ELSEVIER

Journal of Molecular Catalysis B: Enzymatic 10 (2000) 81–86

JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC

www.elsevier.com/locate/molcatb

What evidences were elucidated about photoreactive nitrile hydratase?

Isao Endo *, Masafumi Odaka

Biochemical Systems Laboratory, The Institute of Physical and Chemical Research (RIKEN), Wako, Saitama 351-0198, Japan

Received 6 August 1999; accepted 5 January 2000

Abstract

Characteristic features of a photoreactive nitrile hydratase (NHase) from *Rhodococcus* sp. N-771 were introduced. We have found a new biological function of nitric oxide (NO) that controls the photoreactivity of NHase by association with the non-heme iron active center and photoinduced dissociation from it. We have elucidated the crystal structure of NHase in the nitrosylated state at 1.7 Å resolution. A unique structure of the photoreactive catalytic center of the enzyme was revealed; the center consists of the non-heme iron atom coordinated with the sulfur atoms from three cysteine residues (αCys109, αCys112 and αCys114) and two amide nitrogen atoms in the main chain of αSer113 and αCys114. Two out of the three cysteine residues are post-translationally modified into cysteine sulfinic (αCys112-SO_2H) and -sulfenic (αCys114-SOH) acids. The two oxygen atoms of αCys112-SO_2H and αCys114-SOH form a unique structure, Claw setting, together with the oxygen atom, form αSer113. We have also studied the roles of the hydration water molecules in the enzyme. The hydration water molecules stabilize the catalytic center and the tertiary and quaternary structures of the enzyme. The detailed hydration structure around the active center is also discussed.

Keywords: Nitrile hydratase; Non-heme iron; Post-translational modification; Claw setting; Hydration water molecule

1. Introduction

Nitrile hydratase (NHase), which hydrates various aliphatic and aromatic nitriles to their corresponding amides [1], is important for the industrial production of acrylamide (more than 30,000 tons/year) [2–4]. The enzyme is a soluble metalloenzyme containing a non-heme iron [5] or a non-corrinoid cobalt [6] atom at the catalytic center and consists of two subunits (α and β) with the basic stoichiometry of $\alpha_1\beta_1M_1$ (M, Fe^{3+} or Co^{3+}) [7]. Both subunits have a molecular weight of approximately 23 kDa. Their primary sequences are well conserved while there are no apparent homology between the two subunits [2].

NHase from *Rhodococcus* sp. N-771 belongs to the Fe-type NHase family and is known to have a unique photoreactivity [8,9]. In vivo, the activity of this enzyme disappears during aerobic incubation in the dark for 16 h, but is almost completely recovered by light irradiation (Fig. 1). Interestingly, the photoactivated enzyme cannot be inactivated by darkness in vitro [9].

* Corresponding author. Tel.: +81-48-467-9311; fax: +81-48-462-4658.

E-mail address: endo@cel.riken.go.jp (I. Endo).

1381-1177/00/$ - see front matter
PII: S1381-1177(00)00116-8

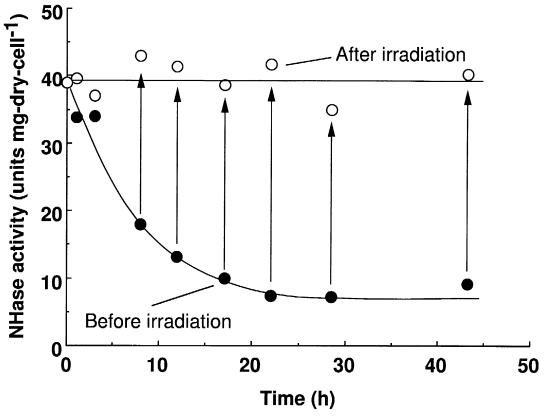

Fig. 1. The dark inactivation and the photoactivation of NHase in *Rhodococcus* sp. N-771. Cells were washed with fresh broth and resuspended with fresh broth at the concentration of 2.0 mg dry cell/ml. The cell suspension was irradiated with white light for 30 min just before incubation in the dark. At each indicated time, cell suspension was sampled, and the activity of hydration of propionitrile to propionamide was assayed in the darkness and after light irradiation. A unit of the activity is defined as the amount of enzyme which produces 1 μmol of propioamide per minute and the specific activity is expressed as units per milligram dry cell.

Similar results have been observed in other NHases from *Rhodococcus* sp. N-774 [10] and R312 [11].

Photoactivation induces UV–visible absorptional changes in NHase (Fig. 2) [9,12]. The inactive NHase shows two absorption peaks at 280 and 370 nm; upon light irradiation, the 370-nm peak almost disappears. Instead, a shoulder at around 400 nm and a small but broad peak at 710 nm appear. Flash photolysis studies show that these spectral changes occur within 50 ns [12]. Spectral changes of electron spin resonance (ESR) [13] and those of Mössbauer [14] were also accompanied by photoactivation. These results mean that the non-heme catalytic center functions as the photoreactive site.

2. New biological function of nitric oxide (NO)

Fourier transform infrared (FT-IR) difference spectra of NHase between before and after photoactivation show large negative bands at around 1850 cm^{-1}, where biochemical compounds do not usually show any signal [15]. The suggestion that NO was responsible for this result was confirmed by its isotope shift using ^{15}N labeling [15]. To prove this hypothesis more clearly, we examined nitrosylation of NHase by NO gas (Fig. 3) [16]. The photoactivated NHase was transformed into the inactive state by binding of exogenous NO and, in turn, the nitrosylated enzyme was re-activated by light irradiation. Besides this result, the interaction between NO and the non-heme iron has been detected directly by resonance Raman spectroscopy [17]. These results indicate that the NO molecule bound to the non-heme iron center is released upon photoactivation, and that the activated NHase can be reconverted to the inactive form by NO binding. The amount of NO release from NHase upon photoactivation has been estimated, using NO scavenger, to be about one NO molecule per enzyme molecule [16]. The quantum yield of the photoactivation has been estimated to be 0.48 by laser flash photolysis studies [16].

It is well known that NO plays various important roles in biological systems; in higher

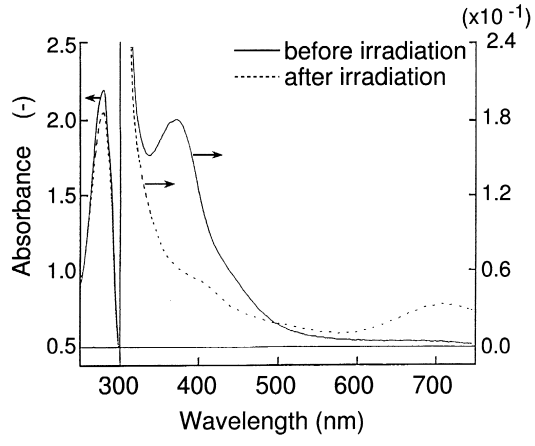

Fig. 2. UV–visible spectra of the photoreactive NHase. NHase is dissolved in 50 mM Hepes–KOH, pH 7.5, containing 20 mM *n*-butyric acid at a concentration of 1.3 mg/ml. The absorption spectra were measured before and after light irradiation for 15 min on ice.

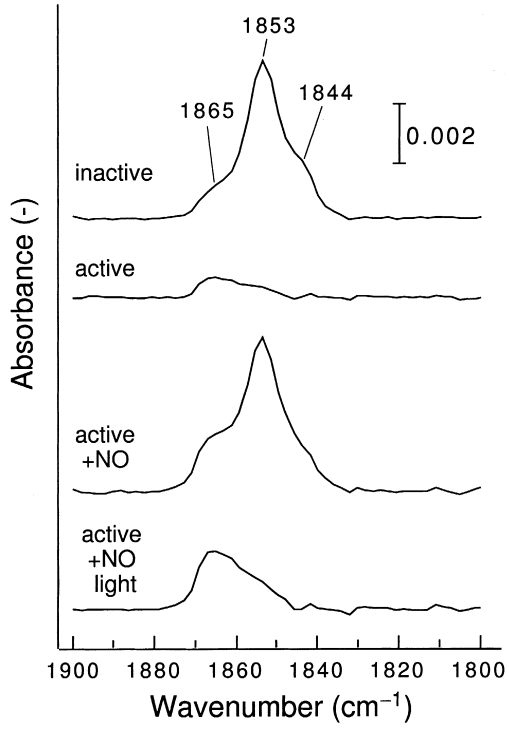

Fig. 3. The effects of exogenous nitric oxide and photoirradiation on FT-IR spectra of NHase. From the top row, inactive NHase, active NHase, active NHase + NO, active NHase + NO (after photoirradiation).

animals such as mammals, these include blood pressure control, neurotransmission and the immure response [18,19]. Also, NO binds to various heme and non-heme iron proteins with high affinity and regulates their biological functions [20–22]. For instance, the activity of guanylyl cyclase is regulated by NO binding to its heme iron [21,22]. Thus, we believe that the finding — that NHase activity is regulated by NO in combination with light in bacteria — provides a new aspect of biological function of NO. Finally, we could picture the activation and inactivation mechanisms of this NHase associated with photoirradiation and NO binding as it was shown in Fig. 4.

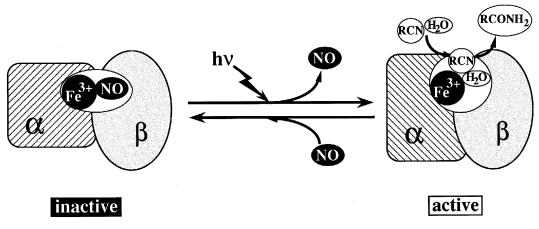

Fig. 4. Scheme of the activity regulation of the photoreactive NHase from *Rhodococcus* sp. N-771 by NO.

3. X-ray crystallographic analysis revealed a unique structure of the active center

The crystal structure of NHase in the inactive state was determined by the authors at a resolution of 1.7 Å [23]. In particular, it was revealed that photoreactive catalytic center consists of the non-heme iron atom coordinated with the sulfur atoms from three cysteine residues (αCys 109, αCys112 and αCys114) and two amide nitrogen atoms in the main chain of αSer113 and αCys114. In the inactive state, an endogenous NO molecule occupies the sixth coordination site. The structure around the active center was shown in Fig. 5. As shown by electrospray mass spectrometry of a tryptic peptide around the iron center isolated from the photoactivated enzyme [24], αCys112 was post-translationally modified to a cysteine sulfinic acid (Cys-SO_2H). Moreover, the crystal structure demonstrated that αCys114 was also post-translationally modified to a cysteine sulfenic acid [23]. This modification of αCys114 was verified by using Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR-MS) against the active center

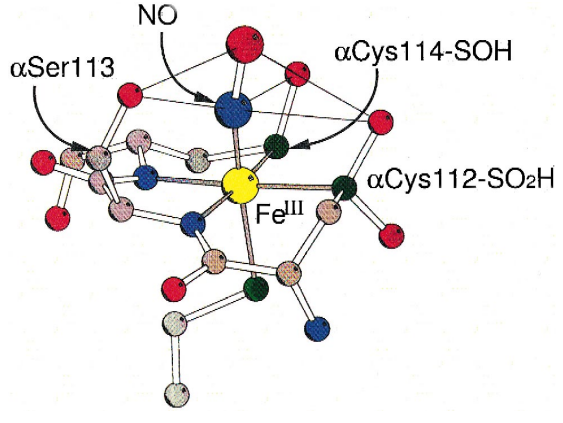

Fig. 5. The ''claw setting'' of the nitrosylated NHase. The carbon atom, the nitrogen atom, the oxygen atom, the sulfur atom and the iron ion are indicated by a gray sphere, a blue sphere, a magenta sphere, a green sphere and a yellow sphere, respectively.

peptide prepared from the inactive enzyme [23]. We note that the X-ray crystal structure analysis is not always sufficient for precise determination of a novel structure having post-translational modifications in a protein. In these cases, confirmation by other investigations such as FT-ICR-MS is ultimately important. The two oxygen atoms of the cysteine sulfenic and sulfinic acids form a unique structure — claw setting — together with the oxygen atom from αSer113 [23]. The structure of the active center is suitable for persistently capturing an endogenous NO molecule.

4. Roles of hydration water molecules in the NHase

In the crystal structure analysis of NHase in the inactive state, we identified more than 800 water molecules both in the interior and at the exterior of the enzyme [25]. These hydration water molecules worked as glue to stabilize the catalytic center, the αβ heterodimer and the $(\alpha\beta)_2$ heterotetramer. Seventy-six hydration water molecules mediated the intersubunit interactions, and 30 out of 76 molecules filled the cavities between two subunits. In addition, we also found that hydration water molecules neutralized the negatively charged surface of the β subunit, and thus enabled the association of the two subunits having the different electrostatic properties at the surfaces.

The distribution of hydration water molecules at the interface was prominent around the active center; 20 hydration water molecules were spanned in a network around the center (Fig. 6). Networks of 57 hydrogen bonds were additionally formed at that interface around the center by the hydration of water molecules, while only six direct hydrogen bonds were observed within protein atoms of the α and β subunits alone. The roles of these hydration water molecules were categorized into three groups with respect to the partner of their hydrogen bonds. (i) Three of them (2, 6 and 17 in Fig. 6; red circles) directly interacted with the atoms in the active center. (ii) Three of them (7, 13 and 14 in Fig. 6; black circles) mediated the hydrogen bond networks between the two subunit. (iii) Thirteen of them (1, 3, 4, 8, 9, 10, 11, 12, 15, 16, 18, 19 and 20 in Fig. 6; blue circles) stabilized the conformation of each subunit. Two hydration water molecules (6 and 17 in Fig. 6) in the first

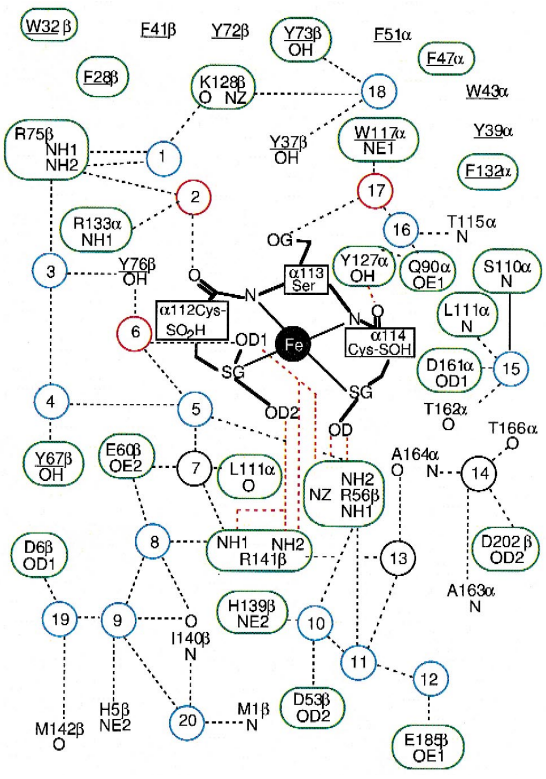

Fig. 6. Schematic diagram for the extensive networks of hydrogen bonds formed around the active center. The atoms forming hydrogen bonds are indicated beside the residue numbers (O: the oxygen atom of main chain amide; OD: the δ oxygen atom of Asp, Cys-SOH or Cys-SO2H; OE: the ε oxygen atom of Glu; OG: the γ oxygen atom of Ser; N: the nitrogen atom of main chain amide; NE: the ε nitrogen atom of Arg; NH: the η nitrogen atom of Arg; NZ: the ζ nitrogen atom of Arg; SG: the γ sulfur atom of Cys). Possible hydrogen bonds are indicated by dotted lines. The black dotted lines are hydrogen bonds formed between hydration water molecules and protein atoms, and the red ones are those between the polar protein atoms alone. Hydration water molecules are indicated with circles. Colors of the circles correspond to the categories of hydration water molecules described in the text. The residues forming the active center are indicated with red boxes. The residues with green boxes are conserved among six Fe-type and Co-type NHases. Those underlined are the members of the aromatic cluster.

group are likely to determine the conformation of the side chains of αCys112-SO_2H and αSer113. Six hydration ones (8, 10, 11, 12, 14 and 19 in Fig. 6) greatly contributed to neutralizing the negatively charged surface of the β subunit. Four molecules (8, 10, 11 and 13 in Fig. 6) fixed the conformation of the side chains of βArg141 and βArg56, which stabilized directly the claw setting structure [23]. Thus, the roles of the hydration water molecules in the subunit interface are summarized as follows: (1) extension of the hydrogen bond networks connecting the two subunits; (2) increasing the complementarity of the surface shape of the two subunits; and (3) moderation of the surface charge of β subunit. We note that the 17 amino acid residues around the active center (shown in Fig. 6) are highly conserved among all known Fe-type [26–29] and Co-type NHases [30,31] and even in the homologous enzyme, thiocyanate hydrolase [32], suggesting structural conservation of the hydration water molecules in the NHase family.

5. Concluding remarks

We have introduced some recent findings on NHase from *Rhodococcus* sp. N-771. This is the first enzyme whose photoreactivity is controlled by NO. Also, the enzyme is the only metalloenzyme that has the post-translationally modified cysteine ligands in its metal site in a native protein. The detailed roles of these modified residues in the photoreaction and the catalysis remain unknown. However, recent mutant studies suggested that these modified side chains were essential for the catalytic activity [33]. The studies on the novel metal active center may provide a new insight for biological functions of post-translationally oxidized cysteine residues.

Acknowledgements

We gratefully acknowledge Drs. M. Nakasako, T. Noguchi, M. Yohda, M. Hoshino, S. Nagashima, M. Tsujimura, J. Honda, N. Dohmae, K. Takio, N. Kamiya, T. Nagamune, Y. Kobayashi and many students for their energetic contribution to these studies. We also thank the Biodesign Research Program, SR Structural Biology Program, Essential Chemistry Project and Promotion of Research in RIKEN, and the Grants-in Aid for Scientific Research on Priority (11116232) from the Ministry of Education, Science, Sports and Culture of Japan for their financial support.

References

[1] Y. Asano, Y. Tani, H. Yamada, Agric. Biol. Chem. 44 (1980) 2251.
[2] M. Kobayashi, T. Nagasawa, H. Yamada, Trends Biotechnol. 10 (1992) 402.
[3] M. Kobayashi, S. Shimizu, Nat. Biotechnol. 16 (1998) 733.
[4] H. Yamada, H. Kobayashi, M. Kobayashi, Biosci. Biotechnol. Biochem. 60 (1996) 1391.
[5] Y. Sugiura, J. Kuwahara, T. Nagasawa, H. Yamada, J. Am. Chem. Soc. 109 (1987) 5848.
[6] B.A. Brennan, G. Alms, M.J. Nelson, L.T. Durney, R.C. Scarrow, J. Am. Chem. Soc. 118 (1996) 9194.
[7] M. Odaka, T. Noguchi, S. Nagashima, M. Yohda, S. Yabuki, M. Hoshino, Y. Inoue, I. Endo, Biochem. Biophys. Res. Commun. 221 (1996) 146.
[8] T. Nagamune, H. Kurata, M. Hirata, J. Honda, A. Hirata, I. Endo, Photochem. Photobiol. 51 (1990) 87.
[9] T. Nagamune, H. Kurata, M. Hirata, J. Honda, H. Koike, M. Ikeuchi, Y. Inoue, A. Hirata, I. Endo, Biochem. Biophys. Res. Commun. 168 (1990) 437.
[10] T. Nakajima, K. Takeuchi, H. Yamada, Chem. Lett. 9 (1987) 1767.
[11] B.A. Brennan, J.G. Cummings, D.B. Chase, I.M. Turner Jr., M.J. Nelson, Biochemistry 35 (1996) 10067.
[12] J. Honda, H. Kandori, T. Okada, T. Nagamune, Y. Shichida, H. Sasabe, I. Endo, Biochemistry 33 (1994) 3577.
[13] J. Honda, T. Nagamune, Y. Teratani, A. Hirata, H. Sasabe, I. Endo, Ann. N. Y. Acad. Sci. 672 (1992) 29.
[14] J. Honda, Y. Teratani, Y. Kobayashi, T. Nagamune, H. Sasabe, A. Hirata, F. Ambe, I. Endo, FEBS Lett. 301 (1992) 177.
[15] T. Noguchi, J. Honda, T. Nagamune, H. Sasabe, Y. Inoue, I. Endo, FEBS Lett. 358 (1995) 9.
[16] M. Odaka, K. Fujii, M. Hoshino, T. Noguchi, M. Tsujimura, S. Nagashima, M. Yohda, T. Nagamune, Y. Inoue, I. Endo, J. Am. Chem. Soc. 119 (1997) 3785.
[17] T. Noguchi, M. Hoshino, M. Tsujimura, M. Odaka, Y. Inoue, I. Endo, Biochemistry 35 (1996) 16777.
[18] C. Nathan, FASEB J. 6 (1992) 3051.
[19] H.H.H.W. Schmidt, U. Walter, Cell 78 (1994) 919.
[20] J.S. Stamler, Cell 78 (1994) 931.
[21] T.G. Taylor, V.S. Sharma, Biochemistry 31 (1992) 2847.

[22] A. Tsai, FEBS Lett. 341 (1994) 141.
[23] S. Nagashima, M. Nakasako, N. Dohmae, M. Tsujimura, K. Takio, M. Odaka, M. Yohda, N. Kamiya, I. Endo, Nat. Struct. Biol. 5 (1998) 347.
[24] M. Tsujimura, N. Dohmae, M. Odaka, M. Chijimatsu, K. Takio, M. Yohda, M. Hoshino, S. Nagashima, I. Endo, J. Biol. Chem. 272 (1997) 29454.
[25] M. Nakasako, M. Odaka, M. Yohda, N. Dohmae, K. Takio, N. Kamiya, I. Endo, Biochemistry 38 (1999) 9887.
[26] M. Nojiri, M. Yohda, M. Odaka, Y. Matsushita, M. Tsujimura, T. Yoshida, N. Dohmae, K. Taiko, I. Endo, J. Biochem. (Tokyo) 125 (1999) 696.
[27] O. Ikehata, M. Nishiyama, S. Horinouchi, T. Beppu, Eur. J. Biochem. 181 (1989) 563.
[28] J. Mayaux, E. Cerbelaud, F. Soubrier, D. Faucher, D. Pétré, J. Bacteriol. 172 (1990) 6764.
[29] M. Nishiyama, S. Horinouchi, M. Kobayashi, T. Nagasawa, H. Yamada, T. Beppu, J. Bacteriol. 173 (1991) 2465.
[30] M.S. Payne, S. Wu, R.D. Fallon, G. Tudor, B. Stieglitz, I.M. Turner Jr., N.J. Nelson, Biochemistry 36 (1997) 5447.
[31] M. Kobayashi, M. Nishiyama, T. Nagasawa, S. Horinouchi, T. Beppu, H. Yamada, Biochim. Biophys. Acta 1129 (1991) 23.
[32] Y. Katayama, Y. Matsushita, M. Kaneko, M. Kondo, T. Mizuno, H. Nyunoya, J. Bacteriol. 180 (1998) 2583.
[33] S.R. Piersma, M. Nojiri, M. Tsujimura, T. Noguchi, M. Odaka, M. Yohda, I. Endo (7), J. Inorg. Biochem (2000) in press.