Photosensitive nitrile hydratase intrinsically possesses nitric oxide bound to the non-heme iron center: evidence by Fourier transform infrared spectroscopy

Takumi Noguchi[a,*], Jun Honda[b], Teruyuki Nagamune[c,*], Hiroyuki Sasabe[b], Yorinao Inoue[a], Isao Endo[d]

[a]*Solar Energy Research Group, The Institute of Physical and Chemical Research (RIKEN), Wako, Saitama 351-01, Japan*
[b]*Frontier Research Program, RIKEN, Saitama, Japan*
[c]*Faculty of Engineering, University of Tokyo, Bunkyo-ku, Tokyo 113, Japan*
[d]*Chemical Engineering Laboratory, RIKEN, Saitama, Japan*

Received 18 November 1994

Abstract Nitrile hydratase (NHase) from *Rhodococcus* sp. N-771 is a photosensitive enzyme that catalyzes hydration of nitriles to the corresponding amides. Light-induced Fourier transform infrared difference spectra between the inactive and active forms of NHase were measured with both the natural (^{14}N) and ^{15}N-labeled NHases. The results showed, for the first time, that NHase intrinsically possesses nitric oxide (NO) molecules bound to the non-heme iron center. The possible role of NO in the photoactivation process of NHase is discussed.

Key words: Nitric oxide; Nitrile hydratase; Fourier transform infrared spectroscopy; Non-heme iron; Photoactivation

1. Introduction

Nitrile hydratase (NHase) is an enzyme that catalyzes hydration of nitriles to the corresponding amides [1]. NHase from *Rhodococcus* sp. N-771, which consists of two subunits, α (M_r = 22,787) and β (M_r = 23,428) [2], possesses two non-heme iron atoms as cofactors [3], and shows a unique manner of photoactivation, i.e. the enzyme is activated by light irradiation [4,5]. This NHase from *R.* sp. N-771 is probably the same as that from *R.* sp. N-774 and *Brevibacterium* sp. R312, because the amino acid sequences are identical among the three [2,6,7]. Although various spectroscopies, including EXAFS [8], EPR [9], ENDOR [10], resonance Raman [8], and Mössbauer [3], have been applied to the NHase from these bacteria, the detailed structure of the di-iron center has not been clarified yet. Also, the mechanism of photoactivation is not well understood except that oxidation of the ferrous iron to the ferric iron takes place within 50 ns upon light irradiation [3,11]. In this study, by means of light-induced Fourier transform infrared (FTIR) difference spectroscopy (for a recent review of this technique, see [12]), we have found that NHase from *R.* sp. N-771 intrinsically possesses nitric oxide (NO) molecules bound to the iron center. The possible role of NO in the photoactivation process will be discussed.

2. Materials and methods

NHase was purified according to the method described previously [11]. For ^{15}N substitution, *R.* sp. N-771 cells were cultured with growth medium described in [3] except that $(^{15}NH_4)_2SO_4$ (Shoko Co., ^{15}N enrichment ~99%) and Celtone-N (Martek Corp., ^{15}N enrichment ~98%) were used as nitrogen sources.

FTIR spectra were measured on a JEOL JIR-6500 spectrophotometer equipped with an MCT detector (EG&G JUDSON IR-DET101) as described previously [13]. The inactive NHase sample (about 200 mg/ml) in 20 mM phosphate buffer (pH 7.5) with 20 mM *n*-butyric acid was placed between a pair of BaF_2 plates and set in a cryostat (Oxford DN1704). The sample amount was controlled so that the absorption peak at about 1650 cm^{-1} due to amide I and water bands was between 0.5 and 1.0. The sample temperature was adjusted to 250K with a temperature controller (Oxford ITC-4). This low temperature was used to avoid fluctuation of the spectral background. FTIR spectra before (inactive form) and after (active form) illumination were measured, and the difference spectrum was obtained by subtracting the former from the latter spectrum. Light illumination was performed by continuous white light from a halogen lamp with a heat-cut filter (60 mW/cm^2 at the sample surface) for 10 s. Each single beam spectrum was an average of 300 scans (150 s accumulation). The spectral resolution was 4 cm^{-1}.

3. Results and discussion

Fig. 1A shows a light-induced FTIR difference spectrum between active (after illumination) and inactive (before illumination) forms of NHase from *R.* sp. N-771 in the 2000–900 cm^{-1} region. In this difference spectrum, only the FTIR bands influenced by the structural perturbations upon photoactivation are exhibited, and the negative and positive bands belong to the inactive and active forms, respectively. A lot of bands were observed overlapping each other in the frequency region lower than 1700 cm^{-1}, which is within the protein absorptive region (< 1750 cm^{-1}) [14,15]; complex structures in the typical amide I (C=O stretching modes of backbone amides) region (1700–1600 cm^{-1}) [16], and strong differential peaks in 1200–1000 cm^{-1} were especially prominent. Besides these bands, to our surprise, there appeared large negative bands at 1855 and 1847 cm^{-1} and a positive band at 1869 cm^{-1} in the region where proteins and amino acids never show their fundamental infra-red bands. It is also highly unlikely that overtones and combination modes of proteins exhibit such strong infra-red intensities. Even in the original FTIR spectrum (inactive form of NHase) before

*Corresponding authors. Fax: (81) (48) 462-4685.

Abbreviations: ENDOR, electron nuclear double resonance; EPR, electron paramagnetic resonance; EXAFS, extended X-ray absorption fine structure; FTIR, Fourier transform infrared; NHase, nitrile hydratase; NOS, nitrile oxide synthase.

SSDI 0014-5793(94)01374-8

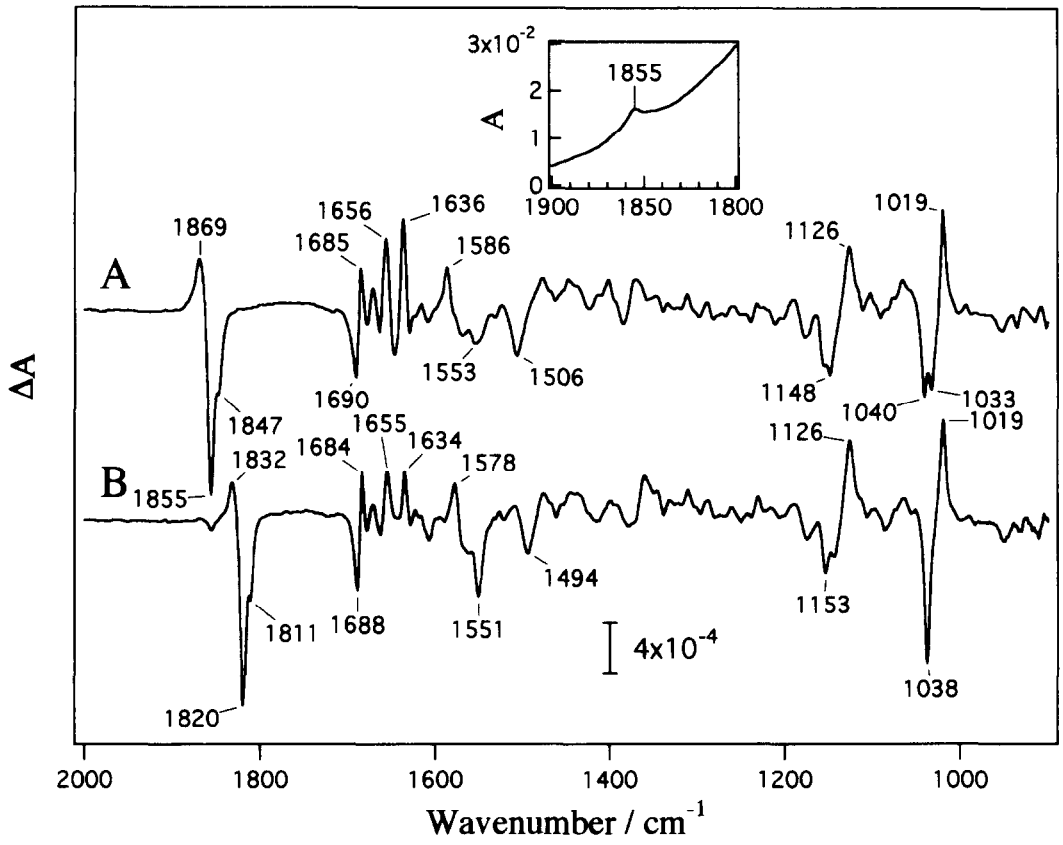

Fig. 1. Light-induced FTIR difference spectra of NHase (active form minus inactive form) measured at 250K. (A) Natural (^{14}N) NHase. (B) ^{15}N-labeled NHase. The inset shows the original spectrum of natural NHase (inactive form) before subtraction in the 1900–1800 cm^{-1} region.

subtraction (Fig. 1, inset), the bands around 1855 cm^{-1} were clearly observed on the foot of the large water and protein bands, indicating that the ~1855 cm^{-1} bands are not some artifact due to spectral substitution.

Nitric oxide is a well-known molecule that has an infrared band due to its stretching mode near 1850 cm^{-1} in nitrosyl metal complexes (free NO has a band at 1876 cm^{-1}) [17]. Cyanide (CN^-), which seems to be an analog of nitrile substrates, cannot be a candidate for these bands, because its metal complexes are known to show bands in 2200–2000 cm^{-1} [17]. Also, other compounds with CN triple- or double bonds show their stretching bands in the region higher than 1990 cm^{-1} (nitriles, isonitriles, nitrile *N*-oxides, cyanamides, cyanates, thiocyanates, isocyanates, isothiocyanates, diazo compounds, and carbodiimides) or lower than 1750 cm^{-1} (imines, oximes, and amidines) [17,18], and are excluded from being candidates. Carbon monoxide (CO) usually shows bands in the region 2100–2000 cm^{-1} in the metal complexes, but it can show bands in the region 1900–1800 cm^{-1} if it forms a bridge between two metals [17].

For definite assignments of the three bands at 1870–1840 cm^{-1} in the NHase FTIR spectrum, ^{15}N-labeled NHase was prepared from *R.* sp. N-771 cells cultured with growth medium in which the nitrogen sources were replaced by ^{15}N-labeled ones, and its light-induced FTIR difference spectrum was measured (Fig. 1B). All the three bands at 1869, 1855 and 1847 cm^{-1} in natural (^{14}N) NHase were shifted to lower frequencies (1832, 1820 and 1811 cm^{-1}) in ^{15}N-labeled NHase (Fig. 2). In the ^{15}N spectrum (Fig. 2, dotted curve), only small bands were observed at around 1855 cm^{-1}, due to unsubstituted ^{14}N species. From their relative intensities, the percentage of substituted ^{15}N was estimated to be about 95%. The observed downshifts clearly indicate that these three bands are attributed to the vibrations including nitrogen. Thus, the bands at 1869, 1855 and 1847 cm^{-1} can be assigned to the stretching mode of NO molecules.

The extent of isotope shifts upon ^{15}N substitution was calculated by assuming a free diatomic NO molecule, and the resultant NO stretching values were compared with the observed ones (Table 1). The agreements between calculated and experimental values were excellent, confirming that these three bands are ascribed to the NO stretching modes. We note that the slightly higher values (by 2–4 cm^{-1}) by diatomic calculation than the observed ones are a general tendency seen in the experimental data of ^{15}N-isotopic shifts in various nitrosyl–metal complexes [19], and are most probably due to the interaction with the metal ion (see below).

The ^{15}N-substitution experiment further proved that the detection of NO in NHase is not due to contamination in the laboratory during purification or measurement procedures; substitution of ^{15}NO for ^{14}NO was achieved, not by direct addition of ^{15}NO, but by bacterial culture with ^{15}N-labeled medium. This indicates that NHase from *R.* sp. N-771 intrinsically contains NO. Very recently, bacterial NO synthase (NOS) has been discovered for the first time in a *Nocardia* species [20]. Since *Rhodococcus* and *Nocardia* are known to be closely related [21], it is probable that *Rhodococcus* sp. N-771 also possess bacterial NOS, and NO in NHase is produced by this NOS.

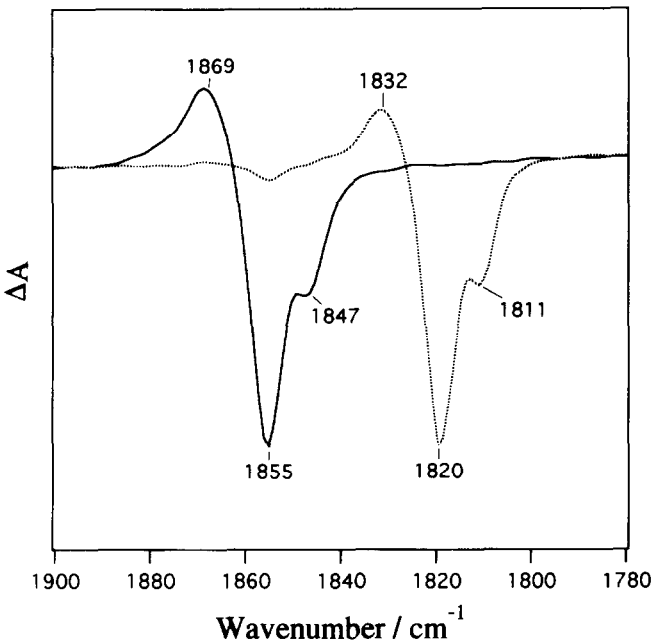

Fig. 2. Expanded view of the FTIR difference spectra between active and inactive forms of NHase in the NO stretching region. Solid curve, natural (^{14}N) NHase; dotted curve, ^{15}N-labeled NHase.

The Mössbauer and magnetic susceptibility studies have shown that the oxidation state of the di-iron center in NHase is changed from Fe(II)Fe(III) to Fe(III)Fe(III) upon photoactivation [3]. Since the frequencies of the NO stretching bands are affected by this redox change, as shown in the FTIR difference spectrum (Fig. 1), it is strongly suggested that the NO molecules are coordinated to the iron atoms. This view is quite reasonable because free NO is unstable in aqueous solution (the lifetime is assumed to be in the order of seconds) and NO readily forms complexes with transition metal ions [22]. Indeed, the interaction of exogenous NO with heme iron has been widely studied [23].

Two negative NO bands belonging to the inactive form of NHase appeared at 1855 and 1847 cm^{-1}, indicating that two NO molecules are included in NHase. However, only one positive band at 1869 cm^{-1}, due to the active form, was observed near this region. Even in the second-derivative spectrum (not shown), no shoulder could be found in the 1869 cm^{-1} band. It has been known that NO exhibits its stretching frequency over a wide range depending on its redox forms; neutral NO is a radical showing a band at 1876 cm^{-1} [17], and it can donate and accept an electron to form NO^+ with a ~2200 cm^{-1} band [17] and NO^- with a ~1300 cm^{-1} band [24], respectively. In nitrosyl complexes, the NO stretching values range between 2000 and 1400 cm^{-1}, and NO with a lower frequency (< ~1700 cm^{-1}) has the NO^- nature [17,25]. Since no band was observed above 1900 cm^{-1} in the FTIR spectrum of NHase (Fig. 1; the region above 2000 cm^{-1} is not shown), it may be possible that another NO band is hidden somewhere in the region lower than 1700 cm^{-1} where numerous protein bands are present, although complex changes of the protein bands upon ^{15}N-substitution hampered its identification.

Table 1
NO stretching frequencies (cm^{-1}) in light-induced FTIR difference spectra of natural (^{14}N) and ^{15}N-labeled NHases

	Natural (^{14}N) NHase	^{15}N-labeled NHase	
	obs.	obs.	cal.[a]
Inactive form	1855	1820	1822
	1847	1811	1814
Active form	1869	1832	1836
	< 1700?		

[a]Isotope shifts were calculated by assuming a free diatomic NO molecule.

The above view might answer the question about the chemical identity of the electron acceptor when Fe(II) is oxidized upon photoactivation [3,11]; the ligand NO may function as an electron acceptor and become NO^- after photoactivation. Such an NO-dependent photoactivation mechanism will be proved when the NO^- band is clearly identified in the FTIR spectrum.

Jin et al. [10] has proposed from the EXAFS, resonance Raman [8] and ENDOR data that the iron ligand donor set of NHase is N_3S_2O. We presume that some of these nitrogen ligands may be provided by the NO molecules found in the present FTIR study. However, further investigations are necessary to obtain the detailed structural model including NO ligands that consistently explains all the spectroscopic results.

Nitric oxide is now known as an important molecule that serves as a neurotransmitter and regulates blood vessel dilatation and immune function [26]. It is generally accepted that NO activates guanylyl cyclase, which catalyses cGMP generation, by binding to the heme iron [23,27]. It has also been suggested that cyclooxygenase, that produces prostaglandin, may be activated by NO [28,29], and hemoproteins in the salivary glands of a blood-sucking insect serve as a reservoir for NO [30]. Besides these hemoproteins, NHase from *R.* sp. N-771 is another type of nitrosyl-iron protein that possesses a non-heme iron center.

Some information other than NO can be obtained from the FTIR difference spectra between active and inactive forms of NHase. The two large differential bands at 1150 1000 cm^{-1} were prominent in the spectra (Fig. 1). Since this region is typical of the strong C–O stretching bands of alcohols [18], and these bands were basically unaffected by ^{15}N substitution (Fig. 1B) (some changes in the negative bands may be due to the ^{15}N effect on the overlapping bands), these could be tentatively assigned to the C–O stretches of Ser and Thr residues. It is presumed that these amino acid residues are involved in interactions with the iron atoms and/or their ligands. Interestingly, Ser and Thr are present in the putative iron binding site of the α subunit [8] with the sequence -Cys_{110}-Ser-Leu-Cys-Ser-Cys-Thr_{116}- [2,6,7]. Also, relatively small band intensities in the amide I region (1700–1600 cm^{-1}) (Fig. 1) indicate that no drastic changes of the protein conformations occur upon photoactivation and hence the changes may be limited to the region nearby the di-iron center.

Acknowledgements: We would like to thank Drs. M. Yohda and M. Odaka for useful discussions, and Mr. Y. Teratani and Ms. K. Kiribuchi for technical assistance of sample preparation.

References

[1] Kobayashi, M., Nagasawa, T. and Yamada, H. (1992) Trends Biotechnol. 10, 402–408.

[2] Honda, J., Nagamune, T., Teratani, Y., Hirata, A., Sasabe, H. and Endo, I. (1992) Ann. NY Acad. Sci. 672, 29–36.
[3] Honda, J., Teratani, Y., Kobayashi, Y., Nagamune, T., Sasabe, H., Hirata, A., Ambe, F. and Endo, I. (1992) FEBS Lett. 301, 177–180.
[4] Nakajima, Y., Doi, T., Satoh, Y., Fujiwara, A. and Watanabe, I. (1987) Chem. Lett. 1767–1770.
[5] Nagamune, T., Kurata, H., Hirata, M., Honda, J., Hirata, A. and Endo, I. (1990) Photochem. Photobiol. 51, 87–90.
[6] Ikehata, O., Nishiyama, M., Horinouchi, S. and Beppu, T. (1989) Eur. J. Biochem. 181, 563–570.
[7] Mayaux, J., Cerbelaud, E., Soubrier, F., Faucher, D. and Pétré, D. (1990) J. Bacteriol. 172, 6764–6773.
[8] Nelson, M.J., Jin, H., Turner Jr., I.M., Grove, G., Scarrow, R.C., Brennan, B.A. and Que Jr., L. (1991) J. Am. Chem. Soc. 113, 7072–7073.
[9] Sugiura, Y., Kuwahara, J., Nagasawa, T. and Yamada, H. (1987) J. Am. Chem. Soc. 109, 5848–5850.
[10] Jin, H., Turner Jr., I.M., Nelson, M.J., Gurbiel, R.J., Doan, P.E. and Hoffman, B.M. (1993) J. Am. Chem. Soc. 115, 5290–5291.
[11] Honda, J., Kandori, H., Okada, T., Nagamune, T., Shichida, Y., Sasabe, H. and Endo, I. (1994) Biochemistry 33, 3577–3583.
[12] Mäntele, W. (1993) Trends Biochem. Sci. 18, 197–202.
[13] Noguchi, T., Ono, T. and Inoue, Y. (1992) Biochemistry 31, 5953–5956.
[14] Venyaminov, S.Y. and Kalnin, N.N. (1990) Biopolymers 30, 1243–1257.
[15] Venyaminov, S.Y. and Kalnin, N.N. (1990) Biopolymers 30, 1259–1271.
[16] Surewicz, W.K. and Mantsch, H.H. (1988) Biochim. Biophys. Acta 952, 115–130.
[17] Nakamoto, K. (1986) in: Infrared and Raman Spectra of Inorganic and Coordination Compounds, pp. 191–371, Wiley, New York.
[18] Socrates, G. (1994) in: Infrared Characteristic Group Frequencies, Wiley, New York.
[19] Quinby-Hunt, M. and Feltham, R.D. (1987) Inorg. Chem. 17, 2515–2521.
[20] Chen, Y. and Rosazza, J.P.N. (1994) Biochem. Biophys. Res. Commun. 203, 1251–1258.
[21] Goodfellow, M. (1986) in: Bergey's Manual of Systematic Bacteriology, Vol. 2 (Sneath, P.H.A., Mair, N.S., Sharpe, M.E. and Holt, J.G., eds.) pp. 1472–1481.
[22] Stamler, J.S., Singel, D.J. and Loscalzo, J. (1992) Science 258, 1898–1902.
[23] Tsai, A. (1994) FEBS Lett. 341, 141–145.
[24] Maricq, M.M., Tanguay, N.A., O'Brien, J.C., Rodday, S.M. and Rinden, E. (1989) J. Chem. Phys. 90, 3136–3144.
[25] McCleverty, J.A. (1979) Chem. Rev. 79, 53–76.
[26] Lowenstein, C.J. and Snyder, S.H. (1992) Cell 70, 705–707.
[27] Ignarro, L.J. (1992) Biochem. Soc. Trans. 20, 465–469.
[28] Salvemini, D., Misko, T.P., Masferrer, J.L., Seibert, K., Currie, M.G. and Needleman, P. (1993) Proc. Natl. Acad. Sci. USA 90, 7240–7244.
[29] Rettori, V., Gimeno, N., Lyson, K. and McCann, S.M. (1992) Proc. Natl. Acad. Sci. USA 89, 11543–11546.
[30] Ribeiro, J.M.C., Hazzard, J.M.H., Nussenzveig, R.H., Champagne, D.E. and Walker, F.A. (1993) Science 260, 539–541.