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Electron transfer mediated decomposition of folic acid by photoexcited dimethoxophosphorus(V)porphyrin

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ABSTRACT

A water soluble porphyrin, dimethoxophosphorus(V)tetraphenylporphyrin (MP(V)TPP) photosensitized folic acid decomposition in aqueous solution, resulting in the formation of a strongly fluorescent pteridine compound. The quantum yield of folic acid decomposition by photoexcited MP(V)TPP could be determined by a flurometry. A possible mechanism of the initial process of this photodecomposition is direct oxidation through an electron transfer from folic acid to the excited singlet state of MP(V)TPP. It is considered that this electron transfer proceeds as a diffusion-controlled reaction. The quantum yields of the electron transfer and the following process of folic acid decomposition could be determined. In deuterium oxide, folic acid decomposition through the photosensitized singlet oxygen generation by MP (V)TPP was also observed. A fluorometry based on the formation of a strong fluorescent compound through the oxidized decomposition of folic acid may be applied to evaluate the biomolecule damaging-activity of photosensitizers. In addition, a protocol of this assay is proposed. This simple photosensitized reaction in aqueous solution may be used for a first screening of a photosensitizer for phototherapy.

1. Introduction

Photochemical processes such as photosensitized singlet oxygen ($^1O_2$) generation and photo-induced electron transfer are important mechanisms of photodynamic therapy (PDT) of cancer [1–3]. In general, PDT process can be explained by the $^1O_2$ generation. Administered photosensitizers in tumor photosensitize $^1O_2$ generation, resulting in oxidative damage to biomacromolecules of cancer cells. However, the PDT effect by $^1O_2$ generation is insufficient, because the oxygen concentration in tumor is lower than that in normal cells [4–6]. Thus, we have studied the development of photosensitizer, which can induce biomolecule damage through photo-induced electron transfer mechanism [7]. Many photosensitizers absorbing ultraviolet ray can oxidize biomacromolecules, such as DNA, through the electron transfer from the targeting molecule to photoexcited state of photosensitizer [8]. However, the oxidative activity of photosensitizer absorbing visible-light is relatively small and the electron transfer-mediated oxidation of biomolecules is difficult. For PDT, porphyrins have been extensively studied as photosensitizers [9–12]. Since porphyrins are visible light photosensitizers, the electron transfer-mediated mechanism is not appropriate. However, phosphorus(V) complex of porphyrins demonstrate a relatively strong oxidative activity in their photoexcited state [7,8,12]. To evaluate the biomacromolecule damaging activity of photosensitizers through electron transfer mechanism, development of a simple analysis method is expected.

Folic acid (Fig. 1), a B vitamin, can be decomposed by oxidation through photo-induced electron transfer [13–18]. Since the highest occupied molecular orbital (HOMO) of folic acid is located on its aminobenzoyl moiety, the electron transfer from this moiety to the photoexcited pteridine moiety can occur [19]. The electron transfer-mediated oxidation of aminobenzoyl moiety leads to the decomposition of folic acid and the formation of strongly fluorescent pteridine compounds [13–18,20,21]. Recently, we reported the decomposition of folic acid through photo-induced electron transfer by rhodamine-6G [22], a fluorescent dye, and the resulting fluorescence enhancement. Although a reaction of PDT occurs in the complex biological system, similar method may be applied to evaluate the activity of PDT photosensitizers simply, for

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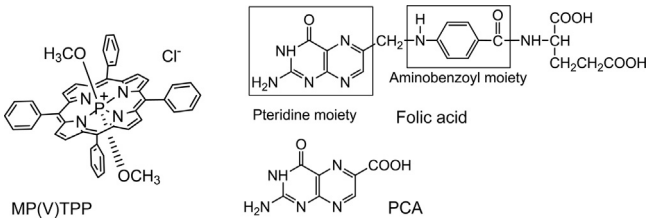

Fig. 1. Structures of MP(V)TPP, folic acid, and PCA.

example, a first screening of PDT drug. In the present study, to develop the simple method to evaluate the activity of electron transfer photosensitizer, the photosensitized decomposition of folic acid by a water soluble porphyrin, dimethoxophosphorus(V) tetraphenylporphyrin (MP(V)TPP, Fig. 1) was examined. MP(V)TPP can photosensitize protein damage through electron transfer and $^1O_2$ generation [23]. The synthesis of this porphyrin is relatively easy and its water-solubility is relatively large. Therefore, MP(V)TPP was used as a model of electron transfer photosensitizer.

2. Experimental

MP(V)TPP was synthesized according to the previous reports [24,25]. Folic acid, sodium azide (NaN_3), and potassium iodide (KI) were purchased form Wako Pure Chemical Industries, Ltd., (Osaka, Japan). 2-Amino-4-oxo-3*H*-pterine-6-carboxylic acid (PCA) was from Sigma–Aldrich Co., LLC. (St. Louis, MO, USA). Sodium phosphate buffer (pH 7.6) was from Nakalai Tesque Inc. (Kyoto, Japan). Distilled water (H_2O) was from Kanto Chemical Co., Inc., (Tokyo Japan). Deuterium oxide (D_2O) was from Acros Organics (Geel, Belgium).

The sample solution containing 5 μM MP(V)TPP and 10 μM folic acid in a 10 mM sodium phosphate buffer (pH 7.6) was irradiated with a light-emitting diode (LED) (λ_{max} = 585 nm, 1 mW cm^{-2}, CCS Inc., Kyoto, Japan). The intensity of the LED light source was measured with a photo-power meter (8230E, ADCMT, Tokyo, Japan). After photo-irradiation, the fluorescence spectrum of folic acid was measured with an F-4500 fluorescence spectrophotometer (Hitachi, Tokyo, Japan). To evaluate the decomposed folic acid by a fluorometry, the fluorescence intensity at 450 nm was measured with a fluorescence photometer 650-60 (Hitachi). The excitation wavelength for these measurements is 350 nm. Because oxygen molecule is required in the decomposition process of folic acid, the fluorescence enhancement of folic acid is not observed under anaerobic condition. Therefore, this assay was performed under air saturated condition. To evaluate the oxygen-independent electron transfer reaction, an effect of $^1O_2$ scavenger, NaN_3, was investigated. The above mentioned sample solution with or without NaN_3 was used for the photosensitized reaction.

Absorption spectra of folic acid, PCA, and MP(V)TPP in a 10 mM sodium phosphate buffer (pH 7.6) were measured with a UV–vis spectrophotometer UV-1650PC (Shimadzu, Kyoto, Japan). Fluorescence decay was measured using a time-correlated single-photon counting method with a Fluorescence Lifetime System TemPro (HORIBA, Kyoto, Japan). Laser excitation at 394 nm (or 571 nm) was achieved by using a diode laser (NanoLED-390 (or NanoLED-570), HORIBA) in a repetition rate of 1.0 MHz. The detection wavelength is >640 nm by using a long-pass filter SCF-50S-64R (SIGMAKOKI Co., Ltd., Tokyo, Japan). The experimental error of this measurement is within 0.01 ns. The sample solution contained 5 μM MP(V)PP and indicated concentration of folic acid in a 10 mM sodium phosphate buffer (pH 7.6).

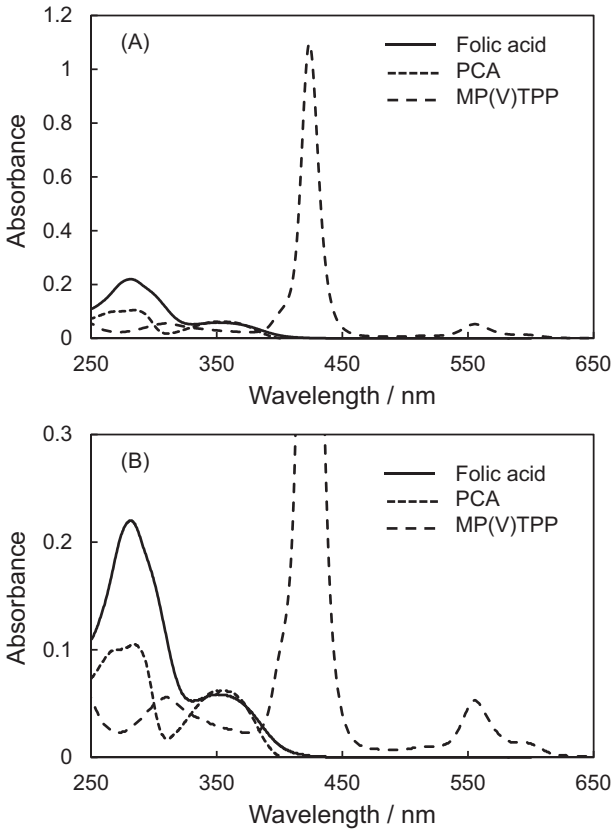

Fig. 2. Absorption spectra of folic acid, PCA, and MP(V)TPP. The sample solution contained 10 μM folic acid, 10 μM PCA, or 5 μM MP(V)TPP in a 10 mM sodium phosphate buffer (pH 7.6). (B) is the magnified figure of (A).

3. Results and discussion

3.1. Photosensitized decomposition of folic acid by MP(V)TPP

The absorption spectra of folic acid, PCA, and MP(V)TPP in a 10 mM sodium phosphate buffer (pH 7.6) were shown in Fig. 2. In general, PDT photosensitizers including porphyrins can absorb light of longer wavelengths than that by folic acid, up to around 600–700 nm. Therefore, selective photoexcitation of porphyrins is possible. The absorption spectrum of MP(V)TPP was not affected by folic acid (data not shown), suggesting that the static interaction between MP(V)TPP and folic acid is negligibly small. Therefore, a diffusion process is necessary for the photosensitized decomposition of folic acid by MP(V)TPP.

The fluorescence intensity assigned to the pteridine moiety of folic acid was increased by the photosensitized reaction of MP(V)TPP (Fig. 3). It was confirmed that MP(V)TPP without photo-irradiation did not enhance the fluorescence of folic acid. Consequently, this fluorescence enhancement indicates the decomposition of folic acid through the photosensitized reaction by MP(V)TPP. The decomposed folic acid was calculated using the calibration curve method on the fluorescence intensity of PCA which is the fluorescent decomposed product of folic acid (Fig. 1 and Supplementary information). The quantum yield of the photosensitized folic acid decomposition in sodium phosphate buffer (pH 7.6, H_2O) (Φ_D) was estimated as follows:

$$\Phi_D = \frac{[PCA] \times V}{\int (1 - 10^{-\mathrm{Abs}(\nu)}) I(\nu) S d\nu \times T} \quad (1)$$

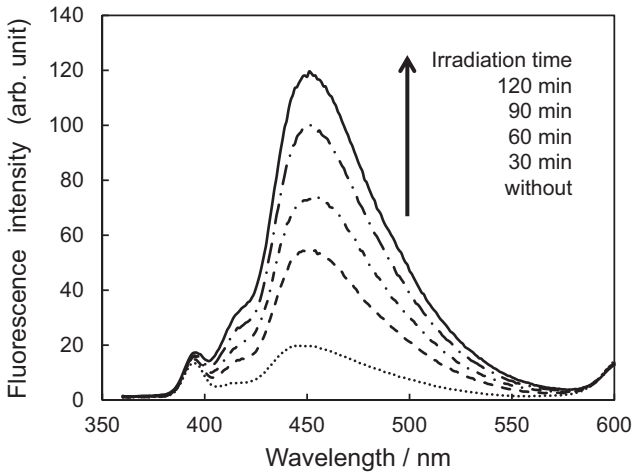

Fig. 3. Fluorescence spectra of folic acid photosensitized by MP(V)TPP. The sample solution containing 5 μM MP(V)TPP and 10 μM folic acid in a 10 mM sodium phosphate buffer (pH 7.6) was irradiated with an LED (λ_{max} = 585 nm, 1 mW cm^{-2}). The excitation wavelength is 350 nm.

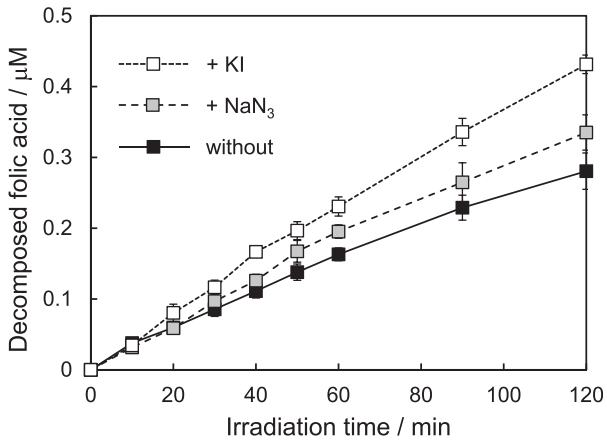

Fig. 5. Effects of sodium azide and potassium iodide on the photosensitized decomposition of folic acid by MP(V)TPP. The sample solution containing 5 μM MP (V)TPP and 10 μM folic acid with or without 1 mM NaN_3 or 1 mM KI in a 10 mM sodium phosphate buffer (pH 7.6) was irradiated with an LED (λ_{max} = 585 nm, 1 mW cm^{-2}). The concentration of decomposed folic acid was determined by a fluorometry. The excitation wavelength is 350 nm.

where, [PCA] represents the concentration of PCA formed, V, the volume of the sample solution (1.2 mL), Abs(ν), the absorbance of MP(V)TPP, $I(\nu)$, the photon fluence of the light source at the indicated light frequency (μmol cm^{-2} min^{-1} s), ν, the light frequency (s^{-1}), S, the irradiated area (1 cm^2), and T, the irradiation time (min). The obtained value of Φ_D was 1.1×10^{-4}.

3.2. Mechanism of the photosensitized decomposition of folic acid by MP(V)TPP

The sample solutions containing 5 μM MP(V)TPP with 10 μM folic acid in H_2O or D_2O were prepared. These samples were irradiated with an LED (λ_{max} = 585 nm, 1 mW cm^{-2}) and the folic acid decomposition was examined by a fluorometry. The decomposed folic acid (formed PCA) increased in a dose-dependent manner in H_2O or D_2O (Fig. 4). This result demonstrated that the photodecomposition rate of folic acid in D_2O is significantly larger than that in H_2O. Because the lifetime of $^1O_2$ is markedly elongated in D_2O (about 3.5 μs in H_2O [26] to more than 70 μs in D_2O [27]), the rate of the $^1O_2$-mediated reaction is accelerated in D_2O. Indeed,

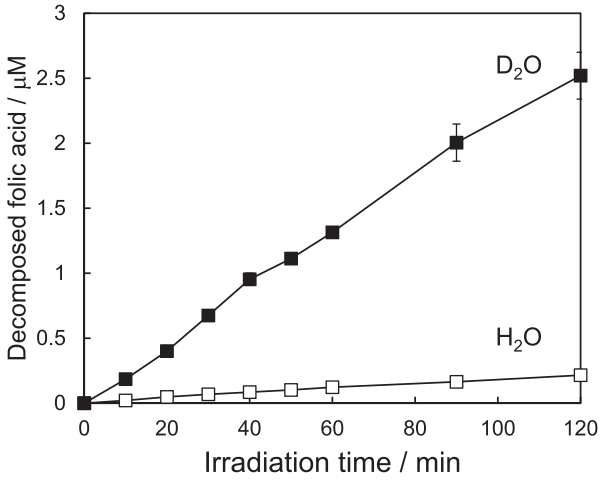

Fig. 4. Solvent deuterium effect on the photosensitized decomposition of folic acid by MP(V)TPP. The sample solution containing 5 μM MP(V)TPP and 10 μM folic acid in H_2O or D_2O was irradiated with an LED (λ_{max} = 585 nm, 1 mW cm^{-2}). The concentration of decomposed folic acid was determined by a fluorometry. The excitation wavelength is 350 nm.

the quantum yield of $^1O_2$ generation by MP(V)TPP is relatively large (0.64) [23]. These findings suggest that $^1O_2$ generation can be an important mechanism of biomolecules damage by MP(V)TPP in a certain condition. However, NaN_3, a typical quencher of $^1O_2$ [28], did not inhibit the folic acid decomposition photosensitized by MP (V)TPP and rather enhanced this photo-decomposition (Fig. 5). Potassium iodide, a typical quencher of excited triplet (T_1) state, also enhanced this photo-decomposition (Fig. 5). These strange results could be reasonably explained as follows: $^1O_2$-mediated damage of folic acid in aqueous solution is negligible and the electron transfer mechanism is mainly responsible for the photo-sensitized folic acid decomposition. Indeed, a previous report demonstrated that folic acid cannot be decomposed by $^1O_2$ in aqueous solution of H_2O due to its short lifetime [29]. Because MP (V)TPP does not statically interact with folic acid, a diffusion process is necessary to decompose folic acid. The lifetime of $^1O_2$ is short in H_2O and most $^1O_2$ generated by MP(V)TPP deactivates in solution. Furthermore, some part of the generated $^1O_2$ around MP (V)TPP molecule induces oxidative self-degradation of MP(V)TPP, resulting in the deactivation. Therefore, the addition of NaN_3 slightly recovered the photosensitized folic acid decomposition by MP(V)TPP. Because, in general, $^1O_2$ is formed through energy transfer from the T_1 state of photosensitizer, the similar effect of KI could be explained by the similar mechanism to that of NaN_3. In addition, the effect of KI suggests that the contribution of the T_1 state of MP(V)TPP to folic acid photo-decomposition is negligible. Since the oxidative activity of T_1 state is smaller than that of excited singlet (S_1) state, the electron transfer-mediated oxidation of folic acid by the T_1 state may be thermodynamically difficult (described in later).

The initial process of photosensitized decomposition of folic acid by MP(V)TPP can be explained by the photo-induced electron transfer from folic acid to the S_1 state of MP(V)TPP. The HOMO of folic acid is located on its aminobenzoyl moiety (Fig. 1) [19], and the electron abstraction from this moiety should trigger the oxidative decomposition of folic acid (Supplementary information) [13–18]. The Gibbs free energy (ΔG) of this electron transfer can be calculated by the following equation:

$$\Delta G = e(E_{ox} - E_{red}) - E_{S_1} \quad (2)$$

where, e is the charge of the electron, E_{ox} and E_{red} are the redox potentials of one-electron oxidation of the aminobenzoyl moiety of folic acid (0.84 V vs. saturated calomel electrode (SCE)) [30] and

one-electron reduction of MP(V)TPP (−0.50 V vs. SCE) [23], respectively, and E_{S_1} is the S_1 energy of MP(V)TPP (2.05 eV, estimated from the fluorescence maximum: 606 nm). The estimated value ($-\Delta G$ = 0.71 eV) supported that electron transfer from folic acid to the S_1 of MP(V)TPP is possible in the terms of thermodynamics.

The $-\Delta G$ value of the electron transfer from folic acid to the T_1 state of MP(V)TPP can be estimated from the similar equation of Eq. (2), of which the E_{S_1} is replaced with the T_1 energy (E_{T_1}). Since the E_{T_1} of similar porphyrin is around 1.5 eV [31,32], the roughly estimated value of $-\Delta G$ is 0.2 eV, suggesting that the electron transfer-mediated oxidation of folic acid by the T_1 state of MP(V)TPP is exothermic reaction. However, in this experimental condition, the photosensitized folic acid decomposition by the T_1 state of MP(V)TPP could not be demonstrated. The driving force of around 0.2 eV might be insufficient (described in Section 3.3).

The MP(V)TPP radical, formed through the electron transfer from folic acid, should be returned to the original cationic form of MP(V)TPP possibly by molecular oxygen under aerobic condition. In the biological system, other oxidative materials such as metal ions may assist the re-oxidation of this MP(V)TPP radical. The photoreaction of MP(V)TPP should proceed catalytically.

The sample solution containing 5 μM MP(V)TPP with or without folic acid in a 10 mM sodium phosphate buffer (pH 7.6) were prepared to measure the time profile of fluorescence intensity. The time profile of MP(V)TPP fluorescence intensity could be analyzed by a single exponential function (Supplementary information). The obtained fluorescence lifetime (τ_f) was 5.2 ns in a 10 mM sodium phosphate buffer (pH 7.6). The electron transfer should occur within this lifetime. To evaluate the electron transfer rate constant, the τ_f with folic acid was measured. A significant change of τ_f was not observed in the presence of relatively small concentration (around 10 μM) of folic acid, which is a similar condition of the photosensitized reaction (Supplementary information). Thus, the values of τ_f with relatively higher concentration of folic acid were measured (Supplementary information). The time profiles of fluorescence decay of MP(V)TPP with high concentration of folic acid could be also fitted by single exponential function. The analyzed values of τ_f were slightly and gradually decreased depending on the concentration of folic acid. To obtain the electron transfer rate coefficient (k_{ET}), these values were analyzed by the Stern–Volmer plot (Fig. 6) using the following equation:

$$\frac{1}{\tau_f} = \frac{1}{\tau_f^0} + k_{ET}[FA] \quad (3)$$

where, τ_f^0 is the τ_f without folic acid and [FA] is the concentration of folic acid. The resulting value of k_{ET} ($4.2 \times 10^9\ M^{-1}\ s^{-1}$) was comparable with the value of diffusion control limit ($k_{dif} = 7.4 \times 10^9\ M^{-1}\ s^{-1}$; Supplementary information). Consequently, the quantum yield of the electron transfer process (Φ_{ET}) can be expressed as follows:

$$\Phi_{ET} = \frac{k_{ET}[FA]}{\frac{1}{\tau_f^0} + k_{ET}[FA]} \quad (4)$$

In this experimental condition ([FA] = 10 μM), the value of Φ_{ET} is 2.2×10^{-4}. The formed charge-transfer (CT) state undergoes further reaction, leading to the decomposition of folic acid [13–18]. Alternatively, some part of the CT state can return to the ground state through reverse-electron transfer. The reaction quantum yield of the CT state to the decomposed product (Φ_r) can be calculated using the following equation:

$$\Phi_r = \frac{\Phi_D}{\Phi_{ET}} \quad (5)$$

The calculated value of Φ_r (0.50) suggests that the efficiency of the reverse-electron transfer (Φ_{RET} = 0.50) is relatively large.

3.3. Protocol to evaluate the activity of photosensitizer using folic acid

This photosensitized reaction of folic acid decomposition and resulting fluorescence enhancement can be applied to the evaluation of the biomolecule damaging-activity of photosensitizers. Because the PDT reaction occurs in the complex biological system, this method may be used for a first screening process of PDT drug. In general, the mechanisms of photosensitized biomolecule damage can be explained by two processes, electron transfer (Type I) and $^1O_2$ generation (Type II) [33]. It has been reported that $^1O_2$ cannot decompose folic acid in aqueous solution, whereas it is possible in D_2O [29]. This study also demonstrated that a $^1O_2$ quencher does not inhibit the photosensitized folic acid decomposition by MP(V)TPP. Therefore, the electron transfer-mediated biomolecule damaging-activity of photosensitizers can be simply evaluated by a fluorometry using folic acid in aqueous solution.

For this fluorometry, a sample solution containing 10–20 μM folic acid and photosensitizers are mixed in aqueous solution. Because folic acid solution exhibits optimum stability at pH 7.6, the pH range 6.0–8.0 is recommend. For example, sodium phosphate buffer (pH 7.6) is appropriate. The concentration of the photosensitizers should be adjusted to the appropriate absorbance of the fluorescence apparatus used for this assay. A visible-light-emitting diode (LED) is recommended as the light source. The sensitivity of this analysis is proportional to the irradiation time, and several minutes (10–30 min) may be sufficient for a general photosensitizer. The biomolecules damaging-activity of photosensitizers can be evaluated by the determination of the Φ_D, which can be estimated by Eq. (1).

Relevantly, photosensitized folic acid decomposition by other compounds, riboflavin [15,17,34] and rhodamine-6G [22] were reported. The folic acid decomposition by these molecules can be explained by electron transfer mechanism. The $-\Delta G$ values of riboflavin and rhodamine-6G are 0.81 eV and 0.34 eV [22], respectively. The $-\Delta G$ value of the case of riboflavin was estimated from the reported redox potential [35] and excitation energy [36] using the Eq. (2). In the case of rhodamine-6G, the reported value of

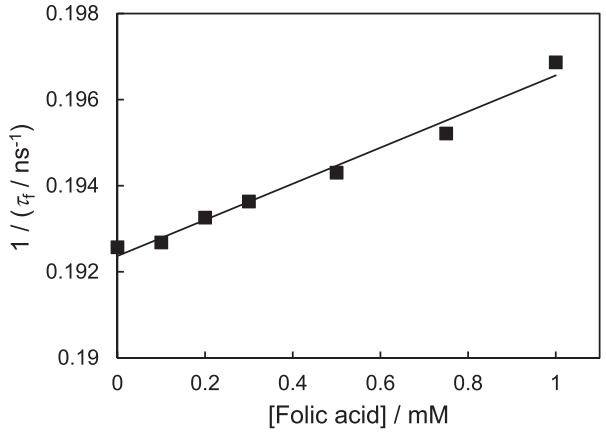

Fig. 6. Stern–Volmer plot of the fluorescence quenching of MP(V)TPP by folic acid. The sample solution contained 5 μM MP(V)TPP and the indicated concentration of folic acid in a 10 mM sodium phosphate buffer (pH 7.6). The excitation wavelength is 571 nm. The detection wavelength is >640 nm.

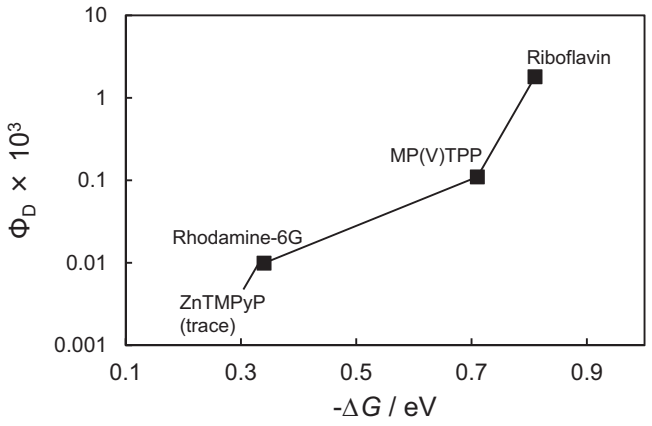

Fig. 7. Relationship between the quantum yields of photosensitized folic acid decomposition by photosensitizers and their driving forces.

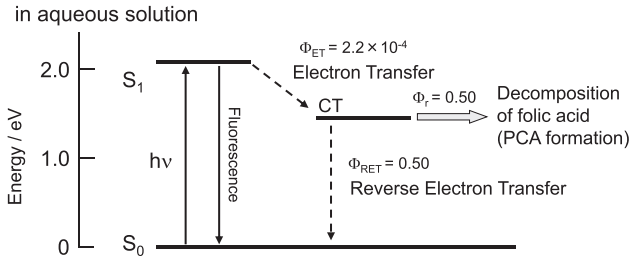

Fig. 8. Relaxation process of photoexcited MP(V)TPP and the photosensitized folic acid decomposition by MP(V)TPP in aqueous solution. The indicated value of Φ_{ET} depends on the concentration of folic acid. The present value is that in the presence of 10 μM folic acid.

Φ_D is relatively small (9.9×10^{-6}) [22]. In addition, the Φ_D of riboflavin was estimated by this method (Supplementary information) and the resulting value is relatively large (1.8×10^{-3}). Thus, we evaluated the Φ_D of zinc tetrakis(*N*-methyl-*p*-pyridinio) porphyrin (ZnTMPyP, Supplementary information), however, the folic acid decomposition was hardly observed and the Φ_D could not be determined. Although, photosensitized $^1O_2$ generation by ZnTMPyP was reported [37], the electron transfer reaction by ZnTMPyP was not strong. The estimated value of $-\Delta G$ for the case of ZnTMPyP from the redox potential [38] and the S_1 energy estimated from the fluorescence maximum in sodium phosphate buffer (625 nm) was 0.29 eV. Fig. 7 shows the summary of the driving force dependence of photosensitized folic acid decomposition. These findings suggest that the threshold of $-\Delta G$ value for this assay is around 0.30 eV. This result supports that the T_1 state of MP(V)TPP cannot oxidize folic acid, because the $-\Delta G$ value of this process is about 0.2 eV. The electron transfer process, which directly depends on the $-\Delta G$, is the initial step of the folic acid decomposition and resulting fluorescence enhancement. Although the Φ_D does not directly depend on the $-\Delta G$ and the comparison between electron transfer rate constant and $-\Delta G$ is more appropriate, this plot could be used to evaluate the threshold of the driving force for photosensitized folic acid decomposition.

4. Conclusions

Folic acid decomposition could be photosensitized by MP(V)TPP, a phosphorus(V)porphyrin photosensitizer. The proposed mechanism of the folic acid photo-decomposition by MP(V)TPP in aqueous solution is the photo-induced electron transfer (Fig. 8). Within the S_1 state lifetime (5.2 ns), electron transfer occurs from the aminobenzoyl moiety of folic acid to the S_1 state of MP(V)TPP. The formed CT state leads to the decomposition of folic acid. The decomposed product of folic acid is a strongly fluorescent compound, PCA, resulting in the fluorescence enhancement [13–18]. In D_2O, $^1O_2$-medided folic acid decomposition was observed. Therefore, $^1O_2$ is also an important species responsible for biomolecule damage by photoexcited MP(V)TPP in a certain condition. Indeed, MP(V)TPP binds to protein and can induce $^1O_2$-medided damage at amino acid residue [23]. In a hydrophobic environment such as cell membrane or protein, $^1O_2$-mediated biomolecule damage by MP(V)TPP might be important. In addition, this method using folic acid may be applied to the fluorometry system to examine the biomolecule damaging-activity of PDT photosensitizer through electron transfer mechanism in aqueous solution.

Acknowledgment

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jphotochem.2015.11.028.

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