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Location of the Non-Heme Iron Center on the α Subunit of Photoreactive Nitrile Hydratase from *Rhodococcus* sp. N-771

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Nitrile hydratase (NHase) from *Rhodococcus* sp. N-771, which possesses a non-heme iron center binding nitric oxide (NO), is activated by light irradiation. To localize the iron center in the protein, we quantified Fe atoms and performed FTIR measurements of the isolated α and β subunits. The native NHase and the isolated α subunit contained about 1.0 and 0.8 mol Fe per mol protein, respectively, whereas the β subunit contained only a trace of Fe. An NO stretching band was observed at 1852 cm^{-1} in the FTIR spectrum of the α subunit, but not in that of the β subunit. Upon light irradiation of the α subunit, the affinity of the Fe atom decreased and the NO band disappeared from the FTIR spectrum. These observations indicate that the non-heme iron center, which is responsible for the photoreaction, is present on the α subunit.

Nitrile hydratase (NHase, EC 4.2.1.84) is a bacterial enzyme which catalyzes hydration of various nitrile compounds to the corresponding amides (for a review, see Ref. 1). The NHase from *Rhodococcus* sp. N-771 exists as active and inactive forms (2). Inactive NHase is converted to active form by light irradiation *in vivo* and *in vitro*. The active form is converted in turn to the inactive one during aerobic incubation of cells in the dark, but this process does not occur *in vitro* (2). This photoreactive NHase consists of α and β subunits (3), with M_rs of 23 kDa, estimated from their nucleotide sequences (4). The sequences have also shown that the NHase from *Rhodococcus* sp. N-771 is probably identical to that from *Rhodococcus* sp. N-774 (5) and *Brevibacterium* sp. R312 (6). These NHases contain a non-heme iron center at the catalytic site (3, 7–9).

Electron spin resonance (ESR), Mössbauer and magnetic susceptibility studies on the NHase from *Rhodococcus* sp. N-771 have suggested that a ferrous ion in the non-heme iron center is oxidized upon light irradiation (10, 11). Fourier transform infrared (FTIR) spectroscopy has revealed that the NHase intrinsically possesses nitric oxide (NO) molecules (12), which have high affinity to heme and non-heme irons in various proteins. FTIR signals of NO stretches from the NHase are responsive to light irradiation, suggesting that the NO molecules are involved in the photoactivation process of this enzyme (12). Isolated subunits, α and β, of the inactive NHase can be re-assembled into the original inactive form by being incubated together in the dark (14). The non-irradiated α subunit shows an absorption spectrum similar to that of the inactive NHase, but upon irradiation, unlike the native enzyme, its characteristic peaks at 280 and 370 nm diminish with no appearance of new absorption peaks. Also, the irradiated α subunit loses its ability to bind the β subunit, whereas irradiation of the β subunit does not prevent reconstitution of the $\alpha\beta$ complex. These results suggested that the photoreactive site in NHase is located on the α subunit (14). In this study, we examined the localization of Fe atoms and NO molecules on the individual subunits and the photoreactions regarding these cofactors as well. The results supported the view that the non-heme iron center with NO, which drives the photoreaction, is located on the α subunit.

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MATERIALS AND METHODS

Preparation of the inactive NHase and its isolated subunits. Rhodococcus sp. N-771 was cultivated as described in (14). The NHase was converted to the inactive form in living cells by aerobic incubation in the dark, then purified in the dark at 4°C by sequential chromatography: anion-exchange DEAE-Sephacel chromatography (Pharmacia), hydrophobic interaction chromatography (Butyl-Toyopearl 650M, Tosoh) and anion-exchange Q-Sepharose FF chromatography (Pharmacia). The subunits were isolated from the inactive NHase by anion-exchange chromatography (Resource Q, Pharmacia) in the presence of 6 M urea in the dark. The detailed procedures are also described in (14). The purified samples were stored as suspensions in 60% saturated ammonium sulfate at 4°C in the dark.

Activation of the NHase by light irradiation and assay of activity. The inactive NHase dissolved in 50 mM Hepes-KOH, pH 7.5, with 20 mM *n*-butyric acid (buffer A) was placed in a glass test 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) for 15 min in an ice bath. The isolated subunits were irradiated in the same manner.

NHase activity was assayed by measuring the hydration of propionitrile to propionamide. To detect photoactivation, the assay was performed in the dark and after light irradiation. The detailed procedures are described in (14). One unit of the activity was defined as the amount of enzyme that produced 1 μmol of propionamide per minute and the specific activity was expressed as units per mg protein (units/mg).

Quantification of Fe atoms in NHase and its subunits. The native NHase and its subunits were dissolved in buffer A in the presence or absence of 2 mM EDTA. Each sample was distributed in two glass test tubes. One was kept in the dark, and the other was irradiated with light as described above. Both samples were thoroughly desalted using a Centriprep10 (Amicon). Iron atoms in each sample were quantified using an inductively coupled radiofrequency plasma spectrophotometer, ICAP-575II (NIPPON Jarrell-Ash). Protein concentrations were determined using an amino acid analyzer (Model 835, Hitachi).

Measurement of FTIR spectra of isolated subunits. FTIR spectra were measured on a JEOL JIR-6500 spectrophotometer equipped with an MCT detector (EG&G JUDSON IR-DET101). The isolated α and β subunits in buffer A were placed between a pair of BaF_2 plates. The sample temperature was controlled in a cryostat (Oxford DN1704) with a temperature controller (Oxford ITC-4). Light-induced FTIR difference spectra were measured as described (12, 15). Briefly, two single-beam spectra (100 s accumulation for each) before and after 10 s of irradiation were measured and the difference spectrum was calculated. White light from a halogen lamp with a heat-cut filter was used for irradiation (60 mW/cm^2 at the sample surface). The resolution of all FTIR spectra was 4 cm^{-1}.

RESULTS AND DISCUSSION

Table I shows the amounts of Fe atoms detected in the native NHase and the isolated subunits. To examine the affinities of Fe atoms for each subunit, some samples were prepared both in the presence and absence of 2 mM EDTA. Addition of EDTA ($\leq$ 5 mM) had no effect on NHase activity (data not shown). Both the inactive (in the dark) and active NHase (after irradiation) contained 1 ± 0.1 mol Fe per mol enzyme. When EDTA was absent, the isolated α subunit possessed 0.81 and 0.74 mol Fe per mol protein in the dark and after light irradiation, respectively, whereas the isolated β subunit contained only a trace amount of Fe (<0.1 mol/mol protein). Even in the presence of EDTA, the isolated α subunit retained 0.78 mol Fe per mol protein in the dark, but most of the iron atoms were lost after light irradiation. This suggests that the affinity of Fe atom

TABLE I
Amounts of Iron Atoms in the Native NHase and Its Isolated Subunits

	EDTA*	In the Dark	After light irradiation
			(mol Fe/mol NHase)
NHase	−	1.0	1.1
	+	N.D.**	0.95
α subunit	−	0.81	0.74
	+	0.78	0.11
β subunit	−	0.05	0.08
	+	N.D.	N.D.

* : The sample was prepared in the presence (+) or absence (−) of 2 mM EDTA.
** : no data.

in the isolated α subunit decreases upon photoreaction. This phenomenon was not seen in the native NHase.

Thus, we concluded that NHase contains one Fe atom in the α subunit. This means that the non-heme iron center, which is thought to be responsible for the photoreaction and probably the catalytic reaction, is located only on the α subunit. The above results also mean that the iron center is rather stable even after subunit dissociation, as long as the isolated α subunit is kept in the dark.

We have reported that the NHase from *Rhodococcus* sp. N-771 includes 2 mol Fe per mol enzyme (3). The inconsistency between the previous work and this study may be due to underestimation of the molar amount of NHase in the previous study. Namely, the Lowry's method used in the previous study was not suitable for absolute determination of the protein concentration. In addition, the M_r was determined by size exclusion chromatography, which gave a value about 20% larger than that calculated from the amino acid sequences. For these reasons, we believe that the molar amount of NHase was underestimated by over 50% in the previous study. Published spectroscopic data on the NHase from *Rhodococcus* sp. N-771, e.g., ESR (11), Mössbauer (10), magnetic susceptibility (10) and FTIR (12), have been interpreted based on this erroneous stoichiometry of two Fe atoms/enzyme. Hence, interpretations of these results (4,10–13) should be reconsidered based on the revised stoichiometry of one Fe atom/enzyme as revealed in the present study.

To characterize the state of the iron atom in the isolated α subunit, ESR spectra before and after light irradiation were measured at 77K. The inactive NHase is ESR silent while the active form is ESR active (11), and its ESR features (g_{max} = 2.222, g_{mid} = 2.147, and g_{min} = 1.967) are characteristic of rhombic low-spin Fe(III) (S = 1/2) (7,11). Our ESR measurements, however, showed that the isolated α subunit remained ESR silent even after light irradiation. This indicates that the iron coordination in the irradiated α subunit is different from that in the active NHase.

Figure 1 shows FTIR spectra of the isolated α and β subunits of the NHase in the typical NO stretching region. The spectrum of the α subunit exhibited a clear band at 1852 cm^{-1} in the dark (Fig. 1A). This band is comparable to the 1855 cm^{-1} band identified in the FTIR spectrum of inactive NHase and assigned to the NO stretching mode by ^{15}N-substitution (12). Upon light irradiation of the α subunit, this NO band disappeared from the spectrum (Fig.1B). On the other

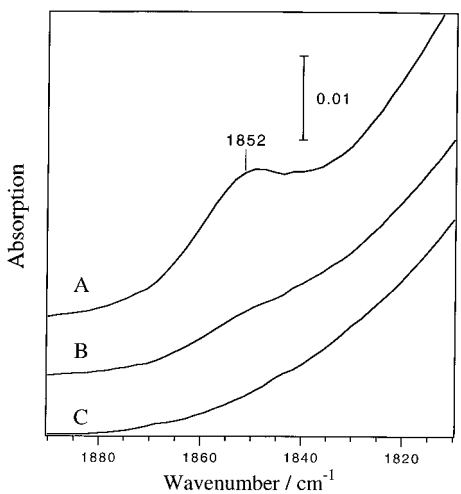

FIG. 1. FTIR spectra of the isolated α and β subunits of the NHase in the NO stretching region. A: α subunit in the dark. B: α subunit after light irradiation. C: β subunit in the dark. The concentration of the α and β subunits were 63 and 87 mg/ml, respectively. The sample temperature was controlled at 5°C. Spectrum (B) was measured with the same sample as (A) after light irradiation for 1 h through a cryostat window.

hand, the β subunit showed no band in this region even in the dark (Fig.1C). These results are consistent with the above observation that the iron atom, to which NO is most probably attached, is present in the α subunit, but not in the β subunit. The sensitivity to light of the NO band in the α subunit is also consistent with our finding that the chromophoric portion of the α subunit undergoes some photoreaction (14). Since no new band appeared in this region to compensate for the negative 1852 cm^{-1} band, NO may be released from the non-heme iron center upon photoreaction of the α subunit.

To investigate the structural changes of protein moieties upon photoreaction in the α subunit, we obtained a light-induced FTIR difference spectrum (after-minus-before irradiation) (Fig.2). The negative band at 1852 cm^{-1} corresponded to the disappearance of the NO band shown in Fig.1. In the amide I region at 1700-1600 cm^{-1} (16), there was only a very small differential signal centered at 1657 cm^{-1}. On the contrary, a number of small complex bands have been observed in this region of the corresponding FTIR spectrum of native NHase (12). These differences found between the α subunit and the NHase indicate that the conformational changes which take place in the NHase upon the photoreaction, no longer occur in the isolated α subunit. The UV-VIS difference absorption spectra upon the photoreaction (14) also show a difference between the NHase and the α subunit; the bands typical of the active NHase at around 400 and 710 nm do not appear in the photoreacted α subunit. It is conceivable that the appearance of these electronic bands requires a conformational change of proteins around the iron center.

The large differential bands at 1114/1157 cm^{-1} and 1010/1050, 1035 cm^{-1} in Fig. 2 probably correspond to the bands at 1126/1148 cm^{-1} and 1019/1040,1033 cm^{-1} in the NHase spectrum, which were tentatively assigned to the C-O stretching modes of Ser and Thr residues (12). Noguchi *et al* (12) pointed out that Ser and Thr were present in the sequence of the putative Fe-binding site in the α subunit (8), i.e., -Cys_{110}-Ser-Leu-Cys-Ser-Cys-Thr_{116}-, and might also be involved in ligating the iron atom to the iron center. The presence of similar FTIR bands even in the isolated α subunit supports this idea.

In conclusion, we have revealed that the non-heme iron center binding NO, which is most probably the photoreactive site of the NHase, is located on the α subunit. The structure of the iron center in the inactive NHase is basically preserved even in the isolated α subunit, while the photoreaction of this subunit differs much from that of the native NHase. The stoichiometry of Fe atom was revised as one Fe/enzyme. Previous spectroscopic results (10–13) should be reinterpreted based on this revised iron stoichiometry so that the mechanism of photoactivation, including the role of NO, can be clarified.

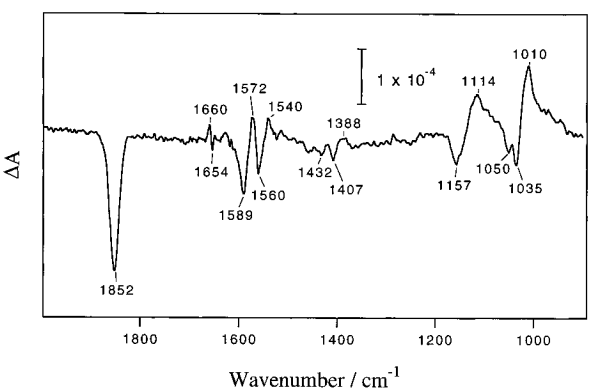

FIG. 2. A light-induced FTIR difference spectrum (after-minus-before irradiation) of the isolated α subunit of the NHase. The sample concentration was about 200 mg/ml. The temperature was controlled at −10 °C. Four difference spectra were averaged.

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