Mechanism for the Production and Reaction of Active-Oxygen Species from Photoexcited Hematoporphyrin Adsorbed on a Biological Heterogeneous System

Hiroyuki Noda,* Hiroaki Ohya-Nishiguchi, and Hitoshi Kamada

Institute for Life Support Technology, Yamagata Technopolis Foundation, 2-2-1 Matsuei, Yamagata 990

(Received July 10, 1996)

The electronic states of hematoporphyrin (HP) adsorbed on the cell wall of yeast [biological heterogeneous (BH) system] have been characterized based on its fluorescence spectra, in comparison with those of the HP monomer and oligomer in a homogeneous system. The formation of some adsorption structures of HP on a BH system was confirmed from the fluorescence excitation spectra. An ESR method has also been applied to clarify the mechanism for the production and reaction of active-oxygen species from photoexcited HP on a BH system. The hydroxyl radical derived through a three-electron reduction of oxygen was the major product. The mechanism for producing the hydroxyl radical from photoexcited HP on a BH system is discussed as a characteristic feature for the formation of a heterogeneous system containing monomeric HP.

Hematoporphyrin (HP) and some of its derivatives (HPD) have been used in the photodynamic therapy (PDT) of various tumors.[1—5] So far, based on an analysis of the results obtained in homogeneous systems, some mechanisms for killing tumor cells by such photoexcited photosensitizers have been proposed involving oxidation induced by singlet oxygen[6—8] produced through an energy transfer from the triplet state of the photosensitizers and/or by hydroxyl radicals produced through the Harber–Weiss reaction catalyzed by iron(III) ions existing in the system.[9] However, the photosensitizers mainly exist in the heterogeneous media of the tumor cells. It is therefore necessary to clarify the reaction mechanism of photosensitizers in heterogeneous media. Recently, the production of singlet oxygen or the hydroxyl radical was confirmed for heterogeneous models and biological systems, such as micelles,[10] lipoproteins,[11] erythrocyte cell ghosts,[12] and human melanoma cells.[13] It thus became clearer that a tumor cell can be killed by singlet oxygen and/or the hydroxyl radical.

The mechanism for producing singlet oxygen and the superoxide ion in a heterogeneous media can easily be understood as being the result of energy and electron transfer from a photoexcited photosensitizer to oxygen as in the homogeneous case. However, the mechanism for producing hydroxyl radicals in heterogeneous media is not clear, though it is thought to be due to the Harber–Weiss reaction catalyzed by the iron(III) ion, as in the homogeneous case. In order to examine in more detail the mechanism for producing and reacting active oxygen species from photoexcited HP on a biological heterogeneous (BH) system, we applied an ESR method for the detecting the radicals from the photoexcited HP adsorbed on the cell wall of yeast. We found that the formation of a heterogeneous system plays a crucial role in the production of the hydroxyl radical.

Experimental

Materials. Hematoporphyrin (HP) dihydrochloride, catalase (6500 units mg^{-1}), D_2O (99.75%), and ethanol (special grade) were obtained from Wako Pure Chemical Industries, Ltd. A two mg cm^{-3} hematoporphyrin oligomer (molecular weight; about 3000) solution, which was prepared by neutralization after dissolving its powder in 0.1 mol dm^{-3} NaOH, was offered by Mr. Sakurai (Seikagaku K. K.). Commercially available dry yeast was used for preparing of the BH system. As a spin-trap reagent, 2,2-dimethyl-3,4-dihydro-2H-pyrrole 1-oxide (DMPO) was purchased from Dojindo Laboratory. Superoxide dismutase (SOD, Cu/Zn, 3000 units) was obtained from Sigma. Sodium azide (NaN_3) and 2,2,6,6-tetramethyl-4-oxopiperidine (TMPD) were purchased from Aldrich Chem. Co., Inc. The cell wall of yeast was prepared by crushing yeast cells by glass beads,[14] followed by centrifugal washing in a phosphate buffer solution (PBS, pH = 7.4, Iatron Laboratories, Inc.) several times at room temperature. Hematoporphyrin and the HP oligomer on the BH system were prepared as follows. The cell wall of yeast was suspended in PBS. A one mmol dm^{-3} HP ethanol solution or 2 mg cm^{-3} HP oligomer solution was added to the suspension. The sample was used for experiments after washing by PBS several times. Hematoporphyrin on silk (offered by Matsuoka Kigyo Co., Ltd.) was prepared by dipping the fiber into a dilute solution of sulfuric acid containing HP at 373 K.

Apparatus. The fluorescence spectra were recorded using a Hitachi Co., Ltd., F-2000 spectrophotometer. A mixture of HP on the BH system and some solutions, such as 0.92 mol dm^{-3} DMPO (or 20 mmol dm^{-3} TMPD), ethanol, SOD, catalase, D_2O, and NaN_3, were fed into a flat quartz ESR cell, which was inserted into the cavity of an ESR spectorometer (JEOL RE-2X). The ESR measurements of HP on silk were conducted using a quartz tissue cell. The sample was irradiated by a 500-W xenon lamp with a 500 nm low-pass filter and an IR cut filter (Toshiba, R-50 and

IRA-20). ESR measurements were conducted at 77 K and at room temperature.

Results

Fluorescence Spectra of HP on the BH System. The electronic state of HP on the BH system is presumed to be different from that in a homogeneous system. It is therefore essential to first clarify the electronic states of HP in both heterogeneous and homogeneous systems for an investigation of their reaction mechanisms. In this experiment their electronic states were characterized by measuring their fluorescence spectra, which have a higher sensitivity and selectivity than does the absorption spectra.

The fluorescence spectra of HP on the BH system (about $1—3\times10^{-5}$ mol dm^{-3}), HP in PBS containing 5% ethanol (5×10^{-5} mol dm^{-3}), and the HP oligomer in PBS (0.2 mg cm^{-3}) are shown in Fig. 1. Figure 1A is a typical fluorescence spectrum of HP in a homogeneous system. The binding of HP to the BH system led to about a 10 nm bathochromic shift and signal broadening, as shown in Fig. 1B. These spectral features indicate that some adsorption structures of HP exist on the BH system. About 5 nm and 15 nm bathochromic shifts and signal broadenings were also observed in the HP oligomer in PBS, and that on the BH system, as shown in Figs. 1C and 1D. The former indicates an effect due to the formation of molecular associates. The latter indicates an environmental effect. Moreover, the oligomer and HP on the BH system led to a decrease of about two orders of magnitude in the fluorescence intensity compared with the case of HP in 5% ethanol.

By fixing the wavelength at some fluorescence positions, the fluorescence excitation spectra were measured in order to confirm the different adsorption structures of HP, as shown in Figs. 2 and 3. The fluorescence excitation spectra of HP in PBS containing 5% ethanol was independent of the emission wavelength of 613 (peak) and 640 nm (shoulder), as shown in Figs. 2A and 2B. About a 10 nm bathochromic shift and signal broadening were observed on the fluorescence excitation spectrum of the HP oligomer in PBS at an emission wavelength of 632 nm (Fig. 2C). The excitation maximum in the Soret region of the HP on the BH system depended on the emission wavelength. Although the fluorescence emitted at 613 nm had an excitation maximum at 394 nm (Fig. 3A), but the fluorescence emitted at 632 nm had a maximum at 399 nm and a shoulder signal (Fig. 3B). A bathochromic shift

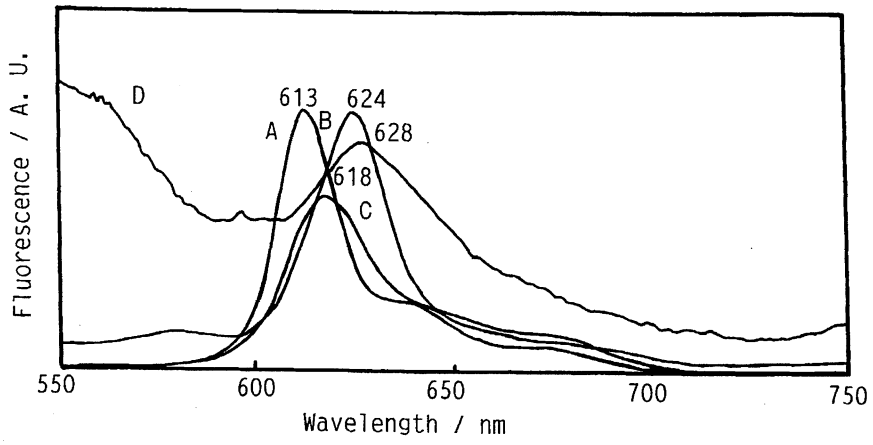


Fig. 1. Fluorescence spectra of HP in 5% ethanol solution (A) and HP on the BH system (B), and the HP oligomer in PBS (C) and that on the BH system (D). Excitation wavelength: 394 nm (A), 398 nm (B), 400 nm (C, D).

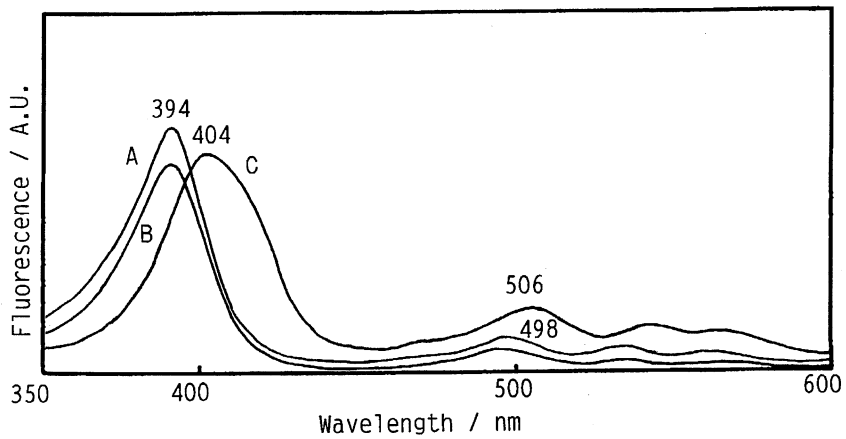


Fig. 2. Fluorescence excitation spectra of HP in 5% ethanol solution (A, B) and the HP oligomer in PBS (C). Emission wavelength: 613 nm (A), 640 nm (B), 632 nm (C).

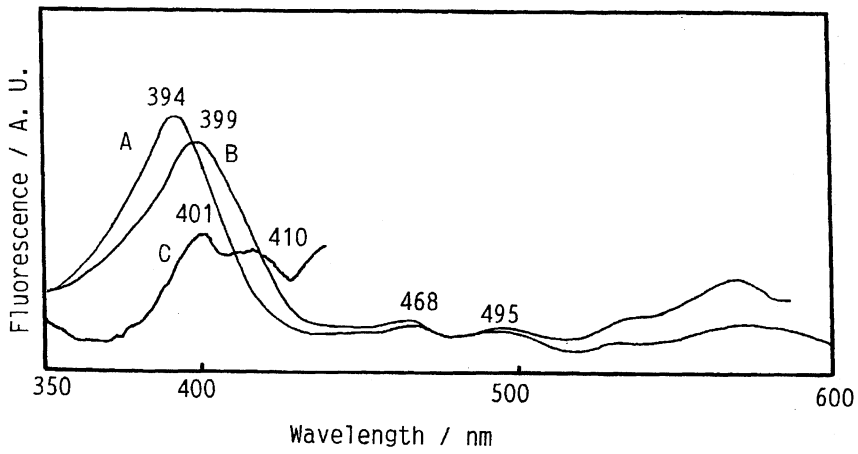


Fig. 3. Fluorescence excitation spectra of HP (A, B) and the HP oligomer (C) on the BH system. Emission wavelength: 613 nm (A), 632 nm (B, C).

similar to the case of the HP oligomer in PBS and signal splitting (401 and 410 nm) were observed in the fluorescence excitation spectra of the HP oligomer on the BH system at an emission wavelength of 632 nm (Fig. 3C).

ESR Detection of Active-Oxygen Radicals. The detection of active-oxygen radicals produced from photoexcited HP on the BH system was attempted by using the ESR spin-trapping method. 2,2-Dimethyl-3,4-dihydro-2*H*-pyrrole 1-oxide (DMPO) was used as a water-soluble spin trap reagent (partition coefficient in 1-octanol–water system is 0.1[15]) in order to trap the radicals produced in hydrophilic media.

Few of the hydroxyl radical adducts were produced from the photoexcited HP in a 5% ethanol solution (not shown). This result agreed with the result of Steveninck et al.,[9] which was obtained from photoexcited HPD in a homogeneous system in the absence of iron(III) ions.

The ESR spectra of the DMPO spin adducts obtained from photoexcited HP on the BH system are shown in Fig. 4. The signal shown in Fig. 4A can be assigned to the superoxide ion (DMPO–OOH) and the hydroxyl radical (DMPO–OH) adducts in view of their hyperfine-splitting constant (hfsc) values ($a_N = 1.41$ mT, $a_H^\beta = 1.13$ mT, and $a_H^\gamma = 0.13$ mT for DMPO–OOH, $a_N = a_H^\beta = 1.49$ mT for DMPO–OH).[16,17] The addition of ethanol to the solution changed the hydroxyl radical adduct into a C-centered radical (DMPO–C-centered) adduct, the adduct of which is shown as a six-line spectrum in Fig. 4B. This signal was also assigned based on its hfsc values.[17] The formation of this adduct means the production of the free hydroxyl radical in our system. Thus, the formation of this adduct strongly suggests a three-electron reduction of oxygen by photoexcited HP, as already reported in the case of chlorophylls in a heterogeneous system.[18] The reaction scheme can be described as follows (Scheme 1).

In order to examine this scheme in more detail, experiments were carried out by adding SOD and/or catalase. The former disproportionates the superoxide ion into O_2 and H_2O_2, and the latter disproportionates H_2O_2 into H_2O and O_2.

The time dependence of the DMPO–OH production is shown in Fig. 5. The addition of SOD led to a decrease in the

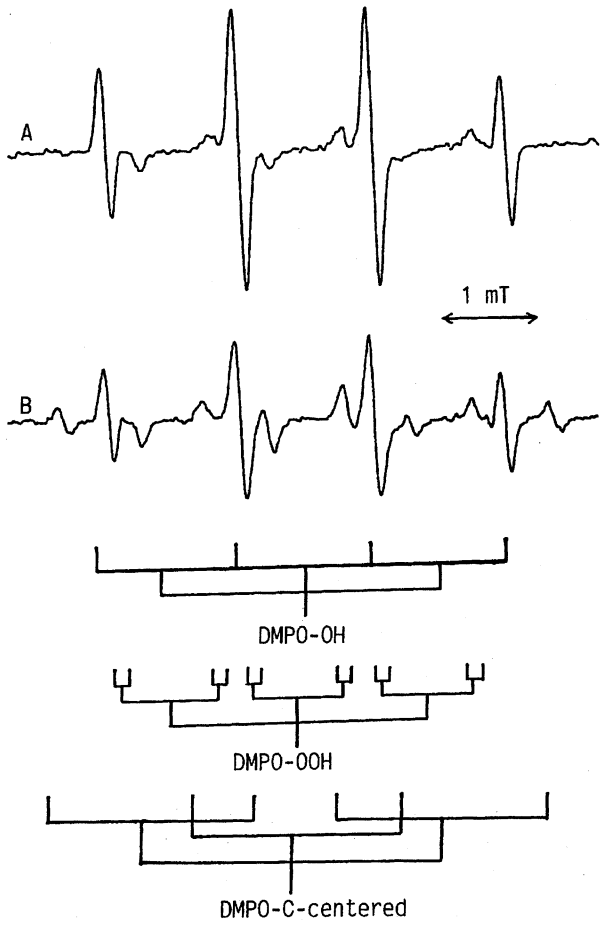
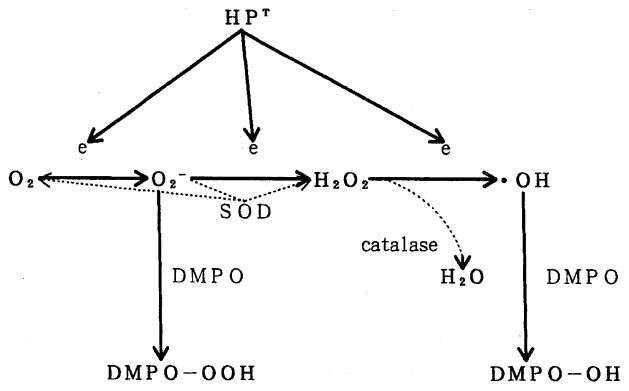


Fig. 4. ESR spectra of the DMPO spin adducts obtained by 30-s irradiation of HP on the BH system. A: control, B: 10% ethanol.

production of DMPO–OH (Fig. 5B). Superoxide dismutase also reacts with other active-oxygen species, such as the singlet oxygen and the hydroxyl radical. Denatured SOD was then used to confirm the fact that the result of Fig. 5B was brought about by the function of SOD. The denatured SOD reacts with singlet oxygen and the hydroxyl radical,



Scheme 1.

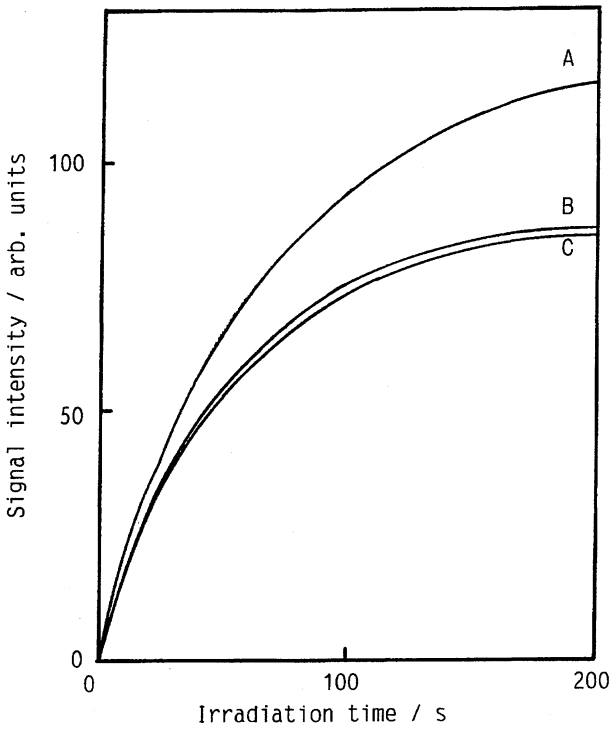


Fig. 5. Time dependence of the ESR signal intensity of DMPO–OH during irradiation. A: control, B: 600 units cm^{-3} SOD, C: B+2000 units cm^{-3} catalase.

but not with the superoxide ion, itself. No decrease in the production of DMPO–OH was confirmed by the addition of denatured SOD (not shown). The addition of catalase had no effect on the formation of DMPO–OH (Fig. 5C). This may have arisen due to the deactivation of catalase by the produced singlet oxygen in the system. The addition of H_2O_2 led to a small increase in the intensity of DMPO–OH (not shown).

In order to exclude the effect of singlet oxygen, such as the deactivation of catalase and the reaction with DMPO,[19] the effect of catalase was studied in the presence of a singlet oxygen quencher NaN_3. The azide is well known to be an inhibitor of catalase. The time dependence of DMPO–OH production in the presence of 20 mmol dm NaN_3 is shown in Fig. 6. The addition of catalase led to a decrease in the

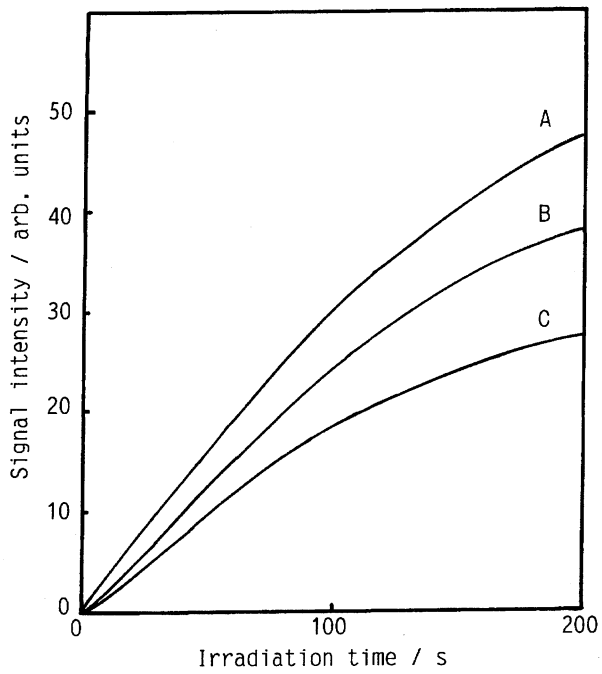


Fig. 6. Time dependence of the ESR signal intensity of DMPO–OH containing 20 mmol dm^{-3} NaN_3 during irradiation. The other conditious are the same as Fig. 5.

production of DMPO–OH (Fig. 6C). This may have arisen due to the competition between the disproportionation of H_2O_2 and the inhibition by NaN_3 (described above). The above results were confirmed by several measurements. An analysis of these results shows that the superoxide ion and the hydrogen peroxide produced in the system are indispensable to produce the hydroxyl radical, as shown in Scheme 1.

Production of Active-Oxygen Radicals from Photoexcited HP on Polypeptide. In general, the formation of the hydroxyl radical in vivo is thought to be due to the Harber–Weiss reaction catalyzed by the iron(III) ion.[9] In our BH system, the iron(III) ion is absent ($< 10^{-6}$ mol dm^{-3}), as was confirmed by the ESR at 77 K. However, the presence of the iron(II) ion in an enzyme cannot be neglected. Therefore, the Fenton reaction also cannot be neglected concerning the production of the hydroxyl radical in our BH system. In a previous study,[18] however, we found that the hydroxyl radical was also produced from photoexcited chlorophyll in a heterogeneous system without the iron (II and/or III) ion. Therefore, this point had to be clarified using some other BH system in which no iron (II and/or III) ion participates. We thus carried out experiments using HP on the polypeptide fiber of silk to clarify the above point.

First no iron(III) ion in silk fiber was observed by an ESR measurement ($< 10^{-6}$ mol dm^{-3}, not shown). Second, the identity of the electronic states of HP on both the BH system and on silk was examined by measuring the fluorescence spectrum. As shown in Fig. 7, a similar bathochromic shift in the fluorescence spectrum of HP on silk was observed compared with that of HP in the homogeneous system (Fig. 1A). Figure 8 shows the fluorescence excitation spectra of HP on

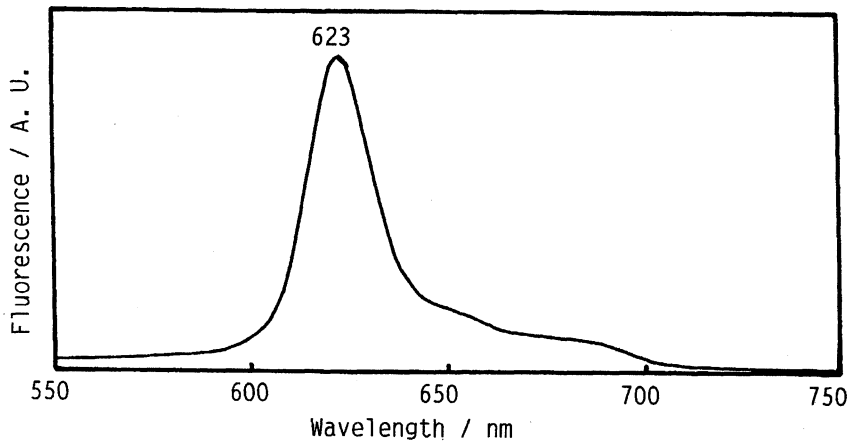


Fig. 7. Fluorescence spectrum of HP on silk fiber upon 400 nm excitation.

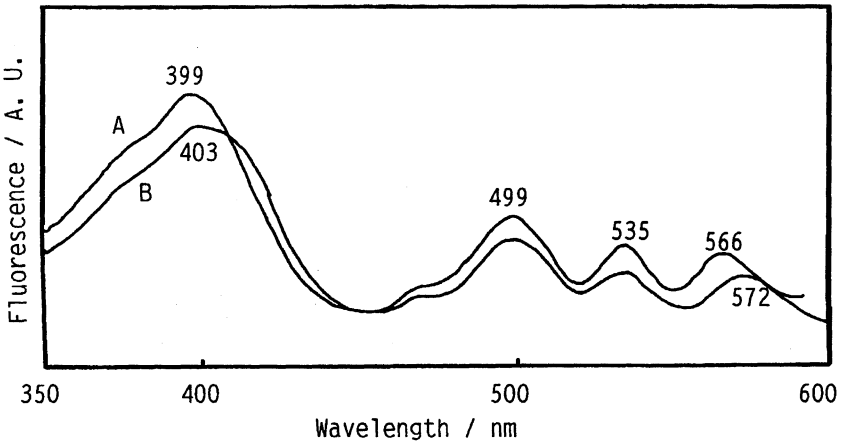


Fig. 8. Fluorescence excitation spectra of HP on silk fiber. Emission wavelength: 613 nm (A), 625 nm (B).

silk. The position of the excitation maximum in the Soret region is dependent on the emission wavelength. The fluorescence wavelength at 613 nm has an excitation maximum at 399 nm, while the fluorescence wavelength at 632 nm has an excitation maximum at 403 nm. These bathochromic shifts were larger than in the case of HP on the BH system.

Finally, the production of active-oxygen species from photoexcited HP on silk was investigated using ESR spin-trapping. As shown in Fig. 9, the signal observed from HP on silk (DMPO–OH and DMPO–OOH) was the same as that observed from HP on the the BH system (Fig. 4A). These results suggest that the hydroxyl radical is produced even in the absence of the iron(III) ion.

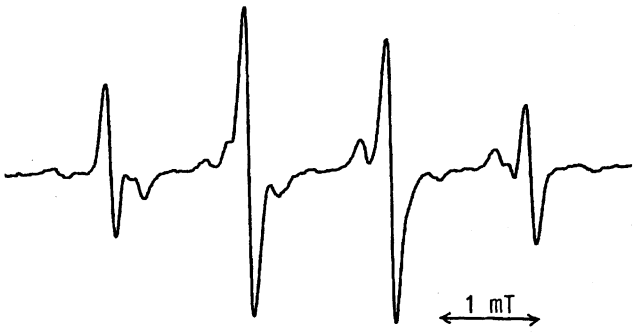


Fig. 9. ESR spectrum of the DMPO spin adduct obtained by 30-s irradiation of HP on silk fiber.

Production of Active-Oxygen Radicals from the Photoexcited HP Oligomer. In order to clarify the effect of the formation of molecular associates on the production of the hydroxyl radical, we attempted experiments to detect active-oxygen radicals from the photoexcited HP oligomer in PBS using ESR spin-trapping.

The ESR spectra of the DMPO spin adduct obtained from the photoexcited HP oligomer in PBS are shown in Fig. 10. The signal of Fig. 10A can also be assigned to DMPO–OOH and DMPO–OH, similar to the case of Fig. 4. The addition of ethanol to the solution changed the hydroxyl radical adduct into a C-centered radical adduct, as shown in Fig. 10B. These behaviors were similar to the case of Fig. 4, though the intensity of DMPO–OH was less than in the case of Fig. 4. From these results, the formation of a molecular associate plays a crucial role in the production of the hydroxyl radical.

ESR Detection of Singlet Oxygen. In order to confirm the production of the singlet oxygen in the system in more detail, spin-trapping experiments using TMPD[20] were carried out to detect the singlet oxygen.

The ESR spectra of 2, 2, 6, 6- tetramethyl- 4- oxo- 1- piperidinyloxyl (TEMPONE), obtained by 600-s irradiation of HP on the BH system, are shown in Fig. 11. A weak

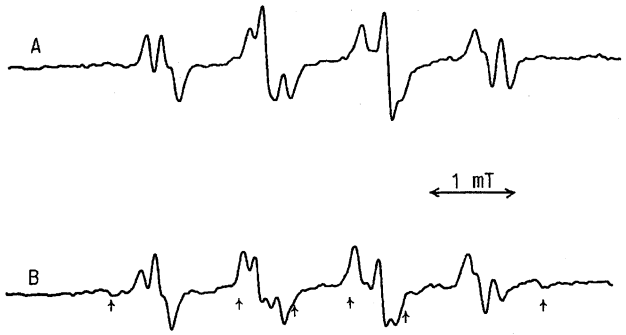


Fig. 10. ESR spectra of the DMPO spin adduct obtained by 60-s irradiation of the HP oligomer in PBS. A: control, B: 10% ethanol, arrow: DMPO–C-centered.

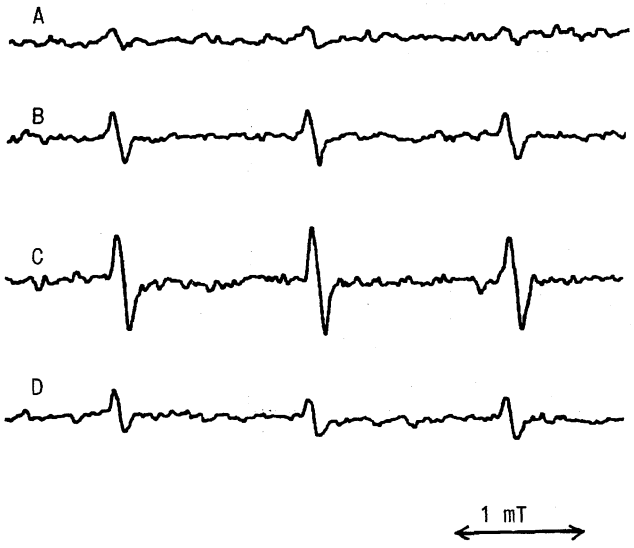


Fig. 11. ESR spectra of TEMPONE produced by irradiation of HP on the BH system. A: before irradiation, B: after 600-s irradiation, C: after 600-s irradiation containing 50% D_2O, D: C+1 mmol dm^{-3} NaN_3.

signal was observed before the irradiation of HP on the BH system, as shown in Fig. 11A. This was regarded as being the background signal. A small increase in the signal intensity of TEMPONE was observed after the irradiation of HP on the BH system, as shown in Fig. 11B. In general, the lifetime of the singlet oxygen becomes longer in the presence of deuterium compounds. Therefore, the effect of deuterium compounds on the signal intensity of TEMPONE was examined using deuterium oxide (D_2O). The presence of 50% D_2O led to a further increase in the intensity of TEMPONE (Fig. 11C). The addition of the singlet oxygen quencher NaN_3 inhibited the formation of TEMPONE (Fig. 11D). From these results, the production of singlet oxygen from photoexcited HP on the BH system was confirmed.

Role of Tryptophyl Residues on the Photodynamic Oxidation Process of the BH System. Figure 12 shows the fluorescence spectra obtained from the BH system upon 290 nm excitation. This fluorescence originates in protein tryptophyl residues, which are strongly affected by binding with HP.[21] In HP on the BH system, a decrease in the fluorescence intensity and blue shift were observed (Fig. 12B). This means that some interactions, such as energy and/or electron transfer, occur between the tryptophyl residues and HP.

Discussion

The focus of our research was to clarify the mechanism for the production and reaction of active-oxygen species from photoexcited hematoporphyrin (HP) on a biological heterogeneous (BH) system. To accomplish our objective we carried out characterization using fluorescence spectroscopy and the detection of active-oxygen species using ESR. In the following we discuss the mechanism for producing and reacting radical species from photoexcited HP on the BH system in relation to the adsorption structures of HP.

Adsorption Structures of HP on the BH System. The bathochromic shift shown in Fig. 1B is caused by some origins, such as the formation of molecular associates (see Figs. 1A and 1C) and an environmental effect (see Figs. 1C, 1D, and 7). Based on the results of the fluorescence excitation spectra, we now discuss the adsorption structures of HP on the BH system in the following in more detail.

In the homogeneous media of HP in 5% ethanol, the fluorescence positions at 613 and 640 nm have the same excitation maximum at 394 nm (see Figs. 2A and 2B), similar

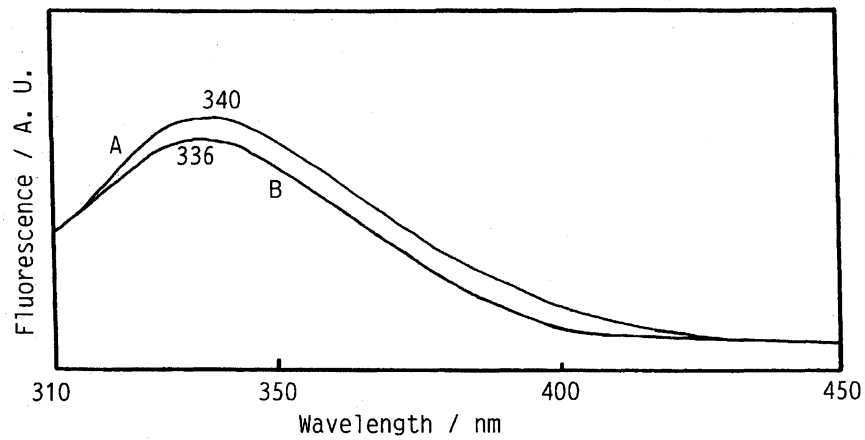


Fig. 12. Fluorescence spectra of HP on the BH system upon 290 nm excitation.

to that observed for HP in aqueous solution by Beltramini et al.[21] This excitation maximum and band width are the typical Soret band for monomeric HP.[22] In the homogeneous media of the HP oligomer in PBS, the fluorescence position at 632 nm has an excitation maximum at 404 nm (see Fig. 2C). This result means that the formation of molecular associates leads to a bathochromic shift of the excitation maximum. In general, the formation of molecular associates in homogeneous media leads to a bathochromic shift of the absorption spectra[23] and/or a decrease in the fluorescence efficiency.[24] Both effects were observed from the HP oligomer in PBS.

For HP on the BH system, the fluorescence emitted at 613 nm has an excitation maximum at 394 nm (see Fig. 3A), which is similar to monomeric HP. About a 5 nm bathochromic shift (399 nm) and a weak shoulder signal were observed based on the fluorescence emitted at 632 nm (see Fig. 3B). This cannot be easily assigned, because a different result was observed on HP on silk. The fluorescence emitted at 613 nm has an excitation maximum at 399 nm, while that emitted at 632 nm has an excitation maximum at 403 nm. Moreover, the same bathochromic shift (400 nm, emitted at 632 nm) has been observed in the case of lipoprotein bound HP by Beltramini et al.,[21] and has been assigned to monomeric HP in nonpolar media. Therefore, the 5 nm bathochromic shift (399 nm) of HP on the BH system may be due to an environmental effect of monomeric HP binding with some protein components, such as glycoprotein or protein in the BH system (see Fig. 12, binding with tryptophyl residue). Monomeric HP on a BH system having a fluorescence maximum at 394 nm might have bound with some components of the BH system except for protein. From the bathochromic shifts of the HP oligomer, both in PBS (404 nm) and on the BH system (401 and 410 nm) and HP on silk (403 nm), the weak shoulder signal (> 400 nm) of HP on the BH system may be due to the formation of molecular associates.

From the spectroscopic properties reported in this paper, we concluded that more than three adsorption structures of HP in addition to monomer exist on the BH system.

Mechanism for the Production and Reaction of Active-Oxygen Species. Generally, hematoporphyrin (HP) is first excited to its singlet state by light irradiation. The singlet state of HP (HP^S) rapidly changes to a triplet state (HP^T) through intersystem crossing [reaction (1)]. There are two processes in the reactions between HP^T and oxygen ($^3O_2$): one is an energy transfer from HP^T to oxygen (called Type II reaction), which results in the production of singlet oxygen ($^1O_2$) [reaction (2)]; the other is an electron transfer from HP^T to oxygen (called Type I reaction), and the superoxide ion (O_2^-) is produced [reaction (3)].

$$HP + h\nu \longrightarrow HP^S \longrightarrow HP^T \quad (1)$$

$$HP^T + {}^3O_2 \longrightarrow HP + {}^1O_2 \quad (2)$$

$$HP^T + O_2 \longrightarrow HP^+ + O_2^- \quad (3)$$

The production of singlet oxygen has been confirmed by the production of TEMPONE (see Fig. 9). The production of the superoxide ion has also been confirmed by using ESR spin-trapping (see Fig. 4).

Based on the ESR results observed from HP on the BH system, the possible mechanisms for producing and reacting active-oxygen species from HP^T on the BH system can be proposed:

$$HP^T + O_2^- + 2H^+ \longrightarrow HP^+ + H_2O_2, \quad (4)$$

$$O_2^- + \cdot O_2H + H^+ \longrightarrow H_2O_2 + O_2, \quad (5)$$

$$2O_2^- + 2H^+ \longrightarrow H_2O_2 + O_2, \quad (6)$$

$$2\cdot O_2H \longrightarrow H_2O_2 + O_2, \quad (7)$$

$$HP^T \text{ (or } HP^+ \cdots HP^-) + O_2^- + H_2O_2 \longrightarrow \cdot OH + OH^- + O_2 + HP \text{ (or } HP_n), \quad (8)$$

$$HP^T + D + H_2O_2 \longrightarrow \cdot OH + OH^- + D^+ + HP, \quad (9)$$

$$HP^+ + \text{Reducing Agent} \longrightarrow HP + \text{Oxidant}. \quad (10)$$

Many reactions are caused by electron transfers from HP^T on the BH system after the reaction (3) has occurred. The existence of hydrogen peroxide in our system was confirmed by the addition of catalase (see Fig. 6C). Hydrogen peroxide may be produced through two processes: an electron transfer from HP^T to the superoxide ion [reaction (4)] and the disproportionation of the superoxide ion and/or its protonated (about 1% of O_2^- in pH=7) [reactions (5)—(7)]. The hydroxyl radical, which is the major product of our system (see Fig. 4), may be produced by a three-electron reduction of oxygen, such as the Harber–Weiss reaction catalyzed by HP^T (or associates: $HP^+ \cdots HP^-$, see Fig. 10) [reaction (8)] and the reaction between HP^T and H_2O_2 in the presence of an electron donor (D), such as tryptophan [reaction (10)]. The former is presumed based on the decrease in the DMPO–OH intensity upon the addition of SOD (see Figs. 5 and 6), and from hydroxyl radical production from HP on silk in the absence of the iron(III) ion (see Fig. 9). The latter is also presumed based on the fluorescence spectra, showing that an interaction exists between tryptophyl residues and HP (see Fig. 12). However, the contribution of the latter may be smaller than that of the former, because only a slight increase in the intensity of the ESR signal was observed upon the addition of H_2O_2 (described in the results section). The three-electron reduction of oxygen can be regarded as being a characteristic feature, not only of the formation of molecular associates, but also the formation of the heterogeneous system containing monomeric HP on protein (see Figs. 4, 9, and 10), similar to the system of chlorophylls on MgO.[18] The occurrence of reaction (10) is expected because of the existence of various electron donors, such as tryptophyl residues or other amino acid in the BH system, though the production of the cation radical was not confirmed by the ESR measurements.

In summary, we have demonstrated that the adsorption structures of HP and the mechanism for producing and reacting active-oxygen species from photoexcited HP on the BH system can be explained based on an analysis of the fluorescence and ESR spectra. The observed results of our report

represent a useful approach to understanding the mechanism for killing tumor cells by active-oxygen species from photoexcited photosensitizers in a heterogeneous system.

References

1) R. C. Benson, J. H. Kinsey, D. A. Cortese, G. M. Farrow, and D. C. Utz, *J. Urol.*, **130**, 1090 (1980).

2) D. Kessel and T-H. Chou, *Cancer Res.*, **43**, 1994 (1983).

3) A. Dahlman, A. G. Wile, R. G. Burns, G. R. Mason, F. M. Johnson, and M. W. Berns, *Cancer Res.*, **43**, 430 (1983).

4) L. Tomio, P. L. Zorat, L. Corti, P. Calzavara, F. Reddi, and G. Jori, *Acta Radiol., Oncol., Radiat. Phys., Biol.*, **22**, 49 (1983).

5) Y. Hayata, H. Kato, C. Konaka, T. Okunaka, and K. Furukawa, "Photodynamic Therapy and Biomedical Lasers," ed by P. Spinelli, M. D. Fante, and R. Merchesini, Elsevier Science Publishers B. V., Amsterdam (1992), pp. 1—5.

6) A. Blum and L. I. Grossweiner, *Photochem. Photobiol.*, **41**, 27 (1985).

7) J. P. Keene, D. Kessel, E. J. Land, R. W. Redmond, and T. G. Truscott, *Photochem. Photobiol.*, **43**, 117 (1986).

8) R. W. Redmond, K. Heihoff, S. E. Braslsvsky, and T. G. Truscott, *Photochem. Photobiol.*, **45**, 209 (1987).

9) J. van Steveninck, K. Tijssen, J. P. J. Boegheim, J. van der Zee, and T. M. A. R. Dubbelman, *Photochem. Photobiol.*, **44**, 711 (1986).

10) M. A. J. Rodgers, "Photodynamic Therapy of Tumors and Other Diseases," ed by G. Jori and C. Perria, Edizioni Libreria Progetto, Padova (1985), pp. 21—35.

11) R. Langlois, H. Ali, N. Brasseur, J. R. Wagner, and J. E. Van Lier, *Photochem. Photobiol.*, **44**, 117 (1986).

12) G. Valduga, S. Nonell, E. Reddi, G. Jori, and S. E. Braslavsky, *Photochem. Photobiol.*, **48**, 1 (1988).

13) N. Miyoshi, T. Kondo, M. Ishihara, M. Kobayashi, M. Komiyama, I. Sakata, and M. Fukuda, "Magnetic Resonance in Medicine No. 6," ed by H. Kamada, H. Ohya-Nishiguchi, and T. Yoshikawa, Nihon Igakukan, Tokyo (1995), pp. 288—290.

14) B. J. Cathey, "Yeast, A Practical Approach," ed by I. Campbell and J. H. Duffus, IRL Press, Washington, D. C. (1988), p. 163.

15) R.Konaka, M. Kawai, H. Noda, M. Kohno, and R. Niwa, *Free Rad. Res.*, **23**, 15 (1995).

16) J. R. Harbour and M. L. Hair, *J. Phys. Chem.*, **81**, 1791 (1977).

17) Finkelstein, G. M. Rosen, and E. J. Rauckman, *J. Am. Chem. Soc.*, **102**, 4994 (1980).

18) H. Noda, K. Oikawa, H. Ohya-Nishiguchi, and H. Kamada, *Chem. Lett.*, **1994**, 1949.

19) J. R. Harbour, S. L. Issler, and M. L. Hair, *J. Am. Chem. Soc.*, **102**, 7778 (1980).

20) Y. Lion, M. Delemile, and A. van de Vorst, *Nature*, **263**, 442 (1976).

21) M. Beltramini, P. A. Firey, F. Ricchelli, M. A. J. Rodgers, and G. Jori, *Biochemistry*, **26**, 6852 (1987).

22) A. Andreoni, R. Cubeddu, S. de Silvestri, P. Laporta, G. Jori, and E. Reddi, *Chem. Phys. Lett.*, **88**, 33 (1982).

23) R. V. Bensasson, E. J. Land, and T. G. Truscott, "Excited States and Free Radicals in Biology and Medicine," Oxford University Press Inc., New York (1993), pp. 322—345.

24) A. Andreoni, R. Cubeddu, S. de Silvestri, P. Laporta, G. Jori, and E. Reddi, *Z. Naturforsch. C*, **38**, 83 (1983).