Activity Regulation of Photoreactive Nitrile Hydratase by Nitric Oxide

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Abstract: Nitrile hydratase (NHase) from *Rhodococcus* sp. N-771 is a novel enzyme that possesses a non-heme iron(III) center binding endogenous nitric oxide (NO). It is inactivated by aerobic incubation of cells in the dark, whereas the inactive form is converted to the active one by light irradiation. To clarify the mechanism of activity regulation in the NHase, we investigated the role of NO. When the inactive NHase was irradiated in the presence of Fe(II) ions and a spin trap, *N*-methyl-D-glucamine dithiocarbamate (MGD), three hyperfine lines from the nitrogen atom of the [$(MGD)_2$-Fe^{II}-NO] complex were resolved, indicating that NO was released from the enzyme upon photoactivation. The amount of NO release was obtained as 0.99 ± 0.12 per enzyme ($n = 4$) by using an NO scavenger, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide. The active NHase was completely inactivated by exogenous NO, and upon photoirradiation 86% of the original activity was restored. FTIR, ESR, and UV−VIS absorption measurements confirmed the view that association of NO restores the original inactive form of the enzyme. The rate constant for nitrosylation of the enzyme activated by a laser pulse asymptotically increased with an increase in NO concentration. A kinetic analysis of the NHase nitrosylation demonstrated that inactivation of the enzyme by NO binding proceeded via an intermediate. The rate constant for inactivation and the quantum yield of photoactivation were determined as 14 s^{-1} and 0.48, respectively. It is thus concluded that the activity of the NHase is regulated by nitrosylation and photoinduced denitrosylation of the non-heme iron center. This finding provides a new aspect regarding biological function of NO, i.e., regulation of the enzymatic activity with the aid of light.

Introduction

Nitrile hydratase (NHase; EC4.2.1.84) is an enzyme that has been found in various microorganisms and that catalyzes the hydration of nitriles to the corresponding amides.[1] All of the known NHases consist of two kinds of subunits (α and β) and contain non-heme iron[2−7] or cobalt atoms[8,9] at the catalytic site. NHase from *Rhodococcus* sp. N-771 shows unique photosensitivity.[7,10] The inactive NHase is converted to the active form by light irradiation both *in vivo* and *in vitro* (photoactivation). The active form, in turn, is transformed to the inactive one by aerobic incubation of cells in the dark (dark-inactivation). The latter process, however, does not occur *in vitro*, suggesting that this process requires some additional factors present in the cell. A similar photoresponse has been observed in NHases from *Rhodococcus* sp. N-774[11] and sp. R312.[12] These NHases are probably identical to that from *Rhodococcus* sp. N-771, because their amino acid sequences are identical.[13−15] However, the subunit stoichiometry of NHase from *Rhodococcus* sp. N-771 is estimated to be a heterodimer, $\alpha\beta$, by size exclusion HPLC,[7] whereas those from the other strains were reported as $\alpha_2\beta_2$ by the same technique.[16] The reason for this discrepancy remains unclear but may be due to the different experimental conditions.

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Our initial studies[15,17] suggested that NHase had a dinuclear iron active site, but we have recently determined the iron-protein stoichiometry to be 1: 1.[18] From electron spin resonance (ESR),[4] extended X-ray absorption fine structure (EXAFS),[16] resonance Raman[16] and electron nuclear double resonance (ENDOR)[19] studies of the NHase in the active form, the ligand-donor set of the iron center was proposed as N_3S_2O,[19] which was also supported by model complex analyses.[20−22] Very recently, detailed studies on the structure of the iron center by using Mims pulsed ENDOR,[23] resonance Raman,[24] and X-ray absorption[25] spectroscopies demonstrated that the active site consisted of two *cis* coordinated cysteine thiolate ligands, three histidines, and an exchangeable solvent ligand, most likely to a hydroxo group.

Nitric oxide (NO) plays several important roles in biological systems including blood pressure control, neurotransmission, and the immune response.[26−28] NO binds to various heme and non-heme iron proteins with high affinity *in vitro* and *in vivo* and regulates their biological functions.[29,30] For instance, the activity of guanylyl cyclase is regulated by NO-binding to its heme iron and prostaglandin H synthase may be activated by NO.[31,32] Although these functions have basically been identified in higher animals such as mammals, the discovery of NO synthase in a bacterium, *Nocardia* sp. NRRL-5646,[33,34] has led to the notion that NO plays some physiological roles even in microorganisms. Recently, Fourier transform infrared (FTIR) spectroscopy showed that the NHase possesses endogenous NO molecules at the iron center.[35] This finding proposed that the bound NO might function as the electron acceptor in the photoactivation process.[35]

In this study, we investigated mechanism of the photoactivation and dark-inactivation of the NHase focusing on the behavior of NO at the non-heme iron center. By means of several spectroscopic techniques including FTIR, ESR, UV−VIS absorption, and laser photolysis, we showed that photodissociation and NO binding play a key role in the activity regulation of NHase.

Experimental Section

Materials. Nitric oxide gas (99.9%) was purchased from Takachiho Chem. Ind. Co. (Japan). MGD was a generous gift from Dr. I. Ueno of RIKEN. Carboxy-PTIO was obtained from Dojindo Laboratories (Japan). Other chemical reagents were products of Sigma (USA) or Nacalai Tesque (Japan).

Preparation of the Inactive and Active NHase. *Rhodococcus* sp. N-771 was cultivated as described previously.[36] NHase was converted to the inactive form by aerobic incubation of cells in the dark (dark-inactivation) and purified in the dark.[36] The purified inactive NHase was stored as suspension in 60% saturated ammonium sulfate at 4 °C in the dark. For spectroscopic measurements, the NHase was dissolved in 50 mM Hepes-KOH (pH 7.5) containing 20 mM *n*-butyric acid (buffer A) and desalted by Centriprep-30 (Amicon, USA) in the dark. For activation, the inactive form of the NHase ($\sim 5.0 \times 10^{-5}$ M) in buffer A was placed in a glass tube (12 × 75 mm) and exposed to 5000 lx of white light (71.0 W/m^2) from a photoreflector lamp (500 W SPOT, Toshiba, Japan) for 15 min in an ice bath (photoactivation) if not specified.

Exposure of the Active NHase to NO Gas. The photoactivated NHase ($\sim 5.0 \times 10^{-5}$ M) dissolved in buffer A was placed in a glass tube (i.d., 15 mm) connected to a vacuum line. The tube was shielded from light with aluminum foil. After degassing the solution in vacuum, NO gas was introduced into the solution at about 200 Torr. NO pressures were measured with a mercury manometer. The reaction mixture was vigorously shaken for 1−2 min to thoroughly expose the enzyme to NO gas. After pumping off the excess NO gas, it was aerated in the dark.

Spectroscopic Measurements. FTIR, ESR, and UV−VIS absorption spectra were measured with a JEOL JIR-6500 spectrophotometer equipped with an MCT detector (EG&G JUDSON IR-DET101), a JEOL JES FE-3AX X-band ESR spectrometer, and a Shimadzu UV-2100PC spectrophotometer, respectively. To obtain a light-induced difference spectrum, the UV−VIS absorption spectra before and after light irradiation were measured, and the former was subtracted from the latter. Other details are described in the figure captions.

Laser Photolysis. Laser photolysis was performed using an Nd:YAG laser (JK Lasers Ltd., Model HY 500) equipped with a third harmonic generator. The pulse width and the energy were ca. 20 ns and 100 mJ/pulse, respectively. NHase samples for laser photolysis (1.2×10^{-4} M) were prepared in a quartz cuvette (light path, 10 mm) by introducing NO gas at various partial pressures. The NO concentration in solutions was calculated from the partial pressure of NO gas and the Bunsen absorption coefficient of NO (4.21×10^{-2} at 1 atom and 293 K). The pressures of NO gas were measured with a mercury manometer. The detection system for transient spectra has been reported previously.[37]

Other Procedures. NHase activity was assayed by measuring the activity of the hydration of propionitrile to propionamide.[36] One unit of the activity was defined as the amount of enzyme which produces 1 μmol of propionamide per min, and the specific activity is expressed as units per mg protein (units/mg). The concentration of NHase was determined by amino acid analysis of a known volume of the enzyme using an amino acid analyzer (Model 835, Hitachi, Japan).

Results

Trapping of NO Release from NHase upon Photoactivation. Photoinduced denitrosylation of the inactive NHase was studied by using a spin trap, [(MGD)$_2$-Fe^{II}]. If free NO is present in a solution containing [(MGD)$_2$-Fe^{II}], a stable complex, [(MGD)$_2$-Fe^{II}-NO], is formed and can be readily detected by ESR spectroscopy at an ambient temperature.[38,39] The NHase in the inactive form was ESR silent at room temperature (data not shown). Addition of [(MGD)$_2$-Fe^{II}] also did not induce any detectable signals in the dark (Figure 1, top curve). However, when the mixture was irradiated with a Xe-lamp for 5 s, three

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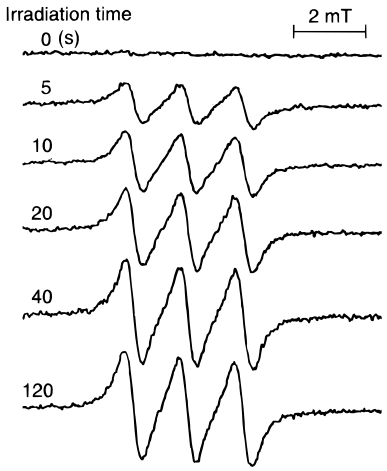

Figure 1. ESR spectra of the [$(MGD)_2$-Fe^{II}-NO] complex formed by trapping NO that was released from the inactive NHase upon light irradiation. The inactive NHase (50 μM) was dissolved in 50 mM Hepes-KOH (pH 7.5) containing 20 mM *n*-butyric acid, and then MGD (75 mM) and $FeSO_4$ (15 mM) were added in the dark. The mixture in a quartz cuvette (light path 0.3 mm) was irradiated with a Xe-lamp for 0, 5, 10, 20, 40, and 120 s, and ESR spectra were measured with the same gain (2×10^2) at room temperature. Instrument settings were as follows: modulation frequency and amplitude, 100 KHz and 0.1 mT, respectively; microwave power, 2.61 mW.

clear ESR lines characteristic of the mononitrosyl-Fe^{II} complex ($a^N = 1.29$ mT and $g_{iso} = 2.04$) were observed (Figure 1, second curve). The three lines well coincided with those of the [$(MGD)_2$-Fe^{II}-NO] complex.[38,39] The signal intensities increased with irradiation up to 40 s and were saturated afterwards (Figure 1). Either the solutions of the NHase alone or [$(MGD)_2$-Fe^{II}] alone did not show any detectable ESR signal even after irradiation (data not shown). These results indicate that the inactive NHase releases NO into the medium upon light irradiation.

The amount of NO release from the NHase upon photoactivation was estimated by using an NO scavenger, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide (carboxy-PTIO).[40−42] Carboxy-PTIO rapidly and selectively reacts with NO to form 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl (carboxy-PTI) and NO_2 in an aqueous neutral solution in a stoichiometric manner as follows:

$$\text{NO} + \text{carboxy-PTIO} \rightarrow \text{NO}_2 + \text{carboxy-PTI}$$

Carboxy-PTIO and carboxy-PTI display the distinct ESR signals and hfc of them are $\alpha^{1,3}{}_N = 0.82$ mT, and $\alpha^1{}_N = 0.98$ mT and $\alpha^3{}_N = 0.44$ mT, respectively.[40] Figure 2 shows ESR spectra of the solution of carboxy-PTIO (250 μM) in the presence of the inactive NHase (104 μM) before and after irradiation with Hg lamp for 3 min, demonstrating that a significant amount of carboxy-PTIO reacted with NO released from the NHase to form carboxy-PTI after light irradiation. The shape of the spectrum was not affected by additional light irradiation, indicating that the reaction of carboxy-PTIO with the photoactivation product is complete, and thus, we inferred that the photoactivation is complete within 3 min (data not shown). The amount of NO photodissociated from the NHase

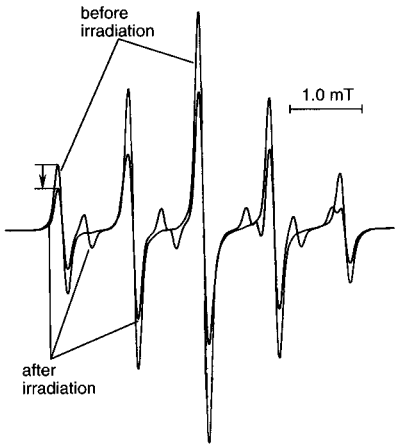

Figure 2. ESR spectra of the solution of 250 μM carboxy-PTIO containing the inactive NHase (104 μM). ESR spectra were measured before and after irradiation with Hg lamp for 3 min in a quartz cuvette (light path 0.3 mm). Buffer used is 100 mM Hepes-KOH (pH 7.5) containing 20 mM *n*-butyric acid. ESR spectra were measured with the same gain (5.0) at room temperature, and instrument settings were as follows: modulation frequency and amplitude, 100 KHz and 0.05 mT, respectively; microwave power, 1.53 mW.

was determined to be 0.99 ± 0.12 per enzyme ($n = 4$) by measuring the decrease of the height of the hyperfine ESR signal of carboxy-PTIO observed at the lowest magnetic field (the arrow in Figure 2), where carboxy-PTI did not display any signals.[40] These results strongly suggest that the inactive form of the NHase possesses one NO molecule per enzyme and release it upon photoactivation.

Nitrosylation of the Active NHase. The activity levels of the photoactivated and inactive NHase at 0 °C were 8.3×10^2 and 6.5 units/mg, respectively. Introduction of NO gas into the solution of the active NHase resulted in the complete disappearance of the activity. However, 86% of the activity (7.2×10^2 units/mg) of the native active enzyme was restored after light irradiation, indicating that the NHase inactivated by NO exposure can be photoactivated like the native enzyme. In other words, the NHase inactivated by exogenous NO is identical to the native inactive form of the enzyme. The small decrease in the yield of photoactivation is probably due to the partial protein denaturation caused by NO, because exposing the inactive NHase to NO gas also brought about a 20% decrease in the activity when measured after light irradiation (data not shown). These observations lead to the view that the active form is converted to the inactive one by nitrosylation.

Spectroscopic Measurements of the Nitrosylated NHase. To confirm the above notions, we monitored the denitrosylation and nitrosylation of NHase by using three spectroscopic techniques. Figure 3 shows the FTIR spectra of the NHase samples in the typical NO stretching region. The inactive NHase displayed a band at 1853 cm^{-1} with shoulders at 1865 and 1844 cm^{-1} (Figure 3A), which had been assigned to the NO stretching modes by ^{15}N-substitution.[35] Upon photoactivation, the 1853 cm^{-1} band and the 1844 cm^{-1} shoulder disappeared, but the small band at 1865 cm^{-1} remained (Figure 3B). When the active NHase was inactivated by introducing extrinsic NO, the signals at 1853 and 1844 cm^{-1} appeared again with mostly recovered intensities (Figure 3C). Like the native enzyme, these NO peaks disappeared again upon subsequent light irradiation (Figure 3D). These results are in agreement with the view that NO is released from the inactive NHase upon photoactivation, and its rebinding converts the active form to the inactive form. The intensity of the 1865 cm^{-1} band also increased after introducing NO (Figure 3C) but remained after

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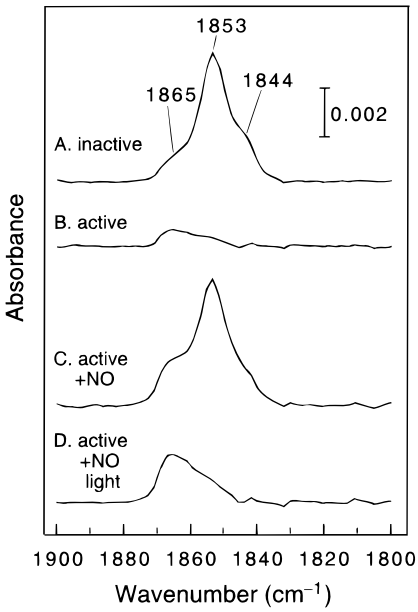

Figure 3. FTIR spectra of NHase in the NO stretching region. A: native NHase in the dark (inactive form). B: native NHase after light irradiation (active form). C: after introducing extrinsic NO into the active form in the dark. D: after light irradiation of C. The NHase samples (~3 mM) were placed between a pair of BaF_2 plates with an aluminum foil spacer (~10 μm). The spectra were measured at room temperature with a spectral resolution of 4 cm^{-1}. The spectra were normalized on the amide II band (1549 cm^{-1}) after subtracting the buffer spectrum.

light irradiation (Figure 3D). This band may be attributed to the NO molecules nonspecifically bound to or trapped within the enzyme. In fact, its intensity remarkably depended much on the storage conditions of the preparation (data not shown).

Figure 4 shows the ESR spectra of the NHase samples measured at 77 K. The inactive NHase showed only several small signals (Figure 4A), whereas the active enzyme gave the ESR signals characteristic of rhombic low-spin ferric iron (g_{max} = 2.25, g_{mid} = 2.13 and g_{min} = 1.97) (Figure 4B).[4,15] The signals corresponding to those of the inactive form did not change after light irradiation, indicating that they were not in association with the photoreactive center. When NO was introduced to the active form, all of the peaks disappeared leaving a slightly enhanced signal at g = 2.04 (Figure 4C). The three ESR signals characteristic of the active form were recovered again by light irradiation (Figure 4D). These results suggest that NO having an unpaired electron binds to the ferric iron in the inactive NHase and makes the iron center diamagnetic, whereas the low spin ferric iron becomes paramagnetic again upon dissociation of NO. The signal at g = 2.04 did not change after irradiation (Figure 4D). This suggests that the signal originates from the NO molecules nonspecifically bound to the enzyme or trapped, as was seen in the FTIR spectra.

The changes of the UV−VIS absorption spectrum of the NHase induced by nitrosylation (Figure 5) were also consistent with the results of the FTIR and ESR studies. Upon introduction of NO to the active NHase, the characteristic peak at 710 nm[43] disappeared, and a peak at 361 nm appeared (Figure 5). The latter peak probably corresponds to the photoresponsive peak at 370 nm of the native NHase in the inactive form,[43] because exposure of the inactive NHase to NO gas also induced a blue-shift of the peak from 370 to 361 nm (data not shown). Although the reason for this blue-shift is unknown, it seems to be related to partial denaturation and nonspecific binding of NO as described above. Nonetheless, the photoinduced difference spectrum of the NHase exposed to NO (after minus before

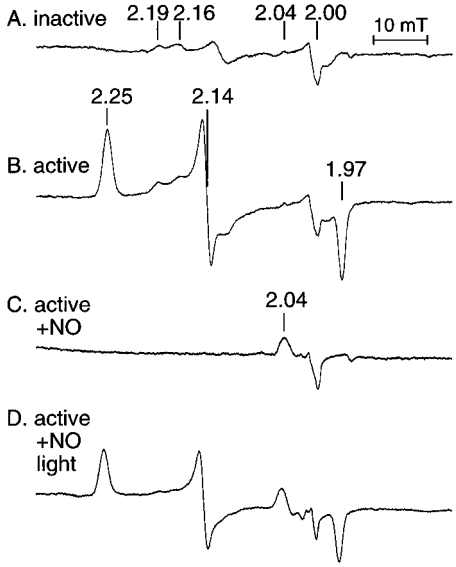

Figure 4. ESR spectra of NHase measured at 77 K. A: native NHase in the dark (inactive form). B: native NHase after light irradiation (active form). C: after introducing extrinsic NO into the active form in the dark. D: after light irradiation of C. The NHase samples (4.9 × 10^{-5} M) were placed in a quartz ESR tubes (i.d., 2 mm). All spectra were measured with the same gain (10^2). Instrument settings were as follows: modulation frequency and amplitude, 100 KHz and 1 mT, respectively; microwave power, 2.50 mW.

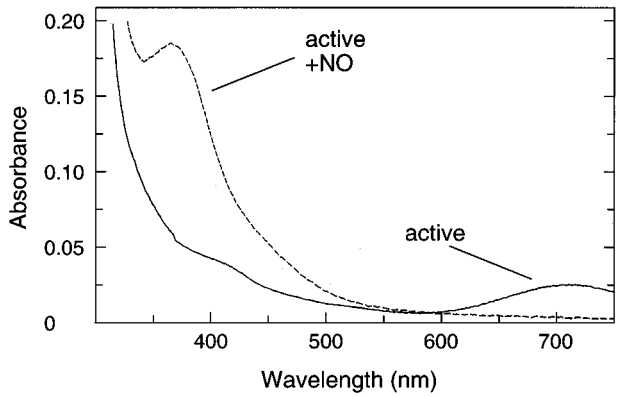

Figure 5. UV−VIS absorption spectra of NHase. Solid curve: the active form of the native NHase. Dotted curve: after introducing extrinsic NO into the active form in the dark. The concentration of the NHase samples was 4.2 ×10^{-5} M. The spectra were measured with a quartz cuvette (light path, 10 mm) at room temperature.

light irradiation) exhibiting a negative peak at 370 nm and a positive peak at 710 nm (Figure 6) was essentially identical to that of the native NHase.[43]

These spectroscopic results indicated that the NO molecule bound to the non-heme iron(III) center is released upon photoactivation, and the activated NHase can be converted again to the original inactive form by NO binding.

Laser Photolysis of the NHase. Laser photolysis studies in the presence of excess NO were carried out in order to elucidate the mechanism of the nitrosylation and photoinduced denitrosylation of NHase. The transient absorption spectrum of NHase in the absence of excess NO at 50 ns after an incident laser pulse is identical to the stationary difference spectrum before and after irradiation, and the transient spectrum does not decay afterwards.[43] Together with the above view, these data can imply that photoinduced denitrosylation occurs within 50 ns after laser pulsing. Here, we estimated the rate constant for the

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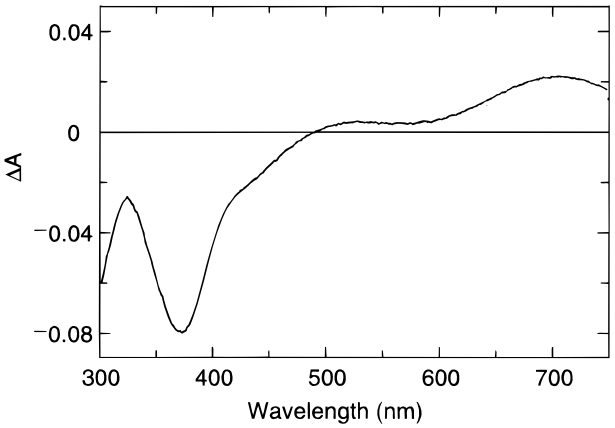

Figure 6. Light-induced UV−VIS difference spectrum (after minus before light irradiation) of the NHase inactivated by introducing extrinsic NO. The concentration of the NHase was 4.2×10^{-5} M.

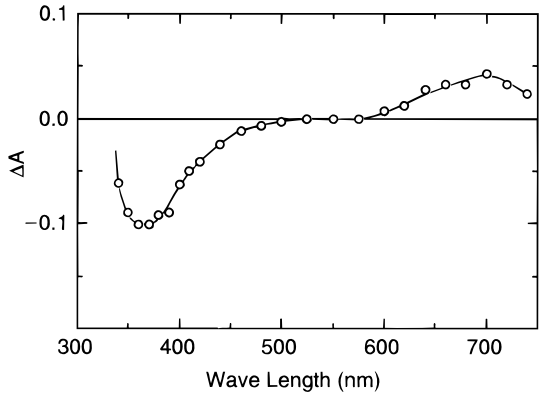

Figure 7. Transient UV−VIS absorption spectrum of the NHase measured at 20 ns after a 355-nm laser pulse in the presence of excess NO (1.3×10^{-3} M). The NHase concentration was 1.2×10^{-4} M.

NHase nitrosylation by detecting the recovery of the inactive form in the presence of excess NO by a 355-nm laser pulse. The 355-nm laser photolysis of the inactive NHase gives the transient spectrum having a negative peak at 370 nm and a positive peak at around 700 nm due to the formation of the active form (Figure 7). The transient spectrum uniformly decayed over the whole wavelength region studied, showing regeneration of the inactive form. From the ratio between the pulse-induced absorbance change and the original absorbance of the enzyme solution at 370 nm, we estimated that about 10% (1.2×10^{-5} M) of the NHase was photoconverted to its active form by a single laser pulse.

The nitrosylation was kinetically analyzed by monitoring the time profile of the absorbance change at 370 nm. The absorbance change, ΔD, followed a pseudo first order kinetics

$$\Delta D = \Delta D_0 \exp(-k_{obs}t) \tag{1}$$

where ΔD_0 is the absorbance change measured 20 ns after the laser pulse and k_{obs} is the pseudo-first-order rate constant for the decay monitored at 370 nm. Figure 8 shows the plot of the k_{obs} represented as a function of NO concentration, [NO]. Obviously, k_{obs} asymptotically increases to a limiting value, k_{lim}, with an increase in [NO]. This result is not explained by simply assuming that the active NHase reacts with NO by a diffusion process. Therefore, we took the regeneration process into account:

$$\mathrm{NHase}_{(active)} + \mathrm{NO} \underset{k_b}{\overset{k_f}{\rightleftharpoons}} \textit{NHase}\cdots\textit{NO} \tag{2}$$

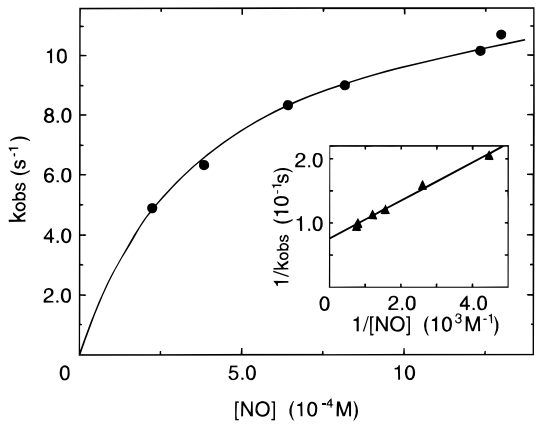

Figure 8. Apparent rate constant for formation of the inactive NHase, k_{obs}, plotted as a function of [NO]. The solutions of the inactive NHase exposed to various NO pressures were irradiated with a 355 nm laser pulse (~20 ns width) to trigger the formation of the active NHase. The decay curve of the absorption change at 370 nm due to the recovery of the inactive form was monitored, and the k_{obs} value was obtained from the pseudo-first-order kinetics. The inset shows the plot of $1/k_{obs}$ as a function of 1/[NO]. The solid curve in the inset is the best fit of eq 5. The fitting parameters were $k_{NO} = 14$ s^{-1} and $K = 2.5 \times 10^3$ M^{-1}. The solid curve in the Figure 8 is calculated from these fitting parameters.

$$K = k_f/k_b \tag{3}$$

$$\textit{NHase}\cdots\textit{NO} \xrightarrow{k_{NO}} \mathrm{NHase}_{(inactive)} \tag{4}$$

The active NHase reacts with NO to yield an intermediate, *NHase···NO*, with an equilibrium constant, *K*. k_f and k_b are the rate constants for the forward and backward reactions between the active form and the intermediate. We assume that the NO molecule in *NHase···NO* is weakly trapped by the enzyme, and *NHase···NO* is intramolecularly transformed to the inactive form with a rate constant, k_{NO}. We consider that NO in the inactive form strongly coordinates to the iron(III) center of the NHase, because the inactive form is stable even under aerobic conditions as long as it is kept in the dark. From eqs 2 and 4, the decay rate constant, k_{obs}, is expressed as

$$k_{obs} = k_{NO}\mathrm{K[NO]}/(1 + \mathrm{K[NO]}) \tag{5}$$

From the slope and intercept of the plot of $1/k_{obs}$ as a function of 1/[NO] shown in the inset of Figure 8, k_{NO} and *K* are determined to be 14 s^{-1} and 2.5×10^3 M^{-1}, respectively. The calculated curve with the use of these k_{NO} and *K* values (solid line) is in good agreement with the experimental data.

Equation 5 is derived on the assumption that the rate constant, k_{eq}, for establishing the equilibrium in eq 2 is much larger than k_{NO}:

$$k_{eq} = k_f[\mathrm{NO}] + k_b \gg k_{NO} \tag{6}$$

Assuming that the formation of *NHase···NO* is controlled by a diffusion process, i.e., $k_f = 10^{10}$ M^{-1}s^{-1}, k_b is estimated as 4.0×10^6 s^{-1}. Since the concentrations of NO used here ranged from 2.5×10^{-4} to 1.3×10^{-3} M, the value of k_f [NO] plus k_b is calculated as 6.5×10^6−2.7×10^7 s^{-1}. This value and k_{NO} fully satisfy eq 6, indicating that the assumption used for deviation of eq 5 is appropriate in the present system.

The quantum yield of photoactivation of the NHase, Φ, was measured also by laser photolysis.[44,45] The absorbance change,

(44) Hoshino, M.; Kogure, M.; Amano, K.; Hinohara, T. *J. Phys. Chem.* **1989**, *93*, 6655−6659.

ΔD^{370}, of the NHase at 370 nm measured at 20 ns after a 355-nm laser pulse is formulated as

$$\Delta D^{370} = \Delta\epsilon^{370}\Phi I_0(1 - 10^{-D}) \quad (7)$$

where I_0 is the intensity of the laser pulse, D is the absorbance of the inactive NHase at the laser wavelength (355 nm), and $\Delta\epsilon^{370}$ is the difference in the molar absorption coefficient at 370 nm between the inactive and active forms. I_0 was evaluated by using benzophenone dissolved in benzene. The absorbance of the benzophenone solution at 355 nm was adjusted to the same value as that of the inactive NHase, D. The triplet yield, Φ_{ST}, and the molar absorption coefficient of the triplet state at 530 nm, ϵ_T, are 1.0 and 7.6×10^3 M^{-1} cm^{-1}, respectively.[46,47] The absorbance, D_T, of the triplet state of benzophenone measured at 530 nm after an incident pulse is represented as

$$D_T = \epsilon_T\Phi_{ST}I_0(1 - 10^{-D}) \quad (8)$$

Equations 7 and 8 give

$$\Phi = \Delta D^{370}(\Delta\epsilon^{370})^{-1}\epsilon_T\Phi_{ST}{D_T}^{-1} \quad (9)$$

By measuring ΔD^{370} and D_T, Φ was determined as 0.48.

Discussion

The present study unveiled the mechanisms of the photoactivation and dark-inactivation of NHase. Noguchi *et al.*[35] have recently found that the NHase from *Rhodococcus* sp. N-771 intrinsically possesses NO at the photoreactive site. It is this NO that plays a key role in these phenomena. The experiment using a spin trap, [$(MGD)_2$-Fe^{II}], and an NO scavenger, carboxy-PTIO, indicated that NO is released from the NHase upon photoactivation (Figures 1 and 2). Also, the photoactivated NHase was inactivated by binding extrinsic NO. We monitored the processes of denitrosylation and nitrosylation by means of FTIR (Figure 3), ESR (Figure 4), and UV−VIS (Figures 5 and 6) spectroscopies and showed that the inactive NHase produced by binding exogenous NO was basically identical to the native NHase in the inactive form and was photoactivated by subsequent irradiation.

The NHase contains one Fe/enzyme.[18] The ESR finding that the iron center in the inactive form of the NHase is diamagnetic, whereas the active form displays low-spin ferric signals (Figure 3) suggests that one NO molecule bound to the Fe(III) atom in the inactive form cancels the spin in the iron center. Quantitative measurements using carboxy-PTIO (Figure 2) demonstrated that the NHase released one NO molecule per enzyme in the process of photoactivation. These indicate that one NO molecule is associated with the mononuclear iron(III) center in the inactive form of the NHase and that light irradiation induces dissociation of NO to activate the enzyme. Since the NO stretching frequency around 1850 cm^{-1} is typical of neutral NO,[48] the oxidation number of the iron atom may be retained at +3 even after coordination of NO. It should be noted that the FTIR spectra showed a shoulder band at 1844 cm^{-1} in addition to the main NO band at 1854 cm^{-1} (Figure 2). The relative intensities of the two bands were highly temperature dependent.[49] Probably, two states having slightly different NO binding are present in the enzyme and exist in equilibrium with each other.

The mechanism described above answers the question why dark-inactivation occurs *in vivo* but not in the isolated NHase.[10] Since NO is an indispensable factor for the inactivation of NHase, *Rhodococcus.* sp. N-771 must have a system for NO production. In fact, about 50 μM of NO_2^- and NO_3^- are accumulated in the broth after cultivation or in the buffered medium after dark-inactivation.[50] Bacterial NO synthase has been isolated from a *Nocardia* species.[33,34] As *Rhodococcus* is closely related to *Nocardia*[51] and does not have denitrification activity,[5] the NO in *Rhodococcus* sp. N-771 may also be produced by a similar NO synthase. The enzyme that catalyzes NO production in *Rhodococcus* sp. N-771 is currently under investigation in our laboratory.

The results of the laser photolysis suggested that, in the process of inactivation, the active NHase initially generates an intermediate denoted as *NHase···NO* before conversion to the inactive enzyme. The equilibrium constant, K, for association of NO is estimated to be 2.5×10^3 M^{-1}, which is comparable to those for association of NO with ferric forms of hemoproteins such as catalase, metmyoglobin, and methemoglobin.[52] This nitrosylation mechanism means that light irradiation of the inactive NHase releases NO from the iron center to the trapping site to form the intermediate. The trapping site would exist in the protein moiety of the enzyme, because the spectrum of the intermediate was similar to that of the active form. The estimated rate constant, k_b, for dissociation of NO from *NHase···NO* is 4×10^6 s^{-1}, while that for the intramolecular transposition of NO, k_{NO}, is 14 s^{-1}, i.e., the former rate constant is 10^5-fold larger than the latter. Therefore, unless a large amount of excess NO is present, *NHase···NO* produced upon irradiation of the inactive enzyme readily releases NO without regeneration of the inactive form. Nelson, Scarrow, Que, Hoffman, and their colleagues[16,19,23−25] have studied the structure of the iron center in the active NHase by using ENDOR, resonance Raman, and X-ray absorption spectroscopies and pointed out that the metal site is $Fe^{III}(Cys^-)_2(His)_3(OH)$. Analyses of model compounds have yielded results consistent with this notion.[18] The fact that the nitrosylation rate constant, k_{NO}, is very small might be explained as follows. When NO is photodissociated from the iron center, the binding site is immediately occupied by a new ligand such as side chains of His or Cys in the active state, and therefore the intramolecular transposition of NO to the iron center is effectively suppressed, leading to a small value of k_{NO}.

The quantum yield of photodissociation of the NO form of the NHase (inactive form) is estimated to 0.48. This is much higher than those of nitrosyl adducts of ferrihemoproteins (∼0.06).[52] Presumably, the trapping site of NO in *NHase···NO* is very shallow in energy in comparison with the heme pocket.

In conclusion, the mechanism of the photoactivation and dark-inactivation of NHase is illustrated in Figure 9. The NHase is inactivated by binding NO to the ferric iron in the non-heme iron center and activated by its photodissociation. The view that NO is directly bound to the iron atom in the inactive enzyme was recently proved by detecting the Fe-NO vibrations by means of resonance Raman spectroscopy.[53] The reason for inactivation of the NHase by NO binding may be attributed to an induced change in protein conformations and/or active site structure as

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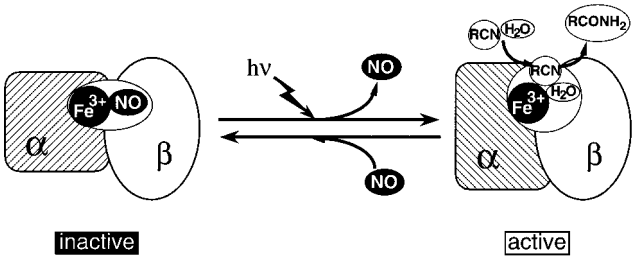

Figure 9. Scheme of the activity regulation of the photoreactive NHase from *Rhodococcus* sp. N-771 by NO.

described above or direct occupation by NO of the substrate binding site. Indeed, complex structural changes including protein conformations and amino acid side chains between the active and inactive forms have been observed by light-induced FTIR difference spectroscopy.[35] The finding of activity regulation of NHase by NO in combination with light provides a new aspect of biological function of NO besides its well-known functions such as activation of guanylyl cyclase and immunological self-defense with reactive oxygen species.[26−32] Further structural studies not only of the iron center but also of the enzyme itself are required for the elucidation of the detailed mechanism of the photoactivation. X-ray crystallographic analysis of the inactive NHase is currently underway.[54]

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