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Thermal Equilibrium of Two Conformations in Photosensitive Nitrile Hydratase Probed by the FTIR Band of Nitric Oxide Bound to the Non-Heme Iron Center

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Nitrile hydratase (NHase) from *Rhodococcus* N-771 is a novel enzyme that is inactive in the dark due to an endogenous nitric oxide (NO) molecule bound to the non-heme iron center, and is activated by its photodissociation. FTIR spectra in the NO stretching region of the dark-inactive NHase were recorded in the temperature range of 270–80 K. Two NO peaks were observed at 1854 and 1846 cm^{-1} at 270 K, and both frequencies upshifted as the temperature was lowered, retaining the peak separation of 8–9 cm^{-1}. The relative intensity of the lower-frequency peak increased with decreasing temperature up to ~120 K, whereas it was mostly unchanged below this temperature. This observation indicates that two distinct conformations with slightly different NO structures are thermally equilibrated in the dark-inactive NHase above ~120 K, and the interconversion is frozen-in at lower temperatures. The intensity ratio of the NO bands changed gradually upon increasing the pH from 5.5 to 11.0, but no specific pK_a value was found. This result, together with the comparison of the light-induced FTIR difference spectra measured at pH 6.5 and 9.0, suggests that the protonation/deprotonation of a specific amino acid group in the active site of NHase is not a direct cause of the occurrence of the two conformations, although several protonatable groups in the protein may influence the energetics of the two conformers. From the previous observation that the isolated α subunit of NHase exhibited a single broad NO peak, it is suggested that interaction of the β subunit forming the reactive cavity is essential for the double-minimum potential of the active-site structure. The frequencies and widths of the two NO bands changed upon addition of propionamide, 1,4-dioxane, and cyclohexyl isocyanide, indicating that these compounds are bound to the active pocket and change the interactions of the iron center or the dielectric environments around the NO molecule. Thus, the NO bands of NHase can also be a useful probe to monitor the binding of substrates and their analogues to the active pocket.

Key words: FTIR, infrared absorption, nitric oxide, nitrile hydratase, non-heme iron.

Abbriviations: Mes, 2-(*N*-morpholino)ethansulfonic acid; NHase, nitrile hydratase.

Nitrile hydratases (NHases) are bacterial enzymes that catalyze the hydration of nitriles to the corresponding amides. They have been used in the industrial production of acrylamide and nicotinamide, and are also important for bioremediation in which nitriles in industrial waste and toxic herbicides can be degraded (*1–3*). NHases consist of α and β subunits and contain either a non-heme iron(III) or a non-corrinoid cobalt(III) atom at the catalytic center (*3*).

Some of the Fe-type NHases, those from *Rhodococcus* N-774, N-771, and R312, exhibit a characteristic phenomenon of photoactivation: they are inactive in the dark and activated by light illumination (*4*). Clarification of the photoactivation mechanism was accelerated by the finding of endogenous nitric oxide (NO) attached to NHase by Fourier transform infrared (FTIR) spectroscopy (*5*). Resonance Raman (*6*) and ESR spin trapping (*7*) measurements further proved that photodissociation of the NO molecule bound to the non-heme iron activates the enzyme. It was also shown that the Fe-type NHase from *Comamonas testosteroni* NI1, which did not exhibit photosensitivity originally, was inactivated by exogenous NO and reactivated by subsequent light illumination (*8*).

The X-ray crystal structures of NHase in the light-active (*9*) and dark-inactive (*10*) forms revealed that the protein ligands of the iron center are three S atoms of cysteine side-chains and two nitrogen atoms of backbone amides, being consistent with earlier spectroscopic results (*11–16*). Furthermore, it was shown that two of the three cysteine ligands, αCys112 and αCys114, are post-translationally modified to cysteine-sulfinic (Cys-SO_2H) and cysteine-sulfenic (Cys-SOH) acids, respectively (*10*, *17*, *18*). Our recent FTIR study showed that the sulfinic acid and most likely sulfenic acid exist as deprotonated Cys-SO_2^- and Cys-SO^- forms, respectively (*19*). These Cys-SO_2^- and Cys-SO^- interact with βArg56 and βArg141, forming a reactive cavity at the interface of the α and β subunits (*10*).

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The reaction mechanism of nitrile hydration in NHase is still controversial (*3*, *9*). One view is that a nitrile substrate is attached to the non-heme iron, which works as a Lewis acid to activate the nitrile, and the activation is followed by the nucleophilic attack of a water molecule on the nitrile carbon. Another view is that a metal-bound water or hydroxide ion either attacks the nitrile carbon or activates a water molecule, which in turn can act as a nucleophile. To clarify the catalytic mechanism of NHase, detailed information about the active site, including the hydrogen-bonding and electrostatic structures and the interactions of substrate in its binding pocket, is essential. The vibrations of NO and CO molecules have been extensively used to probe the active site structure in metalloenzymes like myoglobin (*20–24*). Their frequencies are sensitive to the hydrogen bonding interaction and dielectric environment as well as the electronic structure of the metal center. Analogously, the NO molecule that is endogenously bound to the non-heme iron center in the dark-inactive NHase should provide important information about the active-site structure.

In this study, we have investigated the structure of the active center of NHase using the NO vibration as a probe. To do so, we measured FTIR spectra in the NO stretching region at various temperatures and pHs. In addition, the effects of chemical compounds that are expected to bind to the active cavity of NHase were studied. It was found that two distinct conformations with slightly different NO structures are in thermal equilibrium in dark-inactive NHase.

MATERIALS AND METHODS

Sample Preparation—Dark-inactive NHase was purified from *R*. N-771 cells as described previously (*25*) and dissolved in 50 mM Tris/HCl buffer (pH 7.5). The buffer was replaced with 1 mM Tris/HCl buffer (pH 7.5) and the sample was concentrated to 50 mg/ml using Microcon-50 membranes (Amicon). An aliquot of sample (6 μl) was deposited on a CaF_2 plate (13 mm ϕ) and lightly dried under N_2-gas flow. The sample was then dissolved in 1.5 μl of 50 mM buffer at an intended pH and covered with another CaF_2 plate with a piece of aluminium foil (~15 μm in thickness) as a spacer. The buffers used were citric acid/NaOH (pH 4.0), Mes/NaOH (pH 5.5 and 6.5), Tris/HCl (pH 7.5 and 9.0) and glycine/NaOH (pH 11.0). The sample temperature was controlled in a cryostat (Oxford DN-1704) using a temperature controller (Oxford ITC-5). All procedures were performed under dim red light.

FTIR Measurements and Data Processing—FTIR spectra were recorded using a JEOL JIR-6500 spectrophotometer equipped with an MCT detector (IR-DET101). A Ge filter (OCLI LO2584-9) was placed in front of the sample to cut a He-Ne laser beam from the interferometer. In measurements of the NO stretching region, spectra were obtained with 200 scans (100 s). The spectrum of buffer was subtracted from a sample spectrum to eliminate a steep baseline due to water absorption. Further baseline correction was done in the DX software system provided by JEOL.

Light-induced FTIR difference spectra were measured at 250 K as described in ref. *5* except for the pH values of 6.5 and 9.0 instead of 7.5. Illumination was performed for 10 s by continuous white light from a halogen lamp (Hoya-Schott HL150) with an intensity of 60 mW/cm^2 at the sample surface. All FTIR spectra were measured with a resolution of 4 cm^{-1}.

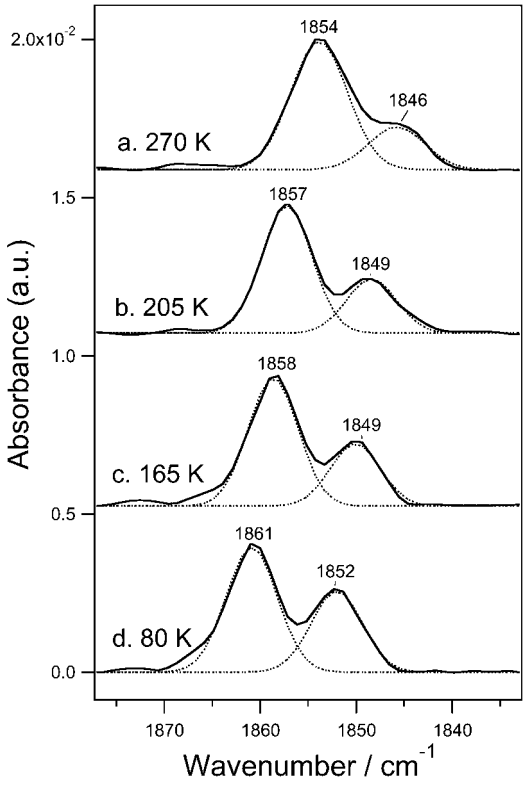

Fig. 1. **FTIR spectra in the NO stretching region of dark-inactive NHase in a pH 7.5 buffer measured at 270 (a), 205 (b), 165 (c), and 80 (d) K.** Dotted curves are two Gaussian functions fitting to the NO bands.

Band fitting was performed using the Igor Pro 4 program (WaveMetrics, Inc.). Two Gaussian functions with the same bandwidth were assumed to fit the two NO bands of each spectrum. This assumption provided more reasonable data than the assumption of independent bandwidths, by which the lower-frequency shoulder observed at a higher pH was sometimes fitted with an unreasonably broad band.

RESULTS

Temperature Dependence of the NO Bands—Figure 1 shows the NO stretching region of the FTIR spectra of dark-inactive NHase at pH 7.5, measured in the temperature range of 270–80 K. Two NO bands with a wavenumber separation of 8–9 cm^{-1} were observed at all temperatures. The peaks were detected at 1854 and 1846 cm^{-1} at 270 K, and both frequencies progressively increased as the temperature was lowered. Throughout this temperature range, the higher-frequency peak exhibited a stronger intensity than the lower-frequency peak, although the relative intensities became more even at lower temperatures.

The ratio (α) of the intensity of the higher-frequency peak (I_{high}) to that of the lower-frequency peak (I_{low}) ($\alpha = I_{high}/I_{low}$) was estimated by band fitting using two Gaus-

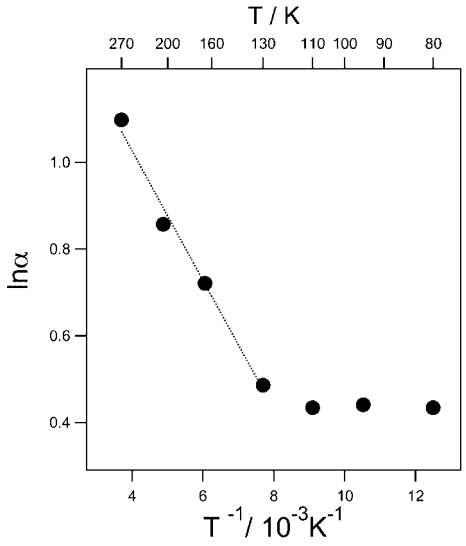

Fig. 2. **The logarithm of the intensity ratio (α) of the higher-frequency NO band with respect to the lower-frequency band as a function of $10^3/T$.** Data were obtained at 270, 205, 165, 130, 110, 90 and 80 K at pH 7.5. The dotted line is the regression line of the data between 130 and 270 K.

sian functions (Fig. 1, dotted lines). The logarithm of α is plotted as a function of $10^3/T$ in Fig. 2. The ln α values changed linearly above ~120 K, whereas they were mostly unchanged below this temperature.

The observation of temperature-dependence suggests that there are two distinct conformations in the dark-inactive NHase, and they exist in thermal equilibrium above ~120 K but the interconversion is frozen-in below ~120 K. The two conformers with higher and lower NO frequencies are hereafter designated as $NHase_{high}$ and $NHase_{low}$, respectively. From the regression line of the data above 130K in the ln α *versus* $1/T$ plot (Fig. 2, dotted line), the ΔH and ΔS values between $NHase_{low}$ and $NHase_{high}$ were estimated to be 1.24 kJ mol^{-1} and 13.5 J mol^{-1} K^{-1}, respectively. $NHase_{high}$ has a higher population than $NHase_{low}$ above ~120 K because of its lower free energy level, which is ascribed to the larger contribution of the $-T\Delta S$ term than the ΔH term. It is noted that since the buffer used in this work does not include a cryoprotectant such as glycerol, the observed phase transition at ~120 K may not be attributed to solvent freezing but rather to glass transition of the protein (*26*, *27*). It is also noted that the frequencies of the two NO peaks progressively changed with decreasing temperature and did not exhibit a phase transition at ~120 K. This observation suggests that the frequency shifts do not originate from the structural perturbation of the protein induced by the temperature change, but rather from anharmonic coupling with a low-frequency mode (*28*).

Band-splitting by Fermi resonance can be excluded as a possible mechanism for the presence of two NO peaks, because two similar NO peaks were previously observed at downshifted frequencies upon both uniform ^{15}N labeling of NHase and ^{15}NO substitution (*5*, *6*). Attachment of another NO molecule to the protein moiety other than the non-heme iron center can also be excluded, because both peaks are similarly bleached upon photoacticvation

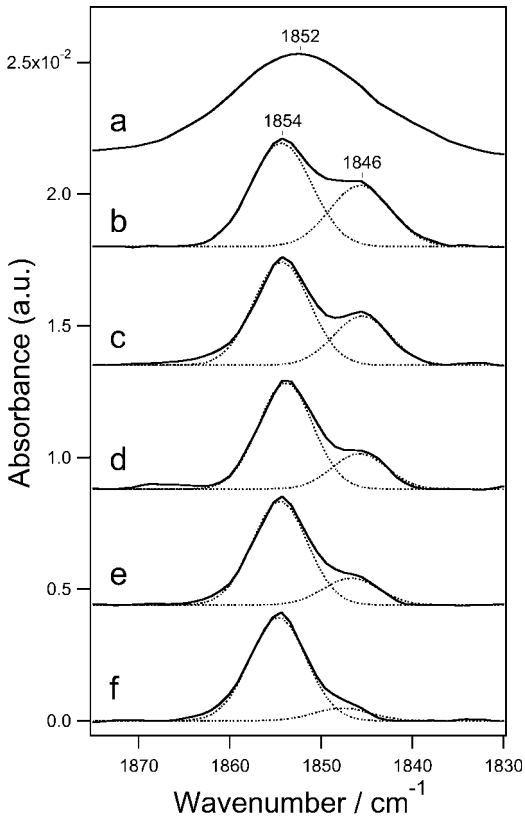

Fig. 3. **The NO bands of dark-inactive NHase at pH 4.0 (a), 5.5 (b), 6.5 (c), 7.5 (d), 9.0 (e), and 11.0 (f).** The spectra were measured at 270 K. Dotted curves are two Gaussian functions fitting to the NO bands.

of NHase (*5*) and because this view is not consistent with the temperature-dependence of the intensity ratio of the NO peaks (Figs. 1 and 2).

pH Dependence of the NO Bands and of the Light-Induced Difference Spectrum—To examine whether protonation/deprotonation of a certain amino acid group is responsible for the presence of two conformers in NHase, pH dependence of the NO bands was studied. Figure 3 shows the NO bands measured at 270 K in buffers at pH 4.0 (a), 5.5 (b), 6.5 (c), 7.5 (d), 9.0 (e), and 11.0 (f). At pH 5.5–11.0, two NO peaks were conserved, and the intensity of the 1846 cm^{-1} peak relative to that of the 1854 cm^{-1} peak decreased with increasing pH, basically without changing the peak positions. At pH 4.0, however, only one broad NO band was observed at 1852 cm^{-1}, which is very similar to that of the isolated α subunit measured at 278 K (*29*), and hence, it seems likely that NHase is denatured and dissociated into the α and β subunits at this low pH. In Fig. 4, the population of $NHase_{low}$, estimated as the relative intensity of the low frequency peak with respect to the total intensity of the two peaks, $I_{low}/(I_{high} + I_{low})$, was plotted against pH. This plot shows that the population of $NHase_{low}$ gradually decreases, without a specific inflection point, as the pH is changed from pH 5.5 to 11.0.

To further study the involvement of specific amino acid groups at the active site in the observed pH dependence, light-induced FTIR spectra were measured at different

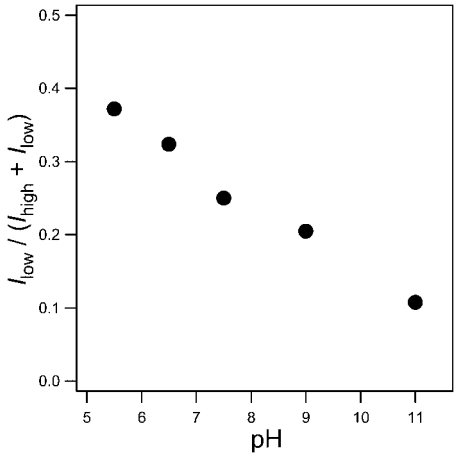

Fig. 4. **The pH dependence of the relative intensity of the lower-frequency NO band with respect to the total intensity of the two NO bands [$I_{low}/(I_{high} + I_{low})$].** The band intensities were obtained from the spectra in Fig. 3 measured at 270 K.

pHs. A light-induced FTIR difference spectrum of NHase reveals structural changes of the protein, especially in the active site, upon photo-conversion from the dark-inactive to the light-active form (*5*). Figure 5 shows FTIR difference spectra of NHase at pH 6.5 (a) and 9.0 (b). The large signals at (1154, 1148)/1126 and (1040, 1034)/1019 cm^{-1} have recently been assigned to the asymmetric and symmetric stretching vibrations, respectively, of the deprotonated SO_2^- group of αCys112-sulfinic acid by ^{34}S isotope labeling and density functional theory calculations (*19*). Note that the two negative doublets at 1154 and 1148 cm^{-1} and at 1040 and 1034 cm^{-1} were attributed to band splitting by Fermi resonance (*19*). In addition, a relatively small signal at 915 cm^{-1} has been assigned to the stretching vibration of the deprotonated SO^- group of αCys114-sulfenic acid (*19*). These SO_2^- and SO^- bands were basically unchanged by the pH difference between

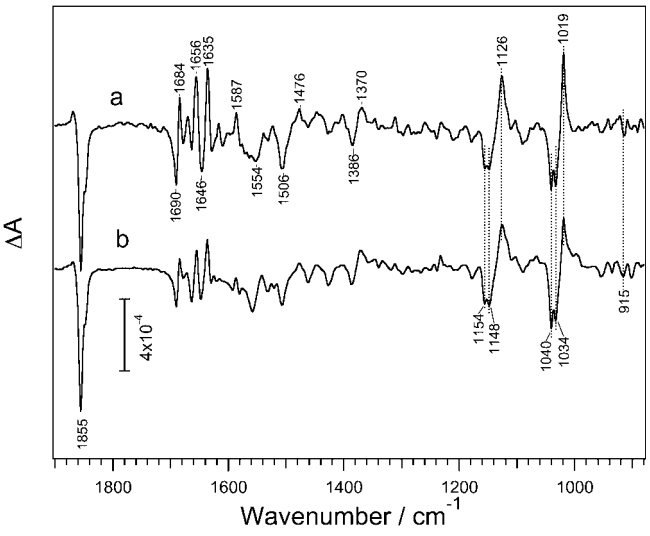

Fig. 5. **Light-induced FTIR difference spectra of NHase at pH 6.5 (a) and 9.0 (b).** The spectra were measured at 250 K. Intensities of the two spectra were normalized to the NO peak at 1855 cm^{-1}.

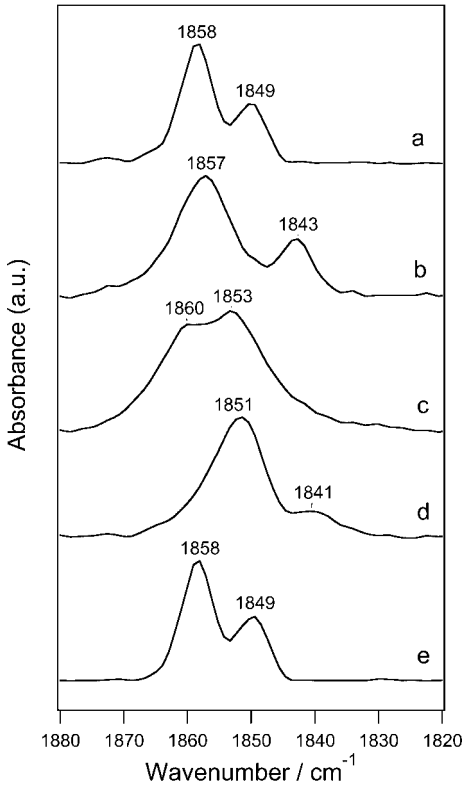

Fig. 6. **NO stretching bands of dark-inactive NHase in 50 mM Tris buffer (pH 7.5) without any addition (a), and in the presence of 200 mM propionamide (b), 200 mM 1,4-dioxane (c), and 200 mM cyclohexyl isocyanide (d), and 20 mM n-butyric acid (e).** The spectra were measured at 165 K.

pH 6.5 and 9.0. Their intensities were rather larger at pH 6.5 than at pH 9.0, suggesting that protonation of these groups at lower pH did not take place in this pH range. In other spectral regions, no significant difference was observed between the two spectra except for the band intensities in the amide I (1700–1600 cm^{-1}) and amide II (~1550 cm^{-1}) regions, which drastically decreased at a higher pH. Since bands in these regions represent changes in the secondary structures of a protein (*30*), this result suggests that at least some overall changes in the protein structure occur in NHase by the pH change.

Effects of the Compounds Bound to the Active Pocket—The effect of chemical compounds, which are expected to interact with the active pocket of NHase, is shown in Fig. 6. The spectra were measured at 165 K in a pH 7.5 buffer. In the presence of a relatively high concentration (200 mM) of propionamide (Fig. 6b), which is a product of a substrate, propionitrile, the NO signal exhibited clear changes: the higher- and lower-frequency peaks downshifted by 1 and 6 cm^{-1}, respectively, and the higher-frequency band was rather broadened. Note that addition of propionitrile resulted in the same spectrum as Fig. 6b, probably because the active form of NHase contaminating the dark-inactive NHase converted propionitrile to propionamide.

The X-ray crystallographic study of the dark-inactive NHase showed that 1,4-dioxane is bound to the cavity above the NO-Fe center (*10*, *31*). The NO bands in the

presence of 1,4-dioxane (Fig. 6c) were observed at 1860 and 1853 cm^{-1}, frequencies upshifted by 2 and 4 cm^{-1} from the untreated ones, with a higher relative intensity of the lower-frequency peak. Cyclohexyl isocyanide is a substrate analogue that was also detected to be bound to the active-site cavity by a recent crystallographic study (Nojiri *et al*., unpublised data). Upon addition of cyclohexyl isocyanide, the higher- and lower-frequency peaks downshifted by 7 and 8 cm^{-1}, respectively, with band broadening and a change in the relative intensities (Fig. 6d).

n-Butyric acid has been used as a protein stabilizer for the active form of NHase (*32*), whereas it also acts as a competitive inhibitor of nitrile hydration (*33*). Upon addition of 20 mM *n*-butyric acid (Fig. 6e), however, features of the two NO bands, including the frequencies, bandwidths and relative intensities, were unchanged from those without any addition (Fig. 6a). This concentration (20 mM) of *n*-butyric acid should be high enough to inhibit the enzyme, which has a K_i value of 0.9 mM at pH 7.2 (*33*), although measurements with a higher concentration of butyric acid were not performed because of the difficulty of pH control in a 50 mM Tris buffer (pH 7.5). The above result suggests that *n*-butyric acid does not interact with the NO-Fe center in NHase, being consistent with the previous EPR results in which butyric acid interacts only with the active form (*33*, *34*) but does not bind to the dark-inactive form (*35*).

DISCUSSION

The FTIR measurements of the NO stretching bands of dark-inactive NHase at various temperatures (Fig. 1) showed that NHase exists as two distinct conformers in solution, $NHase_{high}$ and $NHase_{low}$, which have higher and lower NO frequencies, respectively. These two conformers are in thermal equilibrium above ~120 K with a lower free energy in $NHase_{high}$. The frequency difference between the two NO peaks of 8–9 cm^{-1} indicates that the structure and interaction of the NO molecule at the iron center are somewhat different between $NHase_{high}$ and $NHase_{low}$. For instance, differences in the electrostatic and/or hydrogen-bonding interactions of NO in the binding pockets will affect the NO frequency, as has been extensively discussed for carbonmonoxy or nitrosyl myoglobin (*20–24*). Regarding the hydrogen-bonding interactions, however, both of the NO frequencies exhibited only slight downshifts (<1 cm^{-1}) by H_2O/D_2O exchange (T. Noguchi, unpublished result), suggesting that the NO molecule forms only a weak hydrogen bond in both conformations. From a general correlation between the NO frequency and M-N-O angle in nitrosyl complexes (*36*, *37*), it is also possible that the Fe-N-O angle is slightly smaller in $NHase_{low}$ than in $NHase_{high}$. Such an angle difference could result from a change in the extent of π back donation from the iron atom (*36*) induced by the slight structural changes of other ligands.

It could be further assumed that the two conformers originate from protonation/deprotonation of a certain amino acid group interacting with the non-heme iron center or forming the active-site cavity. Candidates for such amino acid groups are αCys112-SO_2^- and αCys114-SO^-, which provide sulfur ligands to the iron atom (*10*) and exist basically in deprotonated form at pH 7.5 (*19*). Examination of the pH dependence of the NO bands, however, did not provide a straightforward result: the relative intensities of the two NO peaks changed gradually with the pH change from 5.5 to 11.0 (Fig. 3), but a clear pK_a value was not observed in this pH region (Fig. 4). Also, in the light-induced difference spectra (Fig. 5), the intensities of the SO_2^- stretching bands at (1154, 1148)/1126 and (1040, 1034) /1019 cm^{-1} and the SO^- band at 915 cm^{-1} (*19*) were even slightly higher at pH 6.5 than at pH 9.0, contrary to what would be expected as a result of protonation of either of these groups at a lower pH. Other protonatable amino acid residues forming the active site are βArg56, βArg141, αArg167, βTyr72, βTyr76 and βTyr37 (*10*, *31*), none of which can be responsible for the observed pH dependence at neutral and acidic pHs (Fig. 4). Histidines (βHis5, βHis139) and carboxylic amino acids (αAsp161, αGlu165, βAsp53, βGlu60) are involved in the hydrogen-bond network around the active center (*31*), but are located rather far from the iron center (>7.5 Å). Thus, it seems unlikely that the presence of the two conformers directly originates from the protonation state of an amino acid group at the active site. Rather, we prefer the view that protonation states of several amino acid residues in the protein indirectly affect the energetics of the two conformers without changing the active-site structures and cause the observed pH dependence. In fact, band intensities in the amide I and II regions in the light-induced difference spectra became smaller upon the pH increase (Fig. 5), indicating the presence of some perturbation of the overall secondary structure of the protein.

Previous FTIR measurements of the isolated α subunit showed a single broad NO band at 1852 cm^{-1} (*29*). This broad NO band indicates that the α subunit itself has a protein conformation with a single potential minimum but with large structural fluctuation. Thus, two conformations with a double minimum potential may be introduced by the attachment of the β subunit, which produces the reactive cavity through the interactions of αCys112-SO_2^- and αCys114-SO^- with βArg56 and βArg141 (*10*). The previous observation that the NHase mutant in which βArg56 was replaced with Lys showed a single NO peak at 1856 cm^{-1} (*38*) is consistent with this view.

Addition of propionamide, 1,4-dioxane, and cyclohexyl isocyanide drastically affected the NO bands (Fig. 6). In contrast to the case of pH change, these treatments changed not only the relative intensities but also the peak frequencies and the bandwidths. Since the NO molecule is bound to the iron atom, these compounds cannot bind directly to the iron center. As for 1,4-dioxiane (*10*, *31*) and cyclohexyl isocyanide (Nojiri *et al*., unpublished), X-ray crystallography showed that they are bound to the space above the NO-Fe center. Binding of these compounds may change the electrostatic and hydrogen-bonding interactions in the active site and also the secondary structures of the protein, hence affecting the characters of the NO bands. It is worth noting that the NO frequencies were shifted in the opposite directions by binding of dioxane and cyclohexyl isocyanide: dioxane uphifted the NO peaks by 2 and 4 cm^{-1} (Fig. 6c), whereas cyclohexyl isocyanide downshifted them by 7 and 8 cm^{-1} (Fig. 6d). Since the cyclohexyl and dioxane rings occupy basically

the same position in the pocket and the isocyanide group is directed to the opposite side of the NO molecule, these results suggest that the electrostatic repulsion between the oxygen atoms of dioxane and NO may substantially contribute to the upshifts of NO frequencies. Propionamide, which is a product of propionitrile, also seemed to bind to the active pocket, judging from the downshifts of the NO frequencies (Fig. 6b). The lower-frequency band exhibited a relatively large shift of –6 cm^{-1} compared with a small shift of –1 cm^{-1} in the higher-frequency band. Significant broadening of the latter band (Fig. 6b), however, indicates that propionamide was actually bound to $NHase_{high}$. The different behaviours in the NO shifts upon binding of propionamide between $NHase_{high}$ and $NHase_{low}$ may indicate that the active-site structures of these two conformers, such as the NO angle or the position of some protein group in the cavity, are indeed different.

The X-ray crystal structure of dark-inactive NHase at 1.7 Å resolution (*10*) did not reveal the presence of two conformations at the non-heme iron site. This may be because (1) only one of the two conformations is preferred in the crystal state and the thermal equilibrium is realized only in solution, or (2) the structural difference causing the NO frequency change is not large enough to be recognized at the 1.7 Å resolution. In the latter case, the observed crystal structure can be an average structure of the two conformers.

The finding of two conformations in the dark-inactive NHase does not necessarily indicate the presence of two conformations in its light-active form. However, unless the occurrence of two conformations is directly caused by NO binding, it is possible that the light-active NHase also has two conformations. Further detailed studies using various spectroscopic methods are expected to detect such conformations in the active form.

In conclusion, we have found by FTIR measurement of the NO stretching bands that there are two distinct conformations in photosensitive NHase, at least in its dark-inactive form. These two conformations are in thermal equilibrium in solution, and the relative population is affected by pH. To identify the direct origin of the two conformations, however, further studies including the FTIR measurements of various site-directed mutants and X-ray crystallography at a higher resolution are necessary. It was also found that binding of chemical compounds in the active pocket affects the NO frequencies and bandwidths, and thus, the NO bands can be a useful probe to monitor the binding of substrates and their analogues.

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REFERENCES

1. Kobayashi, M., Nagasawa, T., and Yamada, H. (1992) Enzymatic synthesis of acrylamide: a success story not yet over. *Trends Biotechnol.* **10**, 402–408
2. Yamada, H. and Kobayashi, M. (1996) Nitrile hydratase and its application to industrial production of acrylamide. *Biosci. Biotechnol. Biochem.* **60**, 1391–1400
3. Kobayashi, M. and Shimizu, S. (1998) Metalloenzyme nitrile hydratase: Structure, regulation, and application to biotechnology. *Nat. Biotechnol.* **16**, 733–736
4. Nagamune, T., Kurata, H., Hirata, M., Honda, J., Hirata, A., and Endo, I. (1990) Photosensitive phenomena of nitrile hydratase of *Rhodococcus* sp. N-771. *Photochem. Photobiol.* **51**, 87–90
5. Noguchi, T., Honda, J., Nagamune, T., Sasabe, H., Inoue, Y., and Endo, I. (1995) Photosensitive nitrile hydratase intrinsically possesses nitric oxide bound to the non-heme iron center: evidence by Fourier transform infrared spectroscopy. *FEBS Lett.* **358**, 9–12
6. Noguchi, T., Hoshino, M., Tsujimura, M., Odaka, M., Inoue, Y., and Endo, I. (1996) Resonance Raman evidence that photodissociation of nitric oxide from the non-heme iron center activates nitrile hydratase from *Rhodococcus* sp N-771. *Biochemistry* **35**, 16777–16781
7. Odaka, M., Fujii, K., Hoshino, M., Noguchi, T., Tsujimura, M., Nagashima, S., Yohda, M., Nagamune, T., Inoue, Y., and Endo, I. (1997) Activity regulation of photoreactive nitrile hydratase by nitric oxide. *J. Amer. Chem. Soc.* **119**, 3785–3791
8. Bonnet, D., Artaud, I., Moali, C., Petre, D., and Mansuy, D. (1997) Highly efficient control of iron-containing nitrile hydratases by stoichiometric amounts of nitric oxide and light. *FEBS Lett.* **409**, 216–220
9. Huang, W.J., Jia, J., Cummings, J., Nelson, M., Schneider, G., and Lindqvist, Y. (1997) Crystal structure of nitrile hydratase reveals a novel iron centre in a novel fold. *Structure* **5**, 691–699
10. Nagashima, S., Nakasako, M., Dohmae, N., Tsujimura, M., Takio, K., Odaka, M., Yohda, M., Kamiya, N., and Endo, I. (1998) Novel non-heme iron center of nitrile hydratase with a claw setting of oxygen atoms. *Nat. Struct. Biol.* **5**, 347–351
11. Sugiura, Y., Kuwahara, J., Nagasawa, T., and Yamada, H. (1987) Nitrile hydratase. The first non-heme iron enzyme with a typical low-spin iron(III)-active center. *J. Amer. Chem. Soc.* **109**, 5848–5850
12. Jin, H., Turner, I.M., Jr., Nelson, M.J., Gurbiel, R.J., Doan, P.E., and Hoffman, B.M. (1993) Coordination sphere of the ferric ion in nitrile hydratase. *J. Amer. Chem. Soc.* **115**, 5290–5291
13. Doan, P.E., Nelson, M.J., Jin, H., and Hoffmann, B.M. (1996) An implicit TRIPLE effect in Mims pulsed ENDOR: A sensitive new technique for determining signs of hyperfine couplings. *J. Amer. Chem. Soc.* **118**, 7014–7015
14. Nelson, M.J., Jin, H., Turner, I.M., Grove, G., Scarrow, R.C., Brennan, B.A., and Que, L., Jr. (1991) A novel iron-sulfur center in nitrile hydratase from Brevibacterium sp. *J. Amer. Chem. Soc.* **113**, 7072–7073
15. Scarrow, R.C., Brennan, B.A., Cummings, J.G., Jin, H., Duong, D.J., Kindt, J.T., and Nelson, M.J. (1996) X-ray spectroscopy of nitrile hydratase at pH 7 and 9. *Biochemistry* **35**, 10078–10088
16. Brennan, B.A., Cummings, J.G., Chase, D.B., Turner, I.M., and Nelson, M.J. (1996) Resonance Raman spectroscopy of nitrile hydratase, a novel iron-sulfur enzyme. *Biochemistry* **35**, 10068–10077
17. Tsujimura, M., Dohmae, N., Odaka, M., Chijimatsu, M., Takio, K., Yohda, M., Hoshino, M., Nagashima, S., and Endo, I. (1997) Structure of the photoreactive iron center of the nitrile hydratase from *Rhodococcus* sp. N-771: Evidence of a novel post-translational modification in the cysteine ligand. *J. Biol. Chem.* **272**, 29454–29459
18. Murakami, T., Nojiri, M., Nakayama, H., Odaka, M., Yohda, M., Dohmae, N., Takio, K., Nagamune, T., and Endo, I. (2000) Post-translational modification is essential for catalytic activity of nitrile hydratase. *Protein Sci.* **9**, 1024–1030
19. Noguchi, T., Nojiri, M., Takei, K., Odaka, M., and Kamiya, N. (2003) Protonation structures of Cys-sulfinic and Cys-sulfenic acids in the photosensitive nitrile hydratase revealed by Fou-

rier transform infrared spectroscopy. *Biochemistry* **42**, 11642–11650
20. Miller, L.M., Pedraza, A.J., and Chance, M.R. (1997) Identification of conformational substates involved in nitric oxide binding to ferric and ferrous myoglobin through difference Fourier transform infrared spectroscopy (FTIR). *Biochemistry* **36**, 12199–12207
21. Phillips, G.N., Teodoro, M.L., Li, T.S., Smith, B., and Olson, J.S. (1999) Bound CO is a molecular probe of electrostatic potential in the distal pocket of myoglobin. *J. Phys. Chem. B* **103**, 8817–8829
22. Müller, J.D., McMahon, B.H., Chien, E.Y.T., Sligar, S.G., and Nienhaus, G.U. (1999) Connection between the taxonomic substates and protonation of histidines 64 and 97 in carbonmonoxy myoglobin. *Biophys. J.* **77**, 1036–1051
23. Tomita, T., Hirota, S., Ogura, T., Olson, J.S., and Kitagawa, T. (1999) Resonance Raman investigation of Fe-N-O structure of nitrosylheme in myoglobin and its mutants. *J. Phys. Chem. B* **103**, 7044–7054
24. Thomas, M.R., Brown, D., Franzen, S., and Boxer, S.G. (2001) FTIR and resonance Raman studies of nitric oxide binding to H93G cavity mutants of myoglobin. *Biochemistry* **40**, 15047–15056
25. Tsujimura, M., Odaka, M., Nagashima, S., Yohda, M., and Endo, I. (1996) Photoreactive nitrile hydratase: The photoreaction site is located on the α subunit. *J. Biochem.* **119**, 407–413
26. Morozov, V.N. and Gevorkian, S.G. (1985) Low-temperature glass transition in proteins. *Biopolymers* **24**, 1785–1799
27. Doster, W., Cusack, S., and Petry, W. (1989) Dynamical transition of myoglobin revealed by inelastic neutron scattering. *Nature* **337**, 754–756
28. Marks, S., Cornelius, P.A., and Harris, C.B. (1980) A critical test of vibrational dephasing theories in solids using spontaneous Raman scattering in isotopically mixed crystals. *J. Chem. Phys.* **73**, 3069–3081
29. Odaka, M., Noguchi, T., Nagashima, S., Yohda, M., Yabuki, S., Hoshino, M., Inoue, Y., and Endo, I. (1996) Location of the non-heme iron center on the α subunit of photoreactive nitrile hydratase from *Rhodococcus* sp N-771. *Biochem. Biophys. Res. Commun.* **221**, 146–150
30. Haris, P.I. and Chapman, D. (1992) Does Fourier-transform infrared spectroscopy provide useful information on protein structures? *Trends Biol. Sci.* **17**, 328–333
31. Nakasako, M., Odaka, M., Yohda, M., Dohmae, N., Takio, K., Kamiya, N., and Endo, I. (1999) Tertiary and quaternary structures of photoreactive Fe-type nitrile hydratase from *Rhodococcus* sp N-771: Roles of hydration water molecules in stabilizing the structures and the structural origin of the substrate specificity of the enzyme. *Biochemistry* **38**, 9887–9898
32. Nagasawa, T., Ryuno, K., and Yamada, H. (1986) Nitrile hydratase of *Brevibacterium* R312: Purification and characterization. *Biochem. Biophys. Res. Commun.* **139**, 1305–1312
33. Kopf, M.A., Bonnet, D., Artaud, I., Petre, D., and Mansuy, D. (1996) Key role of alkanoic acids on the spectral properties, activity, and active-site stability of iron-containing nitrile hydratase from *Brevibacterium* R312. *Eur. J. Biochem.* **240**, 239–244
34. Doan, P.E., Gurbiel, R.J., Cummins, J.C., Nelson, M.J., and Hoffman, B.M. (1999) Inhibitor binding in nitrile hydratase. Determining the geometry of enzyme-bound butyrate using ENDOR spectroscopy. *J. Inorg. Biochem.* **74**, 116–116
35. Popescu, V.C., Münck, E., Fox, B.G., Sanakis, Y., Cummings, J.G., Turner, I.M., and Nelson, M.J. (2001) Mössbauer and EPR studies of the photoactivation of nitrile hydratase. *Biochemistry* **40**, 7984–7991
36. Nakamoto, K. (1997) *Infrared and Raman Spectra of Inorganic and Coordination Compounds* 5th Edition Part B pp. 149–154, Wiley, New York
37. Quinby-Hunt, M. and Feltham, R.D. (1978) Infrared spectra of linear and nonlinear transition-metal nitrosyls. 2. Dimethyldithiocarbamate and o-phenylenebis(dimethylarsine) complexes of $\{FeNO\}^6$, $\{FeNO\}^7$, and $\{CoNO\}^8$. *Inorg. Chem.* **17**, 2515–2520
38. Piersma, S.R., Nojiri, M., Tsujimura, M., Noguchi, T., Odaka, M., Yohda, M., Inoue, Y., Ambe, F., and Endo, I. (2000) Arginine 56 mutation in the β subunit of nitrile hydratase: importance of hydrogen bonding to the non-heme iron center. *J. Inorg. Biochem.* **80**, 283–288