Resonance Raman Evidence that Photodissociation of Nitric Oxide from the Non-Heme Iron Center Activates Nitrile Hydratase from *Rhodococcus* sp. N-771[†]

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ABSTRACT: Nitrile hydratase (NHase) from *Rhodococcus* sp. N-771, which contains a non-heme iron center in the catalytic site, has been known to be activated by light illumination. Recently, endogenous nitric oxide (NO) was found in this enzyme by FTIR spectroscopy [Noguchi et al. (1995) *FEBS Lett. 358*, 9−12]. In order to directly detect the bonding between NO and the iron atom and the reaction of NO upon photoactivation, resonance Raman spectra of the NHase were measured with 413 nm excitation at 85 K. Two prominent bands at 592 and 570 cm^{-1} were observed in the inactive form, and both of them were completely lost upon photoactivation. Upon subsequent introduction of ^{15}NO, the active NHase was converted to the inactive form again and the above two bands were restored with downshifts by 10 and 12 cm^{-1}, respectively. Also, the excitation profiles of these bands in the 350−500 nm region mostly followed the absorption spectrum arising from the iron center. From these isotopic shifts and the excitation profiles, the two Raman bands were assigned to the Fe−NO stretching and bending vibrations that are probably coupled with each other. The results provided solid evidence that NO is bound to the non-heme iron in the inactive NHase and its photodissociation activates the enzyme.

Nitrile hydratase (NHase)[1] is an enzyme found in microorganisms, which catalyzes hydration of nitriles to corresponding amides (Kobayashi et al., 1992). The NHase from *Rhodococcus* sp. N-771 consists of the α and β subunits and contains a non-heme iron center in the active site that resides on the α-subunit (Odaka et al., 1996). This NHase has been known to show unique reactivity to light (Nagamune et al., 1990a,b); the NHase that is in the inactive form in the dark is activated by light illumination (photoactivation), and is inactivated by aerobic incubation in the dark (dark-inactivation). This dark-inactivation occurs only *in vivo*, whereas photoactivation occurs both *in vivo* and *in vitro*. Similar photoreactivity has been observed also in NHases from *Rhodococcus* sp. N-774 (Nakajima et al., 1987) and *Rhodococcus* sp. R312 (formerly *Brevibacterium* sp. B312) (J. Honda, unpublished observation). These NHases are probably identical to the one from *R.* sp. N-771 because of the same amino acid sequences (Ikehata et al., 1989; Mayaux et al., 1990; Honda et al., 1992a). The structure of the iron center of the NHase in its active form has been investigated by means of EPR (Sugiura et al., 1987), EXAFS (Nelson et al., 1991), resonance Raman (Nelson et al., 1991), and ENDOR (Jin et al., 1993). The ligand donor set of N_3S_2O has been proposed for active NHase from *R.* sp. R312 (Jin et al., 1993). Very recent X-ray absorption (Scarrow et al., 1996) and resonance Raman (Brennan et al., 1996) studies have confirmed this conclusion and detailed analysis of the EXAFS spectra has shown *cis* coordination of the two cysteine ligands, histidine side chains as the nitrogen ligands, and water or hydroxide as the oxygen ligand (Scarrow et al., 1996). On the other hand, the mechanism of photoactivation has been investigated by Mössbauer, magnetic susceptibility, EPR (Honda et al., 1992a,b), and time-resolved absorption spectroscopy (Honda et al., 1994). Although oxidation of the iron atom by an unknown electron acceptor has been proposed as a possible mechanism (Honda et al., 1992a, b), a clear answer must await further investigations.

A breakthrough has been provided by our recent discovery of endogenous nitric oxide (NO) in the dark-adapted NHase by means of Fourier transform infrared (FTIR) spectroscopy (Noguchi et al., 1995). NO was also found in the α-subunit isolated in the dark (Odaka et al., 1996). This NO, which is a radical species in itself, was first proposed as a candidate for the electron acceptor in the photoactivation process (Noguchi et al., 1995). More recently, however, we have found that introducing extrinsic NO gas into the photoactivated NHase can inactivate the enzyme (Odaka et al., submitted). This process was monitored by FTIR, EPR, UV−vis absorption, and flash photolysis, and it was suggested that photodissociation and association of NO control the activity of NHase (Odaka et al., submitted). Since NO has high affinity for metal ions (Tsai, 1994) and free NO is very unstable under the physiological condition, it is assumed that the NO found in the NHase is bound to the non-heme iron. However, evidence for this view should be given by direct detection of the Fe−NO bond. To achieve this purpose, in the present study, resonance Raman (RR) spectroscopy was employed. RR spectroscopy can selectively detect metal−ligand vibrations by using a laser line

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[1] Abbreviations: FTIR, Fourier transform infrared; NHase, nitrile hydratase; NO, nitric oxide; RR, resonance Raman.

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resonant to a specific electronic transition. With the aid of isotopic substitution, we have identified the Fe−NO vibrations in the NHase and clearly shown that photodissociation of NO from the iron atom activates this enzyme.

MATERIALS AND METHODS

R. sp. N-771 cells were cultured as described by Tsujimura et al. (1995). NHase was converted to the inactive form by aerobic incubation of cells in the dark (dark-inactivation) and purified as described also by Tsujimura et al. (1995). The isolated NHase in the inactive form was dissolved in a buffer containing 50 mM Hepes-KOH (pH 7.5) and 20 mM *n*-butyric acid. For activation, this inactive NHase was illuminated for 30 min on ice with white light (∼60 mW/cm^2) from a halogen lamp through a heat-cut filter (photoactivation). NHase activity was assayed by measuring the activity of hydration of propionitrile to propionamide at 0 °C (Tsujimura et al., 1995). A unit of the activity was defined as the amount of enzyme which produces 1 μmol of propionamide per min, and the specific activity was expressed as units per mg of protein.

Introduction of ^{15}NO gas (Shoko Co. Ltd., 99%) into the NHase was performed as follows. The photoactivated NHase solution was placed in a glass tube connected to a vacuum line. After degassing the solution in vacuum, ^{15}NO gas was introduced into the solution at about 200 Torr. The reaction mixture was vigorously shaken for 1−2 min, and then the solution was degassed again to remove excess ^{15}NO.

RR spectra were measured with a JASCO TRS-600/S single monochromater equipped with an 1800 groove/mm grating. A 413.1 nm line from a Kr ion laser (Coherent, Innova 90-K) was used for excitation, and Raman scattering was collected perpendicular to the laser beam. Rayleigh scattering was removed with a holographic Notch filter (Kaiser Optical Systems, Inc.). The dispersed Raman scattering was detected with a CCD detector (Prinston Instruments, Inc., LN/CCD-1100PBUVAR). The NHase samples (∼40 mg/mL) were directly mounted on an aluminum holder and frozen with liquid N_2, and then the sample temperature was kept at 85 K in a cryostat (Oxford DN1704) with a temperature controller (Oxford ITC502). The laser power was 40 mW at the sample point and the laser beam was slightly defocused to avoid heating the sample. The accumulation time for each Raman spectrum was 20 min. The buffer spectrum was subtracted from NHase spectra so as to cancel the ice bands in 320−200 cm^{-1}. For obtaining excitation profiles of the Raman bands of inactive NHase, laser lines of 406.7 and 413.1 nm from a Kr ion laser, 363.8 and 488.0 nm from an Ar ion laser (Coherent, Innova 90-4), and 441.6 nm from a He-Cd laser (Kimmon IK5651R-G) were used, and the Raman band at 990 cm^{-1} from SO_4^-, which was added into the sample solution with concentration of 0.2 M, was used as an internal standard. For wavenumber correction, spectra of carbon tetrachloride and toluene measured at room temperature were used. Spectral resolution was 8 cm^{-1}.

FTIR spectra were measured with a JEOL JIR-6500 spectrophotometer at room temperature with a spectral resolution of 4 cm^{-1}. The NHase samples were placed between a pair of BaF_2 plates with an aluminum foil spacer (∼10 μm). The buffer spectrum was subtracted form each of the NHase spectra to correct the base line.

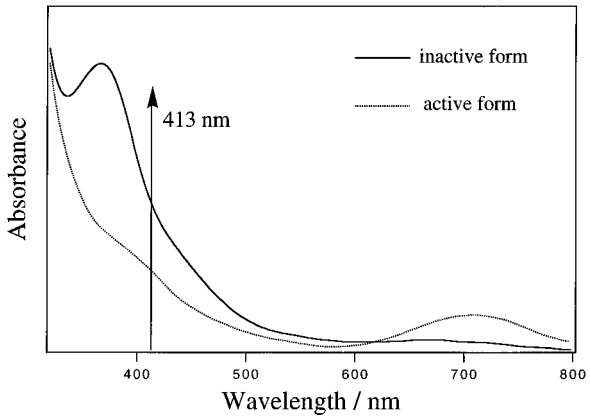

FIGURE 1: Absorption spectra of NHase in the inactive form (solid line) and in the active form (dotted line). The arrow indicates the wavelength of the laser line (413.1 nm) used for resonance Raman measurements.

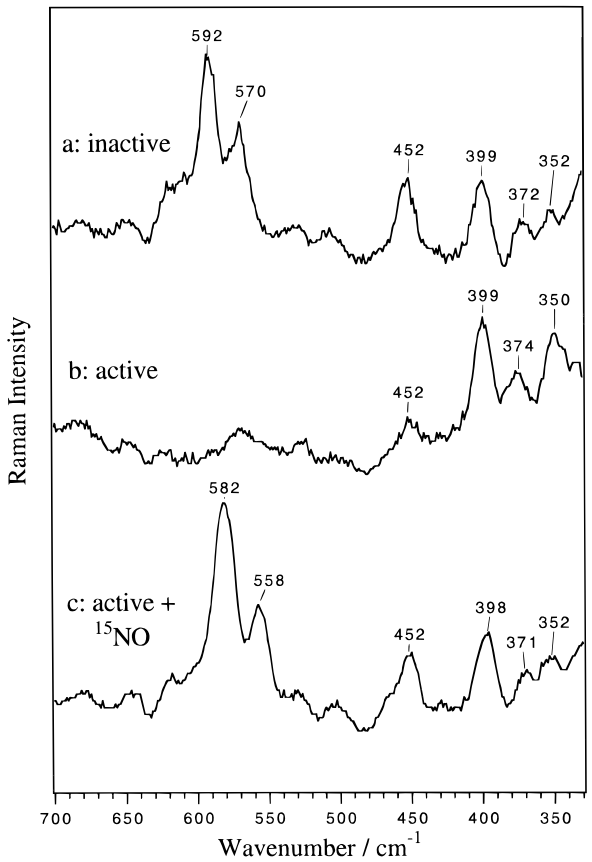

FIGURE 2: Resonance Raman spectra of NHase measured at 85 K. (a) Inactive form. (b) Active form. (c) After introduction of ^{15}NO into the active form.

RESULTS

An absorption spectrum of the NHase in inactive form exhibits a typical band at 370 nm with a gentle shoulder in 400−500 nm (Figure 1, solid line). In the active form, this band is mostly lost leaving a weak band near 400 nm and a new band is observed at 710 nm (Figure 1, dotted line). A laser line at 413.1 nm, which is resonant to these near-UV transitions, was selected to obtain the RR spectra of both the inactive and active NHase.

Figure 2 shows the RR spectra of the NHase samples measured at 85 K. The NHase in inactive form (activity was 6.5 units/mg), which was prepared from dark-adapted

cells, showed the spectrum having two prominent bands at 592 and 570 cm^{-1} and several bands in 460−350 cm^{-1}, i.e., the bands at 452, 399, 372, and 352 cm^{-1} (Figure 2a). In the active NHase (activity was 831 units/mg), which was produced by illumination of the inactive NHase at 0 °C, the bands at 592 and 570 cm^{-1} were completely lost but the lower frequency bands at 452, 399, 374, and 350 cm^{-1} remained with altered relative intensities (Figure 2b). From these apparent differences in spectral features between the inactive and active NHases, it is clear that the inactive form did not convert to the active form by the incident laser beam at this cryogenic temperature of 85 K.

We have recently shown that when extrinsic NO is introduced into the active NHase, it is converted to the inactive form again (Odaka et al., submitted). This NO-introduced NHase (inactive form) recovered 86% of the original activity after subsequent photoactivation (Odaka et al., submitted). In the present experiment, we introduced ^{15}NO into the photoactivated NHase instead of ^{14}NO to observe the isotopic shift. In the RR spectrum of ^{15}NO-introduced NHase (activity was 0.0 units/mg), the prominent two bands were restored at 582 and 558 cm^{-1} with downshifts by 10 and 12 cm^{-1}, respectively (Figure 2c). The lower frequency bands at 452, 398, 371, and 352 cm^{-1} were basically identical to those of the original inactive NHase (Figure 2a). The downshifts of the 592 and 570 cm^{-1} bands by ^{15}NO binding are more clearly shown in Figure 3b.

FTIR spectra in the NO stretching region of the original inactive NHase and the ^{15}NO-introduced NHase (Figure 3a) confirm that the exchange of NO indeed took place by the ^{15}NO introduction after illumination. It is noted that since the excess ^{15}NO gas in the solution was evacuated in vacuum (see Materials and Method), the observed bands originated only from the NO molecules strongly attached to the protein. The main peak at 1853 cm^{-1} and the lower-frequency shoulder at 1844 cm^{-1} downshifted by 35 cm^{-1} to 1818 cm^{-1} and 1809 cm^{-1}, respectively. The higher-frequency shoulder at 1865 cm^{-1} remained at the same position. In fact, only this NO band was left when the inactive NHase was illuminated to produce its active form (Odaka et al., submitted).

Figure 4 shows excitation profiles of the RR bands at 592, 570, 399, and 372 cm^{-1} of the inactive NHase. Although some scatter was seen, the excitation profiles of these bands mostly followed the absorption spectrum in the 350−500 nm region that originates from the iron center of inactive NHase.

DISCUSSION

It has been known that metal−nitrosyl complexes show the M−NO stretching and bending modes in 650−300 cm^{-1} (Quinby-Hunt & Feltham, 1978; Nakamoto, 1986). A larger ^{15}N isotopic shift is usually seen in the bending mode than in the stretching mode (Quinby-Hunt & Feltham, 1978). From the observed ^{15}N isotopic shifts of the 592 and 570 cm^{-1} bands (Figure 3b), it is clear that these two bands correspond to the Fe−NO vibrations. Since the stretching and bending modes are often coupled with each other (Nakamoto, 1986) and in fact the shifts of the two bands were about the same (10 and 12 cm^{-1} for the 592 and 570 cm^{-1} bands, respectively), these bands may have characters of both stretching and bending vibrations. The excitation

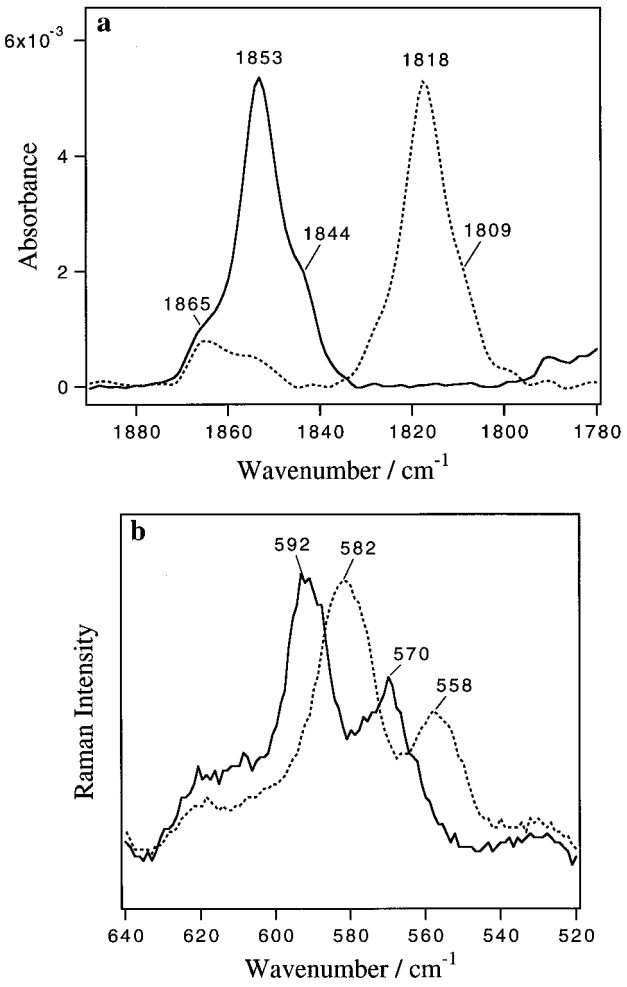

FIGURE 3: FTIR spectra in the NO stretching region (a) and resonance Raman spectra in the Fe−NO stretching and bending region (b) of the native inactive NHase (solid line) and the ^{15}NO-introduced NHase (dotted line).

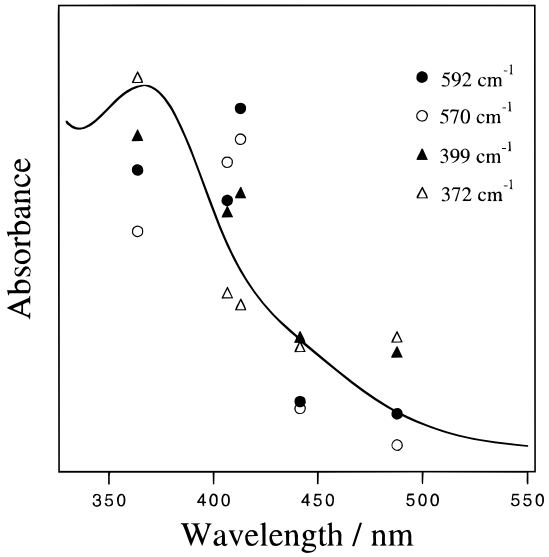

FIGURE 4: Excitation profiles of the resonance Raman bands at 592 (closed circle), 570 (open circle), 399 (closed triangle), and 372 (open triangle) cm^{-1} of the inactive NHase.

profiles of the 592 and 570 cm^{-1} bands obtained in the 350−500 nm region confirm the assignments of these bands to the Fe−NO vibrations. Since the absorption bands in this region are due to the iron center (Tsujimura et al., 1996;

Odaka et al., 1996), the agreement of the Raman excitation profiles with the absorption spectrum indicates that these vibrational modes are related to the iron-ligand vibrations. This result also excludes the idea that the above Fe−NO bands are due to contaminated NO in the NHase sample. The presence of the Fe−NO bands in the inactive NHase indicates that the NO molecule, which has been found in NHase by detecting the NO stretching mode (∼1850 cm^{-1}) by FTIR (Noguchi et al., 1995), is indeed bound to the iron center.

In FTIR measurements two photosensitive NO bands have been observed at 1855 and 1847 cm^{-1} in the inactive NHase at 250 K (Noguchi et al., 1995). These bands were observed at 1853 and 1844 cm^{-1} at room temperature and showed downshifts to 1818 and 1809 cm^{-1}, respectively, by exchanging ^{14}NO for ^{15}NO (Figure 3a). Although these two NO bands were first interpreted as reflecting the presence of two NO molecules attached to a di-iron center (Noguchi et al., 1995), the recently revised Fe stoichiometry (one Fe/enzyme) (Odaka et al., 1996) and the strong temperature dependence of relative intensities of the two NO bands (T. Noguchi, unpublished data) favor another interpretation that one NO molecule is present in inactive NHase and two slightly different states of the NO binding are thermally equilibrated. However, the two RR bands at 592 and 570 cm^{-1} may not correspond to these two NO binding states; no ^{15}N-sensitive band was found except for the 592 and 570 cm^{-1} bands in the 300−700 cm^{-1} region (Figure 2) and one Fe−NO bond should have two modes of stretching and bending vibrations. The above view was supported by the RR measurement of the isolated α-subunit of NHase: two RR bands were observed at 588 and 570 cm^{-1} (data not shown) comparable to the bands at 592 and 570 cm^{-1} of the native NHase, whereas the FTIR spectrum of the α-subunit showed only one NO band at 1852 cm^{-1} (Odaka et al., 1996). Probably the difference between the two NO-binding states has rather minor effect on the Fe−NO vibrations and hence was not resolved in the present RR spectra (8 cm^{-1} resolution).

The loss of the Fe−NO bands in the active form (Figure 2b) indicates that the NO molecule is released from the iron atom upon photoactivation. This disappearance of the Fe−NO bands is not due to the change in the resonance condition caused by the movement of the absorption band from 370 to 710 nm upon photoactivation (Figure 1); The RR spectra of active NHase previously measured by using 640 and 714 nm laser lines (Nelson et al., 1991; Brennan et al., 1996), which are resonant to the 710 nm transition, also did not show the Fe−NO bands. The fact that ^{14}NO was replaced with ^{15}NO by introducing ^{15}NO after illumination (Figure 3) confirmed that the NO release indeed took place upon illumination. The released NO was also captured with an NO spin trapper, *N*-methyl-D-glucamine dithiocarbamate (MGD) with Fe(II) ion (Odaka et al., submitted). The recent EXAFS study by Scarrow et al. (1996) also concluded that NO is not bound to the iron in active NHase, being consistent with the present conclusion. The recovery of the Fe−NO bands upon inactivation of NHase by introducing NO (Figure 2c) proves that NO binding to the iron center is the direct cause of inactivation.

In the FTIR spectra a small NO stretching band at about 1865 cm^{-1} remained in the photoactivated NHase (Noguchi et al., 1995; Odaka et al., submitted; also see Figure 3a). The intensity of this NO band is strongly dependent on the storage condition of the sample, and increases upon exposure to NO gas (Odaka et al., submitted) and sometimes upon illumination (Noguchi et al., 1995). The observation that no Fe−NO band remained in the RR spectrum after illumination (Figure 2b) supports our recent interpretation that this NO is non-specifically bound to the protein and not essential to the photoreaction (Odaka et al., submitted).

The RR spectra of active NHase measured by Nelson and coworkers (Nelson et al., 1991; Brennan et al., 1996) with excitation at 640 and 714 nm showed bands in the 300−500 cm^{-1} region. It was shown by ^{34}S labeling that these bands arise from Fe-cysteine modes coupled with cysteine side-chain deformations (Brennan et al., 1996). The bands at 452, 399, 374, and 350 cm^{-1} in the spectrum of active NHase obtained in this study by 413 nm excitation (Figure 2b) may be interpreted in the same way. Some of the bands may originate from the modes identical to those with excitation into the 710 nm band. Little frequency change between the inactive and active forms (Figure 2) indicates that the structural change upon photoactivation is rather small with respect to the Fe−S structure of the iron center.

The results of excitation profiles for the 592 and 570 cm^{-1} bands assigned to the Fe−NO vibrations and for the 399 and 372 cm^{-1} bands probably arising from the Fe−S vibrations (Figure 4) indicate that the electronic band at 370 nm and the shoulder in 400−500 nm of inactive NHase are due to the transitions including both the Fe←NO and Fe←S charge transfer characters. Also, enhancement of the RR bands in 350−460 cm^{-1} with active NHase by 413 nm excitation (Figure 2b) indicates that the weak electronic band around 400 nm of active NHase (Figure 1) may be due to the Fe←S charge transfer transition as well as the 710 nm band.

In conclusion, the RR measurements provided solid evidence that endogenous NO is bound to the non-heme iron in the inactive NHase and its photodissociation activates the enzyme. It was further demonstrated that association of exogenous NO with the iron atom inactivates the enzyme again. Although the NO production system has not been identified yet in *R.* sp. N-771, a bacterial NO synthase has been recently found in *Nocardia* sp. (Chen & Rosazza, 1994, 1995) that is closely related to *Rhodococcus* (Goodfellow, 1986). It is possible that this bacterium has a similar NO synthase. Dark-inactivation of NHase, which takes place only *in vivo* by aerobic incubation of cells, is likely caused by this NO producing system. Thus, the photosensitive NHase in *R.* sp. N-771 is a novel enzyme whose activity is controlled by combination of NO and light.

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