Spectroscopic Observation of the Intramolecular Electron Transfer in the Photoactivation Processes of Nitrile Hydratase

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ABSTRACT: The photoactivation phenomena of the photosensitive enzyme nitrile hydratase (NHase) was studied by various spectroscopic methods. We have already shown that the photoactivation of NHase accompanies oxidation of an iron atom in the NHase [Honda *et al.* (1992) *FEBS Lett. 301*, 177–180]. From the results obtained in the present study by absorption, action, and fluorescence spectra, we show that the chromophore responsible for the photoactivation process is the iron complex, and the tryptophan residues in NHase induce the oxidation of the iron atom via an energy-transfer process. The nanosecond flash photolysis experiment revealed that this photoactivation process is completed within 50 ns, which suggests that the changes observable in the absorption spectra originate from an intramolecular electron transfer occurring from an electronically excited state. Also the role of a stabilizing reagent, namely, *n*-butyric acid (BA), was investigated using the above methods, which revealed that BA, besides its stabilizing effect, contributes to the increase in apparent photoactivation rate.

Nitrile hydratase (NHase;[1] EC 4.2.1.84) is an enzyme which catalyzes the reaction of hydrating organic nitriles to the corresponding amides [$RCN + H_2O \rightarrow RC(O)NH_2$]. Its existence was first reported by Asano *et al.* (1980), and it is being commercially used *in vivo* in the production of acrylamide from acrylonitrile in the bioreactors of Nitto Chemical Industry Co. Ltd. (Watanabe *et al.*, 1987). There are NHases from various sources, mainly bacteria, and they are well characterized in various laboratories. There are iron-containing NHases and cobalt-containing NHases, and these are summarized in a review by Kobayashi *et al.* (1992). As for cofactors other than metal, Nagasawa and Yamada (1987) showed that NHase contains pyrroloquinoline quinone (PQQ). Recently, however, the presence of PQQ in NHase has become controversial after Duine's report (1991) which indicated the absence of PQQ from several quinoproteins assayed in the same method.

There have thus been many studies to characterize these different NHases, but there has been very little study that deals with the photosensitive property of NHase. The "photosensitive" NHase isolated from *Rhodococcus* sp. N-771 (a generous gift from Nitto Chemical Industry), which we focus our present study on, is composed of two subunits $\alpha\beta$, each with a molecular weight of approximately 22 800 and 23 400 respectively (Nagamune *et al.*, 1991). From DNA sequencing using the PCR method, this NHase was found to be identical to NHase from *Rhodococcus* sp. N-774 (Honda *et al.*, 1992a). It has two iron atoms per molecule as cofactor (Nagamune *et al.*, 1990a). Its unique photosensitive phenomenon has been observed *in vivo* (Nakajima *et al.*, 1987; Nagamune *et al.*, 1990b) and *in vitro* (Nagamune *et al.*, 1990a; Honda *et al.*, 1992b). One of the most interesting features of the actual photoactivation phenomenon of this NHase is that, upon light irradiation, one of the two iron atoms incorporated in this enzyme is oxidized from Fe(II) to Fe(III) (Honda *et al.*, 1992b). In this scheme, a photoinduced electron-transfer reaction is involved in the photoactivation processes of the NHase where the iron atom acts as an electron donor. This in turn implies that there should be an electron acceptor of some form within the NHase molecule, and its presence has already been indicated from magnetic susceptibility measurements (Honda *et al.*, 1992b). Other aspects of the photoactivation phenomenon of this NHase have to date not been examined in detail.

Nagasawa *et al.* (1986) characterized NHase from *Brevibacterium* R312, which has the same DNA sequence as that from *R.* sp. N-774 (Mayaux *et al.*, 1991) and *R.* sp. N-771 (Honda *et al.*, 1992a). In addition, we have observed that the NHase activity of *B.* R312 *in vivo* is affected by light irradiation (unpublished results). Since the amino acid sequence and the photosensitive property are identical, NHases from these sources can be said to be identical, and hence we will hereafter refer to them uniquely as a "photosensitive NHase". Despite these facts, however, the reported absorption spectra of the active form of the photosensitive NHase are different between Nagasawa *et al.*'s (1986) and ours (Nagamune *et al.*, 1990a). There was a notable difference in the characteristic peak at about λ = 700 nm: in Nagasawa's data, λ_{max} = 712 nm, whereas in ours, λ_{max} = 680 nm—shift of over 30 nm. There were also notable differences in the shoulders around λ = 300–500 nm. They reported that this NHase is very labile to dilution, and is stabilized by addition of 20 mM *n*-butyric acid (BA) to the buffer, and performed all experiments with this buffer containing BA (Nagasawa *et al.*, 1987). We reported that the NHase is stable in the inactive form without the addition of BA (Nagamune *et al.*, 1990a). The effect of

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[1] Abbreviations: BA, *n*-butyric acid; ESR, electron spin resonance; ET, electron transfer; EXAFS, extended X-ray absorption fine structure; NAD, nicotinamide adenine dinucleotide; NHase, nitrile hydratase; PQQ, pyrroloquinoline quinone.

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BA other than its stabilizing effect has so far not been studied, and we felt that this may be the key to explaining the differences in the results obtained from the two groups. In this paper, several spectroscopic data are presented in order to shed more light into the photoactivation phenomenon of the photosensitive NHase, and also some interesting roles played by BA on this rare phenomenon.

EXPERIMENTAL PROCEDURES

Preparation of Inactive Nitrile Hydratase. *R.* sp. N-771 was cultivated as described previously (Nagamune *et al.*, 1990b). The purification procedure in the dark has been modified slightly since our previous purification method (Nagamune *et al.*, 1990a). To mention briefly, the cells of *R.* sp. N-771 were suspended in 20 mM potassium/sodium phosphate buffer with 20 mM *n*-butyric acid (stabilizing reagent) and disrupted with an ultrasonic disrupter (UD-201, Tomy Seiko). The cell debris were removed by ultracentrifugation at 190 000*g* for 30 min. The supernatant was then fractionated by ammonium sulfate at 40–60% saturation. The precipitate was dissolved and dialyzed in 0.1 M Tris-HCl buffer (pH 7.5). This enzyme solution was subjected to ion-exchange chromatography [TMAE-650(M), Merck], eluted with a linear gradient of 0.1–0.7 M Tris-HCl (pH 7.5). The fractions having nitrile hydratase activity were collected, concentrated by ultrafiltration, dialyzed in 20 mM phosphate buffer containing 30% ammonium sulfate (pH 7.5), and subjected to hydrophobic chromatography (Toyopearl HW-65F, Tosoh), eluted with a linear gradient of 30–10% ammonium sulfate. Again, active fractions were collected, concentrated, dialyzed in 5 mM phosphate buffer (pH 7.2), and subjected to adsorption chromatography (Bio-Gel HTP, Bio-Rad), eluted with 5–50 mM phosphate buffer. The active fractions, which are electrophoretically pure, were collected and used in a buffer containing 20 mM sodium/potassium phosphate and 20 mM *n*-butyric acid (pH 7.5) throughout the subsequent experiments, except where specified.

Absorption Spectra. In order to observe the influence of *n*-butyric acid (BA) on the absorption spectrum of NHase, two 3-mL samples of inactive NHase (0.5 mg/mL) in buffers with and without 20 mM BA were placed in quartz cuvettes and their absorption spectra were measured with a spectrophotometer (U-3300, Hitachi) at room temperature. They were then photoactivated by white light from a tungsten lamp as described (Nagamune *et al.*, 1990b), and changes in their absorbance were observed against irradiation time.

Also, inactive NHase sample (0.86 mg/mL or $A_{280} = 1.3$) in buffer with 20 mM BA was used to measure the time-dependent spectral change with a steady-state irradiation by methods described (Imamoto *et al.*, 1989). The sample was irradiated in the sample compartment of a spectrophotometer (MPS-2000, Shimadzu) with light from a 1-kW tungsten–halogen lamp (Sanko) passed through a blue-glass filter (V-40, Toshiba), followed by recording of the absorption spectra. The temperature of the sample was kept at 15 °C.

Action Spectrum. In order to clarify what wavelength of light is most effective for photoactivation, an action spectrum was measured where the inactive NHase in buffer (about 60 μg/mL) with 20 mM BA was irradiated with light of various wavelengths (250–400 nm) using a built-in Xe lamp of a fluorescence spectrophotometer (F-4000, Hitachi) with a bandpass of 5 nm. Each NHase sample (1 mL) was placed in a 1-cm path length quartz cuvette and irradiated for 3 min at room temperature. The irradiated NHase samples were reacted in an ice bath for 20 min with propionitrile (final concentration of 450 mM), which is a substrate for the enzymatic reaction. The amount of reaction product (propionamide) formed was assayed by gas chromatography. Initial velocity for the reaction rate was measured from the calibration curve of propionamide formed against time, and this was multiplied by the reciprocal of light intensity of the Xe lamp to obtain the efficiency of photoactivation per quanta. The light intensity of the lamp was measured with a Coherent Fieldmaster equipped with a silicon thermal disk sensor (LM-2 UV).

Fluorescence Spectroscopy. For fluorescence spectral measurements, 2 mL of inactive NHase in buffer (25 μg/mL or $A_{280} \approx 0.04$) was placed in a quartz cuvette, and the fluorescence spectra were measured with a fluorescence spectrophotometer (F-4000, Hitachi). The excitation wavelengths were 280, 370, and 710 nm with a bandpass of 5 nm, and their emission spectra were measured at room temperature. After checking that the sample was not photoactivated significantly by measuring its absorption spectrum, it was fully photoactivated by white light irradiation, and the fluorescence spectra were measured again.

Nanosecond Laser Photolysis. The experimental setup for flash photolysis was a double-beam spectrometer linked with a nanosecond laser as described previously (Okada *et al.*, 1991). NHase sample (about 45 mg/mL or $A_{370} = 6.0$) was irradiated with a 17-ns pulse (full width at half-maximum) from an excimer-pumped dye laser, and a photographic flash lamp was used as a probe light source. The excitation wavelength was 350 nm, and the energy was 0.8 mJ/1.8 mmϕ. A light pulse from the photographic flash lamp was divided into two pulses, one for probing an absorbance change of the sample and the other as a reference. Each pulse was focused onto the slit of each polychromator. Transient spectra of the sample at selected times after excitation were recorded with a spectrometric multichannel analyzer (SMA) system in which two sets of cooled (–20 °C) photodiode arrays were used as detectors. In order to eliminate the effects of rotation and diffusion of the molecules, the angle between the plane of polarization of the excitation and probe lights was kept at 54.7° (magic angle). Data taken with 64 laser shots were averaged.

RESULTS

Influence of n-Butyric Acid on the Photoactivation Processes. The absorption spectra of active NHase showed differences depending on the presence of a stabilizing reagent *n*-butyric acid (BA). The absorption spectra of inactive NHase in the presence and absence of 20 mM BA are shown in Figure 1a, and those of active NHase are shown in Figure 1b. It is clear from Figure 1a that BA has no significant effect on the absorption spectrum of inactive NHase. Both spectra (Figure 1a) show characteristic peaks at $\lambda = 280$ nm and $\lambda = 370$ nm with a long absorption tail up to 900 nm, and their peak amplitudes are almost identical. The absorption spectra of active NHase, on the other hand, show a distinct difference depending on the presence of BA in the buffer. The active NHase in the absence of BA shows the characteristic peak at $\lambda = 676$ nm, and two shoulders at about $\lambda = 370$ nm and $\lambda = 450$ nm, data which are identical with those previously reported (Nagamune *et al.*, 1990a). The absorption spectrum of active NHase in the presence of BA shows the characteristic peak at $\lambda = 710$ nm and only one shoulder at about $\lambda = 400$ nm, data which are identical with those reported by Nagasawa *et al.* (1986). These results suggest that BA has considerable

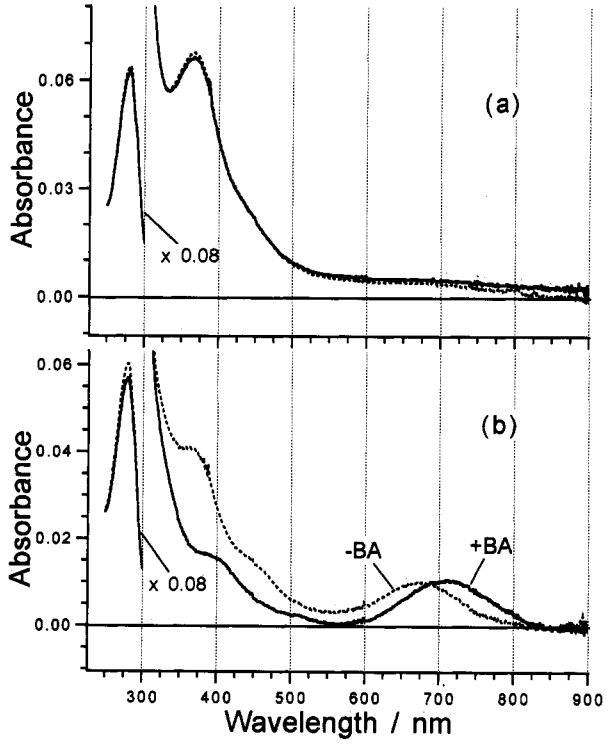


FIGURE 1: Absorption spectra of the inactive (a) and active (b) NHase (0.5 mg/mL) in phosphate buffer (dotted lines) and in phosphate buffer with *n*-butyric acid as stabilizer (solid lines). The inactive NHase (a) was thoroughly irradiated with the white light of a tungsten lamp to form the active NHase (b). The absorption spectra below 300 nm are scaled down by a factor of 0.08.

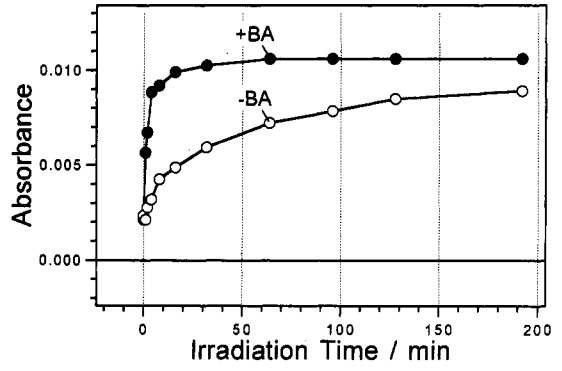


FIGURE 2: Change in absorbance of 680-nm [without *n*-butyric acid (○)] and 710-nm [with *n*-butyric acid (●)] peaks of NHase in buffer (0.5 mg/mL) against time during photoactivation with the white light of a tungsten lamp.

influence on the NHase chromophores. It was ascertained by activity measurement using gas chromatography that both NHase samples maintain their enzymatic activity during the experiment. It should be noted that the absorption bands at $\lambda = 280$ nm decrease during the photoconversion process from inactive to active NHase for both samples.

The change of absorption ($\lambda = 710$ nm for +BA and 680 nm for –BA) against irradiation time during photoactivation of NHase also showed a distinct difference between the NHase samples in two buffer conditions (Figure 2). The time for irradiation, by which half of the total NHase in the sample is photoactivated, is less than 1 min for NHase in a buffer containing BA (+BA) and about 24 min for that without BA (–BA). In Figure 2, the absorbance of +BA NHase at t = 192 min is saturated, but –BA NHase is still rising. When the absorbance of –BA NHase is extrapolated to $t = \infty$, it is slightly lower than the +BA absorbance, probably due to the

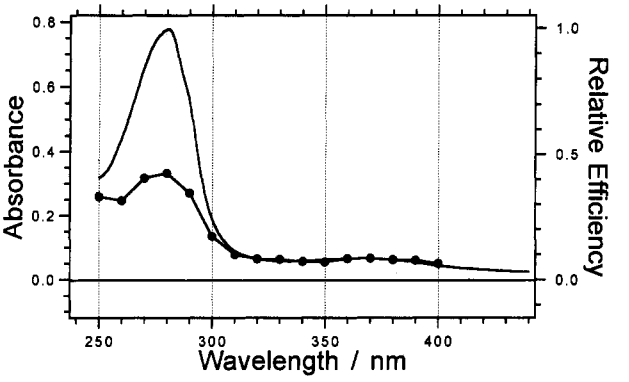


FIGURE 3: Action spectrum of the NHase (60 μg/mL) in phosphate buffer with 20 mM *n*-butyric acid (solid line with filled circles) superimposed onto the absorption spectrum of the inactive NHase (solid line). The vertical axis of the action spectrum represents the relative enzyme activity per quanta (see text).

difference in absorption coefficient between the two buffer conditions apparent in Figure 1b and/or to denaturation of –BA NHase. These results show that BA, besides its stabilizing effect, helps NHase to photoactivate in a shorter time: a factor of greater than 24. Therefore, the experiments to follow were performed for the NHase sample in the presence of BA.

The Action and Fluorescence Emission Spectra. The action spectrum of the NHase is shown in Figure 3 together with the absorption spectrum of the inactive NHase normalized at $\lambda = 370$ nm. There is a large peak at $\lambda = 280$ nm and a small secondary peak at around $\lambda = 370$ nm. This is consistent with the peak wavelengths of the absorption spectrum, but with a different amplitude ratio of 280/370. The peak amplitude of action spectrum at $\lambda = 280$ nm is about 40% of that of absorption spectrum. The absorption peak at $\lambda = 280$ nm is the absorbance of aromatic amino acid residues (mainly tryptophan). Since there is also a major action peak at $\lambda = 280$ nm, tryptophan may be involved in the photoactivation of NHase.

We next measured fluorescence emission spectra with excitation of three absorption bands at $\lambda = 280$ nm (both inactive and active NHases), 370 nm (inactive NHase), and 710 nm (active NHase). However, no detectable fluorescence was observed with excitation wavelengths at $\lambda = 370$ nm for the inactive NHase and $\lambda = 710$ nm for the active NHase (data not shown). On the other hand, fluorescent emission spectra were measured with an excitation wavelength at $\lambda = 280$ nm (Figure 4). The emission spectra of both inactive and active NHase had a maximum at around $\lambda = 340$ nm, which is typical of tryptophan fluorescence. This measurement showed that the photoactivated NHase has 1.2-fold increase in the emission amplitude compared to that of the inactive NHase. This is an indication that the environment of tryptophan in the molecule has changed and/or the lifetime of the excited state of tryptophan is shortened in the inactive NHase.

Steady Light Irradiation Experiment of NHase. Irradiation of the NHase causes marked absorption changes of three bands at $\lambda = 280$, 370, and 710 nm (676 nm without BA), as shown in Figure 1. In order to trace the depletion/formation of the three absorption peaks upon photoactivation, the steady-state illumination was applied to the NHase at 15 °C. In this experiment, the NHase in a buffer with BA was irradiated with a blue filtered light ($\lambda_{max} = 400$ nm) to selectively photoactivate the chromophore having $\lambda_{max} = 370$ nm, and the absorption spectra were recorded after irradiation (Figure

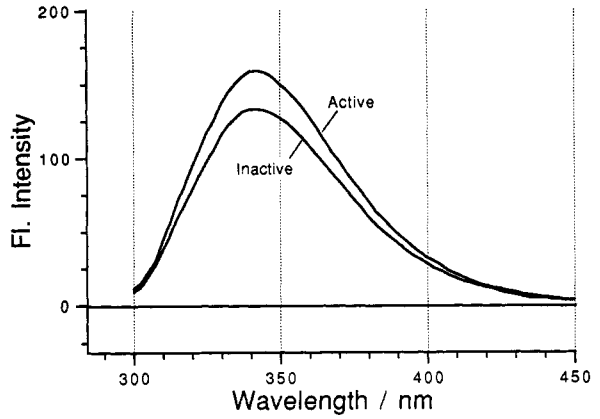


FIGURE 4: Fluorescence emission spectra of the inactive and active NHase in phosphate buffer with *n*-butyric acid (25 μg/mL). The excitation wavelength was 280 nm.

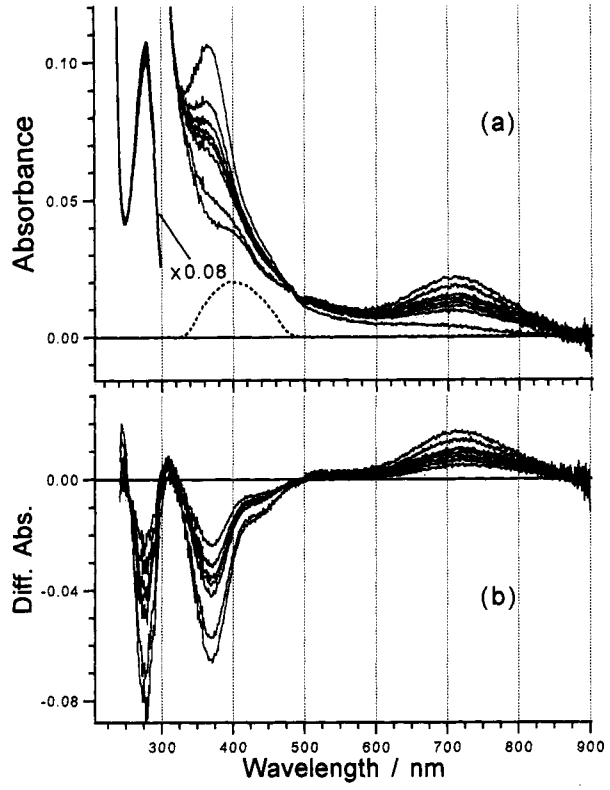


FIGURE 5: Absorption spectra of NHase before and after steady-state irradiation (a) and their difference absorption spectra from that before irradiation (inactive form) (b). The absorption spectra below 300 nm are scaled down by a factor of 0.08. The dotted line indicates the emission spectrum of the filtered light irradiated on the NHase sample. The NHase sample with BA was irradiated for a total of 1, 2, 3, 4, 5, 16, and 60 min until the active NHase was completely formed, and a successive decrease in absorbance at 280 and 320–500 nm, and a successive increase at 500–900 nm, can be observed.

5). After irradiation for 1 min, the absorption at 260–300 and 320–500 nm decreased, while the new absorption band appeared at 500–900 nm. These features are observed for the successive irradiation up to 60 min, where no more absorption change occurred and the spectrum corresponded to that of the active NHase. We confirmed that the absorption spectrum at 60 min is stable for at least 1 h at 15 °C and irradiation of 710-nm chromophore caused no spectral change (data not shown).

The spectral changes in Figure 5 imply the presence of two kinetic components, since the change after 1-min irradiation

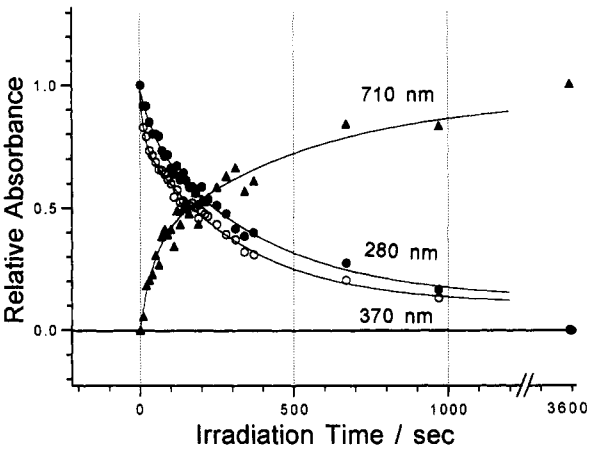


FIGURE 6: Change in absorption at 280 (●), 370 (○), and 710 nm (▲) during blue light irradiation against time. The absorbances at 280 and 370 nm are normalized so that $A(t_0) = 1$ and $A(t_\infty) = 0$, while the absorbance at 710 nm is normalized so that $A(t_0) = 0$ and $A(t_\infty) = 1$. In these kinetics, the fast and slow components can be seen. Solid lines are the double-exponential fitting curves.

is far greater than that after the following irradiation. This is visible in the kinetics of the three absorption bands (Figure 6), where the absorbance changes at λ = 280, 370, and 710 nm against irradiation time during blue light irradiation are shown. Since the kinetics cannot be fitted with a single exponential, we applied double-exponential fitting of the experimental results (solid lines). The decay of the absorption at λ = 280 and 370 nm is different when normalized [$A(t_0) = 1$ and $A(t_\infty) = 0$], as evident from Figure 6. These absorption changes have time constants of 41 ± 22 s (280 nm) and 11 ± 3 s (370 nm) for the fast components. For the slow components, the corresponding values are 390 ± 70 s (280 nm) and 330 ± 30 s (370 nm). For the absorption at λ = 710 nm, we see a rise in amplitude where it was normalized as $A(t_0) = 0$ and $A(t_\infty) = 1$. The time constants were 44 ± 22 s for the fast component and 520 ± 300 s for the slow component. The slow components seem to be identical among the three wavelengths, while the fast component at 370 nm appears to be shorter than the other two.

Flash Photolysis Experiment of NHase. Then, in order to evaluate the rate of change in the absorption spectra in the shorter time scale, nanosecond flash photolysis experiments were conducted. The experiments revealed that, within 50 ns after flash irradiation, a depletion in the absorption band at λ = 370 nm and an increase in the absorption band at about λ = 700 nm were observed (Figure 7). These findings are a clear indication that the active NHase is formed within 50 ns, suggesting that the photoactivation, which accompanies electron transfer, occurs in a shorter time scale. An identical spectrum is observed for the same experiment 1 μs after flash irradiation (Figure 7).

The transient absorption spectrum recorded at 1 μs after flash was compared to the stationary difference spectra between the active and inactive NHase with BA in buffer (Figure 8a) and without BA (Figure 8b). It can be seen that the transient and stationary spectra show almost identical profiles for the NHase in buffer without BA (Figure 8b), but not so for the NHase in buffer with BA (Figure 8a). It must be noted that this transient absorption spectrum is obtained for the NHase with BA in buffer which is identical to that without BA in buffer for 1 μs (Honda *et al.*, 1992a). Thus the present results suggest that after photoactivation, in the presence of BA in buffer, there exists a process for a change in the NHase molecule which leads to this change in the

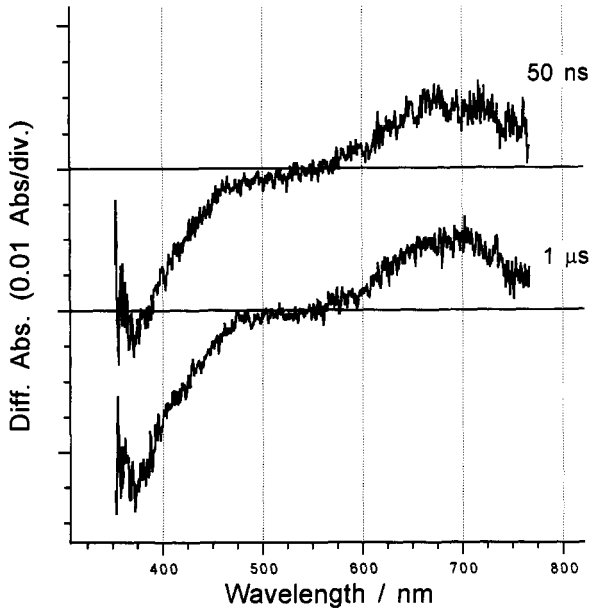


FIGURE 7: Transient difference spectra of NHase. The upper spectrum is that of 50 ns after flash irradiation, and the lower, that of 1 μs after.

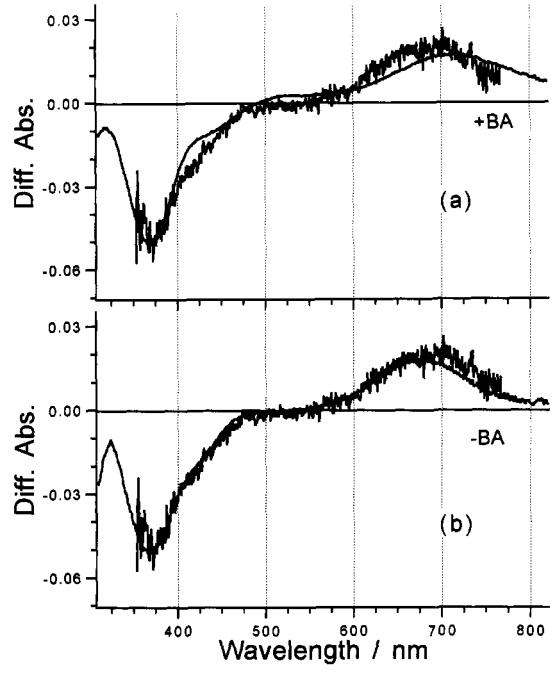


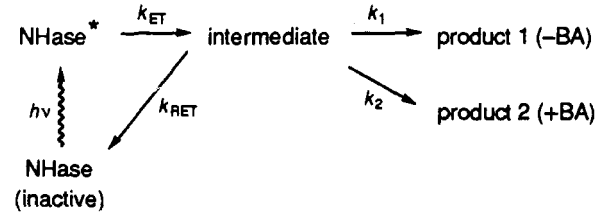
FIGURE 8: Superimposed transient and stationary difference absorption spectra. The transient spectrum is that of 1 μs after flash irradiation, and the other, a mere difference between inactive and active absorption spectra (a) with *n*-butyric acid in buffer and (b) without.

absorption spectrum. No significant change that leads to spectral change occurs for the NHase without BA. This is probably related to the results shown in Figure 2, where the time taken for the formation of the active form during irradiation depends largely upon the presence of BA.

DISCUSSION

Interaction of n-Butyric Acid with the NHase. An absorption spectrum of the active NHase is different depending on the presence or absence of BA (Figure 1). The present results indicate that there is a considerable interaction between the chromophore of the active NHase molecule and BA. The exact mechanism of the interaction of BA on NHase molecule still remains to be investigated, but at this stage we can conclude that two different spectral profiles reported from two different groups (Nagasawa *et al.*, 1986; Nagamune *et al.*, 1990a) originated from different buffer conditions rather than different NHases.

The presence of *n*-butyric acid (BA) in the buffer is extremely effective in preserving catalytic activity of NHase which is otherwise very labile, as reported in detail by Nagasawa *et al.* (1987). We have now shown that BA is also effective in increasing the efficiency of the photoactivation process of NHase (Figure 2). The mechanism of this phenomenon may be explained most simply by considering the electron transfer (Honda *et al.*, 1992b) and its forward and reverse reactions in the photoactivation processes as follows:



Here the wavy and solid lines represent the processes of photon absorption and thermal reaction, respectively. The rate constants of each thermal step are denoted by k_{ET}, k_{RET}, k_1, and k_2. Both unstable and stable states in the photoactivation processes are represented by "intermediate" and "product", respectively. The present nanosecond transient absorption measurements directly revealed that the electron transfer occurs within 50 ns, indicating $(k_{ET})^{-1} < 50$ ns, and the process for the formation of the intermediate seems to be identical between the samples with and without BA because of their identical difference transient spectra. In the present hypothetical scheme, the reverse electron transfer occurs from the intermediate with the rate constant of k_{RET}, while the relaxation after the electron transfer causes the formation of product 1 (–BA) or product 2 (+BA) with rate constants of k_1 and k_2, respectively. After formation of the products, no more absorption change occurs. Thus, under the present scheme, the reverse electron transfer (k_{RET}) competes with the relaxation processes that follow (k_1 or k_2) after the photoinduced electron transfer. By taking $k_{RET} > k_1$ and k_{RET} about the same order of magnitude as k_2, the different efficiency in the product formation (Figure 2) is reasonably explained.

The difference absorption spectrum between the intermediate and the inactive NHase is shown in Figure 7, while those between product 1 or product 2 and the inactive NHase are compared to the former in Figure 8. Interestingly, the transient absorption spectrum of the NHase with BA matches very well with the stationary difference spectrum of the NHase without BA (Figure 8b), but not with BA (Figure 8a). In the absence of BA, only a slight spectral change may take place in the process from intermediate to product 1. It suggests no significant change around the chromophore, although no more reverse electron transfer is possible after formation of product 1. On the other hand, in the presence of BA, the chromophore seems to undergo greater change accompanying the absorption change shown in Figure 8a. This is probably the result of an interaction between BA and the NHase chromophore in the active form, which leads to the stabilization of the active site. Having mentioned that $k_1 < k_2$, suggesting that photoactivation

of –BA NHase is a slower process, we can speculate that this slower process reflects greater change in the NHase molecule to reach its energetically stable state in the absence of BA (although not apparent in the absorption spectrum). This greater change may be the cause of its instability.

The Role of Aromatic Amino Acid Residues in Photoactivation Processes. The peak at about λ = 280 nm in the action spectrum (Figure 3) is suggestive of tryptophan residues playing a role in the photoactivation processes. However, it is unlikely that these tryptophan residues are directly concerned in the photochemical processes of NHase. Rather, they are involved in the energy-transfer processes, as was the case in the photoactivation of urocanase from *Pseudomonas putida* (Hug *et al.*, 1978). Urocanase is a photoactivable enzyme and has an action peak at λ = 280 nm. It is also similar to NHase in that it has a fluorescence emission that increases upon photoactivation. They conclude that this is due to intramolecular energy transfer from tryptophan to NAD sulfite (its modified coenzyme), which is an energy quencher having absorption at λ = 320 nm. Upon photodissociation of sulfite from NAD, the energy transfer no longer occurs, and thus tryptophan fluorescence is enhanced. A similar mechanism may be true for NHase, an acceptor having enough spectral overlapping (λ_{max} = 370 nm) with an emission spectrum of tryptophan to realize the Förster-type energy transfer. The enhancement of an emission of tryptophan upon photoactivation of the NHase (Figure 4) supports the present argument.

It is noted that the absorbance at λ = 280 nm decreases slightly (6%) upon irradiation of the 370-nm chromophore (Figure 5). The change in the 280-nm absorbance can be interpreted as the change in the environment of tryptophan as a result of photoactivation, or it is a part of the absorption band of the iron cluster which also has absorbance at λ = 370 nm. In the latter case, the peak at λ = 280 nm in the action spectrum might partially be attributable to the iron cluster. Even in this case, tryptophan residues are still playing an important role in photoactivation especially in photon acquisition since the chromophore that changes upon photoactivation has 280/370 nm ratio of about 1.4 (Figure 5b), while the action spectrum shows a much greater 280/370 nm ratio of about 5.0 (Figure 3).

From the results of kinetic study of absorption changes in the 280-, 370-, and 710-nm chromophores, it was observed that the time constants of the decreasing absorbances, namely, those of 280 and 370 nm, were different. This difference is considered to be quite significant, especially that of fast components where the difference is more conspicuous. This is an evidence that the 280-nm absorption and the 370-nm absorption originate from two independent chromophores.

The Iron Complex and the Absorption Bands of the NHase. The number of iron atoms per molecule of photosensitive NHase reported from various groups is controversial despite their identity (for evidence of identity, see the introduction). Sugiura's group (1987) reports the number of iron atoms per NHase molecule to be 3, judging from double integration of the ESR spectrum giving 2.9 ± 0.2 spin per NHase molecule. On the other hand, Nelson *et al.* (1991) reports that ratio of iron/protein was 1.7, which means 2 irons per NHase molecule. We reported that the number of iron atoms per NHase molecule is 2 (Nagamune *et al.*, 1990a). The analysis of the ratio of two iron species in the inactive NHase by Mössbauer spectroscopy, which was 1.16, supports this result (Honda *et al.*, 1992b).

The structure of the photoactivated iron complex has been reported by Jin *et al.* (1993), who showed from electron–nuclear double resonance (ENDOR) spectroscopy combined with EXAFS and resonance Raman spectroscopy data (Nelson *et al.*, 1991) that the ligands are 2 sulfurs, 3 imidazoles, and water. Here, they assume that the 2 irons exist in the molecule as equal independent complexes.

As for the absorption bands, the absorption peak at λ = 370 nm is diminished upon irradiation of the inactive NHase, and the new absorption appears at either λ = 676 nm (–BA) or λ = 710 nm (+BA). Nelson *et al.* (1991) explain the peak at λ = 710 nm by an assignment to a mononuclear Fe^{III}-S charge-transfer complex. The change in absorption of the 370-nm chromophore occurs in the same time as the appearance of the 710-nm chromophore during irradiation, as evident from the results of kinetic study on the photoactivation process (Figures 5 and 6), and the result of Mössbauer spectroscopy combined with magnetic susceptibility measurements has shown that one of the two iron atoms in the NHase molecule is oxidized from Fe(II) to Fe(III) and the other iron remains Fe(III) throughout (Honda *et al.*, 1992b). In the inactive form of the NHase, however, this peak at λ = 710 nm is not exhibited despite the presence of one Fe(III) species. Therefore, the assignment of the iron to the absorption bands is not so straightforward. But in any case, the change in absorption, stationary or transient, reflects product formation upon photoactivation which seems to be directly related to the fast (<50-ns) electron-transfer reaction.

Dynamics of the Electron-Transfer Reaction in the NHase. The electron-transfer (ET) reactions in proteins have been extensively investigated both experimentally and theoretically (Boxer, 1990; Moser *et al.*, 1992). The normal ET takes place between the donor and acceptor molecules in their electronic ground state according to their redox potentials. In these cases, the protein–protein interaction (complex formation) or the conformational change of the proteins triggers the ET, and the ET rates vary in a very wide range (k_{ET}^{-1}; from microseconds to seconds). On the other hand, there is one special ET process in the case of the photosynthetic reaction centers, where light absorption is the trigger source of the ET reaction (Deisenhofer & Norris, 1993). In this case, the ET occurs from an electronically excited state, so it is possible for the ET rate to be in the "ultrafast" time scale. In fact, it is well-known that the primary ET of the purple bacteria occurs in 2–3 ps (Breton *et al.*, 1986; Holzapfel *et al.*, 1990; Chan *et al.*, 1991). The ultrafast ET provides efficient usage of the absorbed light energy. So far, the research on photoinduced ET has been focused predominantly on the processes occurring in the photosynthetic reaction centers. Although there has been much research in reconstituting the proteins by incorporating ruthenium and observing the photoinduced ET reaction (Onuchic *et al.*, 1992), there have been fewer studies of the native proteins.

The present NHase can be a good example for studying photoinduced ET in the native protein other than the photosynthetic reaction centers. It was shown by our previous results that the photoinduced ET occurs in the NHase (Honda *et al.*, 1992b). Through the results by nanosecond flash photolysis, we observed the appearance of the 710-nm absorption band within 50 ns (Figure 7). Since the change in these bands represents the ET reaction, intramolecular ET is complete within 50 ns. In addition, no detectable emission from the 370-nm chromophore suggests a rapid decay in its excited state. Thus the ET in NHase seems to occur from the excited state. The real-time observation of the photoinduced ET processes in the NHase by femtosecond spectroscopy is under consideration. This electron liberated from Fe is

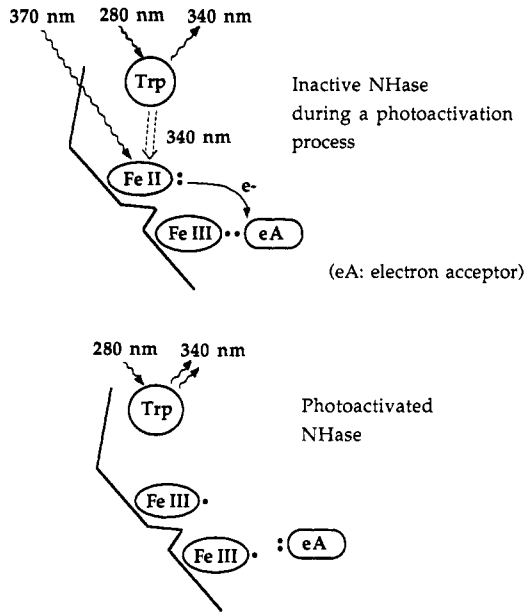


FIGURE 9: Hypothetical scheme for the photoactivation process of the photosensitive NHase.

transferred to an electron acceptor, which at present is unknown. This electron acceptor seems to couple with Fe(III) before photoactivation since the ESR spectrum is not detected in the inactive NHase (Nagamune *et al.*, 1992). This may also be the cause for the absence of 710-nm absorption band in the inactive NHase. For a detailed explanation of the photoinduced ET process, three-dimensional structural information is indispensable, and the structural study of this NHase is in progress by X-ray crystallography (Nagamune *et al.*, 1991).

Photoactivation Scheme of the NHase. We have shown that the spectral changes upon photoactivation reflect the change in the state of iron atoms caused by the electron-transfer reaction, in a time scale of less than 50 ns. Also, we have shown that this photooxidation reaction is triggered by light irradiation either by electronic excitation of Fe complex or by an energy transfer from tryptophan. Then the electron from Fe(II) is transferred to an unknown electron acceptor. Gathering this information together, we propose the model shown in Figure 9 for the photoactivation process of this photosensitive NHase. The iron states are according to the results of Mössbauer and magnetic susceptibility measurements. In the NHase before photoactivation, the Fe(III) is coupled with an electron acceptor which has an unpaired electron, thus canceling out the spin that would give an ESR signal. This explains the silent ESR spectrum of the inactive NHase (Nagamune *et al.*, 1992). Upon photoactivation, an electron transfer occurs from the Fe(II) to the electron acceptor, thus breaking the coupling of Fe(III) and the electron acceptor, and both the irons exhibit the typical ESR spectrum. The exact arrangement of the iron is unknown, but it is likely to be taking part in the enzymatic reaction (Sugiura *et al.*, 1987) and thus should be located around the active site.

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