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# Photoprocesses of chlorin e6 bound to lysozyme or bovin serum albumin

Yazhou Zhang [1], Helmut Görner*

*Max-Planck-Institut für Bioanorganische Chemie, D-45413 Mülheim an der Ruhr, Germany*

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ABSTRACT

The ground and excited state processes of chlorin e6 in aqueous solution were studied in the presence of lysozyme and also bovine serum albumin. Non-covalent binding to proteins was analyzed using fluorescence, UV–visible and circular dichroism absorption spectroscopy. The number of binding sites, $n$, was 1.5–2.4 and the apparent macroscopic dissociation constant, $K_d$ was 0.2–2.5 μM. The binding of chlorin e6 to lysozyme, in contrast to that of bovine serum albumin, imparted fluorescence quenching; circular dichroism spectra revealed a chiral environment upon binding to β-sheets of bovine serum albumin. Time-resolved photolysis showed a longer triplet lifetime upon interaction with the proteins owing to shielding of the dye. The quantum yields for both damage to chlorin e6 ($\Phi_d$) and for protein oxidation ($\Phi_{ox}$) were determined under oxygen-free conditions; $\Phi_d$ was smaller because of shielding by the protein with respect to the self-quenching of the free dye. The major effects concerning photooxidation in the absence and presence of oxygen are discussed.



## 1. Introduction

The primary processes in photosynthesis have been studied in great detail. Many efforts have been made to describe the properties of chlorophyll assemblies [1,2]. The strong electronic interaction of the many chlorophyll molecules in the antenna, however, makes it practically impossible to address a single entity in this large aggregated complex. While monomeric chlorophyll *a* in organic solvents shows both fluorescence and intersystem crossing in substantial quantum yields, the quantum yield of intersystem crossing ($\Phi_{isc}$) of aggregated chlorophyll *a* is small [3]. Another supramolecular aggregate system involves chlorin moieties which play a key role for photosynthesis in green plants and photosynthetic bacteria. The self-assembly of zinc chlorins to an artificial supramolecular light-harvesting device has been intensively investigated [4].

Chlorins are used as sensitizers, their photoredox features are well known and their spectroscopic properties have been investigated widely [5–27]. Chlorin e6 (Chart 1) and chlorin p6 are structurally similar and only the $CH_2COO^-$ group in position 15 is replaced by $COO^-$, respectively. Some features of chlorin e6 in aqueous solution are known, e.g. $\Phi_{isc}$ is large and the fluorescence quantum yield ($\Phi_f$) is moderate. The efficient triplet population is supported by a quantum yield of singlet molecular oxygen production ($\Phi_\Delta$) of 0.65 at pH 7–8 [5,8]. The binding conditions of chlorins to proteins have been examined [18–20].

Another class of photosensitizers is constituted by the porphyrin moiety [28–41]. The amount of knowledge about chlorins is much smaller than about porphyrins and only little is available in the literature concerning chlorin-sensitized photooxidation of proteins. The reactive state of porphyrins towards intermolecular processes is generally the triplet, which is efficiently populated, for e.g. *meso*-tetra(4-sulfonatophenyl)porphyrin (TSPP, see Chart 1) $\Phi_{isc}$ and $\Phi_\Delta$ are 0.5–0.6 [35]. Porphyrin and chlorin dyes are suitable as sensitizers for the photooxidation of proteins, such as bovine (BSA) or human (HSA) serum albumin [42,43]. Three dimensional structures, size, shape, and charge distribution of the two frequently applied proteins are well known. However, both BSA and porphyrins may be subjects of aggregation which generally lowers the reactivity [30,31,42]. A variety of other synthetic molecules have been applied for photooxidation of proteins, e.g. TSPP [31–34], chain-substituted pyrenyl peptides [44], methylene blue [45], cyanine dyes [46] and xanthene dyes, such as eosin, erythrosin and rose bengal [47]. The cyanine dyes have attractive spectroscopic and photochemical features but low $\Phi_{isc}$ and $\Phi_\Delta$.

In this paper we present results on the binding of chlorin e6 in aqueous solution to either lysozyme or BSA and the photosensitized protein oxidation. Lysozyme belongs to the most often studied enzymes [44–46]. We aim at a better understanding of the photoprocesses of chlorin e6, which are induced by binding to the two proteins. Thereby, we elucidate the features and conditions of the photosensitized oxidation of lysozyme and BSA under oxygen-free conditions. The quantum yields of either damage (bleaching, $\Phi_d$) of

* Corresponding author.
*E-mail address:* goerner@mpi-muelheim.mpg.de (H. Görner).
[1] On leave from the Institute of Chemistry, Chinese Academy of Sciences, Beijing 100190, PR China.

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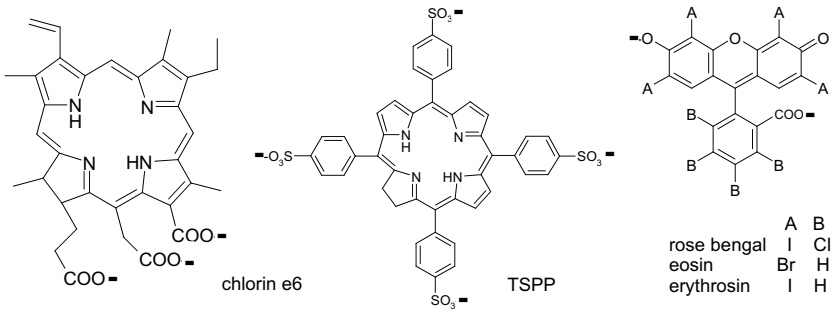


**Chart 1.**

chlorin e6 or of protein oxidation ($\Phi_{ox}$) in argon-saturated aqueous solution, involving free radicals as key intermediates, were compared with those in the presence of air, involving singlet molecular oxygen as reactant. The photophysical and molecular aspects may provide a basis for related photosensitizer–protein interactions.

## 2. Materials and methods

Chlorin e6 (Frontier Scientific) [22] and BSA (Sigma) and lysozyme from egg white (Fluka) [46,47] were the same as used previously. Water was from a millipore (milli Q) system. The stock solutions of proteins, typically 100 μM, were freshly prepared without buffer unless indicated otherwise. The dye solutions were also freshly prepared and the concentrations were adjusted to 5–15 μM. The pH was typically 7.6–8.0 and shifted by addition of protons ($HClO_4$) or hydroxyl ions (NaOH). The UV–vis absorption spectra were recorded on a diode array spectrophotometer (HP, 8453). A spectrofluorimeter (Varian Cary, eclipse) was employed to measure the fluorescence spectra. Irradiation was performed with a 250 W Hg lamp and a filter (Schott UG5) or a 1000 W Xe–Hg lamp and a monochromator. The relative quantum yield $\Phi_d^{rel}$ of decomposition of dyes in argon-saturated aqueous was obtained from the changes in absorption at $\lambda_a^0$ upon continuous irradiation at 380–450 nm. The changes were measured by $(A_0–A_t)/(A_0–A_e)$, where $A_t$ is the absorption at a given time, $A_e$ that at long times and $A_0$ at the beginning. The relative quantum yield of photooxidation of proteins ($\Phi_{ox}^{rel}$) was obtained by the fluorescence intensity ($\lambda_{ex} = 280$ nm) of the tryptophan residues at $\lambda_f = 350$ nm which decreases vs. the irradiation time [37–39]. A variation of $\lambda_{ex}$ between 275 and 295 nm did not reveal a significant change in $\Phi_{ox}^{rel}$. As reference we used $\Phi_d = 2 \times 10^{-3}$ for chlorin e6 in air-saturated buffered aqueous solution at pH 7 [5,17]. The CD spectra were recorded using a Jasco J-715 spectrometer with Hamamatsu R376 photomultiplier and appropriate software: Spectra Manager. The flash photolysis operated at $\lambda_{exc} = 308$ nm [22] or at $\lambda_{exc} = 410–690$ nm (Nd-YAG laser + OPO), the absorption signals were measured by a Luzchem system. The measurements were carried out at 24 °C and refer to air-saturated aqueous (for convenience) unless indicated otherwise. Test measurements revealed that for CD and fluorescence essentially the same results were obtained either in air- or argon-saturated aqueous solution in contrast to both steady-state and time-resolved photolysis.

## 3. Results

The formation of ground state complexes between a dye and a protein leads to changes in fluorescence properties and the UV–vis absorption spectra as well as CD absorption spectra. The various spectral and kinetic features change upon increase of the protein concentration [*P*], keeping the dye concentration [*D*] constant. The results may roughly be distinguished between high loading conditions with many pigments bound to one protein molecule ($[P]/[D] \leq 0.1$) and low loading ($[P]/[D] > 1$) with only one dye molecule per protein or less.

### 3.1. Absorption properties

The absorption maxima of the Soret and Q band of free chlorin e6 in aqueous solution at pH 7.8 are at $\lambda_a^0 = 405$ and 640 nm. The position of the latter maximum is sensitive to the ionic strength since $\lambda_a$ is 14 nm red-shifted on addition of 5–10 mM phosphate buffer [22]. The Q band is red-shifted upon increasing of the protein concentration, e.g. to $\lambda_a^P = 667$ nm when bound buffer-free to BSA. Chlorin e6 in concentrations of <10 μM remains monomeric in aqueous solution at low ion strength [7,8]. One or several isosbestic points, e.g. at 520 nm, demonstrate the presence of only two species, free and bound dye. The data are compiled in Table 1 and examples are shown in Fig. 1. No shift in $\lambda_a^P$ was found for BSA in the presence of 5 mM phosphate buffer, in contrast to lysozyme.

The signal at $\lambda_a^0 = 640$ nm decreases with increasing the [BSA]/[chlorin] ratio and $A_{665}$ increases correspondingly (inset of Fig. 1).

$$n\text{Dye} + \text{Protein} \rightarrow \text{Complex} \quad (1)$$

The effect is described by the characteristic protein/dye concentration ratio for 50% change, $([P]/[D])^a_{1/2}$, which is 0.05–0.2 for BSA and lysozyme (Table 1). Another characteristic parameter is the $A^P/A^0$ ratio, measuring the change in absorbance at an appropriate wavelength due to low loading. $A^P/A^0$ is <0.1 for BSA. In the presence of 5 mM phosphate buffer the maximum change in $A^P$ is smaller for BSA and very small for lysozyme, whereas the characteristic $([P]/[D])^a_{1/2}$ ratio remains unchanged for BSA. The

**Table 1**
Absorption, fluorescence and triplet data of chlorin e6 and data for binding to proteins.[a]

| Parameter | No protein | BSA | Lysozyme |
|---|---|---|---|
| $\lambda_a$ (nm) | 405/640 (654)[b] | 405/667 (668) | 405/667 (654) |
| $\lambda_f^{ex}$(nm) | 640 | 670 | 640 |
| $\lambda_f^{em}$(nm) | 650 | 675 | 648 |
| $A^P/A^0$ | | <0.1 (0.5)[b] | 0.5 (0.8) |
| $([P]/[D])^A_{1/2}$ | | 0.05 (0.2) | 0.2 |
| $\Phi_f^P/\Phi_f^0$ | | 1.2[c] | 0.4[c] |
| $([P]/[D])^F_{1/2}$ | | <0.2 | 0.2 |
| $\Phi_{isc}^P/\Phi_{isc}^0$ | | 0.9 | 0.3 |
| $\tau_T$ (ms) | 0.2 [0.2][d] | 0.5 | 0.4 |

[a] In aqueous solution at pH 7.8 unless indicated otherwise.
[b] Values in parentheses refer to 5 mM phosphate buffer.
[c] Same result using $\lambda_f^{ex} = 380$ or 520 nm.
[d] Value in brackets: pH 4.