Photoreduction of Methemoglobin by Irradiation in the Near-Ultraviolet Region†

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Received June 21, 2000; Revised Manuscript Received September 18, 2000

ABSTRACT: Ferric metHb can be photoreduced to the ferrous state by direct photoexcitation in the near-ultraviolet region. In this research, we studied the mechanism and facilitating conditions for the photoreduction and the resulting restoration of O_2 binding. MetHb in phosphate-buffered saline or pure water in a CO atmosphere was photoreduced to form HbCO by illuminating the N band (365 nm), one of the porphyrin $\pi \rightarrow \pi^*$ transitions, whereas the photoreduction did not occur in Ar, N_2, or O_2. The transient absorption spectrum exhibited the generation of deoxyHb within 30 ns in both the CO and Ar atmospheres; however, only in CO did the subsequent CO binding inhibit the back reaction. The photoreduction rate was dependent on the pH and ligand anions, showing that aquametHb in the high-spin state was predominant for the photoreduction. Axial ligand-to-metal charge-transfer (LMCT) bands overlap with the Soret and Q bands in metHb; however, the excitation of these bands showed little photoreduction, indicating that the contribution of these LMCT bands is minimal. Excitation of the N band significantly contributes to the photoreduction, and this is facilitated by the external addition of mannitol, hyaluronic acid, Trp, Tyr, etc. Especially, Trp allowed the photoreduction even in an Ar atmosphere, and the reduced Hb can be converted to HbO_2 by O_2 bubbling. One mechanism of the metHb photoreduction that is proposed on the basis of these results consists of a charge transfer from the porphyrin ring to the central ferric iron to form the porphyrin π cation radical and ferrous iron by the N band excitation, and the contribution of the amino acid residues in the globin chain as an electron donor or an electron pathway.

The photoinduced charge separation and the electron-transfer reaction in a metal complex embedded in a host protein play key roles in the functions of biological systems, especially in the chlorophyll−protein complex during photosynthesis. In other hemoproteins such as hemoglobin (Hb),[1] myoglobin (Mb), cytochrome oxidase, and P450, which are originally not related to the photoreaction in the biological system, photoexcitation and the resulting photoreduction have been reported since the 1970s (*1*−*5*). However, the photoreduction mechanism even for synthetic hemes is still controversial (*6*−*8*). It is suggested that the electron transfer from a distal histidine to a ferric iron would be the primary step (*9*), which was supported by the facts that the photoreduction of hemin was accelerated with the increasing amount of pyridine as a base (*10*) and that the synthetic heme underwent photoreduction in the presence of 2-methylimidazole (*11*−*14*). In the absence of base ligands, on the other hand, irradiation at a halide anion ligand-to-metal charge-transfer (LMCT) band may induce photoreduction as the result of halide radical formation (*15*−*17*). It has also been confirmed that the addition of alcohols such as 2-propanol or glycerol facilitates the photoreduction of hemoprotein (*8*) and synthetic hemes (*6*, *18*, *19*).

In this study, we have made a significant effort to determine the conditions that facilitate the photoreduction of ferric metHb by the direct excitation of heme in the near-ultraviolet N band as one possible method. The N band is one of the porphyrin $\pi \rightarrow \pi^*$ transitions, and its origin has been extensively studied (*20*−*22*). We analyzed the influence of dissolved gases, irradiation wavelength, pH, radical scavengers, amino acid residues, etc., to elucidate the underlying mechanism of the metHb photoreduction that cannot be explained by the conventional proposed mechanisms.

MATERIALS AND METHODS

Preparation of MetHb. Carbonylhemoglobin (HbCO) was purified from human blood provided by the Hokkaido Red Cross Blood Center as previously reported (*23*, *24*) using ultrafiltration, heat treatment, and dialysis. MetHb was prepared by the reaction of HbCO with an excess amount of potassium ferricyanide. The unreacted ions and ferrocyanide ions were removed by stirring twice with a mixed bed ion-exchange resin (Bio-Rad AG 501-X8), and the solution was then filtered through 0.22 μm disposable filters (Ad-

† This work is supported by Health Sciences Research Grants (Research on Advanced Medical Technology, Artificial Blood Project), the Ministry of Health and Welfare, Japan (H11-BLOOD-007), Grants in Aid for Scientific Research from the Ministry of Education, Science, Sports, and Culture, Japan (Grants 12480268 and 12558112), and Grants from the Mukai Sciences Foundation. E.T. is a CREST Investigator, JST.

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[1] Abbreviations: Hb, hemoglobin; Mb, myoglobin; LMCT, ligand-to-metal charge transfer; PBS, phosphate-buffered saline; NEM, *N*-ethylmaleimide; DMPO, 5,5′-dimethyl-1-pyrroline *N*-oxide; IEF, isoelectric focusing; D, electron donor.

10.1021/bi0014204 CCC: $19.00
Published on Web 10/28/2000

vantec Co.). The extent of metHb conversion was 99.8%, as measured by the modified Evelyn−Malloy method (*25*).

Chemicals. Amino acids (Trp, His, Tyr, Ser, Phe, Thr, Val, and Leu), alcohols (mannitol, glucose, glycerol, ethylene glycol, and sodium citrate), and hyaluronic acid as radical scavengers, halide salts (NaF, NaCl, NaBr, and NaI), KCN, and NaN_3 were purchased from Kanto Chemical Co. *N*-Ethylmaleimide (NEM) and 5,5′-dimethyl-1-pyrroline *N*-oxide (DMPO) were from Sigma. All the reagents were used without further purification.

Photoreduction of MetHb. Three milliliters of phosphate-buffered saline (PBS, pH 5.0−9.0) with a reagent (e.g., amino acids or radical scavengers) or deionized pure water in a quartz cuvette was sealed with a butyl rubber cap. Through the solution was bubbled either Ar, O_2, N_2, or CO for 30 min. A concentrated metHb stock solution (about 3 mM, 10 μL) deaerated by a gentle N_2 flow in another bottle was injected into the cuvette. This procedure avoided bubbling which might induce foaming and metHb denaturation. The final concentration of heme was 10 μM ([Hb] = 2.5 μM). The light source was a super-high-pressure mercury lamp (USH-250D, 250 W, Ushio Co., Tokyo, Japan) with a cutoff filter (U-360, Hoya Co., Tokyo, Japan) to obtain a single beam with a maximum wavelength of 365 nm which is near the wavelength of the laser beam for transient spectrum measurements (355 nm). The cuvette was located 2.5 cm away from the light source, and the light intensity was 89 mW/cm^2, which was measured with a power meter (PSV-3102, Gentec Co.). The extent of conversion of the reaction was calculated from the Soret band peak ratio of 405 nm (metHb) versus 419 nm (HbCO), 430 nm (deoxyHb), or 415 nm (HbO_2), measured with an UV−vis spectrophotometer (V-560, Jasco, Tokyo, Japan). The initial rates of photoreduction (% per minute) were obtained by extrapolation of the plots of the levels of photoreduction (%) during the initial 5 min.

For the analysis of the photoreduction dependence on the irradiation wavelength, a light with a 405 nm maximum wavelength using L-39/HA-50 cutoff filters (103 mW/cm^2) and a light with a range of 240−400 nm using a U-330 filter (152 mW/cm^2) were employed. A halogen lamp (500 W) was also used as the visible light source to irradiate both the Q and Soret bands (1591 mW/cm^2). A ferrioxalate actinometer was used to measure the quantum yield of the metHb photoreduction (*26*).

Transient Spectrum Measurements. A laser flash photolysis system (Tokyo Instrument Co.) was used for the transient spectrum measurements. The sample solution was excited with the third harmonic (355 nm) of a pulsed Nd:YAG laser (SL803G-10, Spectron Laser Systems, Ltd.). The pulse width was 5−8 ns (fwhm), and the interval was 100 ms. A total of 100 accumulations were collected to obtain an acceptable signal-to-noise ratio. The transient spectra were recorded between 350 and 550 nm using a spectrophotometer (MS257, Oliel Instrument Co.) equipped with an ICCD detector (DH520-18F-WR, ANDOR Technology Co.). The resultant spectra were digitalized and manipulated using computer software (LabVIEW) to produce the ultimate difference spectra. A metHb sample solution ([heme] = 10 μM) was placed in a 10 mm quartz cuvette purged with either CO, Ar, or O_2. The effect of the external addition of 1 mM Trp in an Ar atmosphere was also measured.

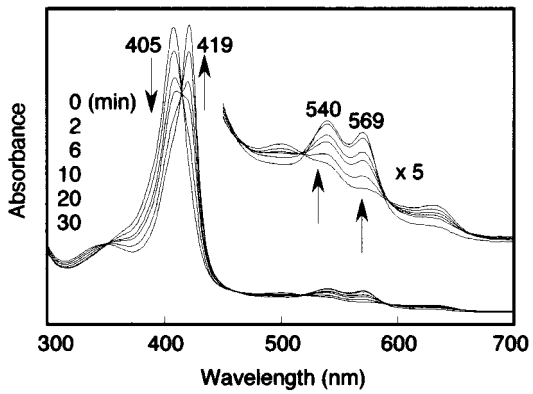


FIGURE 1: Photoreduction of metHb in PBS (pH 7.4) by irradiation with 365 nm light in a CO atmosphere. [heme] = 10 μM, and [mannitol] = 100 mM. The λ_{max} of the Soret band of metHb is 405 nm, and its absorption decreases with photoirradiation. New peaks at 419 nm of the Soret band and 540 and 569 nm of the Q band appear, indicating that metHb is reduced to form HbCO.

Chemical Blocking of Cys and Tyr Residues in Hb. To clarify the contribution of Cys-β93 to the photoreduction system as an electron pathway from the external to the internal Hb (*27*), Cys-β93 was blocked by the reaction with NEM at a Hb-to-reagent ratio of 1:3 (*27*). An HbCO solution (10 g/dL, 5 mL) in 10 mM PBS was mixed with 2.3 mL of a 10 mM NEM solution. The reaction was carried out at 37 °C for 1 h in PBS followed by Sepharose CL-4B chromatography (Pharmacia, Uppsala, Sweden) to separate the Hb from the reagents. The obtained Cys-β93-blocked Hb was oxidized with potassium ferricyanide to convert it to metHb and then deionized in the same manner.

To study the contribution of a Tyr residue as an electron donor or pathway, blocking of the Tyr residues in Hb was performed by iodination (*28*). Briefly, 25 mL of a 1 wt % Hb solution in PBS (pH 9.0) was mixed with 1.55 mL of a cold iodine solution (0.05 M I_2 in 0.24 M KI) and incubated for 15 min. The solution was loaded onto a Sepharose CL-4B column and eluted with PBS (pH 7.4). The Tyr-blocked metHb was prepared by the same oxidation and deionization method.

Isoelectric Focusing (IEF) and Restoration of the Oxygen Binding Property. IEF was performed on PhastGel IEF 5-8 (pH 5−8) using the PhastSystem (Pharmacia). The photoreduced Hbs in CO in the presence or absence of 10 mM Trp were compared with metHb and the purified HbCO. Forty microliters of a sample (1 mg/mL) per one lane was applied to the gel. This was focused and then stained with PhastGel Blue R (Coomassie brilliant blue) in the development unit of the PhastSystem. The marker was the pI calibration kit 3-10 (Pharmacia).

The photoreduced deoxyHb solution in the presence of 10 mM Trp in an Ar atmosphere was bubbled for a few seconds with O_2, and the UV−vis spectrum was measured.

RESULTS

Influences of Dissolved Gas and Irradiation Wavelength (λ_{ex}) on Photoreduction. The photoreduction of metHb did proceed by irradiation at 365 nm in PBS (pH 7.4) with a CO atmosphere as shown in Figures 1 and 2, evidenced by the absorption decrease at a λ_{max} of 405 nm (metHb) and

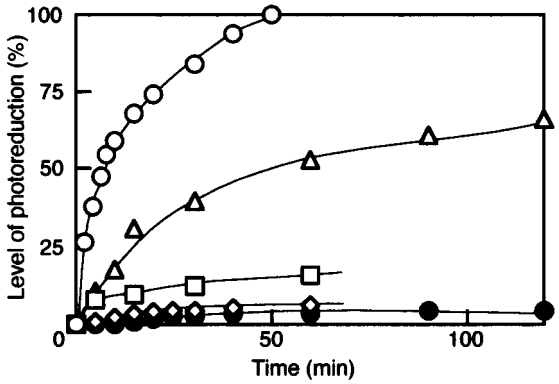


FIGURE 2: Effect of dissolved gases (CO, Ar, N_2, and O_2) on photoreduction of metHb by irradiation with 365 nm light in PBS (pH 7.4) where [heme] = 10 μM: (△) CO, (●) Ar, (◇) N_2, and (□) O_2. Mannitol (100 mM) was added to facilitate the photoreduction (○). In PBS, only the CO atmosphere allows photoreduction, and the addition of radical scavengers such as mannitol significantly facilitates the photoreduction. Photoirradiation in other gases resulted in chemical modification of the heme portions which was evident from the flattening of the absorption (data not shown), resulting in overestimation of the photoreduction level. The levels of photoreduction were calculated from the absorption ratio of metHb (λ_{max} = 405 nm) vs HbCO (419 nm) in CO, deoxyHb (430 nm) in Ar and N_2, and oxyHb (415 nm) in O_2.

increase at 419 nm (HbCO). The photoreduction proceeded even in distilled and deionized pure water in the same way (data not shown). On the other hand, the metHb solutions in PBS saturated with either N_2, Ar, or O_2 gas did not show an increase at 430 nm (deoxyHb) or 415 nm (HbO_2), and the absorption of the Soret band at 405 nm gradually decreased, especially with O_2, indicating that the heme moiety was somehow chemically modified (*8*, *29*). Since the reduction levels were calculated from the ratio of the absorbances at 405 and 430 nm or 415 nm, the decrease in the Soret band absorption may result in an overestimation of the photoreduction level (Figure 2).

The transient absorption difference after flash irradiation (λ_{ex} = 355 nm) in a CO atmosphere showed that after 30 ns, deoxyHb had already appeared with a peak (λ_{max} = 430 nm), and the peak intensity slightly increased up to 50 ns (Figure 3A). Simultaneously, a decrease in the metHb intensity was confirmed from a peak (λ_{max} = 405 nm). The succeeding CO binding to deoxyHb to form HbCO (λ_{max} = 419 nm) was observed, and the HbCO formation was completed within 500 μs. In an Ar atmosphere, on the other hand, metHb was reduced to form deoxyHb by 30 ns, the absorption increased at 50 ns, and it remained until 500 μs. However, the absorption decreased between 500 μs and 5 ms; most of it returned to metHb, and an unknown peak with a λ_{max} of 424 nm was confirmed (Figure 3B). In an O_2 atmosphere, the absorption change was much smaller than in the other gases, and a new unknown peak with a λ_{max} of 424 nm was confirmed, indicating that the heme was chemically modified (Figure 3C).

Figure 4 demonstrates the relation of the photoreduction conversion with the total irradiation energy for each irradiation wavelength, because the light intensity is different under all the irradiation conditions. When the irradiation wavelength (λ_{ex}) was around 405 nm (Soret band) using L-39/HA-50 filters, the photoreduction was significantly slower than the excitation of the N band even in the presence of CO. When the irradiation wavelength range included shorter wavelengths around 240−400 nm using a U-330 filter, the photoreduction rate increased more than 3-fold probably due to the excitation of aromatic amino acid residues such as Tyr or Trp which act as electron donors (*4*). However, the photoreduction level did not reach 100% and tended to decline due to degradation. Irradiation with a wide range of

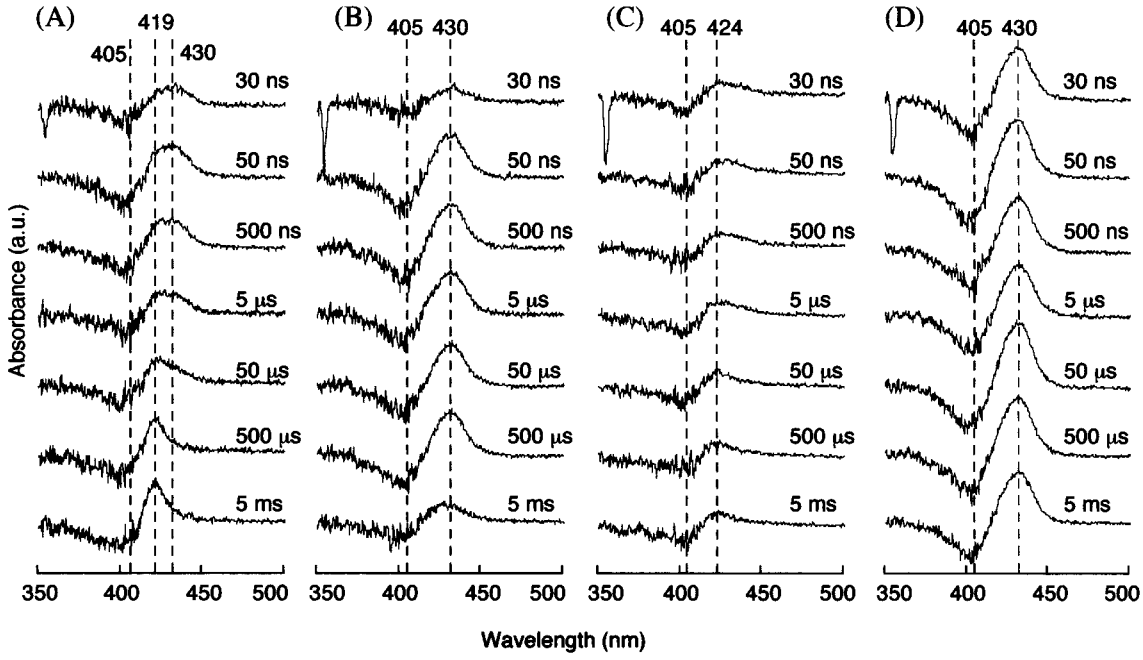


FIGURE 3: Transient difference spectrum of metHb photoreduction in (A) CO, (B) Ar, and (C) O_2 atmospheres and (D) Ar with 1 mM Trp. [heme] = 10 μM. The excitation wavelength was 355 nm. (A) DeoxyHb (430 nm) is formed at 50 ns with a decreasing metHb concentration (405 nm) and binds CO to form HbCO (419 nm) from 50 μs to 5 ms. (B) DeoxyHb is transiently detected in Ar from 50 ns to 500 μs, though it decreased at 5 ms. (C) In O_2, the absorption change was small, and the final product exhibited a λ_{max} of 424 nm, which is not HbO_2 (415 nm). (D) Addition of Trp significantly facilitates the photoreduction even in the Ar atmosphere. DeoxyHb already appeared at 30 ns.

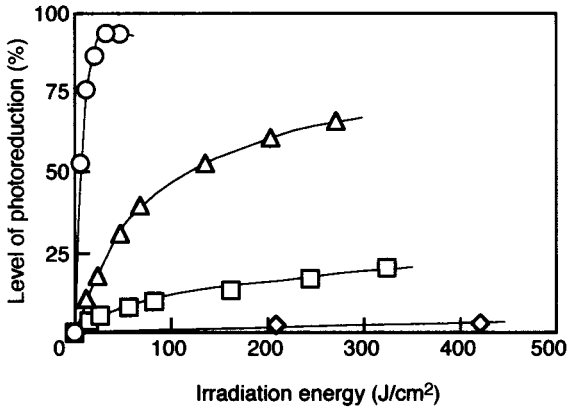


FIGURE 4: Effect of wavelength of irradiation light on the rate of metHb photoreduction in a CO atmosphere. [heme] = 10 μM. The abscissa is the total energy of irradiation (joules per square centimeter) calculated from (irradiation time) × [irradiation strength (watts per square centimeter)]. A super-high-pressure Hg lamp was used with a U-330 filter for the amino acid residues and the N band excitation (○), with a U-360 filter for the N band excitation (△), and with L-39/HA-50 filters for the Soret band excitation (□). A halogen lamp was for the Soret and Q band excitations (◇). Excitation of the N band and amino acid residues markedly facilitates the photoreduction. On the other hand, contribution of the Soret and Q band excitations was minimal.

Table 1: Influence of the Presence of Anions (150 mM) and pH on the Photoreduction Rate of MetHb ([heme] = 10 μM) in 10 mM Phosphate Buffer (pH 7.4) and in a CO Atmosphere

anion	Soret band λ_{max}	Soret band ϵ ($\times 10^5$)	redox potential of anions (E_o)[a] (V) vs NHE	initial rate of photoreduction (%/min)
CN^-	421	1.09	−	0.0
N_3^-	420	1.24	−	0.0
F^-	403	1.30	2.87	0.8
Cl^-	405	1.38	1.36	2.0
Br^-	405	1.69	1.07	2.3
I^-	405	1.39	0.54	3.6

[a] $X_2 + 2e = 2X^-$; from ref *46*.

Table 2: Influence of pH on the Photoreduction Rate of MetHb ([heme] = 10 μM) in a CO Atmosphere[a]

pH	Soret band λ_{max}	Soret band ϵ ($\times 10^5$)	initial rate of photoreduction (%/min)
5.0	403	1.55	14.6
7.4	405	1.38	2.0
9.0	410	1.15	1.0

[a] [NaCl] = 150 mM, in 10 mM phosphate buffer.

strong visible light from a halogen lamp (500 W) with a cutoff filter for the IR region did not induce photoreduction, indicating that Q band excitation did not lead to photoreduction.

Effect of Anions, pH, and Additives. Table 1 summarizes the influence of ligand anions on the Soret band absorption and the initial rate of photoreduction. Among the anions studied, CN^-, N_3^-, and F^- are known to bind to ferric heme. CN^- and N_3^-, which are strongly bound to metHb, did not allow photoreduction. On the other hand, all the halide anions exhibited photoreduction. The rates are in the order of anion size: $F^- < Cl^- < Br^- < I^-$. These are in the order of the spectrochemical series as well as in the order of the redox potential of anions. Table 2 summarizes the pH dependence of the Soret band absorption and the initial photoreduction rate of metHb. The rate of photoreduction was 1.0%/min at pH 9.0 which was increased to 14.5%/min by decreasing the pH to 5.0, where aquametHb in the high-spin state is formed.

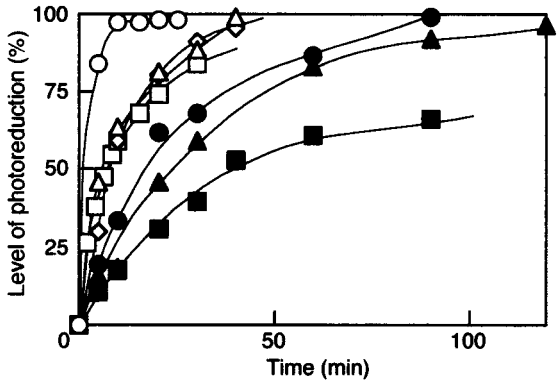


FIGURE 5: Effect of the external addition of 100 mM alcohols [(△) sodium citrate, (◇) glucose, (□) mannitol, (●) ethylene glycol, (▲) glycerol, and (○) hyaluronic acid (5 mM glucose)] on photoreduction of metHb by irradiation with 365 nm light in PBS in a CO atmosphere. (■) No additives. [heme] = 10 μM.

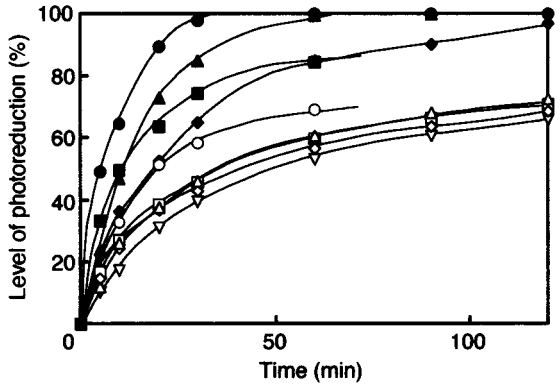


FIGURE 6: Effect of external addition of 1 mM amino acids on the rate of metHb photoreduction in a CO atmosphere: (●) Trp, (■) Tyr, (▲) His, (◆) Ser, (○) Phe, (△) Thr, (□) Val, (◇) Leu, and (▽) no addition. [heme] = 10 μM. The photoreduction was markedly enhanced by the addition of Trp.

The effect of adding 100 mM alcohols was studied (Figure 5). All the alcohols that were tested facilitated photoreduction. Especially, mannitol, glucose, and sodium citrate showed significant facilitation. The addition of hyaluronic acid at a concentration of only 5 mM (glucose units) significantly facilitated photoreduction. The addition of 50 mM DMPO doubled the photoreduction rate (data not shown). However, ESR spectroscopy of the DMPO/metHb solution ([DMPO] = 50 mM, and [heme] = 40 μM) after photoreduction for 5 min and immediate freezing could not detect any radicals.

The effect of the external addition of amino acids on the rate of metHb photoreduction was studied (Figure 6). The amino acids with aromatic groups (Tyr, His, Trp, and Phe) facilitated photoreduction. Especially, the addition of Trp doubled it. On the other hand, Ser, Thr, Val, and Leu did not contribute to the photoreduction. Moreover, the addition of 1 mM Trp allowed photoreduction in the Ar atmosphere, even though the level of reaction did not reach 100%. Increasing the concentration of Trp to 10 mM resulted in a significantly fast photoreduction, which was completed within 5 min. The transient absorption spectrum of the metHb

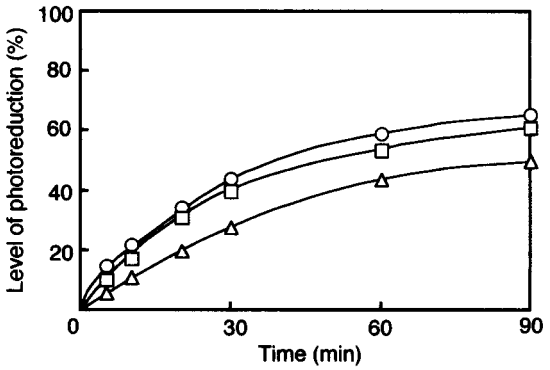


FIGURE 7: Effects of blocking of Tyr (△) and Cys-β93 (○) on the rate of metHb photoreduction by the N band excitation in a CO atmosphere. (□) No blocking. [heme] = 10 μM. Tyr was blocked by iodination, and Cys-β93 was blocked with NEM.

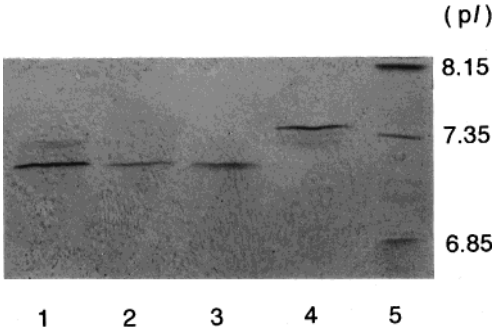


FIGURE 8: IEF of photoreduced HbCO: (1) purified HbCO, (2) photoreduced HbCO, (3) photoreduced HbCO in the presence of 10 mM Trp, (4) metHb, and (5) markers. In the IEF pattern, metHb exhibited an isoelectric point (pI) of 7.4. The photoreduced HbCO exhibited a pI of ~7.0, which was the same for the purified HbCO.

photoreduction in the presence of 1 mM Trp (λ_{ex} = 355 nm) in Figure 3D had already shown a significantly larger peak of deoxyHb (λ_{max} = 430 nm) at 30 ns, and the intensity did not change even after 5 ms. This indicates that the photoreduction was very fast and completed within 30 ns.

The quantum yield of the metHb photoreduction at 10 μM heme by irradiation at 365 nm in the CO atmosphere was 0.003. This was improved to 0.006 in the presence of 100 mM mannitol or 1 mM Trp, as photoreduction was facilitated.

Contribution of Amino Acid Residues. MetHb with a Cys-β93 blockade showed a similar rate of photoreduction with normal metHb (Figure 7), indicating that Cys-β93 does not contribute to the photoreduction system. Iodination of the Tyr residues in metHb resulted in a reduced rate of metHb photoreduction, indicating that Tyr residues should contribute to the photoreduction.

IEF and the Oxygen Binding Property of Photoreduced Hb. In the IEF measurement, metHb exhibited an isoelectric point (pI) of 7.4. The photoreduced HbCO in the presence and absence of Trp exhibited a pI of about 7.0, which was the same as the purified HbCO (Figure 8). These results indicate that the modification of the outer surface of Hb was not detected after photoreduction.

The photoreduction of metHb in the presence of 10 mM Trp was completed within 5 min in an Ar atmosphere. The resulting deoxyHb was exposed to oxygen by gentle bubbling, and UV−vis spectroscopy revealed the generation of oxyhemoglobin (HbO_2) with the characteristic λ_{max} at 415 nm (Figure 9).

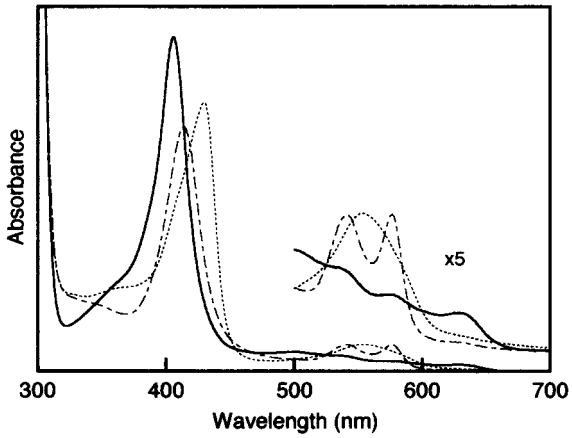


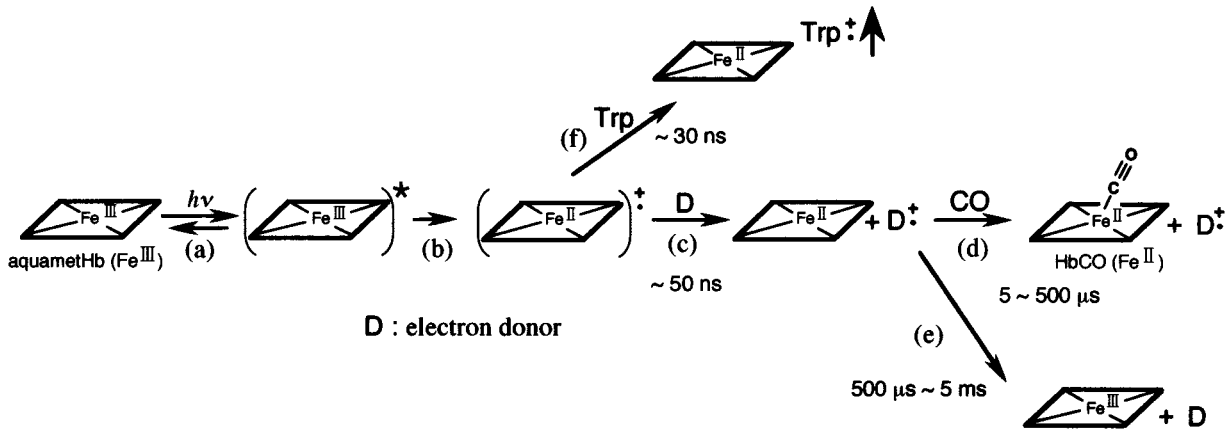
FIGURE 9: UV−vis spectra of metHb (—), photoreduced Hb in the presence of Trp in an Ar atmosphere (•••), and metHb after bubbling with oxygen (— - —). The peak at 415 nm (λ_{max}) is an indication of HbO_2 formation; thus, the oxygen binding property was restored.

DISCUSSION

Our results from the metHb photoreduction support the previous report that the photoreduction of metMb (not metHb) proceeds in the presence of CO but not under aerobic conditions, and that the addition of citrate and glycerol facilitates reduction (*8*). However, in the previous reports of hemoprotein photoreduction (*1*−*5*, *8*), the mechanism was not well-understood. We confirmed that in an Ar and N_2 atmosphere, the photoreduction did not proceed; however, the transient formation of deoxyHb was confirmed in an Ar atmosphere at 30 ns after pulsed excitation, showing a maximum change at 5 μs, and then it reconverted to metHb within 5 ms. In the presence of CO, the binding of CO to the reduced heme was detected, finishing the reaction within 500 μs, and the stable HbCO did not reconvert to metHb. Apparently, the binding of CO to the deoxyHb inhibits a back reaction for conversion to metHb. The rate of photoreduction of metHb to form deoxyHb seems comparable with that of the tetraphenylporphyrin derivatives in organic solvents that showed completion of the reduction within 50 ns (*17*, *30*, *31*). The host macromolecular globin chain does not seem to retard the reaction. The addition of Trp facilitated the photoreduction even in the Ar atmosphere. In the presence of oxygen, on the other hand, the formation of deoxyHb was not confirmed even at 30 ns, and the generation of an unknown peak (λ_{max} = 424 nm) indicated the decomposition of the heme portions probably by the reaction with oxygen or active oxygen species via photooxidation (*29*).

Even though in many reports the irradiation wavelength of 355 nm has been widely used because it matches the third harmonic of the Nd:YAG pulsed laser, little attention has been paid to the relationship of this wavelength and the heme absorption band. Hb has an absorption band at around 360 nm, termed the "N band", a charge-transfer band corresponding to the a'_{2u}, $b_{2u}(\pi) \rightarrow e_g(\pi^*)$ porphyrin π electron transition bands (*20*−*22*). Some of the energy levels of the five iron d orbitals are located between the porphyrin a'_{2u}, $b_{2u}(\pi)$, and $e_g(\pi^*)$ orbitals (*20*) (Figure 10). One simple idea for the mechanism of the photoreduction is that it may be

Scheme 1: Proposed Mechanism of MetHb Photoreduction[a]



[a] (a) Photoexcitation of the N band and radiationless transition. (b) Electron transfer from the porphyrin ring to the central ferric iron and formation of the porphyrin π cation radical. (c) Reaction with an internal or external electron donor D to complete the heme reduction. (d) CO binding to fix the reduced ferrous form. (e) A back reaction of reduced heme with an oxidized electron donor "$D^{+}_{\bullet}$" to form metHb. (f) Rapid reaction with externally added Trp to facilitate photoreduction to form deoxyHb even in the Ar atmosphere.

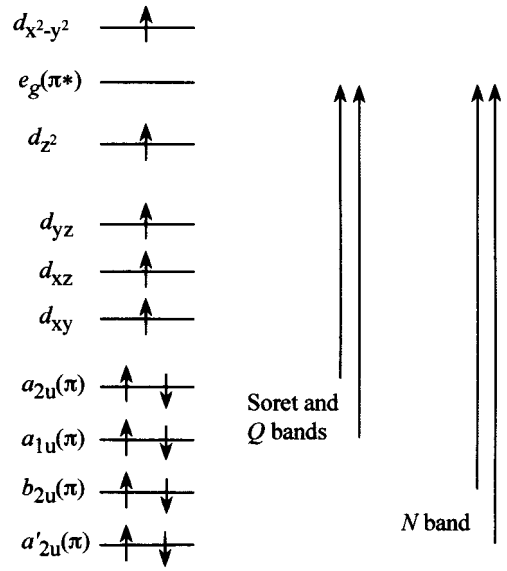


FIGURE 10: Assignment of porphyrin π orbitals for charge-transfer transition bands and iron d orbitals of the high-spin state metHb. The N, Soret, and Q band transitions are all porphyrin (π and π^*) transition bands. After excitation of the N band, the resulting unoccupied a'_{2u} and $b_{2u}(\pi)$ orbitals may be lower than the unoccupied a_{1u} and $a_{2u}(\pi)$ orbitals of the Soret and Q bands.

initiated by an excited electron in the $e_g(\pi^*)$ orbital of a porphyrin ring which moves to the iron d orbitals, thus making the porphyrin ring a π cation radical. Hemoproteins such as horseradish peroxidase, catalase, Hb, and Mb are known to form π cation radicals as active intermediates (*32*, *33*). Studies on the photochemistry of synthetic porphyrins are an indication of the formation of porphyrin π cation radicals with reduction of the central metal (*34*, *35*). From our transient spectra results, the mechanism of photoreduction can presumably be described by Scheme 1, where reaction (a) is the excitation of the N band, reaction (b) is the electron transfer from the porphyrin to the central ferric iron and the formation of the porphyrin π cation radical, reaction (c) is the reaction with an internal or external electron donor "D" and the donor is then oxidized, reaction (d) is the CO binding to fix the reduced ferrous form, and reaction (e) is the back reaction of the reduced heme with an oxidized electron donor to form metHb. The transient absorption spectra in CO and Ar (Figure 3) showed an increase in the absorption from 30 ns until 50 ns, and this period may correspond to reaction (c). This is supported by the report that a synthetic ferrous heme (Fe^{2+}) porphyrin π cation radical prepared by oxidation with iodine showed a significantly decreased and flattened absorption spectrum (*36*), and the ferrous Hb porphyrin π cation radical may be converted to deoxyHb with an increased absorption. In the presence of the externally added Trp, reaction (f) is so fast that it may be completed before 30 ns.

The oxidation potentials (E_o) of Trp and Tyr are 1.05 and 0.94 V versus the normal hydrogen electrode (NHE), respectively (*37*). Alcohols such as mannitol, glycerol, and ethylene glycol exhibit an E_o value of about 0.7 V versus the NHE (*38*) which are higher than that of heme in hemoglobin (0.2 V vs NHE), and there is no reaction between them. However, the E_o of the porphyrin ring is estimated to be around 1.2−1.4 V versus the NHE (*39*, *40*), and the downhill reduction of the porphyrin π cation radical and oxidation of these amino acids and alcohols are possible. Halogen anions Cl^-, Br^-, and I^-, which cannot be an axial ligand in the case of metHb, can also act as electron donors like Trp because their E_o values are between those of the porphyrin ring and heme central iron (*41*). The photoreduction rates in the presence of halide anions are in the same order of the E_o values. However, their efficiency as electron donors (150 mM) is lower than 1 mM Trp. A back reaction may be the oxidation of Fe^{2+} of the heme by "$D^{+}_{\bullet}$" as shown by reaction (e) because the E_o of D is higher than that of the heme. In the presence of CO, reaction (d) may compete with reaction (e). This can explain the transient absorption spectral changes in an Ar atmosphere (Figure 3B), where the reduced deoxyHb reconverts to metHb at 5 ms by the intramolecular back reaction of the reduced heme and $D^{+}_{\bullet}$. The substantially low quantum yield of the photoreduction, 0.003−0.006, is determined by the back reaction of reaction (a) as a radiationless transition from the excited state to the ground state and reaction (e) as a reconversion to metHb.

The Soret band corresponds to the a_{1u}, $a_{2u}(\pi) \rightarrow e_g(\pi^*)$ porphyrin transitions. The absorption of the N band is weaker than that of the Soret band, though the energy levels of a'_{2u}

and $b_{2u}(\pi)$ are lower than those of a_{1u} and $a_{2u}(\pi)$ as shown in Figure 10 (*20*−*22*). Therefore, after excitation, the resulting unoccupied lower a'_{2u} and $b_{2u}(\pi)$ orbitals may find it easier to accept one electron from a donor molecule. This may explain the much faster photoreduction by the excitation of the N band than by excitation of the Soret and Q bands.

A high-spin state ferric heme has more d orbitals with unpaired electrons than does a low-spin state ferric heme. One explanation for the faster photoreduction in the case of aquametHb at low pH than hydroxymetHb at high pH in our study is that the former is in a high-spin state and the latter is in a mixture of low- and high-spin states. MetHbs, which bind CN^- or N_3^-, are known to be in the low-spin state, and they do not cause photoreduction. On the other hand, metHbs in the presence of halides are photoreduced, though F^-, which binds to the heme, retards the photoreduction. The reduction rates ($F^- < Cl^- < Br^- < I^-$) are in the order of the spectrochemical series as well as in the order of redox potentials. Thus, metHb in the low-spin state with more degenerated orbitals exhibits a slower rate of photoreduction.

The results showing that the external addition of amino acids (especially Trp and Tyr) and radical scavengers significantly facilitated the photoreduction indicate two important aspects. One is that amino acid residues in the globin chain may contribute to the photoreduction, and the other is that there may be a direct electron transfer from an external additive to an excited heme. These additives are too large to be inserted into the hydrophobic pocket of the globin chains where hemes are embedded. Even so, two propionic acid groups of protoporphyrin IX face the external aqueous phase. The distance between the central iron and a carboxylic group in a heme is only 4 Å. On the other hand, the nearest Trp and Tyr residues are located 15−20 and 10−14 Å, respectively, from a heme. Therefore, it is possible that water-soluble reagents such as alcohols or amino acids can be located near the activated heme as a radical cation to supply one electron in the same way as the chemical reduction of metHb with reductants such as glutathione. There are indications that a π electron of the porphyrin ring should participate in the initial step of such redox reactions in biological systems (*42*). The hydrophobic nature of hyaluronic acid and Trp may contribute to the enhancement of the affinity with the globin chain and resulting facilitated electron transfer.

The hypothesis that internal amino acid residues such as Tyr or Trp, which are located near a heme, may also contribute to the metHb photoreduction is supported by the result that the photoreduction occurs even in pure water in our study. To confirm the contribution of the Tyr residues to the photoreduction, we studied the effect of Tyr blocking by iodination that inhibits Tyr conversion to the Tyr radical (*28*). Since the iodination of the Tyr residues retarded the metHb photoreduction, they may contribute to some extent to the electron donor or electron pathway to reduce metHb. This is also supported by the oxidative reaction process of metMb with H_2O_2, where an Fe(IV)−oxo porphyrin π cation radical is formed, and this chain reacts with neighboring Tyr or Trp residues to convert them to the corresponding cation radicals (*28*, *43*, *44*). Moreover, cation radical formation leads to the subsequent formation of other amino acid radicals within an electron-transfer process throughout the hemoprotein (*45*). The addition of alcohols known as radical scavengers and a spin trap, DMPO, facilitated photoreduction, indicating that radical formation is involved in the photoreduction system. These external additives as well as amino acids may react with not only the activated heme cation radical but also globin radicals because it was reported that the lifetime of the globin radicals generated by a rapid mixing with metHb and H_2O_2 is significantly shortened with Trp and Tyr (*46*). We tried to detect globin radicals which would be formed during photoreduction, though ESR spectroscopy in the frozen state could not detect any radical at the concentrations that were employed ([DMPO] = 0 and 50 mM, and [heme] = 40 μM). During the time course of photoreduction, the generation and decay of globin radicals may proceed in parallel and the radical concentration may be substantially low for the ESR measurement.

Contrary to our proposed mechanism, it has been suggested that one electron may transfer through an axial LMCT band. A halide anion such as Cl^- as the ligand to a synthetic heme may offer an electron through the axial LMCT band (*15*, *16*, *47*). However, the facts that Cl^- does not bind to metHb and that the photoreduction of metHb proceeds even in pure water support the hypothesis that the axial LMCT band excitation is not the mechanism in the case of metHb. H_2O and OH^- can be the sixth ligand of metHb, and the pH-dependent UV−vis spectrum revealed that the axial LMCT band should overlap with the Soret and Q bands (*48*). However, the significantly small photoreduction by the excitation of these bands suggests that the contribution of the axial LMCT band excitation should be minimal.

Studies on synthetic hemes suggest that charge transfer from a pyridine or imidazole molecule as an axial ligand to the iron may be a key component of the photoreduction process (*10*, *11*, *49*). Sage et al. (*9*) reported the laser wavelength dependence of the photoreduction of metMb coupled with the Soret transition. In contrast, the same group (*8*) later showed an exponential decrease in the yield of the hemoprotein photoreduction as the wavelength increased from 250 to 400 nm, which coincides with our result; we confirmed that light exposure below 300 nm directly excites Trp and Tyr residues and facilitated the photoreduction, and irradiation with 405 nm light showed a significantly slow photoreduction. A Raman investigation revealed that the Fe−N stretching mode of the distal histidine ($Fe-N_{His}$) in Mb and Hb is coupled to the Soret band ($\pi \rightarrow \pi^*$) resonance and not to a separate charge-transfer band (*50*). These results indicate that the direct excitation of the $Fe-N_{His}$ charge transfer may not be the mechanism in the case of the metHb photoreduction.

Without photoirradiation, oxidized hemoproteins slowly undergo autoreduction in the presence of CO via the reaction $CO + H_2O \rightarrow CO_2 + 2H^+ + 2e^-$ (*51*). Bonaventura et al. (*27*) reported that half of the four oxidized hemes (β-hemes) in metHb undergo autoreduction in the presence of CO, and the reaction is mediated by an electron transfer through Cys-β93. We confirmed that the Cys-β93 blockade with NEM did not affect the photoreduction rate. Moreover, the metHb photoreduction proceeds not only in the β-heme but also in the α-heme. Therefore, the photoreduction mechanism is different from autoreduction. This is also supported by the evidence that metHb with Trp induces photoreduction in the absence of CO.

In conclusion, in this study, we clarified the dynamics of the metHb photoreduction in relation to the spin state, irradiation wavelength, electron donors, and globin chain. We proposed a mechanism that includes the generation of a porphyrin π cation radical as an active intermediate by irradiation in the near-UV region. The photoreduced Hb, especially in the presence of Trp, restores the oxygen binding function without the detectable modification of the globin chain. Even though the quantum yield is not high in this system, the knowledge of this study may contribute to a deeper insight into the photoconversion chemistry of hemoproteins and macromolecular metal complexes where the host macromolecule plays a significant role in providing a microenvironment for regulating electron transfer.

ACKNOWLEDGMENT

We greatly acknowledge Prof. K. Tokumaru (Tsukuba University, Japan) for the discussion on the photoreaction process, Dr. T. Komatsu, Dr. T. Yanagimoto, and Dr. A. Nakagawa for the experimental procedures, and Dr. K. Oyaizu (Waseda University) for the discussion on the electrochemical reactions.

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BI0014204