

QUENCHING OF TYPE II PHOTSENSITIZERS IN THEIR TRIPLET STATES BY α -TOCOPHEROL VIA AN ELECTRON TRANSFER REACTION

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Abstract—Occurrence of an electron (or H atom equivalent to one electron plus H⁺) transfer from α -tocopherol (α TOH) to a number of photosensitizers in their triplet states were investigated by monitoring the ESR signal of α -chromanoxyl radical (α TO \cdot) in ethanolic solutions of α TOH and the sensitizers under continuous illumination. Every sensitizer molecule examined, such as protochlorophyllide (Pchl), hematoporphyrin and rose bengal which are generally regarded as efficient type II photosensitizers and thus have long-lived triplet states, was found to actively participate in an electron transfer reaction with α TOH even under air-saturation conditions, generating α TO \cdot . The reaction mechanism seemed to involve a noncovalent triplet sensitizer- α TOH complex as an intermediate in a fashion of Michaelis-Menten type of reaction. For the reaction of α TOH with triplet Pchl, the rate law was derived by applying the steady state approximation for the binary complex, triplet Pchl- α TOH, which turned out to be well consistent with the kinetic data.

INTRODUCTION

Some biological membranes that are routinely exposed to high intensity light are vulnerable to light-induced deterioration which involves lipid peroxidation and oxidative modification of proteins.¹ Such membrane photodamage is believed to largely result from photodynamic reactions promoted by certain endogenous photosensitizers. A good photosensitizer must absorb photon efficiently, it must have a high quantum yield of triplet formation, and the triplet must be long-lived. A category of compounds occurring in plant cells, especially in chloroplasts, which meets these conditions are chlorophyll and chlorophyll-related porphyrins. Chlorophyll in the free state is a well-known type II photosensitizer. In photosynthetic systems, however, the energy transfer and the primary photochemical processes proceed so fast that the chance to populate singlet oxygen (¹O₂), which is produced *via* the interaction between triplet chlorophyll and oxygen, is effectively eliminated.² Notwithstanding, free chlorophyll contained, albeit minimally, in the light harvesting chlorophyll-protein complexes has been suggested to be responsible for photoproduction of ¹O₂ in thylakoids.³ Protochlorophyllide (Pchl) is a precursor of chlorophyll and thus present in leaves under ordinary conditions where chlorophyll

biosynthesis takes place.⁴ Like other porphyrins, Pchl has been regarded as a photogenerator of ¹O₂.⁵

Chloroplasts contain high concentrations of powerful antioxidant molecules in their membranes, such as carotenoids and α -tocopherol (α TOH), which protect against photodynamic damage to membranes.⁶ Photoprotective function of carotenoids is associated with their ability to quench triplet chlorophyll and ¹O₂ very effectively.⁷ Although the major function of α TOH in membranes is inferred from its reactivity with a variety of free radicals at fairly high rates, terminating free radical-mediated chain reactions involving membrane components,⁸ this lipophilic antioxidant also quenches ¹O₂ physically and chemically.⁹ Besides, quenching of triplet type II sensitizer by α TOH has been speculated, but no convincing evidence is available yet. Because α TOH is readily oxidized by chemical species that can abstract an electron (or H atom) from an oxidizable molecule, yielding α -chromanoxyl free radical (α TO \cdot), the triplet quenching process by α TOH, if it indeed occurs, could proceed *via* an electron transfer rather than an energy transfer. This point was the subject of experimental scrutiny in the present study, using several compounds including Pchl that are generally thought as type II photosensitizers. In the case of the triplet Pchl- α TOH reaction, the investigation was further extended to the reaction mechanism and the results were interpreted in terms of Michaelis-Menten type of kinetics.

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[†] Abbreviations: ¹O₂, singlet oxygen; ³Pchl, triplet protochlorophyllide; ³Sen, triplet photosensitizer; ALA, 5-aminolevulinic acid; ESR, electron spin resonance; Pchl, protochlorophyllide; RNO, N,N-dimethyl-4-nitrosoaniline; Sen, photosensitizer; TEMP, 2,2,6,6-tetramethylpiperidine; TEMPO, 2,2,6,6-tetramethylpiperidine-N-oxyl; α TO \cdot , α -chromanoxyl radical; α TOH, α -tocopherol

MATERIALS AND METHODS

Preparation of Pchl. Pchl was isolated from the shoots of rice (*Oryza sativa* L.) seedlings, grown hydroponically in Hoagland

solution for 12 days in a growth chamber (temperature = 25°C, relative humidity = 60%, light regimen = 14 h light / 10 h dark at 30 Wm⁻²), sprayed with an aqueous solution of 5-aminolevulinic acid (ALA, 20mM) and then kept in the dark for 24 h. Separation of Pchl was performed according to the method of Tripathy and Rebeiz,⁴ which may be briefly described as follows. The homogenates of Pchl-accumulated tissues were extracted with 10 volume of basic acetone (acetone plus 0.1 N NH₄OH at the ratio of 9/1, v/v) and filtered. The filtrate was washed with equal volume of n-hexane to remove fully esterified tetrapyrroles, carotenoids and lipids. Pchl contained in the aqueous fraction was partitioned into diethyl ether by addition of equal volume of diethyl ether, 1/17 volume of saturated NaCl and 1/70 volume of 0.5 M K-phosphate buffer (pH 7.0), and the ether fraction was evaporated to dryness under vacuum. The dried residue was dissolved in methanol and subjected to the final step of Pchl purification by HPLC using a semipreparative Econosil C₁₈ column (250 × 10 mm).

Electron spin resonance (ESR) spectroscopic measurements. Formation and disappearance of free radicals in reaction mixtures were monitored by ESR using a JEOL TE 200 spectrometer at operating frequency of approximately 9.4 GHz at ambient temperature. The instrumental parameters were: microwave power, 10 mW; amplitude, 500; time constant, 0.1–0.2 s; modulation frequency, 100 KHz; modulation width, 0.1 mT; sweep width, 10 mT; and sweep time, 2 min. Kinetic studies were done at the fixed magnetic field of 335.1 mT for αTO· and at 331.1 mT for 2,2,6,6-tetramethylpiperidine-N-oxyl (TEMPO) radical, respectively.

Photolysis. The ethanolic sample solutions, either air-saturated, or purged thoroughly with ethanol-saturated argon, in a flat cell (0.4 mm thickness) were irradiated *in situ* through a condenser lens placed on the front of the ESR cavity. A wide band light (500 nm–690 nm) obtained from a halogen lamp by use of a cut-off filter (Y-50, Toshiba) plus a 1 cm layer of 4% CuSO₄ solution, was used for photolysis.

Chemicals. ALA and 2,2,6,6-tetramethylpiperidine (TEMP) were purchased from Aldrich and αTOH, rose bengal, hematoporphyrin, sodium azide and d₆-ethanol were obtained from Sigma. The chemicals were used as supplied.

RESULTS AND DISCUSSION

Pchl as a photodynamic generator of ¹O₂

Pchl is an anabolic intermediate of chlorophyll synthesis in plant cells.⁴ The biosynthetic pathway of chlorophyll comprises light-independent (the earlier phase) and light-dependent (the latter phase) processes; Pchl is the product of the earlier phase reactions. In light, however, Pchl is rather rapidly converted finally into chlorophyll and therefore its level in photosynthetic cells usually remains very low. Supplying plants with ALA exogenously in the dark, however, can result in an accumulation of Pchl in chloroplasts. ALA-treated plants suffer from severe oxidative damage when subjected to exposure to bright light,

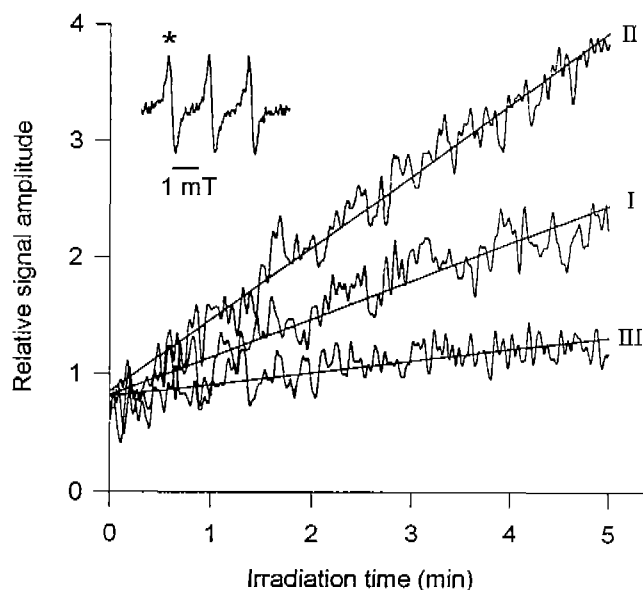


Figure 1. Photodynamic production of ¹O₂ in an air-saturated ethanolic solution of Pchl as a function of irradiation time. ¹O₂ was trapped with TEMP, yielding TEMPO radical whose formation was monitored by ESR spectroscopy at the fixed magnetic field of 331.1 mT as indicated by the star mark (*) in the radical spectrum (inset). Measurements were done in the basal reaction mixture containing 0.7 μM Pchl and 33 mM TEMP (I) and in that supplemented with 33% d₆-ethanol (II) or 3.3 mM sodium azide (III). A flat cell (0.4 mm in light path) containing samples was inserted into the ESR cavity and light was provided *in situ* through a condenser lens placed on the front of the cavity. A cut-off filter (Y-50, Toshiba) and a heat filter (4% CuSO₄ solution, 1 cm thickness) were used to obtain a wide band light (500 nm–690 nm) from a halogen lamp. The light intensity was adjusted by use of a neutral density filter (ND-4, Topaz) to 350 Wm⁻² at the sample position.

which has been ascribed to photodynamic sensitization *via* ¹O₂.^{5,10} Nonetheless, *in situ* photogeneration of ¹O₂ by Pchl has yet to be assayed. In this respect, it would be timely to provide direct evidence for Pchl being a type II photosensitizer.

TEMP is known to react highly specifically with ¹O₂, producing a stable nitroxide free radical, TEMPO, and therefore, ¹O₂ photoproduction in samples containing various photosensitizers has frequently been measured by ESR technique, using this sterically hindered amine as an efficient ¹O₂ trap.^{11,12} Because the commercial product of TEMP usually contains TEMPO as an impurity that is hardly eliminated by purification, an ESR signal corresponding to that of TEMPO is often observed before the occurrence of any reaction. However, the contamination does not interfere with ¹O₂ detection when the photochemical reaction is kinetically accessed. As shown in Fig. 1, the formation of TEMPO occurred in an aerated ethanolic solution of Pchl and TEMP under continuous illumination, which was found to follow pseudo-first order reaction kinetics. The reaction rate was significantly increased by a partial (33%) deuteration of the medium and

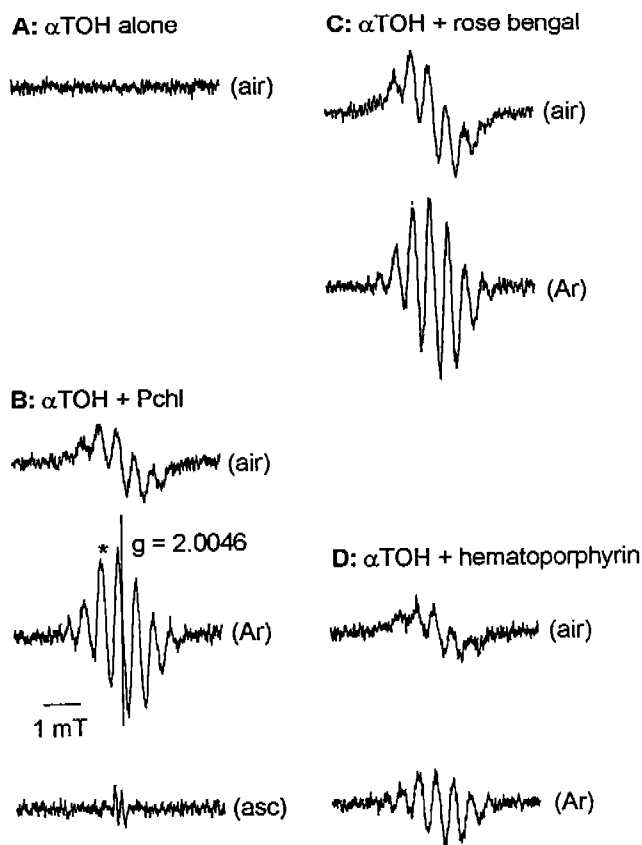


Figure 2. ESR spectra of $\alpha\text{TO}\cdot$ formed in the photolysis system under various conditions. The spectra were measured in ethanolic solution of αTOH (33 mM) alone (A) as well as in those additionally containing such photosensitizers as 0.7 μM Pchl (B), 1.0 μM rose bengal (C) and 1.0 μM hematoporphyrin (D), all of which were irradiated for 3 min. Samples were either air-saturated (air) or deaerated by purging with ethanol-saturated argon (Ar) prior to light exposure. The effect of ascorbate (asc, 33 mM) was also examined and the typical result was shown in panel B. Irradiation conditions were the same as in Fig. 1. The star mark (*) in panel B indicates the magnetic field of 335.1 mT that was used for the ensuing kinetic study on the appearance and disappearance of $\alpha\text{TO}\cdot$.

decreased by the presence of azide (3.3 mM) to an almost complete suppression of the reaction. Virtually same results were obtained in a complementary experiment, employing the imidazole plus N,N-dimethyl-4-nitrosoaniline (RNO) method developed by Kraljić and Mohsni¹³ (data not shown), in that $^1\text{O}_2$ first reacts with imidazole and produces an unstable trans-annular peroxide which in turn reacts with RNO, bleaching it. Those observations can be taken as evidence that $^1\text{O}_2$ is indeed formed through the Pchl-oxygen interaction in light.

Formation of $\alpha\text{TO}\cdot$ promoted by type II photosensitizers

The triplet states of photosensitizer molecules typically have much longer lifetimes than the singlet excited states. Thus, triplet sensitizers will undergo large numbers of collisions with other molecules during their lifetimes and, as a result, can undergo their primary reactions with molecules

in its vicinity either by an electron (or H atom) transfer process or by an energy transfer reaction with oxygen. In a given system, both types of processes can take place simultaneously in a competitive fashion. Therefore, even a compound which is generally considered as a type II photosensitizers may also be involved in an electron transfer process. Such would be particularly the case where substrates that are readily oxidized or reduced are present in the photolysis systems at high concentrations.¹⁴

As a quinoid, αTOH can undergo one-electron oxidation to $\alpha\text{TO}\cdot$. A well-known example of this reaction occurring in biological milieu is the scavenging process of lipid peroxy radical ($\text{LOO}\cdot$) by αTOH in membranes.¹⁵ Another example is found in its reaction with oxoferryl complex ($\text{Fe}^{\text{IV}}=\text{O}$) in myoglobin and hemoglobin^{16,17}, $\alpha\text{TOH} + \text{Fe}^{\text{IV}}=\text{O} \rightarrow \alpha\text{TO}\cdot + \text{Fe}^{\text{III}} + \text{HO}\cdot$. The most relevant to the present investigation is, however, the observation by Jore *et al.*¹⁸ that $\alpha\text{TO}\cdot$ is produced by H-abstraction from αTOH by triplet state ketones in anoxic media. In fact, we were able to demonstrate photoproduction of $\alpha\text{TO}\cdot$ in ethanolic solutions of αTOH and various type II sensitizers, such as rose bengal, hematoporphyrin and Pchl, even under aerobic conditions. Fig. 2 shows the seven-line spectra which appear to bear ESR characteristics of $\alpha\text{TO}\cdot$ in g-value as well as in the hyperfine pattern with respect to the splittings and relative amplitudes of peaks.¹⁹ Pre-deaeration of samples brought about a significant enhancement of $\alpha\text{TO}\cdot$ formation during photolysis, indicating that the photochemical reaction was not mediated by $^1\text{O}_2$ which was apparently produced in aerated samples. The deaeration effect rather suggests the existence of a competition between oxygen and αTOH for reactions with triplet sensitizers. The addition of ascorbate to samples resulted in a complete disappearance of the ESR spectrum of $\alpha\text{TO}\cdot$ and an appearance a slight signal of ascorbate radical,²⁰ again supporting the occurrence of $\alpha\text{TO}\cdot$ formation in the photolysis system because ascorbate effectively scavenges $\alpha\text{TO}\cdot$.²¹ The $\alpha\text{TO}\cdot$ formation process did not involve the electronic excitation of αTOH , for the irradiating light used for photolysis did not contain shorter wavelength photons that are absorbable by αTOH .

Reaction mechanism of the αTOH -triplet Pchl electron transfer

In order to take insights into the reaction mechanism of photosensitizer-dependent $\alpha\text{TO}\cdot$ formation, a kinetic study was conducted. Considering its physiological relevance to high light stress in plants, we focused firstly on the photochemical reaction by Pchl. Upon the onset of illumination of $\alpha\text{TOH}/\text{Pchl}$ samples, an immediate production of $\alpha\text{TO}\cdot$ occurred and the radical concentration reached a steady state level within 100 s under our experimental conditions, as shown in Fig. 3. The initial rates (v_0) of $\alpha\text{TO}\cdot$ formation were measured with samples containing various concentration of αTOH (30 - 210 mM). In doing so, changes in the signal amplitude during the first 5 s were used for regression to straight lines. Plotting v_0 against αTOH concentration

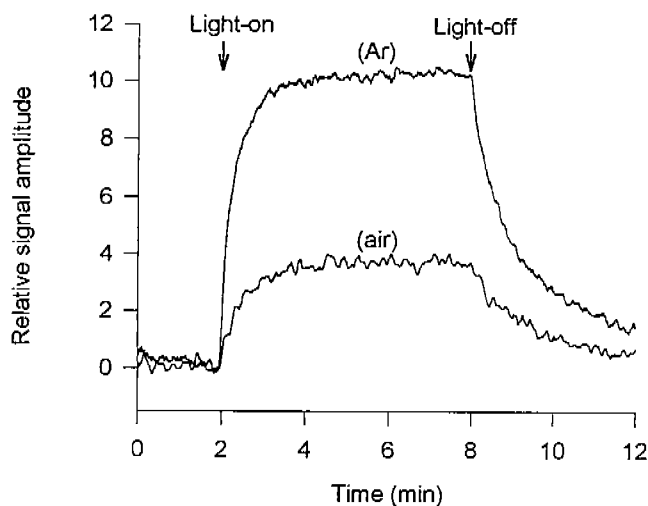
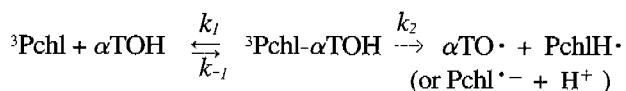


Figure 3. Time-courses of the Pchl-promoted photogeneration and the decay of $\alpha\text{TO}\cdot$ under aerobic and anaerobic conditions. The ethanolic solution of 33 mM αTOH plus 0.7 μM Pchl was either air-saturated (air) or purged with argon (Ar) and subjected to the ESR measurements. Changes in the signal amplitude of $\alpha\text{TO}\cdot$ were monitored at 335.1 mT as indicated in Fig. 2. Irradiation conditions were the same as in Fig. 1.

produced hyperbolic curves; interestingly enough, the double reciprocal plots ($1/v_0$ vs $1/[\alpha\text{TOH}]$) appeared to be linear, as shown in Fig. 4. Because such kinetic behavior is usually seen in Michaelis-Menten type reactions, the reaction of triplet Pchl ($^3\text{Pchl}$) with αTOH may be assumed to proceed *via* a noncovalent $^3\text{Pchl}\text{-}\alpha\text{TOH}$ complex: Accordingly, the reaction mechanism in the simplest form could be formulated as



By applying the steady state approximation for the $^3\text{Pchl}\text{-}\alpha\text{TOH}$ complex, the rate law is obtained.

$$v_0 = \frac{k_2[^3\text{Pchl}]_t [\alpha\text{TOH}]}{K + [\alpha\text{TOH}]} \quad \text{and}$$

$$\frac{1}{v_0} = \frac{K}{k_2[^3\text{Pchl}]_t [\alpha\text{TOH}]} + \frac{1}{k_2[^3\text{Pchl}]_t}$$

$$\text{where } K = \frac{k_{-1} + k_2}{k_1} \quad \text{and } [^3\text{Pchl}]_t = [^3\text{Pchl}] + [^3\text{Pchl}\text{-}\alpha\text{TOH}]$$

It would be reasonably expected that, during the earliest stages of the reaction, there is virtually no change in total concentration of Pchl and hence $[^3\text{Pchl}]_t$ remains constant. Then the above rate law can be re-written as

$$v_0 = \frac{k[\alpha\text{TOH}]}{K + [\alpha\text{TOH}]} \quad \text{and} \quad \frac{1}{v_0} = \frac{K}{k[\alpha\text{TOH}]} + \frac{1}{k}$$

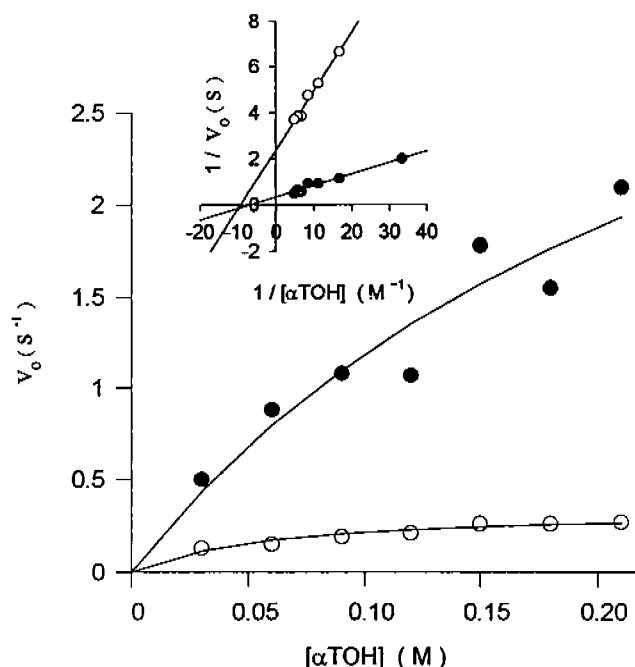


Figure 4. Dependence of the initial rate (v_0) of $\alpha\text{TO}\cdot$ formation by Pchl photosensitization on the concentration of αTOH . The ethanolic solutions of 0.7 μM Pchl plus various concentrations (30 - 210 mM) of αTOH were either air-saturated (open circle) or purged with argon (closed circle) and subjected to the ESR measurements, as in Fig. 3. Changes in the signal amplitude during the first 5 s of irradiation were used for regression to straight lines and the initial rates were determined. The inset shows double reciprocal plots obtained with the same data.

which is well consistent with the experimental results as shown (Fig. 4). The formation of the binary complex between αTOH and $^3\text{Pchl}$ as a reaction intermediate is feasible, for the probability of an electron (or H atom) transfer within the complex would increase if it occurs within the donor-acceptor complex. The double reciprocal plots also show that the values of the constant K for the reactions occurring under both aerobic and anaerobic conditions, obtained from the intercepts on $1/[\alpha\text{TOH}]$ axis, are practically identical, suggesting that the presence of oxygen does not affect the noncovalent interaction between $^3\text{Pchl}$ and αTOH *per se*. Oxygen appears only to quench the triplet states of Pchl, resulting in a decrease in $[^3\text{Pchl}]_t$.

While $\alpha\text{TO}\cdot$ is relatively long-lived even at room temperature as can be inferred from the relatively slow decay curves traced right after the cessation of sample illumination (Fig. 3), Pchl radical ($\text{Pchl}^{\cdot-}$ or $\text{PchlH}\cdot$) in solution seems extremely unstable because no ESR signal other than that of $\alpha\text{TO}\cdot$ was observed in the photolysis system. Note that $\alpha\text{TO}\cdot$ is resonance-stabilized, having several canonical structures.¹⁹ In biological systems $\alpha\text{TO}\cdot$ is rather effectively recycled, being reduced back to αTOH by numerous molecules, of which ascorbate appears to be the most important particularly in plant cells^{8,21}; as a result, the antioxidative capacity of αTOH would be sustained.

CONCLUSIONS

The present study provides evidence that membrane protection by α TOH against photodynamic oxidation reactions is associated with not only its function as an effective active oxygen scavenger but also its ability to quench photosensitizers in their triplet states via an electron transfer reaction, ${}^3\text{Sen} + \alpha\text{TOH} \rightarrow \text{SenH}\cdot$ (or $\text{Sen}\cdot^- + \text{H}^+$) + $\alpha\text{TO}\cdot$.

Various sensitizer molecules presently examined, are generally accepted as efficient type II photosensitizers that transfer their triplet energy to oxygen, producing ${}^1\text{O}_2$. Nevertheless, the formation of $\alpha\text{TO}\cdot$ by the reaction of αTOH with those molecules in light was clearly detected even under air-saturation conditions, indicating that the photosensitized electron transfer reactions proceed at high rates, sufficient enough to compete with the ${}^3\text{Sen}$ -oxygen energy transfer reactions.

The electron (or H atom) transfer seems to occur within the binary complex of ${}^3\text{Sen}$ - αTOH , which is formed as a reaction intermediate. At least in the reaction of αTOH with ${}^3\text{Pchl}$ which was found to follow Michaelis-Menten kinetics, the rate law, derived by assuming a reaction mechanism involving ${}^3\text{Pchl}$ - αTOH and applying the steady state approximation for the binary complex, turned out to be consistent with the experimental results.

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