Electronic and Stereochemical Characterizations of the Photoinduced Intermediates of Nitrosyl Complexes of Metal \( (S = 5/2) \)-substituted Hemoproteins Trapped at Low Temperature*

Hiroshi Hori†
From the Department of Biophysical Engineering, Faculty of Engineering Science, Osaka University, Osaka 560, Japan

Masao Ikeda-Saito
From the Department of Physiology and Biophysics, Case Western Reserve University School of Medicine, Cleveland, Ohio 44106

George Lang
From the Department of Physics, College of Science, The Pennsylvania State University, University Park, Pennsylvania 16802

Takashi Yonetani
From the Department of Biochemistry and Biophysics, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104-6098

Low temperature photolysis of nitric oxide from the nitrosyl complexes of ferric myoglobin \( \text{NO-Fe(III)Mb} \) and manganesc(II)-porphyrin-substituted myoglobin \( \text{NO-Mn(II)Mb} \) was examined by electron paramagnetic resonance (EPR) spectroscopy in order to elucidate the electronic and structural natures of the photoinduced intermediates of these hemoprotein-ligand complexes trapped at low temperature. The photoproduct of \( \text{NO-Fe(III)Mb} \) at 5 K exhibited entirely new X-band EPR absorptions in the magnetic field strength from 0 to 0.4 tesla. The widespread absorption together with distinct, sharp zero-field absorption was consistently observed in the photoproduct of the isoelectronic \( \text{NO-Mn(II)Mb} \). These novel ERP signals indicate a spin-coupled pair with an effective spin of \( S = 5/2 \) and the photodissociated NO \( (S = 1/2) \) trapped adjacent to the metal center. On the other hand, the photolyzed form of nitrosyl complexes of Fe(III)- and Mn(II)-Glycerina hemoglobins, in which the distal histidine of Mb is replaced by a leucyl residue, exhibited somewhat broader EPR absorptions similar to those of the corresponding native Fe(III)- or unliganded Mn(II)-Glycerina hemoglobins, respectively, indicating that the photodissociated NO molecule moved farther away from the metal center in the heme pocket. These observations show the importance of the interaction of the distal residue with the ligand in determining the nature of the photolyzed states.

The photoinduced dissociations of ligands such as oxygen \( (O_2) \), carbon monoxide \( (CO) \), and nitric oxide \( (NO) \) from Mb,

* This investigation has been supported in part by Research grants GM-39492 and GM-39392 (to M. I.-S.) and HL 14508 (to T. Y.) from the National Institutes of Health and by Grant DMB 87-16976 (to G. L.) from the National Science Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom correspondence should be addressed.

‡ The abbreviations used are: Mb, myoglobin; Hb, hemoglobin; EPR, electron paramagnetic resonance; M, metal; T, tesla.

Hb, and other hemoproteins are well-known. Flash photolysis techniques have been widely used in kinetic studies of interactions of hemoproteins with these ligands. Upon the advent of considerable technological advances in recent years, two distinctly different approaches have emerged in this field. The first approach is to measure transient kinetics of photodissociation and recombination processes on the faster and/or slower time scales, using advanced laser and transient spectroscopic techniques (1–4). These kinetic techniques have successfully demonstrated the involvement of multiple steps in photoinduced dissociation and recombination processes even in simple hemoproteins like Mb. Several interesting concepts such as molecular tunneling and geminate processes have been introduced to interpret the observed multiphasic kinetics (5, 6). The second approach in photolysis studies is to trap the photolyzed intermediates at low temperature for spectroscopic characterization (7–10). Electron paramagnetic resonance (EPR) spectroscopy is one of the extremely powerful analytical methods used to probe the electronic structure of paramagnetic metal centers, their immediate environments and their interaction with ligands. Unfortunately, ferrous O2- or CO-hemoprotein derivatives are EPR-silent and their deoxy derivatives have been generally considered to be EPR nonobservable. However, it becomes amenable to EPR examination by either the use of paramagnetic ligands such as NO or the substitution of the EPR-silent ferrous hemes with EPR-visible metalloporphyrins such as Co(II)-, Mn(II)-, and Fe(III)-porphyrins. This is why not only natural Fe(II)-containing hemoproteins but also artificial metal-substituted hemoproteins have been extensively used in this report.

Previously, we reported that the nitrosyl complex of cobalt-substituted myoglobin \( \text{NO-Co(II)Mb} \) is readily photolyzed upon illumination at 4.2 K and the recombination process of the photoproduction was retarded below 20 K (11). The visible absorption spectrum of the photoproduction was found to be indistinguishable from the corresponding deoxy spectrum. However, the photoproduction of \( \text{NO-Co(II)Mb} \) exhibited new EPR signals at \( g = 4.3 \) and a very broad signal around \( g = 2 \), indicating a spin-coupled system between the spins of the cobaltous ion and the dissociated NO molecule. An analogous experiment with nitrosyl complex of Co(II)-Glycerina Hb indi-
cated that its photoproduct had a broad EPR signal around \( g = 2.33 \). The observed line-broadening was interpreted to be due to a magnetic dipole-dipole interaction between the deoxy-Co(II) ion and the dissociated NO. The average distance between the Co(II) ion and the dissociated NO was estimated to be approximately 4 Å (11). Therefore, the unusual EPR signals appeared in the photoproduct of NO-Co(II)Mb was considered that the photodissociated NO ligand might be present very close to the paramagnetic metal center less than 4 Å by the effect of steric hindrance of the distal histidyl residue. Previously, we also reported the photolysis of nitrosyl complex of ferric myoglobin by light absorption and EPR studies at cryogenic temperatures (12). The photoproduct of NO-Fe(III)Mb exhibited a broad EPR absorption at \( g = 5.8 \) together with a broad absorption in the range \( g = 6-2 \). This EPR spectrum did not resemble the corresponding native Fe(III)Mb spectrum. We concluded, then, that the heme of the photoproduct of NO-Fe(III)Mb is present in the pentacoordinated form. However, the broad EPR absorption in the range \( g = 6-2 \) appearing in the photoproduct could not be explained by the pentacoordinated ferric heme.

In this paper, we have prepared nitrosyl complexes of natural Fe(III)-containing hemoproteins such as Mb and Glycera Hb as well as their isoelectronic Mn(II)-porphyrin-substituted apohemoprotein complexes and examine the photoinduced processes of dissociation from and recombination to these hemoproteins of NO by low temperature EPR methods in recombination with EPR, light absorption, and Mössbauer spectroscopic techniques in order to determine the electronic and stereochemical nature of the photolyzed intermediates of these hemoprotein-ligand complexes trapped at low temperature.

**EXPERIMENTAL PROCEDURES**

A lyophilized preparation of myoglobin from sperm whale muscle was obtained from Calbiochem and used without further purification. Whole blood of Glycera dibranchiata was a gift from Dr. E. A. Padlan of the National Institutes of Health, Bethesda, MD, and was purified by a method as described elsewhere (13). Apoproteins of these ferric hemoproteins were prepared by a modification of Teale’s acid butanone technique (14) as described in detail elsewhere (15). Iron into protoporphyrin IX and the 57Fe-enriched Mb were prepared as described previously (16). Preparations of Mn(II)-porphyrin and its substituted sperm whale Mb and Glycera Hb complexes with nitric oxide upon illumination with a weak white light for 10 min at liquid helium temperature. The absorption spectra of the nitrosyl Fe(III) and Mn(II) complexes were strikingly similar. Upon illumination, the NO-Mn(II)Mb spectrum changed completely to that of the corresponding deoxy or unliganded form. The other photoproduct spectra were intermediate between the nitrosyl form and the corresponding unliganded state, suggesting incomplete (50%) photolysis. We therefore compared difference spectra, photolyzed minus liganded NO forms, and unliganded minus liganded NO forms. These were very similar to each other, with the exception of NO-Fe(III)Mb in which the photoproduct exhibited a broad absorption band from 500 to 680 nm, somewhat different from that of native Fe(III)Mb as reported previously (12). The reversion of these photoproducts to the original ligand (NO) forms was retarded below 20 K.

EPR Study—In the absence of NO natural Fe(III) and artificial Mn(II)Mbs are paramagnetic (\( S = 5/2 \)) and EPR-visible as shown in Fig. 2, A and B (upper solid lines). Their nitrosyl complexes are EPR-silent due to spin pairing as shown in Fig. 2, A and B (lower broken lines). Upon photolysis at 5 K for 5–20 min, all of these nitrosyl complexes exhibit entirely new broad EPR absorptions at the X-band microwave frequency in the magnetic field from 0 to 0.4 T. The distinct.

**RESULTS**

**Optical Study**—Fig. 1 illustrates the changes in the visible absorption spectra of natural and artificial manganese-substituted sperm whale Mb and Glycera Hb complexes with nitric oxide upon illumination with a weak white light for 10 min at liquid helium temperature. The absorption spectra of the nitrosyl Fe(III) and Mn(II) complexes were strikingly similar. Upon illumination, the NO-Mn(II)Mb spectrum changed completely to that of the corresponding deoxy or unliganded form. The other photoproduct spectra were intermediate between the nitrosyl form and the corresponding unliganded state, suggesting incomplete (50%) photolysis. We therefore compared difference spectra, photolyzed minus liganded NO forms, and unliganded minus liganded NO forms. These were very similar to each other, with the exception of NO-Fe(III)Mb in which the photoproduct exhibited a broad absorption band from 500 to 680 nm, somewhat different from that of native Fe(III)Mb as reported previously (12). The reversion of these photoproducts to the original ligand (NO) forms was retarded below 20 K.

**EPR Study**—In the absence of NO natural Fe(III) and artificial Mn(II)Mbs are paramagnetic (\( S = 5/2 \)) and EPR-visible as shown in Fig. 2, A and B (upper solid lines). Their nitrosyl complexes are EPR-silent due to spin pairing as shown in Fig. 2, A and B (lower broken lines). Upon photolysis at 5 K for 5–20 min, all of these nitrosyl complexes exhibit entirely new broad EPR absorptions at the X-band microwave frequency in the magnetic field from 0 to 0.4 T. The distinct.
sharp zero-field absorption is consistently observed in the photoproducts of these nitrosyl complexes as illustrated in Fig. 2, A and B (lower solid lines). Some structures can be observed in the zero-field EPR signal for the photoproduction of NO-Mn(II)Mb at 5 K as shown in Fig. 2B. In order to test whether this structure is a hyperfine structure due to the coupling of the unpaired electron to the $^{55}$Mn nucleus with $I = 5/2$ or not, powder EPR spectra at S- and Q-band microwave frequencies were measured. Although we expected better spectral resolution of this hyperfine structure at S-band operation, no hyperfine structure could be observed in the zero-field region but broad EPR absorption in the magnetic field from 0 to 1 T was observed. In the Q-band EPR spectrum, on the other hand, broad and more complex absorptions could be observed (spectra not shown).

The signal intensity of the broad absorption at $g = 5.8$ in the photoreaction of NO-Fe(III)Mb, which was reported previously (12), is very weak compared with the other broad absorption. Thus, this $g = 5.8$ species might be a minor component in the photolyzed species at 5 K. When the illumination is performed at higher temperature slightly above 5 K, however, this $g = 5.8$ signal dominates over the other broad absorption as shown in Fig. 3A. The apparent signal intensity increases with increasing illumination time, suggesting that the EPR spectrum of the photoproduct of NO-Fe(III)Mb is derived from at least two magnetically different species. However, in the photoproduct of NO-Mn(II)Mb, this type of minor EPR signal at approximately $g = 5.8$ was not observed at 5 K. However, upon illumination above 5 K a weak and broad EPR signal with poorly resolved $^{55}$Mn hyperfine structure appears around $g = 6$ as illustrated in Fig. 3B.

The EPR spectra of the native Fe(III)-Glyceru Hb, in which the distal histidine residue of myoglobin is replaced by leucyl residue (22), and its photolyzed and nonphotolyzed products of nitrosyl complexes are illustrated in Fig. 4A. Similar, but somewhat broader EPR absorption to that of the corresponding ferric form could be observed (Fig. 4A, lower solid line). No zero-field resonance signal could be detected in the photoproduct of NO-Fe(III)-Glyceru Hb. Broader EPR absorption with poorly resolved $^{55}$Mn hyperfine structure is observed at $g \sim 6$ at 5 K in the photoproduct of NO-Mn(II)-Glyceru Hb as shown in Fig. 4B. When the temperature of this sample was raised to ~15 K or alternately the illumination was performed at ~15 K, similar but weak EPR absorption to that of the corresponding deoxy-Mn(II)-Glyceru Hb was observed (spectrum not shown). By elevating the temperature over 20 K the EPR signal disappears.

EPR measurement was also performed with the photoproducts of nitrosyl complex of ferrous myoglobin (NO-Fe(II)Mb). Upon illumination at 5 K the signal intensity corresponding to NO-Fe(II)Mb decreased with concomitant increase of broad absorption with the 0.2-T line width centered at $g = 2$ as shown in Fig. 5. No EPR absorption could be observed in the zero-field region at several microwave power ranges from 10 $\mu$W to 100 mW at 5 K.

Mössbauer Study—The Mössbauer spectrum of NO-Fe(III)Mb exhibited a quadrupole pair having an isomer shift of 0.08 mm/s relative to metallic iron and a quadrupole splitting of 1.62 mm/s at 77 K. The same spectrum was observed at 4.2 K. The isomer shift of 0.08 mm/s is much less than the typical 0.3-0.4 mm/s for ferric low spin hemoproteins (23, 24) and/or the typical of 0.2-0.3 mm/s for carbon monoxide complex of ferrous hemoproteins (10). The line positions are very near to those of compound ES of cytochrome c peroxidase (H$_2$O$_2$-oxidized cytochrome c peroxidase) (25) and of the peroxide compounds of horseradish peroxidase (26).
DISCUSSION

Electronic State of NO Fe(III)Mb - When NO reacts with ferric hemoprotein to form a diamagnetic compound, iron might either donate one electron to NO or accept one electron from the liganded NO. In the former case the iron would be oxidized to the ferryl state Fe(IV) and NO reduced to the positive nitrosyl ion. In the latter case the iron would be reduced to the ferrous state, Fe(II)-NO+, rather than in a ferrous state, Fe(II)-NO-. Even though the electronic configuration of nitrosyl-Fe(III)Mb is isoelectronic to that of CO-Fe(II)Mb, one would expect its Mössbauer parameters to be similar to those of CO-Fe(II)Mb with an isomer shift of 0.273 mm/s and a quadrupole splitting of 0.368 mm/s. The central doublet indicates the residual nitrosyl form due to incomplete photolysis.

Upon photolysis for 2 min by illuminating a slide projector through the Dewar window while the sample was immersed in liquid helium, the intensity of the central doublet was reduced, presumably with the buildup of a widely spread magnetic spectrum. Its presence was verified by the difference magnetic field as shown in Fig. 6.

Electronic States of the Photolysed Intermediates - The visible absorption spectra of the photoproducts of the nitrosyl complexes of Fe(III)- and Mn(II)-Mbs were found to be apparently identical to the corresponding native or deoxy states. In addition, a widely spread Mössbauer spectrum appearing in the photoproduct of NO-Fe(III)Mb by application of a small magnetic field was interpreted as being derived obviously from an iron complex having a half-integer spin, probably a ferric high spin Fe(III)Mb analogue. However, these photoproducts exhibited entirely new EPR signals which do not resemble those of the corresponding native or Mössbauer absorption spectra. The photolysed intermediates of hemoprotein-ligand complexes are electronically not identical to the corresponding unliganded states. Since Mössbauer spectroscopy provides evidence of an interaction between the nucleus of iron and an internal magnetic field, it appears that the photolyzed intermediate has a half-integer spin, thus, $S = 5/2$. While visible absorption spectra of metalloporphyrins are dominated by transitions of the porphyrin π system and are sensitive to the coordinated states, the photoproducts may have the pentacoordinated structures in high spin states ($S = 5/2$) as pointed out previously (12). On the other hand, EPR spectra reflect the magnetic interaction between unpaired electrons of the central paramagnetic metal ion ($S = 5/2$) and of the photodissociated NO ($S = 1/2$). If the dissociated NO molecule is trapped far away from the paramagnetic metal center in the heme pocket and the magnetic interaction between these paramagnetic centers is weakened and definitely undetectable, the EPR spectrum of the photoproduct should be expected to be similar or somewhat broader to that of the corresponding unliganded state. Indeed, upon illumination at higher temperature slightly above 5 K, the EPR spectra of the photoproducts exhibit broad absorption and also exhibit a poorly resolved hyperfine structure for the Mn*+ complex around $g \sim 6$ indicating magnetic dipole-dipole interaction between metal ion ($S = 5/2$) and dissociated NO ($S = 1/2$). However, distinct zero-field absorption and broad absorption (~400 mT) observed in the photoproducts at 5 K cannot be explained by magnetic dipole-dipole interaction. In recent years, a number...
of novel EPR signals from iron with integer spin have been reported in literature. Integer-spin resonances have been observed from deoxy-Fe(II)/Mb (33), the photodissociated carbonmonoxy- and oxy-Mbs (33), the two-iron sites of deoxy hemerythrin-azide complex (34), and the iron(Fe$_{a3}$)-copper (Cu$_b$) sites of cytochrome c oxidase from beef heart (35, 36). All these EPR spectra exhibit broad zero-field absorptions which are interpreted as being derived from the transitions between non-Kramer's doublet of "S = 2" spin states (37). Of these, it is of interest that the oxidized cytochrome c oxidase contains a spin-coupled pair of high spin heme $a_1$ ($S_{a1} = 5/2$) and Cu$_{a1}^+$ ($S_{cu} = 1/2$) with an effective spin of "S = 2." The spin-coupling implies that the two paramagnetic centers are close together. If the photolysed intermediate of NO-Fe(III)/Mb contains pure high spin Fe(II)- or Fe(IV)-heme ($S = 2$), its visible absorption spectrum should be similar to that of the corresponding deoxy- or ferryl-Mb. This is inconsistent with our present result. We propose that the novel EPR signals observed in the photoproduce at 5 K are due to a spin-coupled pair of ferric high-spin heme ($S = 5/2$) and photodissociated NO ($S = 1/2$) on the analogy of the oxidized cytochrome c oxidase. Although a quantitative interpretation of the novel EPR signals is still lacking this proposal can be supported by the results that a similar EPR pattern is observed in the photoproduce of NO-Mn(II)/Mb having an isoelectronic state with NO-Fe(III)/Mb. In contrast, the zero-field EPR absorptions have not been observed in the photoproduits of nitrosoyl complexes of Fe(II)- and Co(II)-Mbs, where the metal centers in the photoproduits have spin states of $S = 2$ and 1/2, respectively. Thus, a spin-coupled pair of $S = 5/2$ (metal) and $S = 1/2$ (NO) might be essential for the observation of the zero-field signal.

Interaction of the Distal Residue with Photodissociated NO Molecule—The photoproduits of nitrosoyl complexes of Fe(III)- and Mn(II)-Glycera Hb exhibited similar but somewhat broader EPR absorptions to those of the corresponding native Fe(III)- or unliganded Mn(II)-Glycera Hb. Neither zero-field absorption nor broad absorption could be detected in these photodissociated intermediates. The observed line broadening in these photoproduits implies a magnetic dipole-dipole interaction between the metal ion ($S = 5/2$) and the dissociated NO ($S = 1/2$) rather than a spin-coupled pair system. Previously, a similar magnetic dipole-dipole interaction was found in the photoproduce of NO-Co(II)-Glycera Hb, where the average distance between the deoxy-Co(II) ion and the dissociated NO was estimated to be $\sim 4 \, \text{Å}$ (31). Therefore, it may be concluded that the dissociated NO molecule is trapped in the heme pocket about 4 Å away from the metal center in the photoproduits of nitrosoyl complexes of Fe(III)- and Mn(II)-Glycera Hbs. From the recent X-ray crystallographic study of Glycera Hb, the substitution of leucine for the distal histidine residue creates an unusually hydrophobic ligand binding pocket in this hemoprotein (38). The lack of the steric effect of distal residue may cause the dissociated NO molecule to move farther away from the metal center. In contrast, the distal histidine residue (E7) in myoglobin may sterically hinder the dissociated NO molecule to move farther away from the metal center. The existence of the steric interaction between the distal residue and NO molecule in NO-Fe(III)/Mb was previously confirmed by resonance Raman study (19). Thus, by this steric hindrance of the distal histidine residue, the photodissociated NO molecule and the central metal ion are kept close together, thereby forming a weakly spin-coupled system at the initial process in photolysis. Analysis of the extended X-ray absorption fine structure data on the photoproduce of CO-Fe(II)/Mb at low temperature also indicated that the photodissociated CO molecule was still present near the active site (8). Upon increasing the temperature of photolysis, the dissociated NO molecule may move farther away from the metal center by thermal perturbation to cause the magnetic dipole-dipole interaction. At this higher temperature photolysis, however, the recombination process might be also accelerated to counter this dissociation process. This can be confirmed from the decrease in the total EPR signal intensity due to a formation of the initial strong spin-coupled diamagnetic species. The existence of the zero-field absorption and/or the broad $g \sim 6$ signal in the photoproduits of the nitrosoyl complexes can be an important "probe" of the steric interaction of the distal residue with the ligand in determining the nature of the photolysed states, as well as calculating molecular dynamics.

Relevance of EPR Results to Ligand Binding Properties—It may be useful to consider the functional relevance of the room temperature ligand binding results to our present low temperature EPR results. The oxygen affinity of Glycera Hb is 10-fold lower than that of sperm whale Mb, and this decrease is predominantly due to an extremely fast dissociation rate. Glycera Hb also exhibits an anomalously larger association rate constant for CO when compared to sperm whale Mb (39). Rohlfs et al. (40) have shown that the salient features of the ligand binding properties of Glycera Hb are essentially reproducible in the mutant sperm whale Mb with a leucine residue at the E7 position. They suggested that the equilibrium constant for oxygen binding involves primarily hydrogen-bonding interaction through the distal E7 residue which stabilizes the polar iron-oxygen complex, whereas that for CO binding is influenced by both steric hindrance and polarity effects. X-ray structural study for Glycera Hb has suggested that the substitution of leucine for the distal histidine residue in Glycera Hb creates an unusually hydrophobic ligand-binding pocket (38). The hydrophobicity of the ligand-binding pocket probably is an important factor in the increased affinity of Glycera Hb for CO, which is relatively apolar, and the reduced affinity for oxygen, which is a more polar ligand. Furthermore, in the case of oxygen, a bent Fe-O-O geometry is allowed, whereas for CO, a linear Fe-C-O conformation perpendicular to the heme plane is preferred. The close proximity of the distal histidine required for hydrogen-bonding favors a bent geometry for the bound ligand over a linear orientation. Thus, the distal histidine is thought to reduce the affinity of sperm whale Mb for CO. Our present EPR results suggest that the distal pocket in sperm whale Mb is more restricted than in Glycera Hb, even in the frozen state. If the electronic state of NO-Fe(III)/Mb and/or NO-Mn(II)/Mb with a linear M-N-O linkage is assumed to be isoelectric to that of CO-Fe(II)/Mb, a similar ligand binding kinetic process for CO-Fe(II)/Mb might be expected for those of the nitric oxide complexes. Indeed, the NO binding rate in the $\alpha _{Fe}^{III}$ chains of Opossum Hb, which also lacks the distal histidine residue, is much faster than sperm whale Mb (41).

The EPR results for the complete reversibility in the recombination process of the photoproduits upon increasing temperature strongly indicate that the photodissociated NO ligand is still trapped in the distal pocket of hemoproteins in different intermediate states and does not step out of the protein matrix at low temperature. The different photolyzed intermediates observed in the EPR spectra trapped at low temperature might be a reflection of the multiphasic kinetics of photodissociation and recombination processes observed at higher temperature. The zero-field signal and/or broad absorption may be an indication of a nongeminate dissociated state, whereas the broad $g \sim 6$ signal may indicate the gemi-
nate dissociated state. EPR measurements at lower temperatures (≤4.2 K) and quantitative analyses will be required in order to facilitate the further theoretical interpretation of the photoproducts.

Acknowledgments—We thank Professors T. Inubushi and H. Morimoto for helpful discussions.

REFERENCES