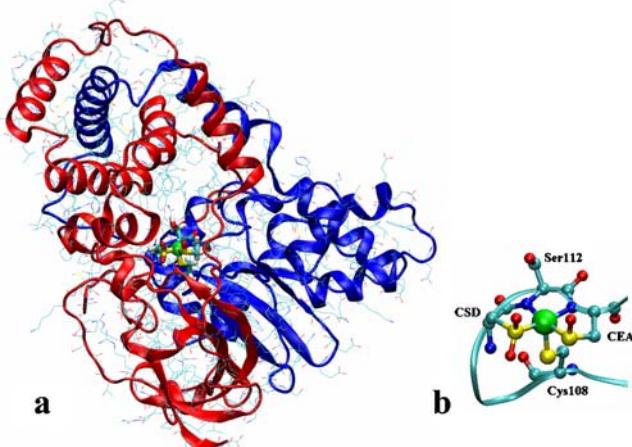
Fig. 2 Crystalographic structure of *Pseudonocardia thermophila* JCM 3095 nitrile hydratase (**a**) and the non-standard active site (**b**)

calculations in 6–31G* basis set. The model of the active centre was the same as in the paper [12]. All the other charges in the enzyme were standard Gasteiger like charges as implemented in AutoDock. For cobalt ion standard AutoDock 3.0.5 metal (Me) van der Waals parameters were used.

Geometries of isolated substrates and products were optimized using Gaussian 98 code with the HF method and 6–31G* basis set. The docked ligands were flexible, nonpolar hydrogens were united with carbons and charges were determined using the Gasteiger method. The choice of the quantum chemical method was compatible with the Charmm27 [20] force field. Postions of oxygen atoms from water molecules observed in protein 1IRE crystal structure were retained and were assumed to be the same for all ligands. Hydrogen atoms were added using ***psfgen*** tool from NAMD 2.5 suite [21]. We have checked that optimization of hydrogen atom positions does not affect results of substrate/product docking in the vicinity of the active site.

For each ligand + NHase system 256 starts of Lamarckian genetic algorithm were performed. In total 2048 binding places have been obtained, but we focused only on the 157 positions the closest to the active site, i.e. ligands with nitrogen atoms located less than 7 Å from the metal ion.

Analysis and figures of the enzyme were prepared using the VMD 1.8.5 code [22].

Results and discussion

Numbers of close lying hits near active site (Co^{3+}-N_{ligand} distance < 7 Å) and respective energies are presented in Table 1. Clearly aliphatic nitriles have much higher

Table 1 Comparison of the chosen parameters from AutoDock output and kinetic parameters for docked ligand

Ligand	Number of docks with dist <7	Closest distance Co-N	Energy of the closest dist. Co-N	Min energy for dist. <7	Max energy for dist. <7	Min energy	Max energy	Kinetic parameters [10]	
								k_{cat} (s^{-1})	K$_m$ (mM)
CNA	42	5.18	−3.09	−3.16	−2.98	−3.43	−2.48	1910	3.6
ACA	31	4.40	−3.59	−4.92	−3.34	−5.12	−3.10	−	−
MAN	30	5.42	−4.21	−4.30	−4.03	−4.74	−3.19	1000	0.49
MAA	36	4.40	−4.67	−4.90	−4.12	−4.90	−2.82	−	−
BNT	5	6.43	−4.81	−4.83	−4.78	−4.91	−3.29	123	0.02
BAM	3	4.82	−4.95	−5.54	−4.95	−5.54	−3.16	−	−
NCN	8	6.40	−4.96	−4.97	−4.74	−4.97	−3.27	131	0.12
NCA	2	5.96	−5.49	−5.49	−5.42	−5.72	−3.42	−	−

Distances are in Å and energies in kcal mol^{-1}.

propensity towards docking close to the metal ion. A number of "successful" docks is around 6 times higher than that for aromatic ones. One may expect that aromatic nitriles and amides are much more restricted in terms of possible low energy binding sites in the close vicinity to Co^{3+}. The number of docks determined in this study correlates quite well with the experimental activity of NHase shown in the last two columns of Table 1. Thus spatial restrictions in the Co-NHase cavity surrounding its active site are probable cause of this selective catalysis. Our observation of rather large conformational freedom for aliphatic nitriles is in agreement with results of Monte Carlo conformational search performed for Fe-NHase by Desai and Zimmer [23].

It is not possible to visualize all orientations of docked ligands. As representative information the closest lying binding sites are presented in Figs. 4, 5, 6 and 7. In figures (a) or (b) substrates or products are shown respectively.

Figure 4a presents a dock for ACN exhibiting the closest distance to the Co^{3+} centre. The nitrile group interacts with

αSer112 and βTyr68. There exists a possibility of creating a H-bond with one of these residues. Side chain of βTrp72 restricts the movement of the nitrile group. The acrylic group is surrounded by αGln89, αTrp116 and βLeu48.

Close lying docked ACA molecules show quite different favoured orientations in space (Fig. 4b). The amine group has close contacts with residues αCSD111 and αCEA113; also βArg52 may interact with this group. The amide group of ACA is stabilized by βLeu48 (not shown in the picture, it is located above βArg52). There is a possibility of H-bond formation between the amide oxygen and αGln89. Acrylic group is encompassed by βLeu48, βPhe51 and βTrp72 residues.

Locations of docked ligands the closest to the active site for MAN, an aliphatic ligand more bulky than ACN, is shown in Fig. 5a. The nitrile group is stabilized by αGln89. Interestingly, this residue is critical for maintaining catalytic activity of NHase [24]. Residues αTyr114 and αPro122 are perhaps stabilizing MAN, too. The methacrylic group in MAN (see Fig. 5a) is strongly interacting with αTrp116 and βTrp72.

**Fig. 4** A result of docking ACN (**a**) and ACA (**b**) into NHase
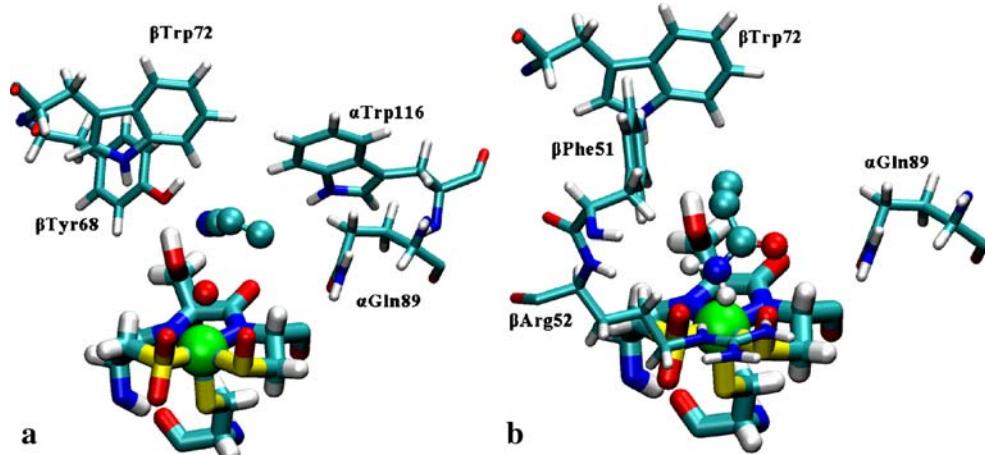
Fig. 5 A result of docking MAN (a) and MAA (b) into NHase
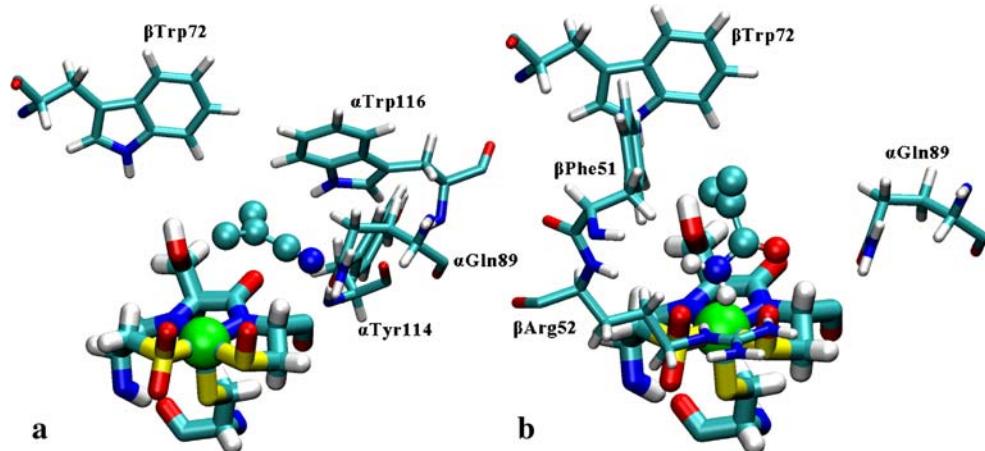
Despite the presence of the methyl group in MAA this amide docks identically as ACA (see Fig. 5b), orientations in space of common atoms are the same in both cases.

It was very interesting to observe different docking preferences for aromatic compounds. Aromatic nitriles and amides show a smaller number of orientations close to the active site but, in contrast to the aliphatic molecules, those docked aromatic molecules represent usually the best energetic scores. BNT has different orientation than ACN (see Fig. 6a). The nitrile group is probably stabilized by αCSD111, αCEA113, βLeu48 (not shown) and βArg52. Phenyl ring is almost parallel to the active site plane and is surrounded by αGln89, αTrp116, βPhe51, βTyr68 and βTrp72.

In contrast to aliphatic amides, in aromatic BAM preferred docked positions the amine group interacts with αSer112, αTrp116 and βTyr68 (see Fig. 6b). There is a possibility of stabilizing interaction between amide's oxygen and βTrp72. Contrary to the BNT case here a phenyl ring is almost perpendicular to the active site plane. It is stabilized by αGln89, αTrp116, βPhe37and βLeu48 (not shown).

NCN docks identically like BNT (compare Fig. 6a and Fig. 7a), but in this case the pyridine ring has a nitrogen

atom. This nitrogen can create a weak H-bond with αSer112. The rest of interactions are the same as for BNT. Also a dock for NCA is similar to BAM, but the amide group is rotated by 180° (see Fig. 7b). The oxygen from the amide group may create H-bond with αSer112 and one of amine hydrogens can form another H-bond with βTyr68. The amide group interacts also with βPhe51 and βTrp72. The pyridine ring is stabilized by the same residues like in the BAM case. The aromatic nitrogen is close to βLeu48 but it cannot create any H-bonds.

In Fig. 8 interactions of ACN with water molecules are shown. This arrangement of nitrile may help to determine the proper catalytic mechanism. The water molecule closest to metal ion is probably substituted by a hydroxide ion, since the oxydized cysteine residues change pK_a of the bound water [25]. Hydroxide ion which is slightly bent towards deprotonated CSD111 could perform nucleophilic attack on carbon from the nitrile (the postulated mechanism M2). Then anionic amide intermediate, temporarily bound to metal ion by oxygen, would be formed. The carbon atom from this transient molecule could be attacked by one of five close lying water molecules to form an amide. These waters in 1IRE PDB entry have numbers: 62, 73, 33, 163,

Fig. 6 A result of docking BNT (a) and BAM (b) into NHase
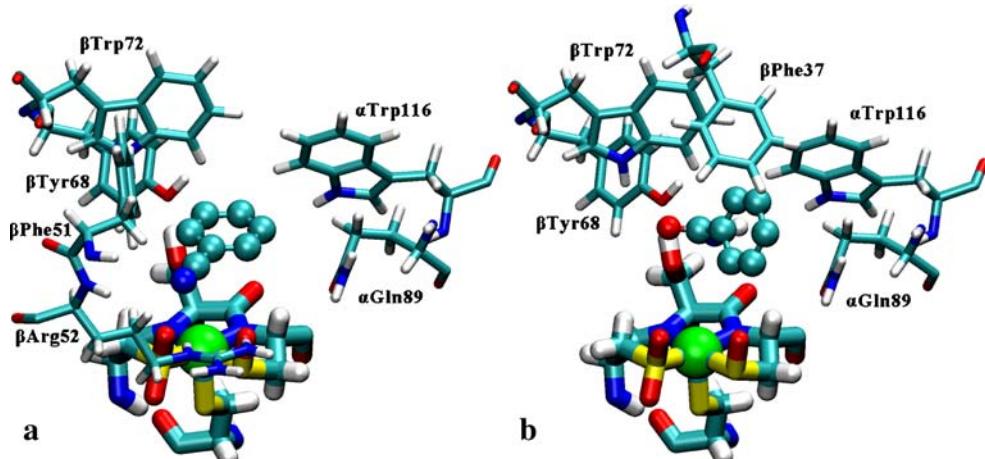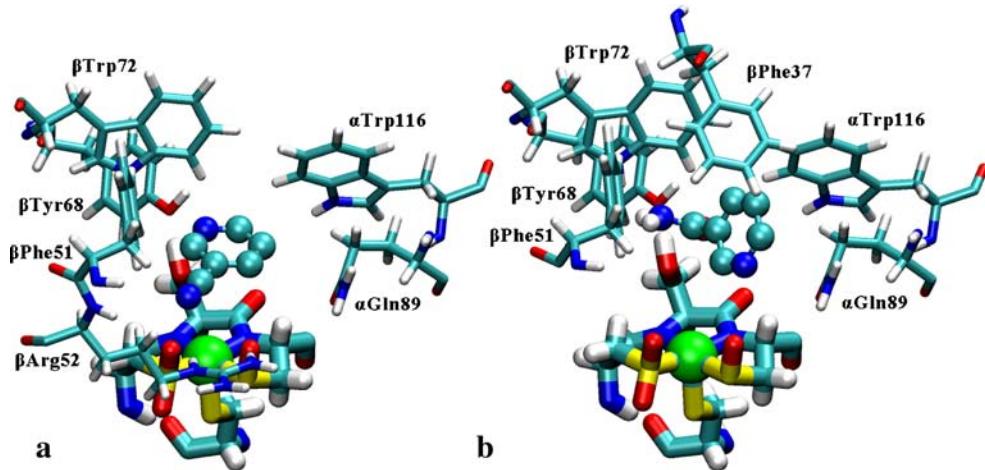
237. The most probable position of the attacking water molecule seems to be represented by water 33.

The other postulated mechanism, involving hydroxide activated water (M3), is less probable, because of the absence of any close lying waters near the water molecule directly connected to the metal ion in the crystallographic structure.

To check the first hypothetical mechanism M1 involving direct interaction of nitrile with the metal ion different docking calculations without coordinated water should be performed.

Finally, it is worth noting that in all studied (aliphatic and aromatic) amides many energetically favoured locations were oriented towards αSer112 by the amide group (as in Figs. 6b and 7b). However, only small aliphatic amides were interacting with sulfinic and sulfenic oxygens from a claw setting of NHase postulated by Nagashima et al. [1]. This may be related to experimentally observed preference of *Pseudonocardia thermophila* NHase to aliphatic nitriles.

Conclusions

Docking of aliphatic and aromatic nitriles (substrates) and amides (products) to a crystal structure model of industrially important enzyme nitrile hydratase from *Pseudonocardia thermophila* JCM 3095 shows remarkable differences in preferred docking sites. Under similar computational conditions aliphatic ligands dock much easier to the vicinity of the enzyme active site. A number of productive docks correlate well with experimental activity. Particularly favorable docking positions are observed for acrylonitrile. Binding mode of aliphatic amides (the amide group oriented towards αCys113-SO$^-$ and αCys111-SO$_2^-$) is quite different than aromatic ones (amide group towards αSer112). Analysis of relative positions of crystallographic waters and the best docked acrylonitrile indicates that molecular arrangements are consistent with M2 postulated mechanism of NHase catalytic activity which involves hydrolysis by a metal bound hydroxide.

Further docking studies of ligands directly bound to the metal center are necessary in order to verify an alternative mechanism (M1). Present report makes a good starting point for elaborate QM/MM calculations for alternative reaction paths in Co-NHase.
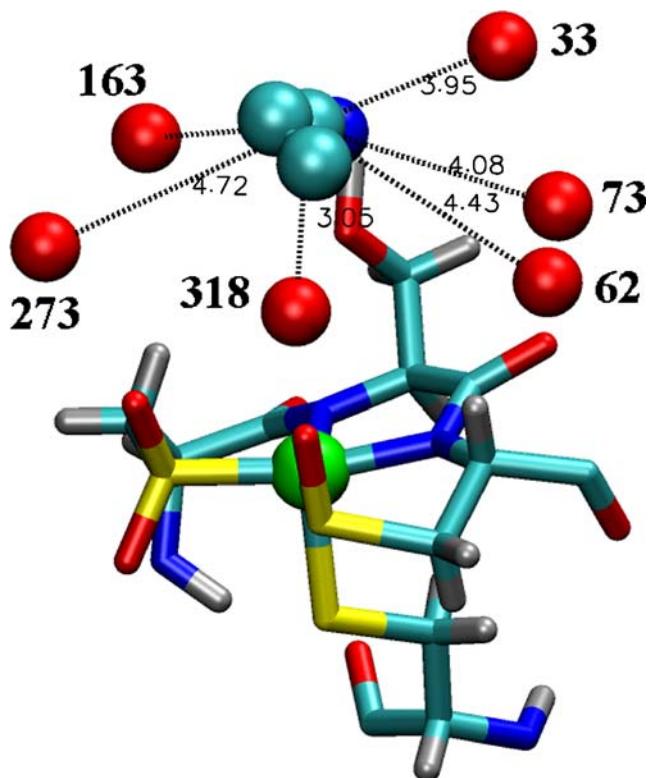

Fig. 8 Arrangement of water molecules near docked NCA. Distances (in Å) from nitrile carbon to oxygens are shown

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