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Photoreactive Nitrile Hydratase: The Photoreaction Site Is Located on the α Subunit[1]

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Nitrile hydratase (NHase) from *Rhodococcus* sp. N-771 exists in active and inactive forms. The inactive NHase is immediately activated by light irradiation and changes to the active form. To characterize the photoreactive center, the inactive NHase was denatured by 6 M urea, and two kinds of subunits (α and β) were separated and purified by anion-exchange chromatography. In a manner similar to the native NHase, the isolated α subunit showed two absorption peaks at 280 and 370 nm, which were diminished by light irradiation. However, irradiation failed to elicit the appearance of absorption peaks at around 400 nm and at 710 nm, which were characteristic of the activated enzyme. The β subunit seemed not to possess any photoreactive chromophore because its absorption spectrum was not altered by light irradiation. Neither of the subunits showed NHase activity before or after light irradiation, but the inactive NHase was reconstituted by incubating the two subunits together in the dark at 4°C for 1 h. Light irradiation of the β subunit did not affect subsequent complex formation or NHase activity. However, the irradiated α subunit could not assemble with the β subunit, and no activity was recovered. These results demonstrate that the chromophore(s) responsible for the photoactivation of NHase are entirely located on the α subunit, and imply that light irradiation induces conformational change of the α subunit.

Key words: nitrile hydratase, non-heme iron center, photoreactive enzyme, reconstitution, subunit isolation.

Nitrile hydratase (NHase) [EC 4.2.1.84] catalyzes hydration of various nitrile compounds to the corresponding amides (*1*). It has been isolated from a number of bacteria. NHase is a soluble metalloenzyme that contains a non-heme iron center (*2-7*) or a cobalt-containing cofactor (*8, 9*) at the catalytic site and consists of two kinds of subunits (α and β). Both subunits have a molecular weight of about 23 kDa (*1*). The primary sequences of both subunits are well conserved among all known NHases. There is no apparent homology between the two subunits (*1*). The catalytic site is thought to be located on the α subunit (*1, 10-12*). NHase from *Rhodococcus* sp. N-771, whose subunit stoichiometry is $\alpha\beta$, possesses unique reactivity to light (*3, 13*). *In vivo*, the NHase activity of *Rhodococcus* sp. N-771 cells decreases during aerobic incubation in the dark and almost disappears (dark-inactivation). The activity is immediately recovered after light irradiation (*13*). The photoreactivity of the NHase is intrinsic to the enzyme because the inactive NHase purified from the dark-inactivated cells is also reactivated by light irradiation (*3*). However, the activated NHase cannot be inactivated *in vitro*. This suggests that other factors, such as one or more enzymes, are required for the dark-inactivation. NHases from *Rhodococcus* sp. N-774 (*14*) and *Brevibacterium* R312 (Honda, J. *et al.*, unpublished observation) are also activated by light irradiation. These NHases are identical with that from *Rhodococcus* sp. N-771 at the level of the nucleotide sequence (Yohda, M. and Endo, I., unpublished observation; *15, 16*).

The iron ions in the non-heme iron center on the activated NHase were shown to be low-spin ferric ions using electron spin resonance (ESR) (*2, 17*). Honda *et al.* reported that the inactive NHase contained low-spin ferrous and ferric ions and that the low-spin ferrous ion was oxidized to a low-spin ferric ion by light irradiation, based on Mössbauer and magnetic susceptibility studies (*10*). Recently, Noguchi *et al.* showed that the NHase purified from the dark-inactivated cells intrinsically possessed nitric oxide (NO) molecules bound to the non-heme iron center and that the bound NO molecules were affected (probably released) by light excitation, using Fourier-transform infrared spectroscopy (*18*). Photoactivation induces a change of the absorption spectrum of the NHase (*3*). Flash photolysis studies showed that these spectral changes occurred within a period

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Abbreviations: NHase, nitrile hydratase; PQQ, pyrroloquinoline quinone; PAGE, polyacrylamide gel electrophoresis; ESR, electron spin resonance.

of 50 ns, suggesting that the chromophores responsible for these spectral changes function as a trigger of the photoactivation (*19*). The absorption spectrum of the active enzyme is quite similar to that of the iron-containing NHase from *Pseudomonas* B23, which shows no photoreactivity (*7*). These results suggest that the photoreactive chromophore is the non-heme iron center. However, the possibility that other chromophores are also involved in the photoreaction site cannot be excluded. For instance, Honda *et al.* suggested that the Trp residues seemed to play a role in photoactivation using several spectroscopies (*19*). The presence of a covalently bound pyrroloquinoline quinone (PQQ) was suggested in the *Brevibacterium* R312 enzyme by hydrazine methods and fluorescence spectroscopy (*20*). Significant interactions between this PQQ and the non-heme iron center were demonstrated by ESR studies (*21*). However, the existence of PQQ in the NHase is still unclear because recent studies have revealed that several enzymes identified as quinoproteins previously by the same methods contain not PQQ, but some modified aromatic residues as the prosthetic groups (*22*). Recent analyses suggested the absence of PQQ in the *Rhodococcus* sp. N-771 NHase (J.A. Duine, personal communication). To elucidate the structure of the photoreaction site, identification of the chromophores required for the photoactivation is essential. However, even the locations of the photoreactive chromophores have not been identified directly.

We have developed methods for the isolation of the individual subunits and the reconstitution of the inactive NHase from these subunits. Using these methods, we have studied the location of the chromophores that are excited by light irradiation and induce activation of the NHase.

EXPERIMENTAL PROCEDURES

Cultivation of Rhodococcus sp. N-771—Rhodococcus sp. N-771 was a generous gift from Nitto Chemical Industry. This bacterium was cultivated aerobically with YM-broth containing ($g \cdot liter^{-1}$): 10 glucose, 5 polypepton, 3 yeast extract, 3 malt extract, 1×10^{-2} $FeSO_4 \cdot 7H_2O$, and 2×10^{-1} antiform (Pluronic L-61, Asahi Denka Kogyo) (pH 7.2). Seed culture (1 liter) was cultivated for 20–30 h at 30°C aerobically. The seed culture was inoculated into 300 liters of YM-broth in a 500-liter jar fermenter and cultivated at 30°C with an aeration rate of 10 liters/min and an agitation speed of 300 rpm for 36 h. About 4 kg of cells were harvested from 300 liters of the culture by centrifugation.

Inactivation of the Cellular NHase Activity by Aerobic Incubation in the Dark (Dark Inactivation)—Inactivation of NHase in the harvested cells was carried out as described in Ref. *12*. About 200 g of the harvested cells was resuspended in 1 liter of 40 mM phosphate-buffered saline containing 20 mM *n*-butyric acid in a 5-liter flask with baffles and incubated aerobically at 4°C in the dark for 15 h. After the incubation, the activity of the cells decreased to about 0 units per mg-dry-cells. The inactivated cells were harvested in the dark, shielded with aluminum foil from the light and stored at −80°C.

Purification of the Inactive NHase—We have purified the inactive NHase *via* the following 4 steps at 4°C in the dark.

Step 1. Preparation of crude extract: About 50 g of the dark-inactivated cells were thawed and resuspended in 160 ml of 50 mM Tris-HCl, pH 7.5, containing protease inhibitors (1 μg/ml Pepstatin A, 2 μg/ml Antipain, and 100 μg/ml phenylmethylsulfonyl fluoride). The cells were disrupted by sonication (Ultrasonic Processor VP-30, Taitec). Insoluble materials were removed by centrifugation at 40,000 × *g* for 60 min.

Step 2. First anion exchange chromatography (DEAE-Sephacel, Pharmacia): The crude extract was applied to a DEAE-Sephacel column (2.5 cm I.D. × 20 cm) equilibrated with 50 mM Tris-HCl, pH 7.5, containing 150 mM NaCl and protease inhibitors as described above. The column was thoroughly washed with the same buffer. NHase was eluted with 150–500 mM NaCl linear gradient at a flow rate of 1.5 ml/min.

Step 3. Hydrophobic interaction chromatography (Butyl-Toyopearl, Tosoh): The pooled fractions were collected and brought to 30% saturation of ammonium sulfate. The precipitate was removed by centrifugation at 40,000 × *g* for 10 min and the supernatant was applied to a Butyl-Toyopearl column (2.5 cm I.D. × 15 cm) equilibrated with 50 mM Tris-HCl, pH 7.5, containing 30% saturated ammonium sulfate. The column was washed with the same buffer and NHase was eluted with a linear gradient of 30% saturated ammonium sulfate to 0% at a flow rate of 1.5 ml/min. NHase in the intrinsically active fractions was precipitated by addition of ammonium sulfate to 60% saturation. The supernatant was removed by centrifugation at 40,000 × *g* for 10 min and the precipitate was dissolved in 50 mM Tris-HCl, pH 7.5, containing 50 mM NaCl and dialyzed against the same buffer to remove ammonium sulfate.

Step 4. Second anion exchange chromatography (Q-Sepharose, Pharmacia): The dialyzed solution was applied to a Q-Sepharose column (2.5 cm I.D. × 10 cm) equilibrated with 50 mM Tris-HCl, pH 7.5, containing 200 mM NaCl. The column was washed with the same buffer and NHase was eluted with 200–500 mM NaCl linear gradient at a flow rate of 1.5 ml/min. The purity of the obtained NHase was estimated to be more than 99% by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The purified NHase was stored at 4°C in the dark as a suspension in 60% saturated ammonium sulfate.

Absorption Spectra under Native and Denaturing Conditions—All absorption spectra were measured with a spectrophotometer (Shimadzu, UV-2100 PC). The spectra of the native NHase and its isolated subunits were measured in buffer A (50 mM Hepes-KOH, pH 7.5, containing 20 mM *n*-butyric acid). The spectra of the unfolded NHase were measured in the denaturing buffer (buffer A containing 6 M urea and 2 mM 2-mercaptoethanol). To avoid aggregation of the denatured subunits due to formation of intermolecular disulfide bonds, 2-mercaptoethanol was included in the denaturing buffer. Addition of 2-mercaptoethanol at a concentration of less than 10 mM had no effect on the activity.

Isolation of the Subunits of NHase—All manipulations were executed in the dark to prevent light irradiation of the subunits. Isolation of the subunits from the inactive enzyme was carried out by anion-exchange chromatography using a Resource Q column (Pharmacia, 6.4 mm i.d. × 30 mm) connected to a HPLC system (8010 model, Tosoh). The inactive NHase was denatured by incubation in 50 mM Tris-HCl, pH 7.5, containing 6 M urea and 2 mM 2-mer-

captoethanol for 1 h at 4°C. The gradient conditions were as follows, solvent A: 50 mM Tris-HCl, pH 7.5, containing 6 M urea and 2 mM 2-mercaptoethanol; solvent B: A+500 mM NaCl. The content of B was increased as follows; from 0 to 2 min, 0%; from 2 to 22 min, 35%; from 22 to 23 min, 100%; from 23 to 25 min, 100%. Elution of the subunits was monitored by measuring the absorbance at 280 nm. The fractions containing each subunit were dialyzed against buffer A containing 2 mM 2-mercaptoethanol to remove urea. To avoid irreversible aggregation during dialysis, the β subunit was diluted to less than 0.05 mg/ml. The isolated subunits were stored as suspensions in 60% saturated ammonium sulfate at 4°C in the dark.

Activation of the Photoreactive NHase by Light Irradiation (Photoactivation)—The inactive NHase purified from the dark-inactivated cells was activated by the light irradiation in the following manner. An aliquot of the inactive NHase in buffer A in a 1.5 ml polypropylene tube was exposed to 5,000 lx white light (71.0 W/m^2) from a photoreflector lamp (500 W Spot, Toshiba) for 15 min in an ice bath. Light irradiation of the denatured NHase and the isolated subunits was performed according to the same procedure.

Assay of NHase Activity—The activity of NHase was assayed by measuring the activity of hydration of propionitrile to propionamide. In order to detect photoactivation of the NHase and its isolated subunits, the activity was assayed both in the dark (before irradiation) and after light irradiation as described above (after irradiation). The reaction was started by addition of 0.45 ml of 1 M ice-cold propionitrile to 0.5 ml of an NHase solution. After 5 min incubation in an ice bath, 0.05 ml of 2 N HCl was added to terminate the reaction. The amount of propionamide was measured by gas chromatography (GC-9A, Shimadzu) as described (*3*). A unit of the activity is defined as the amount of enzyme which produces 1 μmol of propionamide per minute and the specific activity is expressed as units per mg protein. Typically, the activity of the inactive NHase was less than 10 units/mg while that of the activated one was 750-900 units/mg.

Reconstitution of the Inactive Form of the NHase from the Isolated Subunits—Reconstitution of the inactive form of the NHase was performed by the following two methods. All manipulations were performed in the dark.

Method I: Each isolated subunit stored in 60% saturated ammonium sulfate was centrifuged at 40,000×g for 10 min and the precipitate was dissolved in an appropriate volume of buffer A. Equivalent amounts of both isolated subunits were mixed together and incubated at 4°C for 1 h.

Method II: An equivalent amount of each subunit stored in 60% saturated ammonium sulfate was centrifuged at 40,000×g for 10 min and the precipitate was dissolved in the denaturing buffer at 4°C to a protein concentration of 1.3 mg/ml. Both subunits were mixed together and incubated at 4°C for 1 h to be denatured completely. This mixture was then diluted by addition of 24 volumes of buffer A and incubated at 4°C for 1 h.

The reconstituted NHase was analyzed or purified by size exclusion chromatography (G3000SW$_{XL}$, Tosoh). The column running buffer was 50 mM Hepes-KOH, pH 7.0, containing 20 mM n-butyric acid, 2 mM 2-mercaptoethanol, and 100 mM KCl, and the flow rate was 1.0 ml/min. Elution was monitored by measuring the absorbance at 280 nm.

Other Procedures—The concentration of the NHase was estimated from the absorbance of the inactive form at 280 nm. Its adsorption coefficient is $\varepsilon_{280}=1.7$ ml·mg^{-1}·cm^{-1} [as determined from the amino acid analysis (*23*) of a known volume of the purified inactive form and its absorbance at 280 nm]. The concentrations of the isolated subunits were measured using Coomassie Brilliant Blue dye reagents (*24*). SDS-PAGE was performed by the method of Laemmli (*25*). N-terminal sequence analysis of the isolated subunits was done using a protein sequencer (Model 610A, Applied Biosystems) and amino acid analysis using an amino acid analyzer (Model 835, Hitachi).

RESULTS AND DISCUSSION

Absorption Spectra of the Native and Denatured NHase—The absorption spectra of the native NHase (the inactive and active forms) are shown in Fig. 1A (*3*). The inactive NHase showed two absorption peaks at 280 and 370 nm (the solid line in Fig. 1A). The adsorption coefficients were $\varepsilon_{280}=1.7$ ml·mg^{-1}·cm^{-1} and $\varepsilon_{370}=1.4\times10^{-1}$ ml·mg^{-1}·cm^{-1}, respectively. Upon light irradiation, the intensity of absorbance at 280 nm decreased by 12-15% and the peak at 370 nm almost disappeared. Instead a shoulder around 400 nm and a small and broad peak at 710 nm ($\varepsilon_{710}=2.7\times10^{-2}$ ml·mg^{-1}·cm^{-1}) appeared (the dotted line in Fig. 1A). To examine the conformation dependence of the photoreactive chromophores responsible for these absorption changes, the inactive NHase was unfolded by incubation in the denaturing buffer. The unfolded NHase had no activity even after light irradiation (data not shown). Unfolding in the denaturing buffer had little effect on the absorption spectrum of the inactive NHase (the solid line in Fig. 1B). The peaks at 280 and 370 nm diminished (15 and 82%, respectively) after light irradiation, but neither the shoulder around 400 nm nor the peak at 710 nm appeared at all (the dotted line in Fig. 1B). When the activated NHase was dissolved in the denaturing buffer, both the shoulder and peak also disappeared (data not shown). Identical results were observed when the NHase was dissolved in buffer A containing 6 M guanidine hydrochloride instead of 6 M urea (data not shown). Thus, the photoreactive chromophores in the inactive NHase, which are related to the changes of the peaks at 280 and 370 nm, are stable and able to respond to light even in the unfolded state. However, the chromophores responsible for the appearance of the shoulder around 400 nm and the peak at 710 nm after photoactivation are influenced by the conformation of the NHase.

Isolation of the Subunits from the Inactive NHase—To localize the photoreactive chromophores on the subunits, we attempted to isolate the subunits from the inactive NHase. The inactive NHase was unfolded in the denaturing buffer in the dark and subjected to anion-exchange chromatography in the presence of 6 M urea before light irradiation (Fig. 2, A and C) or after light irradiation (Fig. 2, B and D). Figure 2A shows the elution profile of the non-irradiated inactive NHase monitored in terms of absorbance at 280 nm. Two major peaks at 6.2 and 7.6 min were detected. These peaks were identified as those of the α and β subunits, respectively by N-terminal amino acid sequence analysis (data not shown). The small peaks at 5.4, 8.5, and 10.5 min were those of the aggregated complexes

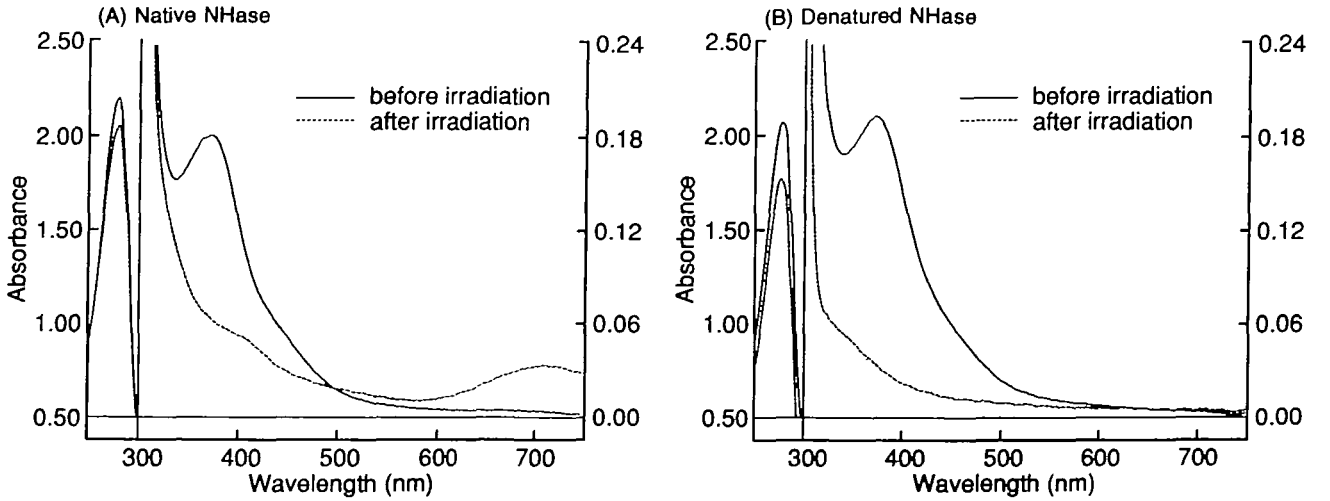

Fig. 1. **Absorption spectra of NHase under native and denatured conditions.** (A) the native NHase (1.3 mg/ml). —, before light irradiation (inactive form); ·····, after light irradiation (active form). (B) the denatured NHase (1.2 mg/ml). —, before light irradiation; ·····, after light irradiation. The buffers used were (A) 50 mM Hepes-KOH, pH 7.5, containing 20 mM *n*-butyric acid; (B) 50 mM Hepes-KOH, pH 7.5, containing 20 mM *n*-butyric acid and 6 M urea. Light irradiation of the native and denatured NHase was performed as described under "EXPERIMENTAL PROCEDURES."

composed of the α and β subunits linked *via* intermolecular disulfide bonds. The peak area of the α subunit was 2.6 times that of the β subunit (Fig. 2A), which can be attributed to the high Trp residue content of the α subunit. After light irradiation, the retention time of the α subunit was shortened by 0.2 min while that of the β subunit remained unchanged (Fig. 2B). The peak intensity of the α subunit, unlike that of the β subunit, diminished by about 15% after light irradiation. Figure 2, C and D, shows the elution profiles monitored at 370 nm. When the denatured NHase was applied to the column without light irradiation, a small peak was observed at the same retention time as the α subunit (6.2 min) (Fig. 2C). This peak disappeared after light irradiation (Fig. 2D). Preparatory-scale isolation of the subunits was carried out in the dark on the same column as described in "EXPERIMENTAL PROCEDURES." The absorption spectrum of the isolated α subunit was similar to that of the inactive NHase denatured with 6 M urea (Fig. 3A). On the other hand, the absorption spectrum of the isolated β subunit showed only a peak at 280 nm and light irradiation did not induce any spectral change (Fig. 3B). These results show that the photoreactive chromophores of the inactive NHase are entirely located on the α subunit.

Reconstitution of the NHase from Its Isolated Subunits— The NHase purified from the dark-inactivated cells showed very little activity, but after photoactivation it hydrated propionitrile with a specific activity of 8.8×10^2 units/mg (Table I, lane 1). The isolated α subunit as well as the β subunit had no activity even after light irradiation (Table I, lanes 2 and 3). However, when equimolar amounts of the α and β subunits were mixed and incubated at 4°C for 1 h (Method I in "EXPERIMENTAL PROCEDURES"), a specific activity of 2.6×10^2 units/mg was recovered after light excitation (corresponding to 30% of the native NHase) (Table II, lane 1). This means that significant amounts of the obtained α and β subunits are correctly refolded and able to assemble together to reconstitute the NHase. The extent of recovery of the activity depended on the freshness of the isolated β subunit. Probably, this is due to irreversible aggregation of the isolated β subunit during storage. When the unfolded subunits were mixed and refolded by dilution (Method II in "EXPERIMENTAL PROCEDURES"), a specific activity of 5.9×10^2 units/mg was recovered after light excitation (Table I, lane 4). Addition of cofactors was not required for the reconstitution. This indicates that the chromophores essential for the photoactivation and catalysis, including the non-heme iron center, are very tightly bound to the subunits and not lost even in the unfolded condition. The efficiency of reconstitution by Method II depended on the protein concentration of the mixture after dilution. To reconstitute the inactive form in high yield, the protein concentration after dilution should be less than 0.05 mg/ml (data not shown).

In order to confirm that the reconstituted NHase was identical to the inactive one, the reconstitution mixture was analyzed by size exclusion chromatography. Because Method II was more efficient than Method I, Method II was used in these experiments. Elution profiles of the inactive NHase and the isolated subunits are shown in Fig. 4, A-C, respectively. The inactive NHase was eluted at 8.3 ml (Fig. 4A). Although the isolated α subunit was eluted at 9.4 ml, the isolated β subunit was eluted as a broad peak at 6.8 ml, suggesting that the isolated α subunit was a monomer while the isolated β subunit existed as highly aggregated complexes (Fig. 4, B and C). Light irradiation of the inactive form and isolated subunits did not alter the elution profiles (data not shown). When the reconstitution mixture was applied to the column, most protein eluted as a single peak at 8.6 ml, in addition to small peaks corresponding to the α and β subunits (Fig. 4D). The elution volume of the main peak was similar to that of the native NHase. When the fraction at the main peak was re-applied to the column, it was eluted at the same elution volume as a single peak (Fig. 4E). This peak was found to be an equimolar complex of the α and β subunits by SDS-PAGE (data not shown). The purified $\alpha\beta$ complex showed activity of 7.3×10^2 units/mg

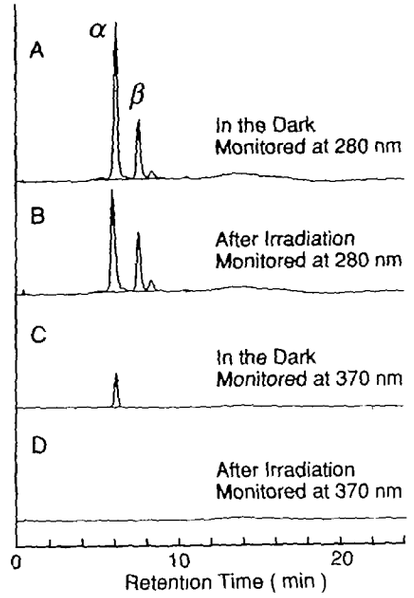

Fig. 2. **Isolation of the subunits from the inactive form of NHase by anion-exchange chromatography.** Elution was monitored by measuring the absorbance at 280 nm (A, B) or at 370 nm (C, D). The inactive form of NHase denatured in solvent A (see below) in the dark was analyzed with an anion-exchange column (Resource Q, Pharmacia) connected to an HPLC system (8010 model, Tosoh). (A) 46 μg, without light irradiation. (B) 46 μg, after light irradiation. (C) 93 μg, without light irradiation. (D) 93 μg, after light irradiation. The buffers used were solvent A: 50 mM Tris-HCl, pH 7.5, containing 6 M urea and 2 mM 2-mercaptoethanol, and solvent B: A+500 mM NaCl. Before application to the column, the inactive form was unfolded in solvent A at 4°C for 1 h. The gradient condition was as follows, from 0 to 2 min, 0%; from 2 to 22 min, 100%; from 22 to 25 min, 100%.

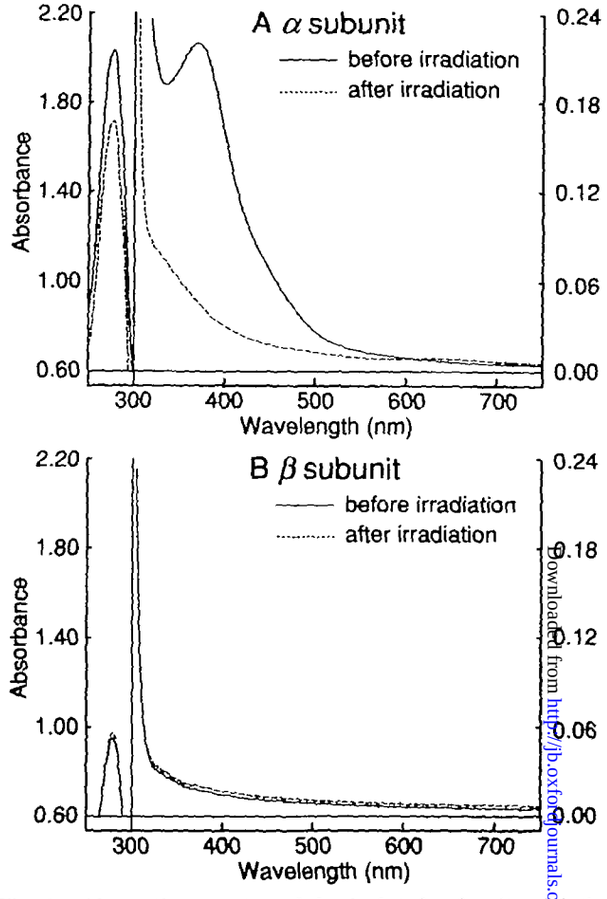

Fig. 3. **Absorption spectra of the isolated subunits.** (A) the isolated α subunit (0.56 mg/ml). —, before light irradiation; ··, after light irradiation. (B) the isolated β subunit (0.56 mg/ml). —, before light irradiation; ··, after light irradiation. The buffer used was 50 mM Hepes-KOH, pH 7.5, containing 20 mM *n*-butyric acid. Light irradiation of each isolated subunit was performed as described under "EXPERIMENTAL PROCEDURES."

after light irradiation, which corresponded to 83% of that of the activated NHase (Table I, lane 5). As shown in Fig. 5, the absorption spectra of the $\alpha\beta$ complex before and after light irradiation were almost identical to those of the native NHase. The adsorption coefficient at 710 nm was 2.3×10^{-2} ml·mg^{-1}·cm^{-1}, which was 85% of that of the active form. From these results, we conclude that the reconstituted NHase has the same properties as the native one.

Effects of Light Irradiation of Each Isolated Subunit on Reconstitution and Activation of the Photoreactive NHase—The irradiated α subunits could not renature but formed non-specific aggregates after unfolding by 6 M urea (data not shown). This result suggests that the light irradiation induces conformational change of each subunit. Then we examined the effect of light irradiation of each subunit on reconstitution and activation of the NHase. In the following experiments, reconstitution was done by Method I to avoid non-specific aggregation of the α subunit.

Figure 6A shows the elution profile of the reconstitution mixture of the non-irradiated α and β subunits on size exclusion chromatography. The reconstitution efficiency was low compared with Method II (Fig. 4D), as described above (Tables I and II). The α subunit partly eluted together with the β subunit at 7.5 ml. Because this peak showed no activity after light irradiation, the α subunit might aggregate with the β subunit. The peak intensity and mobility depend on the content of the α subunit in the

TABLE I. **Activity of the isolated subunits and the NHase reconstituted by method II.** $\alpha+\beta$, the reconstitution mixture of the α and β subunits. Both of the subunits were isolated from the inactive form of NHase. The purified reconstituted NHase was the fraction of the peak at 8.6 ml in Fig. 4D. The experimental conditions are described under "EXPERIMENTAL PROCEDURES."

	NHase activity (10^2 units/mg)	
	Before irradiation	After irradiation
NHase	0.0	8.8
α	0.0	0.0
β	0.0	0.0
$\alpha+\beta$	0.0	5.9
Reconstituted NHase	0.0	7.3

aggregate and depend markedly on the experimental conditions. When the irradiated β subunit was used for reconstitution, the elution profile was essentially identical (data not shown) and the specific activity was 270 units/mg after photoactivation (Table II, lane 2). This value is similar to the activity of the reconstitution mixture prepared without light irradiation (Table II, lane 1). This clearly

TABLE II. **Activity of the NHase reconstituted by method I.** $\alpha+\beta$, the reconstitution mixture of the α and β subunit; $\alpha+$irradiated β, the reconstitution mixture of the α subunit and the irradiated β subunit; irradiated $\alpha+\beta$, the reconstitution mixture of the irradiated α subunit and the β subunit. Both of the subunits were isolated from the inactive form of NHase. The experimental conditions are described under "EXPERIMENTAL PROCEDURES."

	NHase activity (10^2 units/mg)	
	Before irradiation	After irradiation
$\alpha+\beta$	0.0	2.6
$\alpha+$Irradiated β	0.0	2.7
Irradiated $\alpha+\beta$	0.0	0.0

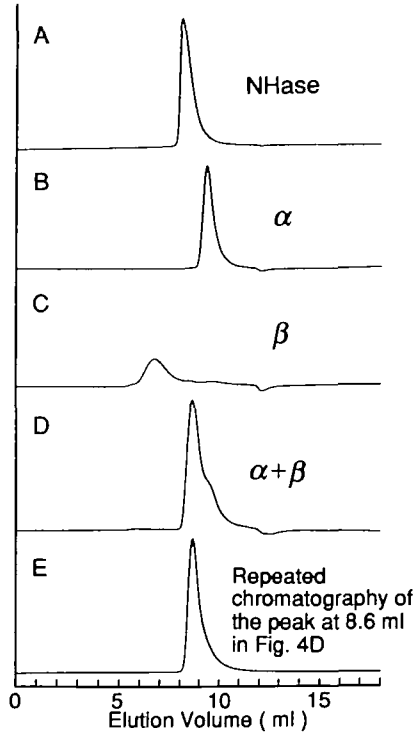

Fig. 4. **Detection and purification of the reconstituted NHase using Method II by gel permeation chromatography.** (A) the inactive NHase. (B) the α subunit. (C) the β subunit. (D) the reconstitution mixture using Method II. (E) repeated chromatography of the peak at 8.6 ml in (D). Both of the subunits were isolated from the inactive NHase. All manipulations were performed in the dark. The absorbance scales are arbitrary. Other experimental conditions are described under "EXPERIMENTAL PROCEDURES."

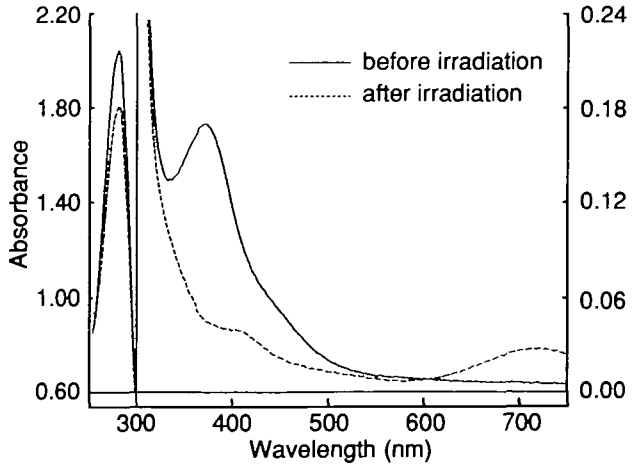

Fig. 5. **Absorption spectra of the reconstituted NHase.** The reconstituted NHase was purified by gel permeation chromatography as described under Fig. 4. —, before light irradiation; · ···, after light irradiation. The concentration of the reconstituted NHase was 1.2 mg/ml. The buffer used was 50 mM Hepes-KOH, pH 7.5, containing 20 mM *n*-butyric acid. Light irradiation of the reconstituted NHase was performed as described under "EXPERIMENTAL PROCEDURES."

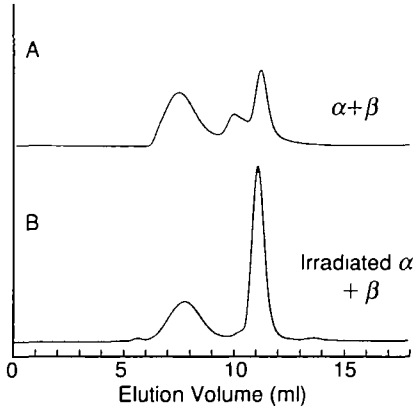

Fig. 6. **Detection of the reconstituted NHase using Method I by gel permeation chromatography.** (A) the reconstitution mixture of the α and β subunits; (B) the reconstitution mixture of the irradiated α subunit and the β subunit. Both of the subunits were isolated from the inactive NHase. All manipulations were performed in the dark. Because a guard column was connected in line with the gel permeation column (G300SW$_{XL}$, Tosoh), the inactive form was eluted at 10.0 ml. The absorbance scales are arbitrary. Other experimental conditions are described under "EXPERIMENTAL PROCEDURES."

indicates that irradiation of the β subunit has no effect on the reconstitution. On the other hand, when the isolated α subunit was irradiated prior to reconstitution, the peak corresponding to the reconstituted NHase was not detected by size exclusion chromatography (Fig. 6B) and the reconstitution mixture did not show any activity (Table II, lane 3). Even after overnight incubation no activity was recovered (data not shown). The same result was obtained when both subunits were irradiated with light (data not shown).

We also isolated the subunits from the active NHase in the same manner. The subunits isolated from the active NHase had the same characteristics on reconstitution as those which were isolated from the inactive NHase and irradiated with light (data not shown). Ikehata *et al.* demonstrated that the active NHase was able to be reconstituted from the insoluble aggregates of the *Rhodococcus* sp. N-774 NHase expressed in *Escherichia coli* by solubilizing them in buffer containing 8 M urea and dialyzing against alkaline buffer in the presence of Fe^{2+} and PQQ (*15*). A high alkaline pH during dialysis and the addition of Fe^{2+} and PQQ seemed to be critical for their method. However, even when the *Rhodococcus* sp. N-771 NHase in the denaturing buffer was irradiated with light and dialyzed against 50 mM Tris-HCl (pH 10.0) in the presence of Fe^{2+} and PQQ, no activity was recovered (data not shown). This discrepancy may be due to the different experimental conditions.

Our results show that the photoreaction of the isolated α

subunit results in loss or decrease of the ability to form the $\alpha\beta$ complex. It is possible that the photoreaction of the isolated α subunit causes some conformational change.

To elucidate in detail the mechanism of the photoactivation process, determination of the three dimensional structures of the NHase both in the inactive and active state will be essential. X-ray crystallography of the inactive NHase is in progress (*26*).

Concluding Remarks—In this study we demonstrated that the 280 and 370 nm chromophores, which are involved in the putative photoreaction site, are entirely located on the α subunit. This is the first report describing direct identification of the location of the photoreaction site. We are studying the chromophores in the isolated subunits and already have some evidence for the presence of an iron atom and an NO molecule in the α subunit, not the β subunit. These results will be described in detail elsewhere. Our results show that the β subunit does not contain any chromophore responsible for photoactivation. It should be noted, however, that the isolated α subunit showed neither the absorption peak at 710 nm nor the NHase activity, while the reconstituted $\alpha\beta$ complex showed both of them (Figs. 3 and 5 and Table I). The β subunit is likely to contribute in some way to the chromophores responsible for the photoactivation of the NHase.

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