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Mechanism of photodynamic activity of pheophorbides

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Abstract. Plasmid DNA is efficiently photocleaved by sodium pheophorbides (Na–Phdes) a and b in the absence of oxygen as well as in the presence of oxygen. Fluorescence microscopic observation shows a rapid incorporation of Na–Phde a into nuclei, mitochondria, and lysosome of human oral mucosa cells. In contrast Na–Phde b is incorporated only into the plasma membrane. The photodynamic activity of these pigments in living tissues is probably determined by the monomeric pigment molecules formed in hydrophobic cellular structures and involves two types of reactions: (i) direct electron transfer between DNA bases (especially guanine) and pheophorbide singlet excited state, and (ii) indirect reactions mediated by reactive oxygen species, including singlet oxygen whose production from molecular oxygen is sensitized by the Na–Phdes triplet state. A preliminary report has appeared in ''Photodynamic Therapy of Cancer II,'' Proc. SPIE **2325**, 416–424 (1994). [DOI: 10.1117/1.1352750]

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1 Introduction

Porphyrins have been used for over 2 decades in the photodynamic treatment of tumors.[1] Photodynamic therapy utilizes the apparent selective retention of certain porphyrins by tumors[1,2] with subsequent light-induced photosensitized destruction of the tumor. Hematoporphyrin derivative (HpD) and Photofrin®, clinically are the most frequently used porphyrins.[3] However, although they are among the most effective preparations to date, they are complex mixtures and thus interpretation of their localization and functional mechanism is difficult. New drugs that are pure and have strong absorption in the red spectral region are being sought, because transmission of tissues for light considerably increases with increasing wavelength. Pheophorbide (Phde) a, a derivative of chlorophylls, is one of the favorable candidates because it can be easily prepared and purified from chlorophyll a and has a high extinction coefficient in the red region.

Phde a has been reported to kill tumor cells much more efficiently than HpD *in vitro*, and its mechanism was proposed to be membrane destructive by singlet oxygen $(O_2(^1\Delta g))$.[4,5] But it is likely that pheophorbides can be located in other sites of the cell such as mitochondria, nuclei, and lysosomes, and thus be responsible of even more fatal damage by radical and/or singlet oxygenation.

In this paper, a brief account of the results of experiments on the incorporation of water-soluble pheophorbides (sodium pheophorbides, Na–Phdes) a and b in human buccal cells by fluorescence microscopic observation, and the photocleavage of plasmid pBR 322 DNA by these pigments in the absence and the presence of oxygen is first reported.[6] Then, (a) the photophysical properties of Na–Phdes are studied, particularly the quantum yield γ_Δ of singlet oxygen generation in different media by using direct and indirect methods, and (b) direct evidence for the electron capture from nucleic acid bases, adenine and guanine, by porphyrins in the singlet excited states is presented. In conclusion, the mechanism of photodynamic action by pheophorbides is discussed.

2 Experiment

Pheophorbides a and b were prepared by acid treatment of chlorophyll a and b, extracted from *Chlorella* and spinach[7] and then purified by normal-phase HPLC.[8(a)] The crude pigments were purified by reversed-phase HPLC, and finally converted to sodium salt to be soluble directly in water. The porphyrin derivatives covalently bounded to nucleobases were synthesized as described before.[8(b)]

Fluorescence microscopy was performed as described previously.[9] Briefly, nonkeratinized epithelial cells were collected by scraping the oral buccal mucosa, and were incubated with 1–4 mg/8 mL of Na–Phde in physiological saline at 37 °C for 15–120 min in the dark. After incubation, the cells were washed with physiological saline several times, and observed under the fluorescence microscope with an excitation light source (a superhigh-pressure Hg lamp, 100 W) coupled with a band-pass filter (395–415 nm). The trypan blue exclusion test indicated that more than 90% of the cells remained intact during microscopic observation.

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Plasmid pBR 322 DNA was purchased from Boehringer Mannheim Co., containing 0.25 μg/μL, 10 mM Tris-HCl buffer, and 1 mM EDTA. The commercial product was used without further purification. Solutions of DNA and Na–Phde mixtures were prepared in the dark by adding 18 μL of a Na–Phde aqueous solution at several concentrations to 2 μL of pBR 322 DNA stock solution in a glass tube. If necessary, oxygen was thoroughly removed from the solution by repeated (three times) freeze-and-thaw cycles. The solution was then irradiated with visible light (350–800 nm) isolated from a xenon lamp (Cermax LX300F, maximum 300 W, multilayered dielectric coating that rejects radiation below 350 nm) with a cold mirror (Kenko CM1 + CM2) and water filter (30 mm). Electrophoresis was performed with 0.8% agarose gel. After migration the gel was stained with ethidium bromide for the densitometric quantification of DNA photocleavage.

Detection of $O_2(^1\Delta g)$ by time-resolved luminescence spectroscopy was made at the Center for Fast Kinetics Research, University of Texas at Austin. The procedure related to this method was as follows. Solutions were adjusted to possess an absorbance of about 0.20 at 532 nm and excited with a single pulses (10 ns) delivered with a frequency-doubled Nd:YAG laser. Singlet oxygen was detected via the 0,0 phosphorescence band using a Judson germanium photodiode. Approximately 50 individual laser shots were averaged for each measurement and the yield of $O_2(^1\Delta g)$ was determined by computer extrapolation to the center of the laser pulse. The laser intensity was varied over a wide range using crossed polarizers. The detector was calibrated by reference to hematoporphyrin IX ($\gamma_\Delta = 0.75$) in d_1-methanol.[10]

The oxygen consumption method was used to determine γ_Δ according to γ_Δ = (number of moles of O_2 consumed) / (number of einsteins absorbed by the chlorin sensitizer). All photo-oxygenations were carried out in a cylindrical photoreactor that is a replicate of the device designed originally by Gollnick et al.[11] It consists of a closed system containing 40 mL of solution of sensitizer and $O_2(^1\Delta g)$ acceptor. A vigorous O_2 gas stream produced by a gas pump provides for rapid circulation of the solution and supplies simultaneously that amount of dissolved O_2 which is consumed in the reaction vessel during irradiation. The photoreactor is irradiated by an HBO 200 mercury arc lamp. Wavelengths below 530 nm were removed with an aqueous solution of potassium dichromate (5 g/L) and all irradiations were made using the combined mercury emission lines at 546 and 578 nm. The O_2 consumption rate is measured by a burette connected to the oxygen atmosphere. Tetramethylethylene and furfuryl alcohol, which is water soluble, were used as substrate at a concentration of 0.15 and 0.05 mol/L, respectively, so that generated $O_2(^1\Delta g)$ is completely trapped by chemical reaction. Light intensities were calibrated using the photo-oxygenation of tetramethylene in O_2-saturated methanol with methylene blue ($\gamma_\Delta = 0.50$) as a sensitizer.[12]

Fluorescence emission and excitation spectra of nucleobase-attached porphyrins in air-saturated CH_2Cl_2 were measured by using a Jasco FP-777 model.

3 Results and Discussion

3.1 Photodynamic Activity of Pheophorbides

3.1.1 *Fluorescence Microscopic Observation of Na–Phdes-Treated Cells*

After incubation of buccal mucosal cells with Na–Phdes a or b in physiological saline, the cells were observed during irradiation with purple light (395–415 nm), which can excite the sensitizers by means of the fluorescence microscope. For cells treated with Na–Phde a, bright and strong red fluorescence is observed in the nuclei, mitochondria, and lysosome. Fluorescence intensity in these organelles increases after increasing the concentration of Na–Phde a and/or the incubation time. The red fluorescence from cytoplasmic membranes is very weak and the bulk of the cytoplasmic region is basically not fluorescent. Fluorescence of Na–Phde a rapidly fades during microscopic observation, indicating that some prompt photochemical reaction occurs in the organelles.

In contrast, Na–Phde b shows very weak red fluorescence only in the cytoplasmic membrane even after 120 min incubation. The nuclear, mitochondrial, and lysosomal regions and the bulk of the cytoplasmic region are basically not fluorescent. Fluorescence spectra of Na–Phde b in the membrane cannot be obtained due to the too weak intensity.

3.1.2 *Photocleavage of DNA by Na–Phdes a and b*

Plasmid DNA is efficiently photocleaved by Na–Phdes a and b in the absence of oxygen (30% and 50%, respectively) as well as in the presence of oxygen (20% and 100%, respectively). Na–Phde b shows remarkably efficient photocleavage in the presence of oxygen at high concentration. In the case of Na–Phde a, the photocleavage efficiency in the absence of oxygen is slightly but significantly higher than that in the presence of oxygen. This result is surprising since DNA degradation by porphyrins has been believed to occur predominantly *via* a singlet oxygen mechanism.[13–15] Therefore it is desirable to know the efficiency of $O_2(^1\Delta g)$ generation by Na–Phdes in different media and to examine the eventuality of an electron transfer in the anaerobic photoalteration of DNA.

3.2 Mechanism of Pheophorbides Sensitized Photodamages

There are several methods to monitor concentrations of $O_2(^1\Delta g)$ produced *via* photosensitization. The most direct technique involves measurement of the infrared luminescence (IRL) emitted by $O_2(^1\Delta g)$, and can be made by both time-resolved or steady-state methods. Other time-resolved techniques, such as thermal lensing or optoacoustic calorimetry, are less direct and rely on the measurement of the heat released by nonradiative deactivation of $O_2(^1\Delta g)$. Photostationary state methods involve the trapping of intermediate $O_2(^1\Delta g)$ with a highly reactive substrate and relate $O_2(^1\Delta g)$ concentrations to depletion of reactants (substrate or O_2) or formation of particular products (e.g., peroxy compounds).

Investigations of the efficiency of $O_2(^1\Delta g)$ generation by Na–Phdes in different media were performed by using both time-resolved luminescence spectroscopy and oxygen consumption methods. By using IRL spectroscopy, yields of sin-

Table 1 Singlet oxygen quantum yields of Na–Phdes a and b determined by oxygen consumption method (γ_Δ^{SS}) and time-resolved infrared luminescence spectroscopy (γ_Δ^{TR}).

Sensitizer	Concentration (mol/L)	Solvent	γ_Δ^{SS}	γ_Δ^{TR}
Na–Phde a	5×10^{-5}	MeOH	0.60	
	2.5×10^{-5}	MeOH	0.62	
	1.1×10^{-5}	MeOH	0.61	
	2.8×10^{-5}	MeOH		0.63
	3.0×10^{-6}	toluene	0.85	
	1.5×10^{-5}	phosphate buffer (*p*H 7.4)	≤0.01	
	5.2×10^{-6}	phosphate buffer (*p*H 7.4)	≤0.01	
	1.02×10^{-6}	phosphate buffer (H 7.4)	≤0.01	
	7.6×10^{-6}	Tris-HCl/EDTA (*p*H 7.4–7.9)	≤0.01	
	1.0×10^{-6}	Tris-HCl/EDTA (*p*H 7.4–7.9)	≤0.01	
Na–Phde b	3.4×10^{-5}	MeOH	0.68	
	6.8×10^{-5}	MeOH	0.68	
	3.1×10^{-5}	MeOH		0.65
	3.6×10^{-5}	Phosphate buffer (*p*H 7.4)	≤0.02	
	7.2×10^{-5}	Phosphate buffer (*p*H 7.4)	≤0.02	
	8.0×10^{-5}	Phosphate buffer (*p*H 7.4)	≤0.02	
	1.05×10^{-4}	Tris-HCl/EDTA (*p*H 7.4–7.9)	≤0.01	

glet oxygen were measured as a function of laser intensity over regions where γ_Δ was directly proportional to the incident photon density. The experimental conditions for the oxygen consumption method were chosen so that the quantum yield of oxygenation is equal to the quantum yield of singlet oxygen generation. Therefore, we selected very reactive substrates which: (i) do not interact with the excited states of the sensitizer, and (ii) do not absorb at the excitation wavelength of the sensitizer, and (iii) can be used in a concentration so that all generated singlet oxygen is quantitatively intercepted prior to its nonradiative deactivation. Besides the experimental parameters were adjusted so that the rate of photooxygenation process, which is a gas–liquid reaction involving interphase mass transfer, corresponds to a chemical regime. Under such conditions, it is possible to obtain values of γ_Δ with an error of less than 3% and the sensitivity of the method is the same in aqueous and nonaqueous media.

Quantum yields of $O_2(^1\Delta g)$ production (γ_Δ) by Na–Phdes a and b under visible light are reported in Table 1. The investigated sensitizers produce singlet oxygen with high efficiency in organic solvents. The values obtained in alcoholic media by the indirect method based on oxygen consumption measurements are in good agreement with those obtained by time-resolved IRL spectroscopy. Table 1 shows that γ_Δ in methanol is constant whatever the compound and its concentration. This finding is consistent with the dye persisting in a monomeric form in methanol solution.

In contrast, singlet oxygen production sensitized by Na–Phdes a and b is insignificant in aqueous media. This inefficiency may be ascribed mainly to the formation of aggregates. In general $O_2(^1\Delta g)$ production yields for aggregates of sensitizers would be significantly lower than those given for the monomeric form. For example, it was reported that γ_Δ for hematoporphyrin IX (Hp) and HpD depends on concentration (γ_Δ values decrease by a factor of ~2 when concentration of the sensitizers increases from 10^{-5} to 5×10^{-4} mol/L), and it was shown that this variation for Hp is consistent with a monomer–dimer equilibrium, both species producing singlet oxygen but with very different quantum yields, $\gamma_\Delta^M=0.74$ for the monomer and $\gamma_\Delta^D=0.12$ for the dimer.[10] However, for tetrakis-(4-sulfonato-phenyl) porphyrin in an aggregated state, the quantum yield for formation of $O_2(^1\Delta g)$ $\gamma_\Delta^D=0.51$ remains on the same order as that of the corresponding monomer in methanol, $\gamma_\Delta^M=0.70$. Thus it appears that for porphyrins, the activity of the aggregate to sensitize formation of $O_2(^1\Delta g)$ depends on the total electronic charge resident on the molecule, decreasing with decreasing negative charge.[16]

In the absence of oxygen it is well known that a few types of sensitizers induce the photoalteration of biomolecules; in

some cases the reaction is even inhibited by oxygen. For example, some psoralens intercalate between the base pairs of DNA and RNA. After illumination under anaerobic conditions, covalent photoadducts are formed between the psoralen and pyrimidine residues in the nucleic acid. Some dyes may also promote DNA damages such as single strand breaks via an electron transfer occurring from a nucleobase to the sensitizer in the excited state.[17] This process generates base radical cations that may undergo deprotonation or dehydration to form neutral radicals.[18] Close proximity and/or binding of sensitizers to biomolecules, which is particularly common in living organisms, strongly promotes hydrogen atom and/or electron transfer reactions, i.e., the probability of a type I mechanism is significantly enhanced.

In order to mimic this situation and to examine the probability of such reactions, four porphyrin derivatives having nucleobases [adenine (A), guanine (G), thymine (T) and cytosine (C)] as the peripheral moieties were synthesized,[8(b)] and the effect of the nature of the nucleobase on the fluorescence yield was observed.[19] G-attached porphyrin shows the strongest reduction of fluorescence. A-attached porphyrin also shows significantly weaker fluorescence than a porphyrin having no nucleobase (reference porphyrin), while T- and C-attached porphyrins show almost the same emission intensity as that of the reference, namely the magnitude of fluorescence quenching caused by an attached nucleobase is in the order $G>A>T\geqslant C$, which agrees with the order of the oxidation potentials E_{OX} of these bases (+1.09, +1.19, +1.29, and 1.44 V versus NHS, respectively.[20]) Using the values of −0.56 V versus NHE,[21] 1.86 and 1.36 eV[22] for the reduction potential E_{RED}, the singlet excited state energy E_S, and the triplet state energy E_T, respectively, the thermodynamic driving force for photoinduced electron transfer from nucleobase to sensitizer singlet $\Delta G_s^0 = E_{OX} - E_{RED} - E_S$ or triplet $\Delta G_T^0 = E_{OX} - E_{RED} - E_T$ may be evaluated. While net electron transfer to the triplet state is unfavorable for all nucleobases, it becomes favorable from guanosine to singlet excited state ($\Delta G_S^0 = -0.21$ V versus NHE). This electron transfer is probably the first step in the oxygen-independent photocleavage of DNA by pheophorbides.

In aerobic conditions, the results are more difficult to analyze, because several mechanisms may occur simultaneously. The singlet excited state of pheophorbides may not only interact with DNA, directly or indirectly via ground state complexation or intercalation (Phde...DNA), but also undergoes the classical photophysical deactivation processes, i.e., fluorescence, internal conversion, quenching by oxygen, and intersystem crossing. Singlet oxygen, which is produced from triplet state quenching by molecular oxygen, induces type II photodamages. As these last processes have the same efficiency for both Phde a and b, the differences of photocleavage effectiveness have to be ascribed to the oxygen independent process. Since the efficiency of photocleavage of Phde a in the absence of oxygen is slightly but significantly higher than in the presence of oxygen, the binding affinity of the photosensitizers to DNA is probably higher for Phde a than for Phde b.

For other possible targets of Na–Phdes, which have been characterized by fluorescence microscopy, such as mitochondria, lysosome, and plasmic membrane, a type II photo-oxygenation process is likely. Indeed killing of tumor cells by a mechanism involving membrane destruction by singlet oxygen was already reported.[4,5] Thus the monomeric Na–Phde formed in hydrophobic cellular structures may be responsible for the photodynamic activity in living tissues.

4 Conclusions on the Mechanisms of Photodynamic Activity of Pheophorbides

The main photophysical properties of Phdes a and b are similar. Monomeric in organic media, their quantum yields of production of triplet state and of singlet oxygen are important and always higher than 60%. By contrast, in aqueous media, they are insignificant (<0.02) as a consequence of aggregates formation. Thus the photodynamic activity of pheophorbides in living tissues is probably due only to the monomeric pigment molecules formed in hydrophobic cellular structures. The mechanism of photodynamic action induced by these monomeric pigment molecules depends on several parameters, and among others on the nature of the site where the sensitizer is localized, on the possible complexation (Phde...target) of the two species, and on the oxygen concentration. In anaerobic media, photocleavage of plasmid DNA is essentially due to a direct electron transfer between DNA bases (especially guanine) and Phde singlet excited states, while in the presence of oxygen, type II photo-oxygenation processes are likely to compete with this reaction. The differences in the photoactivities of Phdes a and b may probably be attributed to a much larger binding affinity of Phde a to DNA. For other cellular sites, for which electron transfer processes are endergonic (such as membranes), the photodynamic activity results from reactions mediated by reactive oxygen species including singlet oxygen.

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