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Photochemistry of Nitric Oxide Adducts of Water-Soluble Iron(III) Porphyrin and Ferrihemoproteins Studied by Nanosecond Laser Photolysis

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Abstract: Water-soluble iron(III) porphyrin and ferrihemoproteins (methemoglobin, metmyoglobin, oxidized cytochrome c, and catalase) associate with NO to yield the nitric oxide adducts. The equilibrium constants for association of ferrihemoproteins and NO are 1 order of magnitude larger than that of the water-soluble iron(III) porphyrin which is free from protein, suggesting that the proteins offset the forward and backward reaction rates in the equilibrium reactions. Nanosecond laser photolysis studies of the nitric oxide adducts of metmyoglobin, oxidized cytochrome c, and catalase, (NO)MblII, (NO)CytilII, and (NO)CatIII, have been carried out. The transient detected after laser flash photolysis of (NO)CatIII is identified as CatIII. However, the transients observed for (NO)MblII and (NO)CytilII at 50 ns after laser pulse are ascribed to MblIIIp and CytilIIIp, respectively, with the absorption spectra different from those of uncomplexed MblII and CytilII. In particular, the absorption spectrum of CytilIIIp markedly differs from that of the uncomplexed CytilII. The species MblIIIp and CytilIIIp are found to change to MblIII and CytilIII, respectively, within a few microseconds. The quantum yields for the photodissociation of NO from nitric oxide adducts of ferrihemoproteins are 1 order of magnitude less than that from the NO adduct of the water-soluble iron(III) porphyrin, probably due to fast geminate recombination reaction of NO and ferrihemoprotein in a heme pocket. The photochemistry of the nitric oxide adducts of hemoproteins and water-soluble iron(II) porphyrin is also described on the basis of laser photolysis studies.

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2 University of California.


Iron(II) and iron(III) porphyrins, FeIIIP and FeIIIIP, interact with nitric oxide, NO, to yield the adducts (NO)FeIIIP and (NO)FeIIIIP.1-3 It has been established by ESR experiments that (NO)FeIIIP and (NO)FeIIIIP are respectively paramagnetic and diamagnetic: the spin state of the former is S = 1/2 and the latter S = 0.4-6 Recently, nitric oxide has been recognized to have important bioregulatory roles as the endothelium-derived relaxing factor in blood vessels, in the cytotoxic action of macrophages, and in neuronal communication in the brain.7-11 Nitric oxide is known to be synthesized in vivo from L-arginine by the enzyme NO synthase.12,13 Because NO is highly reactive toward both ferric and ferrous forms of hemoproteins, it is expected that NO reacts in vivo with both ferric and ferrous forms of hemoproteins, giving rise to the formation of their nitrosyl adducts. Thus, quantitative studies on the interaction between NO and natural iron porphyrins assume increased importance.

The photochemistry of the O2 adducts of hemoglobin and myoglobin has been extensively investigated by nanosecond and picosecond laser flash photolysis in order to elucidate the nature of the FeII-O2 bond as well as the role of proteins in photodissociation of O2 from the O2 adducts.14-19 The CO and NO adducts of both hemoproteins and synthetic iron(II) porphyrins have also been subjected to the laser photolysis studies.20-37 However, the photochemistry of the O2 adducts of hemoproteins and water-soluble iron(II) porphyrin is also described on the basis of laser photolysis studies.

Iron(III) Porphyrin and Ferrijemoproteins

Experimental Section

Water-soluble iron porphyrin, [Fe(III)(H2O)(TPPS)]2+, was synthesized from the reaction of (Na14H2TPPS) and iron(III) sulfate in water and purified by cation exchange columns according to the literature.15 Oxidized forms of hemoglobin (human), myoglobin (white skeletal muscle), and cytochrome c (horse heart) and (Na14H2TPPS) supplied by Sigma were used without further purification. Catalase from bovine liver with the activity 4000 units/mg was supplied by Wako Pure Chemical Ind. Ltd. Distilled water (pH 6.5) was used as a solvent. The aqueous solutions were not buffered in order to avoid the effects of ions on the photochemical reactions of the nitrosyl complexes.

Optical absorption spectra were recorded on a Hitachi 330 spectrophotometer. Laser photolysis studies were carried out with second (355 nm), third (393 nm), and fourth (420 nm) harmonics and a Nd:YAG laser (HY 500 from JK Lasers, Ltd.): the duration of a laser pulse is ca. 20 ns, and the energies of laser pulses are 200, 100, and 70 mJ per pulse at 352, 355, and 266 nm, respectively. The detection system of transient spectra has been reported elsewhere.16

All samples were prepared with the use of a vacuum line to avoid contamination by oxygen: aqueous solutions of hemoproteins were degassed on the vacuum line without freezing the solutions to avoid denaturing of the proteins, and nitric oxide gas was introduced into the solution after degassing. The concentrations of NO in solutions were calculated from the partial pressure of NO gas and a Bunsen absorption coefficient of NO (4.21 × 10⁻² at 1 atm and 293 K). The pressures of the nitric oxide gas were measured by a mercury manometer.

Results

Absorption Spectroscopic Studies. (1) Fe(III)TPPS and Fe(II)TPPS. The absorption spectrum of Fe(III)TPPS in an aqueous solution exhibits peaks at 393 and 528 nm with the molar absorption coefficients 1.55 × 10⁶ and 1.22 × 10⁶ M⁻¹ cm⁻¹, respectively. Figure 1 shows the absorption spectral changes observed for an aqueous solution of 1.0 × 10⁻³ M Fe(III)TPPS at various NO concentrations. The spectrum is reversible. With changing [NO], the spectra display isosbestic points, indicating that the equilibrium is simply expressed as

\[
\text{Fe(III)TPPS} + \text{NO} \rightleftharpoons \text{Fe(II)TPPS} \quad (\text{NO})\text{Fe(III)TPPS} \quad (1)
\]

The equilibrium constant, K, was determined from the spectral changes. With the use of the molar absorption coefficients (\(\epsilon_{\text{Fe}}\) and \(\epsilon_{\text{NO}}\)) and the concentrations ([Fe] and [NO]) of Fe(III)TPPS and

\[
(\Delta \epsilon) \text{NO} = (\epsilon_{\text{Fe}} - \epsilon_{\text{NO}}) [\text{NO}] = \frac{\text{Fe(III)TPPS} + \text{NO}}{\text{Fe(II)TPPS} (\text{NO})\text{Fe(III)TPPS}}
\]

The reaction proved to be irreversible, and the resulting spectrum
of (NO)Fe\textsuperscript{II}TPPS displayed peaks at 412 and 542 nm with molar absorption coefficients 1.55 \times 10^2 and 9.56 \times 10^4 M^{-1} cm^{-1}, respectively.

(2) Methemoglobin and Hemoglobin. The spectrum of methemoglobin, Hem\textsuperscript{III}, has an absorption peak at 403 nm, while that of hemoglobin, Hem\textsuperscript{II}, has peaks at 428 and 553 nm. When the aqueous solution of Hem\textsuperscript{III} was exposed to NO gas (250 Torr), new bands appeared at 417 nm in the Soret band region and 532 and 563 nm in the Q band region, owing to the formation of (NO)Hem\textsuperscript{III}. After a few minutes, the latter bands were red-shifted to 542 and 570 nm, while that at 417 nm decreased only slightly in intensity. The overall spectral change was interpreted as the reductive nitrosylation of Hem\textsuperscript{III}, since the spectrum of (NO)Hem\textsuperscript{II} produced by the reaction of Hem\textsuperscript{II} and NO also has absorption peaks at 417, 542, and 570 nm. Thus, the reaction of Hem\textsuperscript{III} and NO is apparently represented by

$$\text{Hem}^\text{III} + \text{NO} \rightleftharpoons (\text{NO})\text{Hem}^\text{III}$$

(Similar reductive nitrosylation has been reported for the reaction of Cl\textsuperscript{II}TPPS (TPP = tetraphenylporphyrin) in ethanol.\textsuperscript{40})

(3) Metmyoglobin and Myoglobin. The electronic spectrum of metmyoglobin, Mb\textsuperscript{III}, in an aqueous solution exhibits absorption maxima at 408 (\(\epsilon = 1.5 \times 10^8\) M\(^{-1}\) cm\(^{-1}\)) and 500 nm (\(\epsilon = 1.0 \times 10^9\) M\(^{-1}\) cm\(^{-1}\)). Figure 3 shows the reversible spectral changes observed for the aqueous solution of Mb\textsuperscript{III} at various concentrations of NO.

$$\text{Mb}^\text{III} + \text{NO} \rightleftharpoons (\text{NO})\text{Mb}^\text{III}$$

The absorption peaks of (NO)Mb\textsuperscript{III} are located at 418 (\(\epsilon = 2.0 \times 10^9\) M\(^{-1}\) cm\(^{-1}\)), 536, and 570 nm. The equilibrium constant, \(K = k_\text{f}(\text{Mb}^\text{III})/(\text{NO})\text{Mb}^\text{III} = k_\text{f}(\text{Mb}^\text{III})/(\text{NO})\text{Mb}^\text{III} = 1.41 \times 10^{10}\) M\(^{-1}\). The spectrum of myoglobin, Mb\textsuperscript{II}, in an aqueous solution has bands at 432 (\(\epsilon = 1.16 \times 10^9\) M\(^{-1}\) cm\(^{-1}\)) and 555 nm. The introduction of NO gas into the aqueous solution led to the irreversible formation of (NO)Mb\textsuperscript{III} with absorption maxima at 420 (\(\epsilon = 1.29 \times 10^9\) M\(^{-1}\) cm\(^{-1}\)), 545, and 578 nm.

(4) Oxidized and Reduced Cytochrome c. The absorption spectrum of oxidized cytochrome c, Cyt\textsuperscript{III}, in an aqueous solution displays peaks at 408 (\(\epsilon = 1.0 \times 10^7\) M\(^{-1}\) cm\(^{-1}\)) and 528 nm

Iron(III) Porphyrin and Ferrihemoproteins

Table I. Equilibrium Constants for Association of Axial NO*

<table>
<thead>
<tr>
<th>reaction</th>
<th>equilibrium constant (M⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fe³⁺ + NO = (NO)Fe³⁺TPPS</td>
<td>K(Fe³⁺) = 1.1 × 10³</td>
</tr>
<tr>
<td>Mb³⁺ + NO = (NO)Mb³⁺</td>
<td>K(Mb³⁺) = 1.4 × 10⁴</td>
</tr>
<tr>
<td>Cyt³⁺ + NO = (NO)Cyt³⁺</td>
<td>K(Cyt³⁺) = 1.6 × 10⁴</td>
</tr>
<tr>
<td>Cat³⁺ + NO = (NO)Cat³⁺</td>
<td>K(Cat³⁺) = 1.8 × 10⁴</td>
</tr>
<tr>
<td>Cyt³⁺ + NO = (NO)Cyt³⁺</td>
<td>K(Cyt³⁺) = 2.89 × 10⁵</td>
</tr>
</tbody>
</table>

*The equilibrium constants for Hem³⁺, Hem²⁺, Mb²⁺, and Cat²⁺ could not be obtained (see text). Experimental errors are ±10%.

Figure 5. Absorption spectral changes of 2.1 × 10⁻⁴ M Cat³⁺ in an aqueous solution in the presence of NO: 1, [NO] = 0 M; 2, [NO] = 1.77 × 10⁻⁵ M; 3, [NO] = 2.28 × 10⁻⁵ M; 4, [NO] = 7.57 × 10⁻⁴ M.

Figure 6. Transient absorption spectrum observed for an aqueous solution of (NO)Mb³⁺ in the presence of 1.15 × 10⁻³ M NO, 50 ns after 355-nm laser pulsing.

M⁻¹. The chemical reduction of Cat³⁺ by Na₂S₂O₄ was unsuccessful, so no studies could be done for Cat³⁺.

The equilibrium constants, K, obtained in the present study are listed in Table I.

Laser Flash Photolysis Studies. (1) (NO)Fe³⁺TPPS and (NO)-Fe³⁺TPPS. Figure 6 shows the transient absorption spectrum observed 50 ns after an aqueous solution of (NO)Fe³⁺TPPS at 1.15 × 10⁻³ M NO was subjected to a laser pulse at 355 nm. The transient is in good agreement with the difference spectrum obtained by subtracting the spectrum of (NO)Fe³⁺TPPS from that of Fe³⁺TPPS. The photoreaction, therefore, is expressed as

(NO)Fe³⁺TPPS + hv → Fe³⁺TPPS + NO (14)

The transient spectrum decays uniformly over the whole wavelength range studied according to first-order kinetics with a rate constant of 1.58 × 10⁻⁵ s⁻¹. No permanent photoprocess was detected. These results indicate that the transient Fe³⁺TPPS returns to (NO)Fe³⁺TPPS according to reaction 1. It, therefore, would be expected that the rate constant, k₁, for the decay of the transient Fe³⁺TPPS in the presence of excess NO is represented by

k₁ = k₄(Fe³⁺)[NO] + k₅(Fe³⁺) (15)

Figure 7 shows the straight line plot of the first-order decay rate constant, k₁, as a function of [NO]. From the slope and the intercept of the line, k₄ and k₅ are respectively determined as 7.2 × 10⁴ M⁻¹ s⁻¹ and 6.8 × 10⁵ s⁻¹. The equilibrium constant, K(Fe³⁺), was calculated from the rate constant ratio k₄(Fe³⁺)/k₅(Fe³⁺) to be 1.06 × 10³ M⁻¹, in agreement with that (1.1 × 10³ M⁻¹) obtained from the spectroscopic studies. Photodissociation of NO from (NO)Fe³⁺TPPS was also found to occur upon excitation at 532 nm.

As mentioned above, Fe³⁺TPPS irreversibly reacts with NO to yield (NO)Fe³⁺TPPS. The laser photolysis studies revealed that (NO)Fe³⁺TPPS photodissociated NO, resulting in the formation of Fe³⁺TPPS according to the difference spectra. The decay of Fe³⁺TPPS follows first-order kinetics in the presence of excess NO to regenerate (NO)Fe³⁺TPPS. The plot of the first-order rate constant for the decay of Fe³⁺TPPS vs [NO] is a straight line, and the slope and the intercept give k₄(Fe³⁺) = 1.8 × 10⁻⁵ M⁻¹ s⁻¹ and k₅(Fe³⁺) < 10⁻¹⁵ s⁻¹, respectively.

(2) (NO)Hem³⁺ and (NO)Hem²⁺. Because (NO)Hem³⁺ spontaneously reduces to (NO)Hem²⁺, no laser photolysis studies could be made for (NO)Hem³⁺. The laser photolysis of an aqueous solution of (NO)Hem²⁺ confirmed that only a small amount of Hem³⁺ is photochemically produced from (NO)Hem²⁺; the yield, Φ, was roughly estimated as <10⁻¹ upon excitation at both 355 and 532 nm. Because of the low yield of Hem³⁺, the rate constants for association between NO and Hem²⁺ and dissociation of NO from (NO)Hem²⁺ could not be obtained.

Femtosecond photolysis of (NO)Hem²⁺ has shown that the major product is a geminate pair in which NO is trapped at the heme pocket. The geminate pair disappears within a few hundred picoseconds by geminate recombination. Since the duration of the laser pulses used in the present study is ca. 20 ns, the transient such as geminate pairs with a lifetime shorter than 20 ns could not be detected. Therefore, the quantum yields reported here are concerned with longer lived species, i.e., those where NO is fully dissociated from the heme.

(3) (NO)Mb³⁺ and (NO)Mb²⁺. Figure 8 shows the transient absorption spectra observed for an aqueous solution of (NO)-Mb³⁺ plus 1.0 × 10⁻³ M NO for time delays of 50 ns and 4.5 μs after 355-nm laser pulse. There were modest differences between...
these two transient spectra, particularly in the short-wavelength region ($\lambda < 420$ nm). Since the spectrum detected at 4.5 $\mu$s after puling closely resembles the difference spectrum ($\text{Mb}^{III} - (\text{NO})\text{Mb}^{III}$), one is tempted to suggest that the photoreaction of $(\text{NO})\text{Mb}^{III}$ is represented by the following sequence:

$$\begin{align*}
(\text{NO})\text{Mb}^{III} + h\nu & \rightarrow \text{Mb}^{III} + \text{NO} \\
\text{Mb}^{III} & \rightarrow \text{Mb}^{III}\n
\end{align*}$$

where $\text{Mb}^{III}$, which is produced from $(\text{NO})\text{Mb}^{III}$ immediately after laser pulsing, is assumed to be a species slightly different from $\text{Mb}^{III}$, perhaps a different protein conformation. The time-dependent spectral change of the transient observed in the time interval 50 ns - 4.5 $\mu$s after pulsing would then be attributed to the conformation change which transforms $\text{Mb}^{III}$ to $\text{Mb}^{III}$. The further slow decay of $\text{Mb}^{III}$ to give $(\text{NO})\text{Mb}^{III}$ followed first-order kinetics with a rate constant of $k_2 = 2.1 \times 10^3$ s$^{-1}$ at 1.0 $\times 10^{-3}$ M NO. The rate constant $k_2$ increases with an increase in the NO concentration:

$$k_2 = k_3(\text{Mb}^{III})[\text{NO}] + k_4(\text{Mb}^{III})$$

From the plot of $k_2$ vs [NO] the value of $k_4(\text{Mb}^{III})$ is obtained as $k_4(\text{Mb}^{III}) = 1.9 \times 10^7$ M$^{-1}$. From this value and the equilibrium constant $K(\text{Mb}^{III})$ (Table I), $k_4(\text{Mb}^{III})$ was calculated to be 13.6 s$^{-1}$.

Laser photolysis of an aqueous solution of $(\text{NO})\text{Mb}^{III}$ resulted in the formation of $\text{Mb}^{III}$ with a low quantum yield, $\Phi < 0.1$. No determination of the rate constants for association and dissociation of NO could be made.

4. $(\text{NO})\text{Cyt}^{III}$ and $(\text{NO})\text{Cyt}^{III}$. Figure 9 shows the initial transient absorption spectrum observed for an aqueous solution of $(\text{NO})\text{Cyt}^{III}$ in the presence of $1.5 \times 10^{-3}$ M NO 50 ns after a 355-nm laser pulse. This disagrees markedly with the difference spectrum $(\text{Cyt}^{III} - (\text{NO})\text{Cyt}^{III})$, particularly in the short-wavelength region ($\lambda < 405$ nm). By contrast, the transient spectrum recorded 200 $\mu$s after the pulse is in good accord with the difference spectrum $(\text{Cyt}^{III} - (\text{NO})\text{Cyt}^{III})$. The absorbance changes between the two transient spectra follow first-order kinetics with a rate constant of $(5.5 \pm 0.3) \times 10^1$ s$^{-1}$. Since the rate constant is independent of [NO] in the range $2.1 \times 10^{-4} - 1.5 \times 10^{-3}$ M, the reaction with free NO cannot be responsible for the spectral change of the transient. Another explanation may be that the initial transient is $\text{Cyt}^{III}$, with a protein conformation different from that of uncomplexed Cyt$^{III}$. According to that model, the photochemistry of $(\text{NO})\text{Cyt}^{III}$ would be represented by the sequence of events

$$\begin{align*}
(\text{NO})\text{Cyt}^{III} + h\nu & \rightarrow \text{Cyt}^{III} + \text{NO} \\
\text{Cyt}^{III} & \rightarrow \text{Cyt}^{III}\n
\end{align*}$$

and the time-dependent spectral change of the initial transient would be ascribed to reaction 20. Since the transient spectrum is produced by the aqueous solution of $(\text{NO})\text{Cyt}^{III}$, Cyt$^{III}$ reacts further with NO to regenerate $(\text{NO})\text{Cyt}^{III}$. The decay of Cyt$^{III}$ followed first-order kinetics with the rate constant 1.12 s$^{-1}$ at 1.5 $\times 10^{-3}$ M NO.

According to reaction 10, the rate constant, $k_3$, for the decay of Cyt$^{III}$ is formulated as

$$k_3 = k_5(\text{Cyt}^{III})[\text{NO}] + k_6(\text{Cyt}^{III})$$

From the plot of $k_3$ vs [NO], $k_5(\text{Cyt}^{III})$ is obtained as $7.2 \times 10^7$ M$^{-1}$ s$^{-1}$. With the use of the $k_5(\text{Cyt}^{III})$ value and the equilibrium constant $K(\text{Cyt}^{III})$ (Table I), $k_5(\text{Cyt}^{III})$ was calculated to be $4.4 \times 10^{-2}$ s$^{-1}$.
**Table II.** Rate Constants for Association and Dissociation of Axial NO

<table>
<thead>
<tr>
<th>Reaction</th>
<th>Rate Constant $(k_d, M^{-1}\text{s}^{-1}; \epsilon_d, \text{s}^{-1})$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fe$^{III}$TPPS + NO → (NO)Fe$^{III}$TPPS</td>
<td>$k_d(\text{Fe}^{III}) = 7.2 \times 10^5$</td>
</tr>
<tr>
<td>Mb$^{III}$ + NO → (NO)Mb$^{III}$</td>
<td>$k_d(\text{Mb}^{III}) = 1.9 \times 10^5$</td>
</tr>
<tr>
<td>Cyt$^{III}$ + NO → (NO)Cyt$^{III}$</td>
<td>$k_d(\text{Cyt}^{III}) = 7.2 \times 10^2$</td>
</tr>
<tr>
<td>Cat$^{III}$ + NO → (NO)Cat$^{III}$</td>
<td>$k_d(\text{Cat}^{III}) = 3.0 \times 10^7$</td>
</tr>
<tr>
<td>Fe$^{II}$TPPS + NO → (NO)Fe$^{II}$TPPS</td>
<td>$k_d(\text{Fe}^{II}) = 1.8 \times 10^8$</td>
</tr>
<tr>
<td>Mb$^{II}$ + NO → (NO)Mb$^{II}$</td>
<td>$k_d(\text{Mb}^{II}) = 1.7 \times 10^7$</td>
</tr>
<tr>
<td>Cyt$^{II}$ + NO → (NO)Cyt$^{II}$</td>
<td>$k_d(\text{Cyt}^{II}) = 8.3$</td>
</tr>
<tr>
<td>(NO)Fe$^{III}$TPPS → Fe$^{II}$TPPS + NO</td>
<td>$k_d(\text{Fe}^{III}) = 6.8 \times 10^{2}$</td>
</tr>
<tr>
<td>(NO)Mb$^{III}$ + Mb$^{II}$</td>
<td>$k_d(\text{Mb}^{II}) = 13.6$</td>
</tr>
<tr>
<td>(NO)Cyt$^{III}$ + Cyt$^{II}$</td>
<td>$k_d(\text{Cyt}^{II}) = 1.1 \times 10^{2}$</td>
</tr>
<tr>
<td>(NO)Cat$^{III}$ + Cat$^{II}$</td>
<td>$k_d(\text{Cat}^{II}) = 1.7 \times 10^{2}$</td>
</tr>
<tr>
<td>(NO)Fe$^{II}$TPPS + Fe$^{II}$TPPS + NO</td>
<td>$k_d(\text{Fe}^{II}) = 0$</td>
</tr>
<tr>
<td>(NO)Mb$^{II}$ + Mb$^{II}$</td>
<td>$k_d(\text{Mb}^{II}) = 4.6 \times 10^{5}$</td>
</tr>
<tr>
<td>(NO)Cyt$^{II}$ + Cyt$^{II}$</td>
<td>$k_d(\text{Cyt}^{II}) = 2.8 \times 10^{5}$</td>
</tr>
</tbody>
</table>

* The rate constants for Hem$^{III}$, Hem$^{II}$, Mb$^{II}$, and Cat$^{II}$ could not be obtained (see text). Experimental errors are ±10%.  

**Quantum Yield Measurements for Photodissociation of NO.** Quantum yields for photodissociation of NO adducts were measured with the use of the laser photolysis method.  

When the photoreaction is expressed as

\[(\text{NO})\text{FeP} + h\nu \rightarrow \text{FeP} + \text{NO}\]  

the yield, $\Phi_{\text{dis}}$, is formulated as

$$\Phi_{\text{dis}} = \frac{\Delta \text{OD}}{\Delta \epsilon d Q_{\text{abs}}(D,\lambda)V^{-1}}$$  

where $\Delta \text{OD}$ is the initial absorbance change observed after laser pulsing, $\Delta \epsilon$ is the difference in the molar absorption coefficient between FeP and (NO)FeP, $D$ is the absorbance of (NO)FeP at the laser wavelength, $\lambda$ is the optical path length of observation, $V$ is the volume of the solution irradiated by the laser pulse, and $Q_{\text{abs}}(D,\lambda)$ is the number of quanta absorbed by (NO)FeP. The actinometric method of determining $Q_{\text{abs}}(D,\lambda)$ was to measure the absorbance at the laser excitation wavelength. With the use of the molar absorption coefficient, $\epsilon_\lambda$, of the triple Zn$^{II}$TPP at 470 nm and the triplet yield, $\Phi_{\text{ST}}$, $Q_{\text{abs}}(D,\lambda)$ is expressed as

$$Q_{\text{abs}}(D,\lambda) = \frac{\text{OD}_T}{(\epsilon_\lambda d V^{-1} \Phi_{\text{ST}})}$$  

where OD$_T$ is the initial absorbance of [Zn$^{II}$TPP]$^*$ at 470 nm

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References:

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**Discussion**

Physical and chemical properties of hemoproteins are markedly affected by the nature of proteins surrounding the heme. Therefore, the photochemical properties of the protein-free complexes, (NO)Fe$^{III}$TPPS and (NO)Fe$^{II}$TPPS, were investigated for the appropriate comparisons.

The absorption spectrum of Fe$^{III}$TPPS in an aqueous solution exhibits a peak maximum at 393 nm in the Soret band region. The peak is red-shifted from 393 to 420 nm by forming the nitric oxide adduct, (NO)Fe$^{II}$TPPS. This result has been interpreted.
in terms of a charge-transfer interaction from NO to the metal center such as (NO)FeIIITPPS.\textsuperscript{1,2} On the other hand, the absorption peak of FeIIITPPS at 425 nm is blue-shifted to 412 nm by forming (NO)FeIIITPPS. Analogous shifts of Soret bands were observed for the ferric and ferrous hemoproteins on exposure to NO. The Soret bands of ferric forms shift toward red, but those of the ferrous forms shift toward blue.

The equilibrium constants for association of NO are in the order CatIII (1.8 \times 10^4 \text{ M}^{-1}) > CytIII (1.6 \times 10^4 \text{ M}^{-1}) \sim MbIII (1.4 \times 10^4 \text{ M}^{-1}) > FeIIITPPS (1.1 \times 10^4 \text{ M}^{-1}).\textsuperscript{1} The fact that the equilibrium constants of CatIII, MbIII, and CytIII are an order of magnitude larger than that of FeIIITPPS suggests that the surrounding proteins regulate the rates for both association and dissociation processes of NO from the heme.

Photodissociation of NO from (NO)FeIIITPPS gives a transient spectrum identical with the difference spectrum (FeIIITPPS - (NO)FeIIITPPS), indicating the photochemical formation of FeIIITPPS. No change in the transient spectrum is observed during the course of the decay. Similar observations were made for (NO)FeIIITPPS and (NO)CatIII.

In contrast, laser photolysis of (NO)MbIII affords MbII\textsubscript{NO}, as an initial transient which relaxes to MbIII. The spectrum of MbII\textsubscript{NO} resembles but is different from that of MbII.\textsuperscript{47} According to the crystal structures of MbIII, the fifth and sixth axial positions of the heme in MbIII are occupied by imidazole moieties of both distal and proximal histidine residues. The central iron(II) atom in MbII is considered to interact with the remote imidazole of the distal histidine more weakly than with the close one. When the imidazole moiety of the histidine residue is replaced by NO, there would be a resulting change in the protein conformation of MbII to minimize the strain. The initial product, MbII\textsubscript{NO}, from the photolysis of (NO)MbIII may be assumed to have a protein conformation similar to that of (NO)MbIII. The resulting strained MbII\textsubscript{NO} undergoes relaxation within a few microseconds to give MbIII. The Soret absorption band of MbII\textsubscript{NO} is slightly blue-shifted in comparison with that of MbIII. The similar conformation change in the protein of MbIII has been suggested on the basis of the laser photolysis studies of the dioxygen adduct of MbIII.\textsuperscript{48}

According to the X-ray structure, the fifth and sixth axial positions of the central iron(III) atom in CytIII are occupied by a histidine imidazole moiety and a methionine sulfur, respectively.\textsuperscript{49} When NO reacts with CytIII, it replaces an axial ligand, presumably the methionine sulfur, which is a weaker ligand than imidazole. Thus, the conformation of the protein in (NO)CytIII should differ from that in CytIII. The initial transient spectrum observed for (NO)CytIII 50 ns after laser pulsing was different from the (CytIII - (NO)CytIII) difference spectrum, particularly in the short-wavelength region (\lambda < 405 nm). This observation can be interpreted in terms of the protein conformations: laser photolysis of (NO)CytIII would yield, as the initial product, CytIII\textsubscript{NO}, with a protein structure close to that of (NO)CytIII. Recoordination of the axial methionine sulfur may require changes in the protein conformation, and the relatively slower relaxation of the spectrum of CytIII\textsubscript{NO} to that of CytIII may reflect this process. Transformation of CytIII\textsubscript{NO} to CytIII takes place with the first-order rate constant 5.5 \times 10^3 \text{ s}^{-1}.

Since the sixth axial ligand of CytIII, methionine S, is tightly bound to the central iron atom, the protein conformational changes incurred by CytIII upon nitrosylation may be much larger than those by MbIII.\textsuperscript{50} If, as mentioned above, the protein conformations of CytIII\textsubscript{NO} and MbII\textsubscript{NO} are close to those of their respective nitrosylation analogs, the conformational differences between CytIII\textsubscript{NO} and CytIII are likely to be larger than those between MbII\textsubscript{NO} and MbIII. This proposal is supported by the finding that the rate for CytIII\textsubscript{NO} \rightarrow CytIII transformation is slower than that for MbII\textsubscript{NO} \rightarrow MbIII. Furthermore, the spectral differences between MbII\textsubscript{NO} and MbIII are much less than those between CytIII\textsubscript{NO} and CytIII. However, the latter observation must be quantified by noting that the studies here are limited by the time window for detection (ca. 50 ns). Thus, spectral changes may be influenced by differing rates and efficiencies of geminate pair recombination for the various systems, processes which require picosecond or femtosecond detection methods to be explored in detail.\textsuperscript{1,4,8,51}

The rate constants for the relaxations of CytIII\textsubscript{NO} \rightarrow CytIII and MbII\textsubscript{NO} \rightarrow MbIII do not depend on [NO] below ca. 1 \times 10^{-3} M. This indicates that the rate for the reaction between CytIII\textsubscript{NO} and free NO is slower than that for the relaxation of CytIII\textsubscript{NO} to CytIII.

The transient spectrum observed for (NO)CatIII after laser pulsing is almost identical with the difference spectrum (CatIII - (NO)CatIII). This observation suggests that differences in the protein structure between (NO)CatIII and CatIII are very small. Since CatIII is an enzyme which catalyzes the thermal decomposition of H_{2}O_{2} into O_{2} and H_{2}O, the sixth axial position, which is free from a ligand, is expected to be exposed to the water phase in order to allow the access of H_{2}O_{2}. Nitrosylation of CatIII, therefore, should readily occur at the sixth axial position without significant protein structure changes.

The rate constants, \textit{k}_{t} for the nitrosylation follow the order CatIII (3.0 \times 10^{-7} \text{ M}^{-1} \text{ s}^{-1}) > FeIIITPPS (7.2 \times 10^{-7} \text{ M}^{-1} \text{ s}^{-1}) > MbIII (1.9 \times 10^{-7} \text{ M}^{-1} \text{ s}^{-1}) \gg CytIII (7.2 \times 10^{-5} \text{ s}^{-1}). CytIII gives the smallest rate constant for nitrosylation, probably due to the occupation of the sixth axial position by a sulfur atom in the methionine moiety. It is noteworthy that \textit{k}_{t}(CatIII) is ca. 40 times larger than \textit{k}_{t}(FeIII). In contrast to the cases of MbIII and CytIII, where the protein retards nitrosylation rates, the rate for nitrosylation of CatIII is strongly accelerated by the protein in CatIII.

The rate constants for denitrosylations follow the order (NO)FeIIITPPS (6.8 \times 10^{-3} \text{ s}^{-1}) > (NO)CatIII (1.7 \times 10^{-2} \text{ s}^{-1}) > (NO)MbIII (1.36 \times 10^{-2} \text{ s}^{-1}) > (NO)CytIII (4.4 \times 10^{-2} \text{ s}^{-1}). In each case, the ferric hemoproteins release NO with a rate constant smaller than that of (NO)FeIIITPPS, suggesting that the proteins retard the denitrosylation process.

The laser photolysis studies demonstrate that (NO)FeIIITPPS efficiently photodissociates to give FeIIITPPS. The reaction constant for nitrosylation, \textit{k}_{t}(FeIII), is determined to be 1.8 \times 10^{-6} \text{ M}^{-1} \text{ s}^{-1}, reasonably close to the rate constant, 5.2 \times 10^{6} \text{ M}^{-1} \text{ s}^{-1}, for bimolecular reaction between NO and FeIIITPPS in benzene.\textsuperscript{5} The value of \textit{k}_{t}(FeIII) was found to be 2.5 \times 10^{3} times larger than \textit{k}_{t}(FeII), and the equilibrium constants must be much larger for NO adduct formation. These results indicate that the binding energy between NO and FeII in FeIIITPPS must be stronger than that between NO and FeIII in FeIIITPPS. Similarly, the equilibrium constant for formation of (NO)CytIII is larger than that for formation of the (NO)CytIII analog (Table I).

Yields for photodissociation of NO from (NO)HemIII, (NO)MbIII, and (NO)CytIII are so small (\textit{< 10^{-3}}) that the present nanosecond laser photolysis method could not be used for the measurements of the rate constants for the association of NO with HemIII, MbIII, and CytIII. However, the rate constants, \textit{k}_{t} (CytIII) and \textit{k}_{d} (CytIII), could be obtained with the measurement of the time-dependent absorption spectral changes of CytIII in the presence of NO and of (NO)CytIII in the absence of NO, respectively. As shown in Table II, it is found that \textit{k}_{t}(CytIII) < \textit{k}_{t}(CytIII). This result is very contrary to the cases of FeIIITPPS and MbIII, \textit{k}_{t}(FeIII) > \textit{k}_{d}(FeIII) and \textit{k}_{d}(MbIII) > \textit{k}_{t}(MbIII), and suggests that the sixth ligand in CytIII is more strongly bound to the central iron atom than that in CytIII.
The quantum yield, $\Phi_{\text{dis}}$, for the photodissociation of NO from \((\text{NO})\text{Fe}^{III}\text{TPPS}\) is obtained as 0.37. As listed in Table III, this value is an order of magnitude larger than those of \((\text{NO})\text{Mb}^{III}\), \((\text{NO})\text{Cyt}^{III}\), and \((\text{NO})\text{Cat}^{III}\), indicating that the effects of the proteins surrounding the heme reduce the quantum yield. The decrease in $\Phi_{\text{dis}}$ caused by surrounding proteins is also observed for \((\text{NO})\text{Mb}^{II}\) and \((\text{NO})\text{Cyt}^{II}\): the yields for photodissociation of NO from \((\text{NO})\text{Mb}^{II}\) and \((\text{NO})\text{Cyt}^{II}\) are 2 orders of magnitude less than that from \((\text{NO})\text{Fe}^{III}\text{TPPS}\).

Recent studies\(^{18,30}\) of nitric oxide adducts of hemoglobin by femtosecond and picosecond laser photolysis have shown that (1) NO is photodissociated from \((\text{NO})\text{Hem}^{II}\) within less than 50 fs in the heme pocket and (2) ca. 99% of the NO produced undergoes geminate recombination with Hem\(^{II}\) to regenerate \((\text{NO})\text{Hem}^{II}\) in the heme pocket within a few hundred picoseconds. Less than 1% NO escapes the heme pocket. Such results indicate that the geminate pair of Hem\(^{II}\) and NO would not be detected by the nanosecond laser photolysis system. The present nanosecond laser photolysis study confirmed that the yields for NO photodissociation from the nitric oxide adducts of both oxidized and reduced hemoproteins are very small in comparison with the yields obtained with \((\text{NO})\text{Fe}^{III}\text{TPPS}\) and \((\text{NO})\text{Fe}^{III}\text{TPPS}\). It is likely that most of the NO photodissociated from the nitric oxide adducts of ferric and ferrous hemoproteins is trapped in the heme pocket and undergoes geminate recombination, leading to small quantum yields for full photodissociation of NO.

Studies of the pH effects on nitrosylferrihemoproteins in buffered aqueous solutions are in progress.

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