Effect of Aggregation on Bacteriochlorin a Triplet-state Formation: A Laser Flash Photolysis Study

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ABSTRACT

Bacteriochlorin a (BCA) is a potential photosensitizer for photodynamic therapy of cancer. It has been shown previously that the photoefficiency of the dye is mainly dependent on singlet oxygen (1O₂) generation. Nanosecond laser flash photolysis was used to produce and to investigate the excited triplet state of the dye in methanol, phosphate buffer and dimiristoyl-L-α-phosphatidylcholine (DMPC) liposomes. The transients were characterized in terms of their absorption spectra, decay kinetics, molar absorption coefficients and formation quantum yield of singlet–triplet intercrossing. The lifetime of the BCA triplet state was measured at room temperature. The triplet-state quantum yield is quite high in methanol (0.7) and in DMPC (0.4) but only 0.095 in phosphate buffer. In the last case, BCA is in a monomer–dimer equilibrium, and the low value of the quantum yield observed was ascribed to the fact the triplet state is only formed by the monomers.

INTRODUCTION

Bacteriochlorin a (BCA), a derivative of bacteriophyll a, is a potential photosensitizer for photodynamic therapy (PDT) of cancer (1–3). Indeed, BCA shows preferential tumor-tissues retention (3) and has a high absorption coefficient in the visible region of the spectrum, mainly in the phototherapeutic window (absorption maximum at 760 nm where the skin penetration of light is optimal) (4). Light-activated BCA can transfer energy (a) by interaction with the solvent or substrate through an electron or a proton transfer, leading to the generation of reactive oxygen species (ROS) like hydroxyl radicals (OH•) and superoxide anion (O₂⁻) (Type-I reaction) (5); and (b) directly to molecular oxygen to yield 1O₂ (Type-II reaction), an important mediator of photocellular cell damage (5). Previous electron spin resonance experiments and absorption measurements indicated that BCA in aqueous solutions is a 50:50 (Type-I/Type-II sensitizer (5). Moreover, its singlet-oxygen quantum yield Φ(1O₂) in this medium is equal to 0.05 (6). When BCA was incorporated within dimrystoyl-L-α-phosphatidylcholine (DMPC) liposomes, spin-trapping experiments using 5,5-dimethyl-1-pyrroline-N-oxide and specific quenchers showed that the production of ROS, like OH• and O₂⁻, remains weak, whereas the yield of singlet oxygen (1O₂) increases, reaching a value of 0.33 (6,7).

The medium where BCA is solubilized influences not only the 1O₂ production but also the dye aggregation properties (8). In methanol, BCA is monomeric, and no aggregation is observed below 5 × 10⁻⁵ M (8). In phosphate buffer (pH 7), BCA is strongly aggregated in its dimeric form. The dimerization constant was estimated to be 10⁶ M⁻¹ (7,8). The solubilization of BCA within DMPC liposomes induces dye monomerization. When the lipid-to-BCA ratio is greater than 125, BCA is completely incorporated in its monomeric form inside the lipid bilayers near the polar head groups (8).

From the previous results it seems that the increase of Φ(1O₂) in methanol and DMPC liposomes with respect to its value in phosphate buffer is concomitant with the monomerization of the dye (7). Considering that the generation of 1O₂ from BCA results, to a great extent, from an energy transfer between the sensitizer triplet state and molecular oxygen, it appears worthwhile to check the influence of BCA aggregation on triplet-state formation. To achieve these purposes, laser flash photolysis experiments were carried out in methanol, in DMPC liposomes and in phosphate buffer (pH 7). As a complement to the already reported 1O₂ studies (6,7), the triplet quantum yields were compared with the singlet-oxygen quantum yields.

MATERIALS AND METHODS

Chemicals. DMPC was obtained from Sigma (Leuven, Belgium). Methanol and chloroform were obtained from J. T. Baker (Deventer, The Netherlands). All other chemicals were of analytical grade and used without further purification.

Preparation of BCA. BCA was prepared as described before (6–8). In short, bacteriophyll a was extracted from the photosynthetic anaerobic bacterium Rhodospirillum Rubrum. It was purified according to the method of Omata and Murata (9). Saponifying bacteriophyll a, as described by Oster et al. (10), yielded bacteriophyllin a. To remove the Mg central ion, bacteriophyllin a was subjected to acid hydrolysis with sodium acetate (pH 2.0). The BCA formed was extracted with ethyl acetate, which subsequently was evaporated under reduced pressure. The BCA was then lyophilized overnight and stored at −20°C in.

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Table 1. Concentrations of BCA with the corresponding monomer (Cm) and dimer (Cd) concentrations used for the laser flash photolysis experiments carried out in phosphate buffer, pH 7. Cm and Cd were calculated using Eqs. 2–4.

<table>
<thead>
<tr>
<th>Cm (M)</th>
<th>Cd (M)</th>
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<tbody>
<tr>
<td>$5 \times 10^{-6}$</td>
<td>$1.35 \times 10^{-6}$</td>
</tr>
<tr>
<td>$1 \times 10^{-5}$</td>
<td>$2 \times 10^{-6}$</td>
</tr>
<tr>
<td>$2.5 \times 10^{-5}$</td>
<td>$3.3 \times 10^{-6}$</td>
</tr>
<tr>
<td>$5 \times 10^{-5}$</td>
<td>$4.76 \times 10^{-6}$</td>
</tr>
</tbody>
</table>
formation of only one species. The dependence of the transient signal at 400 nm on the laser energy is linear up to an incident fluence of 2 mJ/cm²; then, it tends to saturate according to the occurrence of a monophotonic process (Fig. 3) in agreement with an assignment of the transient absorption to the lowest triplet state of BCA. Furthermore, this transient spectrum was efficiently quenched by 1 atm of oxygen with a rate constant of $1.6 \times 10^{10}$ M⁻¹ s⁻¹, demonstrating again that it can be attributed to the lowest triplet state of BCA.

The decay rate of the excited triplet state of BCA in the absence of oxygen was measured as a function of BCA concentration (from $4.5 \times 10^{-6}$ to $1.25 \times 10^{-5}$ M). The results reveal that the triplet lifetime does not depend on BCA concentration. The intrinsic rate constant of BCA triplet-decay state was $k_0 = 7.14 \times 10^8$ s⁻¹. At the end of the triplet-state relaxation, the absorption of the BCA ground state was recovered, excluding the formation of stable photoproducts in the absence of oxygen.

Complete conversion of the BCA ground state to the triplet state was not achieved but could be simulated through multiplication of the transient absorption measurements (Fig. 1) by an appropriate coefficient $x$ (12–15). Different values of $x$ were tried. The resulting absolute absorption spectrum of the triplet was then compared with that of the ground state of the molecule. Under the assumption that the triplet absorption spectrum was positive and different from that of the ground state, acceptable $x$ values lay in the range 0.9–1. Under these conditions the triplet absorption coefficients of BCA were estimated with a relative error of ~20%. As shown in Fig. 1, the triplet-state spectrum of monomeric BCA is characterized by two bands centered at 390 nm ($\epsilon \sim 48 500$ M⁻¹ cm⁻¹) and 590 nm ($\epsilon \sim 13 000$ M⁻¹ cm⁻¹). The triplet quantum yield of BCA ($\Phi_T^{BCA}$) was determined using the comparative method described in Materials and Methods; its value was found to be equal to 0.7 (Table 2).

The triplet-state properties of BCA incorporated in DMPC liposomes were then studied with a lipid-to-BCA ratio greater than 125, ensuring the complete incorporation of BCA, in its monomeric form, in the lipid bilayers (8). The spectrodynamical evolution of BCA in DMPC liposomes after a 355 nm laser pulse excitation is not very sensitive to the presence of liposomes (Fig. 2). The triplet-state formation is still effective but with a lower quantum yield ($\Phi_T^{BCA} = 0.4$, Table 2) than the one measured in methanol.

Table 2. BCA quantum yields of singlet-triplet intersystem crossing ($\Phi_T^{BCA}$) and singlet oxygen generation ($\phi(\text{O}_2)$) in various solvents. The relative error is evaluated to 10%

<table>
<thead>
<tr>
<th>Solvent</th>
<th>$\Phi_T^{BCA}$</th>
<th>$\phi(\text{O}_2)$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methanol</td>
<td>0.7</td>
<td>0.36 (6)</td>
</tr>
<tr>
<td>Phosphate buffer (pH 7.0)</td>
<td>0.095</td>
<td>0.05 (6)</td>
</tr>
<tr>
<td>Liposomes</td>
<td>0.4</td>
<td>0.33 (6)</td>
</tr>
</tbody>
</table>

In a deaerated solution the decay of the triplet state also occurs on the microsecond time scale according to a first-order kinetic ($k = 1.15 \times 10^5$ s⁻¹).

The triplet absorption spectrum was determined by the same procedure as in methanol. The $x$ values obtained were in the range of 0.5–0.6. The comparison of the triplet-state absorption spectrum in methanol and DMPC liposomes reveals that the shape is not influenced by the incorporation into lipids bilayers: it presents two major bands with maxima at 400 nm ($\epsilon \sim 41 500$ M⁻¹ cm⁻¹) and 600 nm ($\epsilon \sim 15 000$ M⁻¹ cm⁻¹).

Although BCA incorporated into DMPC liposomes is present only as a monomer, as is in methanol, its triplet quantum yield is lower than in methanol (Table 2). Viscosity is a factor that could influence the intersystem crossing quantum yield of BCA. Indeed, it is well known that, for many dyes, the quantum yield of fluorescence ($\Phi_F$) increases with increasing viscosity (16). Electron spin resonance experiments using $\pi$-dioxyl stearic acid as spin-label revealed that BCA is incorporated only in the polar head region of DMPC bilayers (8). There, the local viscosity is at least hundred times higher than in water and in methanol (17). Consequently, this high viscosity should favor the fluorescence decay of the excited BCA. As we could expect, for the same absorbance at the excitation wavelength, the fluorescence emission of BCA at 775 nm (the maximum of emission in methanol) is almost twice higher in DMPC than in methanol (data not shown). This behavior is already reported for many dyes when they are incorporated within liposomes (18,19). Consequently, the weaker triplet-state quantum yield formation in DMPC is probably correlated to the increase of the BCA fluorescence emission.

Table 2 also reveals that the ratio of quantum yields for the formation of triplet-state and singlet-oxygen $S_A$ determined previously ($\phi(\text{O}_2)/\Phi_T$) varies between 0.5 in methanol and 0.9 in DMPC liposomes. The conversion of triplet-state energy into $\text{O}_2$ yield is particularly good in liposomes but remains quite high in methanol. These differences in $S_A$ may be related to the photobleaching of BCA and to the method used to measure the $\phi(\text{O}_2)$ value. Indeed, previous experiments have shown that BCA in solution can react with $\text{O}_2$ to generate photoproducts (20). Now, $\phi(\text{O}_2)$ has been determined by quantitative analysis of absorbance loss of a specific probe. Because a fraction of $\text{O}_2$ is quenched by BCA itself, this comparative technique leads to an underestimation of the singlet-oxygen quantum yield. In DMPC liposomes, $\text{O}_2$ spends only 70% of its lifetime in the vesicular phase where BCA is located (6), so the photobleaching process is less effective in bilayers and the $\phi(\text{O}_2)$ value is closer to its true value.

Aggregated BCA in phosphate buffer

In phosphate buffer the absorbance of BCA does not follow the Beer–Lambert law (8). The dye is in a monomer–dimer
equilibrium, and the dimerization constant was estimated to be $\sim 10^6 \, M^{-1}$ (7), which means that in the range of concentration used in our experiments (from $5 \times 10^{-6}$ to $5 \times 10^{-3} \, M$), it was not possible to have only one species of BCA in solution (see Table 1). The concentrations of monomers and dimers in solution were calculated by the procedure discussed in Materials and Methods.

In an argon-saturated phosphate buffer solution, the transient spectrum measured at the end of the laser pulse has the same features as in methanol and DMPC liposomes: two maxima around 420 and 580 nm and negative signals centered at 350, 520 and 760 nm (Fig. 4). Therefore, this transient is attributed to the triplet-state formation of BCA. Its quenching by oxygen with a rate constant close to the diffusion limit ($k = 2 \times 10^9 \, M/s$) and the linear variation of the absorbance with the laser fluences (Fig. 3) support this assignment.

Examination of Figs. 1, 2 and 4 shows that the differential absorption spectra of BCA after the laser pulse have the same shape in the three media. But previous absorption and fluorescence experiments have demonstrated that BCA solubilized in methanol or in DMPC lipids bilayers is only in its monomeric form, whereas in phosphate buffer the dye is strongly aggregated (8). This absence of any transient spectral difference, together with the fact that BCA is only monomeric in methanol and in DMPC liposomes, provides support for the following hypothesis: the BCA triplet state is only formed from the BCA monomers even in phosphate buffer. Furthermore, the negative bands of the transient absorption spectrum in phosphate buffer (Fig. 4) correspond to the absorption band of ground-state monomer (Fig. 6).

In an attempt to confirm our hypothesis, the differential absorption spectrum at the end of the laser pulse was recorded for different BCA concentrations corresponding to different monomer–dimer ratios (Table 2). In the concentration range studied, no modification of the shape of the differential absorption spectrum was observed but only variations in the intensities of the transient signals (Fig. 4). Figure 5 shows the dependence of the transient signal intensity on the BCA concentration of monomers (Fig. 5a) and of dimers (Fig. 5b) and on total dye concentration (Fig. 5c). To explain the dependence of $\Delta A$ on the concentration of two BCA species, the following relations were developed.

The absorbance of the sample before the laser pulse ($A_{\text{BEFORE}}$) is given by

$$A_{\text{BEFORE}} = \varepsilon_m C_m l + \varepsilon_d C_d$$

where $\varepsilon_m$ and $\varepsilon_d$ are the extinction coefficients of the monomer and the dimer, respectively, $C_m$ and $C_d$ are the concentration of monomers and dimers, respectively, and $l$ is the optical path length.

After the laser pulse, assuming that only the monomers go through intersystem crossing, the absorbance ($A_{\text{AFTER}}$) is given by

$$A_{\text{AFTER}} = (1 - \beta)\varepsilon_m C_m l + \beta \varepsilon_T C_m l + \varepsilon_d C_d$$

where $\varepsilon_T$ is the extinction coefficient of the triplet and $\beta$ the fraction of monomers converted into triplet state. The differential absorbance $\Delta A$ is thus expressed by

$$\Delta A = A_{\text{AFTER}} - A_{\text{BEFORE}} = \beta (\varepsilon_T - \varepsilon_m) C_m l$$

Under these conditions, $\Delta A$ varies linearly with $C_m$, as observed in Fig. 5a. Under this assumption the analytical dependence of

Figure 2. Ground-state (—) and calculated triplet-state (■) absorption spectra of BCA in DMPC liposomes. Inset: differential end-of-pulse transient absorption spectrum measured upon excitation of BCA ($10^{-5} \, M$) by a 354.7 nm laser pulse in a deaerated solution containing DMPC liposomes (lipids) = 1.5 $\times$ $10^{-3}$ M. Under these conditions BCA was completely incorporated into the lipid bilayers.

Figure 4. Differential absorption spectra measured on 354.7 nm photolysis of four aqueous solutions containing various concentrations of BCA. The spectra were recorded at the end of the laser pulse, and the fluence was equal to 11 mJ/cm$^2$. The arrows indicate the evolution of the spectra for increasing BCA concentrations.
The dimerization constant $K_d$ is defined by the following equation:

$$K_d = \frac{C_d}{C_m^2}$$  \hfill (8)

Using Eqs. 7 and 8,

$$\Delta A = \beta (\epsilon_T - \epsilon_m) I \sqrt{\frac{C_d}{K_d}}$$  \hfill (9)

By combining Eqs. 3, 7 and 8, $\Delta A$ is obtained as a function of the total concentration of BCA ($C_t$):

$$\Delta A = \beta (\epsilon_T - \epsilon_m) I \left(1 + \frac{8K_dC_t - 1}{4K_d}\right)$$  \hfill (10)

The experimental data from Fig. 5b,c are perfectly fitted by the theoretical Eq. 9 and Eq. 10, respectively, confirming the hypothesis that the BCA triplet state only occurs from the monomers. When the experimental data are fitted considering that only dimers (Fig. 5b) or the total concentration of BCA (Fig. 5c) contribute to $\Delta A$ variations, the expressions of $\Delta A$ are then given by Eq. 11 and Eq. 12, respectively.

$$\Delta A = \beta (\epsilon_T - \epsilon_m) I C_d$$  \hfill (11)

$$\Delta A = \beta (\epsilon_T - \epsilon) I C_t$$  \hfill (12)

where $\epsilon_d$ is the extinction coefficient of the dimers and $\epsilon$ is the extinction coefficient of BCA (including both dimers and monomers). Under the assumption that only dimers or both monomers and dimers of BCA go through intersystem crossing, $\Delta A$ must vary linearly with $C_d$ or with $C_t$. Examination of Fig. 5b,c reveals no linear correlation between $\Delta A$ and $C_d$ or $C_t$, ascertaining our first hypothesis that only monomeric BCA is able to generate the triplet state.

Moreover, several experimental results allow us to exclude mechanisms where the excited dimers may dissociate to yield a monomer in the triplet state and a monomer in the ground state (21). Indeed, upon excitation, the photon energy is also absorbed by the dimers of the photosensitizer, but is localized on a single chromophore because the exciton coupling is very weak. Thus, an excited dimer may dissociate, yielding one monomer in the triplet state and another in the ground state. The monomer in the ground state may associate again with another ground-state monomer existing in the solution, which can be excited again. According to this mechanism the final result must be that transient absorption intensity does not depend on the monomer–dimer equilibrium, which is not the case for BCA in phosphate buffer (Fig. 4). All of these results taken together are in agreement with the conclusion that BCA triplet state originates only from the monomer of the molecule.

To obtain the triplet-state spectrum of BCA in phosphate buffer, we used the simulation method described above. We used the spectrum of the BCA monomers as reference for the ground-state spectrum. The values of $x$ employed were in the interval 0.9–1. Irrespective of the BCA concentration employed, the shape of the triplet spectrum did not change. It has two bands, as in methanol
(Fig. 6), centered at 400 nm ($\varepsilon \sim 44,500 \, M^{-1} \, cm^{-1}$) and 600 nm ($\varepsilon \sim 15,600 \, M^{-1} \, cm^{-1}$). $\phi_{T}^{BCA}$ was determined by using the method described in Materials and Methods. The value obtained is equal to 0.095 (Table 2), in agreement with the low monomer concentration in this medium.

The triplet-state quantum yield of BCA in phosphate buffer $\phi_{T}^{BCA}$ is particularly weak, whereas $\phi_{T}^{BCA}$ has a high value in methanol and in DMPC liposomes where BCA is only in its monomeric form. Examination of Eq. 1 (see Materials and Methods) shows that $\phi_{T}^{BCA}$ is defined by the ratio of the number of molecules excited in the triplet state to the total number of absorbed photons. Now in phosphate buffer, in the range of BCA concentration studied (from $5 \times 10^{-6}$ to $5 \times 10^{-5}$ M), because of the high dimerization constant, the concentration of monomers varies in solution from $1.3 \times 10^{-6}$ to $4.8 \times 10^{-6}$ M. So, a great percentage of incident photons was absorbed by the dimers which did not contribute to the triplet-state formation. Again, the comparative study of $\phi_{T}^{BCA}$ in methanol, in DMPC liposomes and in phosphate buffer supports our hypothesis that aggregation influences the BCA triplet-state formation: only the monomers undergo intersystem crossing.

CONCLUSIONS

In summary, we have clearly demonstrated that only monomeric BCA may be excited in the triplet state, $^{1}\text{O}_{2}$, which is the most cytotoxic species in PDT of cancer, results from a reaction between the dye triplet-state and ground-state oxygen. Consequently, the solubilization of BCA in its monomeric form is a way to explore the increase of the dye efficiency in PDT.

The present work now allows us to understand the solvent effect on BCA $^{1}\text{O}_{2}$ production (Table 2). Indeed, because the dye triplet-state is the precursor of singlet-oxygen formation, $\phi(^{1}\text{O}_{2})$ has a high value in solvents where BCA is monomeric, and $\phi(^{1}\text{O}_{2})$ is weak in media where BCA is aggregated.

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REFERENCES