Photochemistry and Photobiology, 2002, 76(5): 480–485

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

Xavier Damoiseau*[1], Francis Tfibel[2], Maryse Hoebeke[1] and Marie-Pierre Fontaine-Aupart[2]

[1]Center of Oxygen Research & Development (CORD), Department of Physics, University of Liège, Belgium and [2]Photophysique Moléculaire, Université de Paris-Sud, Orsay, France

Received 14 June 2002; accepted 20 August 2002

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 ($^1O_2$) 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 bacteriochlorophyll *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 hydroxyls radicals ($OH^{\cdot}$) and superoxide anion ($O_2^{-\cdot}$) (Type-I reaction) (5); and (b) directly to molecular oxygen to yield $^1O_2$ (Type-II reaction), an important mediator of photochemical 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 $\phi(^1O_2)$ in this medium is equal to 0.05 (6). When BCA was incorporated within dimyristoyl-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 $OH^{\cdot}$ and $O_2^{-\cdot}$, remains weak, whereas the yield of singlet oxygen ($^1O_2$) increases, reaching a value of 0.33 (6,7).

The medium where BCA is solubilized influences not only the $^1O_2$ 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 10^6 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 $\phi(^1O_2)$ 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 $^1O_2$ 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 $^1O_2$ 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, bacteriochlorophyll *a* was extracted from the photosynthetic anaerobic bacterium *Rhodospirillum Rubrum.* It was purified according to the method of Omata and Murata (9). Saponifying bacteriochlorophyll *a,* as described by Oster *et al.* (10), yielded bacteriochlorophyllin *a.* To remove the Mg central ion, bacteriochlophyllin *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

¶Posted on the web site on 16 September 2002.

*To whom correspondence should be addressed at: Center of Oxygen Research & Development (CORD), Department of Physics, Institute of Physics (B5), University of Liège, 4000 Liège, Belgium. Fax: 32-4-366-28-13; e-mail: x.damoiseau@ulg.ac.be

Abbreviations: BCA, bacteriochlorin *a;* DMPC, dimiristoyl-L-α-phosphatidylcholine; MLV, multilamellar vesicles; $^1O_2$, singlet oxygen; PDT, photodynamic therapy; ROS, reactive oxygen species; $\phi(^1O_2)$, singlet-oxygen production quantum yield; ϕ_T, triplet-state formation quantum yield.

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

C_t (M)	C_m (M)	C_d (M)
5×10^{-6}	1.35×10^{-6}	1.825×10^{-6}
1×10^{-5}	2×10^{-6}	4×10^{-6}
2.5×10^{-5}	3.3×10^{-6}	1.085×10^{-5}
5×10^{-5}	4.76×10^{-6}	2.26×10^{-5}

the dark and under nitrogen. Throughout the extraction and synthesis procedures, care was taken to work at 4°C and under reduced light.

BCA was first dissolved in methanol to a stock concentration of 1.22×10^{-3} M. The BCA concentration in methanol was determined by absorption measurement using a value of 39 000 M^{-1} cm^{-1} for the extinction coefficient at 760 nm (5,7). Then, the dye was added to phosphate buffer or liposomes solutions at the desired concentration.

The samples were deaerated by bubbling argon through the solution before the experiments. The oxygenated samples were obtained by maintaining 1 atm O_2 upon the solution. All the gases were from Alphagaz with a purity of 99.99%. The measurements were carried out at room temperature (298 K).

Preparation of liposomes. DMPC (5 mg/mL) was dissolved in chloroform, and the solution was dried under vacuum in a rotary evaporator for at least 0.5 h. Multilamellar vesicles (MLV) were prepared by mechanical stirring (vortex mixer) of the lipid film suspended in phosphate buffer (pH 7) above the DMPC phase-transition temperature (24°C). Unilamellar liposomes were formed by extrusion of the MLV solution through polycarbonate filters (0.1 μm pore size, Nucleopore Corp., Pleasanton, CA), using a commercial extruder apparatus. This procedure was repeated at least 10 times, which led to unilamellar liposomes whose mean size was about 90 nm diameter and whose polydispersity was very low (11). The stock solution of liposomes was prepared at a lipid concentration of 7.38×10^{-3} M. BCA in methanol was then incubated with the liposomes at the desired lipid-to-BCA ratio for 30 min in the dark. In our experiments the final methanol concentration was always lower than 2.5%.

Absorption measurements. Ground-state absorption spectra were recorded at room temperature with a PC-controlled Cary 210 (Varian) spectrophotometer using 1 cm quartz cuvettes. To ensure equilibrium conditions, the solutions were kept for 0.5 h in the dark before recording the spectra (8).

Nanosecond transient absorption measurements were obtained using a laser photolysis equipment described previously (12,13). Briefly, the excitation source was a Q-switched Nd/YAG laser (Quantel YG 441) of 3 ns full width at half maximum with third harmonic (355 nm) generation. The 355 nm beam was directed onto one side of a square silica cell containing the sample. The transient transmission variations were monitored at right angles to the excitation in a crossbeam arrangement using a xenon flash lamp, a monochromator, a photomultiplier and a digitized oscilloscope interfaced with a microcomputer. The time and spectral resolutions of this setup were 2 ns and 1 nm, respectively. The fluence of the incident laser pulse in the sample was obtained by calibration of a joulemeter receiving a small fraction of the laser light, using anthracene in deaerated cyclohexane as a triplet actinometer (14). The latter was also used to determine, by the comparative method (14,15), the quantum yield of singlet–triplet intersystem crossing according to the following equation:

$$\phi_T^{BCA} = \phi_T^{Anthracene} \frac{A_T^{BCA}}{E^{BCA}} \frac{E^{Anthracene}}{A_T^{Anthracene}} \frac{\varepsilon_T^{Anthracene}}{\varepsilon_T^{BCA}} \frac{A_S^{Anthracene}}{A_S^{BCA}} \quad (1)$$

where $\phi_T^{Anthracene}$ and ϕ_T^{BCA} were the triplet quantum yields for anthracene and BCA, respectively, $A_T^{Anthracene}$ and A_T^{BCA} the maximum absorbances for anthracene and BCA triplet absorption, respectively, $E^{Anthracene}$ and E^{BCA} the respective excitation fluences, $\varepsilon_T^{Anthracene}$ and ε_T^{BCA} the respective triplet molar extinction coefficients and $A_S^{Anthracene}$ and A_S^{BCA} the respective absorbances of the ground state at the laser excitation wavelength. This method of determining quantum yields is valid if only a small fraction of the molecules are excited so that the absorbance obtained remains linear with the laser energy (14). In these measurements, less than 10% of the BCA and anthracene molecules were converted into the triplet state. To determine ϕ_T^{BCA}, the triplet absorption of anthracene and BCA were monitored at their maxima (422 and 600 nm, respectively) corresponding to triplet extinction coefficients of 6.47×10^4 M^{-1} cm^{-1} for anthracene and 13 000 M^{-1} cm^{-1} for BCA in methanol (see below, in Results). The triplet quantum yield value of anthracene was 0.71 (14,15).

Estimation of the BCA monomers and dimers concentration in phosphate buffer. The determination of the BCA monomers and dimers concentration in phosphate buffer was also evidenced by absorption spectroscopy. At each wavelength of the BCA absorption spectrum, the measured absorbance (A_λ) for an optical path length of 1 cm can be written as

$$A_\lambda = \varepsilon_{m\lambda} C_m + \varepsilon_{d\lambda} C_d \quad (2)$$

where $\varepsilon_{m\lambda}$ and $\varepsilon_{d\lambda}$ are the extinction coefficients of the BCA monomer and dimer at wavelength λ, respectively, and C_m and C_d the monomer and dimer concentrations of BCA, respectively. According to the law of mass conservation, the total concentration of BCA (C_t) can be related to C_m and C_d by the following expression:

$$C_t = C_m + 2C_d \quad (3)$$

According to Eqs. 2 and 3, A_λ can also be expressed as

$$A_\lambda = (\varepsilon_{m\lambda}\alpha + \tfrac{1}{2}\varepsilon_{d\lambda}(1-\alpha))C_t \quad (4)$$

where $\alpha = C_m/C_t$.

The values employed for ε_m and ε_d were 38 000 and 27 000 M^{-1} cm^{-1} at 760 nm respectively (7). For the different total concentrations of BCA used in this study, the corresponding C_m and C_d concentrations calculated from Eq. 4 are summarized in Table 1.

RESULTS AND DISCUSSION

To gain information on the influence of aggregation of BCA on the properties of its triplet state, the excited state of the dye was first studied under conditions where only monomers are present, *i.e.* either in methanol or DMPC liposomes (8). The results obtained were then compared with those in aqueous buffer solutions where monomers and dimers are present.

Monomeric BCA in methanol and in DMPC liposomes

The steady-state absorption properties of BCA in methanol have already been reported by Hoebeke *et al*. (8). In this solvent the absorption spectra did not reveal any aggregation below 10^{-4} M dye concentration. Hence, over the concentration range used in this study (lower or equal to 5×10^{-5} M), the sensitizer exists only as a monomer in its ground state. Its absorption spectrum is characterized as for all bacteriochlorin-type pigments by a strong Soret band centered at 355 nm with a shoulder at 385 nm (ε = 69 000 M^{-1} cm^{-1}) together with two weaker bands at 525 nm (ε = 16 000 M^{-1} cm^{-1}) and 760 nm (ε = 39 000 M^{-1} cm^{-1}) (Fig. 1).

Laser flash photolysis of BCA in argon-flushed methanol solutions was carried out to detect the triplet-state formation of the dye. To obtain a substantial depletion of the ground state needed for the estimation of the triplet-state extinction coefficients (see below), the dye was photoexcited in its Soret band at 355 nm.

Figure 1 inset shows the differential absorption spectrum recorded at the end of a laser pulse (10 ns) under anaerobic conditions. This spectrum was characterized by negative bands due to ground-state depletion (around 360, 525 and 750 nm) and two positive bands with maxima around 420 and 580 nm, respectively. The spectral shape does not vary with elapsing time. The time evolution of the absorbance changes is monoexponential at any wavelength, with τ = 14 μs. These facts are consistent with the

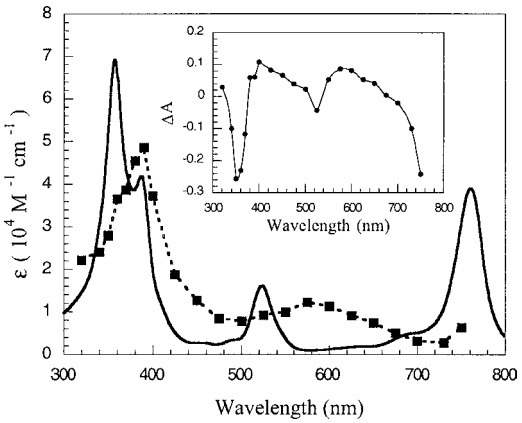

Figure 1. Ground-state (—) and calculated triplet-state (■) absorption spectra of BCA in methanol. The inset shows the differential transient absorption spectrum recorded at the end of the laser pulse on 354.7 nm photolysis of BCA (7.5×10^{-6} *M*) in deaerated methanol solutions.

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/cm^2; 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 1.6×10^{10} *M*/s, demonstrating again that it can 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 4.5×10^{-6} to 1.25×10^{-5} *M*). The results reveal that the triplet lifetime does not depend on BCA concentration. The intrinsic rate constant of BCA triplet-state decay was $k_0 = 7.14 \times 10^4$ 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 ($\varepsilon \sim 48\,500\ M^{-1}$ cm^{-1}) and 590 nm ($\varepsilon \sim 13\,000\ M^{-1}$ cm^{-1}). The triplet quantum yield of BCA (ϕ_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 spectrodynamic 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 ($\phi_{Triplet}$) and singlet oxygen generation ($\phi[^1O_2]$) in various solvents. The relative error is evaluated to 10%

Solvent	$\phi_{Triplet}$	$\phi(^1O_2)$
Methanol	0.7	0.36 (6)
Phosphate buffer (pH 7.0)	0.095	0.05 (6)
Liposomes	0.4	0.33 (6)

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 ($\varepsilon \sim 41\,500\ M^{-1}$ cm^{-1}) and 600 nm ($\varepsilon \sim 15\,000\ M^{-1}$ 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 (ϕ_F) increases with increasing viscosity (16). Electron spin resonance experiments using *n*-doxyl 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_Δ determined previously ($\phi(^1O_2)/\phi_T$) varies between 0.5 in methanol and 0.9 in DMPC liposomes. The conversion of triplet-state energy into $^1O_2$ yield is particularly good in liposomes but remains quite high in methanol. These differences in S_Δ may be related to the photobleaching of BCA and to the method used to measure the $\phi(^1O_2)$ value. Indeed, previous experiments have shown that BCA in solution can react with $^1O_2$ to generate photoproducts (20). Now, $\phi(^1O_2)$ has been determined by quantitative analysis of absorbance loss of a specific probe. Because a fraction of $^1O_2$ is quenched by BCA itself, this comparative technique leads to an underestimation of the singlet-oxygen quantum yield. In DMPC liposomes, $^1O_2$ 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 $\phi(^1O_2)$ 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

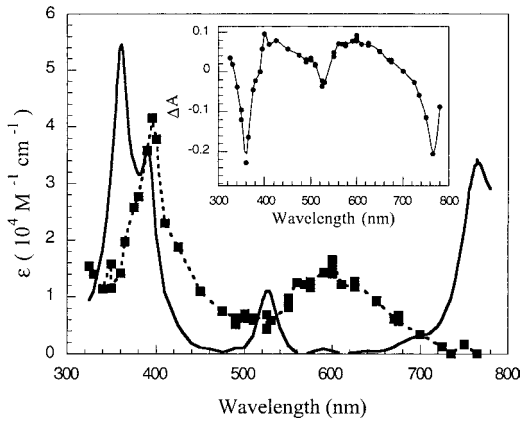

Figure 2. Ground-state (—) and calculated triplet-state (■) absorption spectra of BCA in DMPC liposomes. Inset: differential end-of-pulse transient absorption spectrum measured upon excitation of BCA (10^{-5} *M*) by a 354.7 nm laser pulse in a deaerated solution containing DMPC liposomes ([lipids] =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

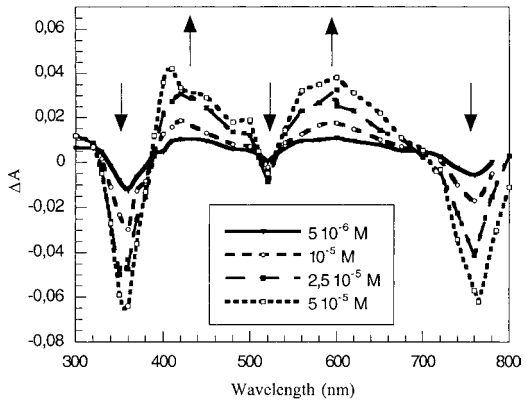

Figure 4. Differential absorption spectra measured on 354.7 nm photolysis of four aqueous solutions containing various concentrations of BCA. The spectra were recorded at the end of the laser pulse, and the fluence was equal to 11 mJ/cm^2. The arrows indicate the evolution of the spectra for increasing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

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 5×10^{-6} to 5×10^{-5} *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

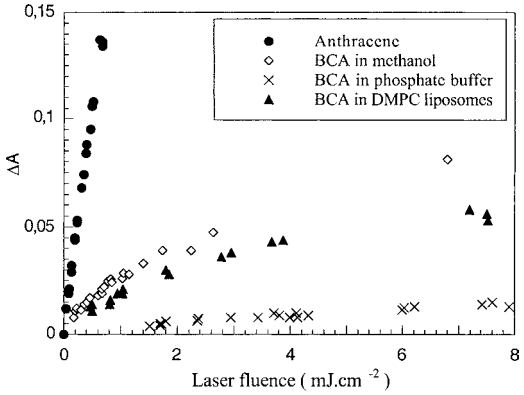

Figure 3. Transient absorption measured at 422.5 nm (•) because of triplet population on laser excitation of deoxygenated anthracene (absorbance at 354.7 = 0.82) in cyclohexane, at 400 nm (◇) because of the triplet population of deoxygenated BCA (7.5×10^{-6} *M*) in methanol, at 600 nm (×) because of the triplet population of deoxygenated BCA (10^{-5} *M*) in phosphate buffer pH 7.0, at 600 nm (▲) because of the triplet population of deoxygenated BCA (10^{-5} *M*) in DMPC liposomes (lipids = 1.5 mM).

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 ΔA on the concentration of two BCA species, the following relations were developed. The absorbance of the sample before the laser pulse (A_{BEFORE}) is equal to

$$A_{BEFORE} = \varepsilon_m C_m l + \varepsilon_d C_d l \quad (5)$$

where ε_m and ε_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_{AFTER}) is given by

$$A_{AFTER} = (1 - \beta)\varepsilon_m C_m l + \beta\varepsilon_T C_m l + \varepsilon_d C_d l \quad (6)$$

where ε_T is the extinction coefficient of the triplet and β the fraction of monomers converted into triplet state. The differential absorbance ΔA is thus expressed by

$$\Delta A = A_{AFTER} - A_{BEFORE} = \beta(\varepsilon_T - \varepsilon_m) C_m l \quad (7)$$

Under these conditions, ΔA vary linearly with C_m, as observed in Fig. 5a. Under this assumption the analytical dependence of

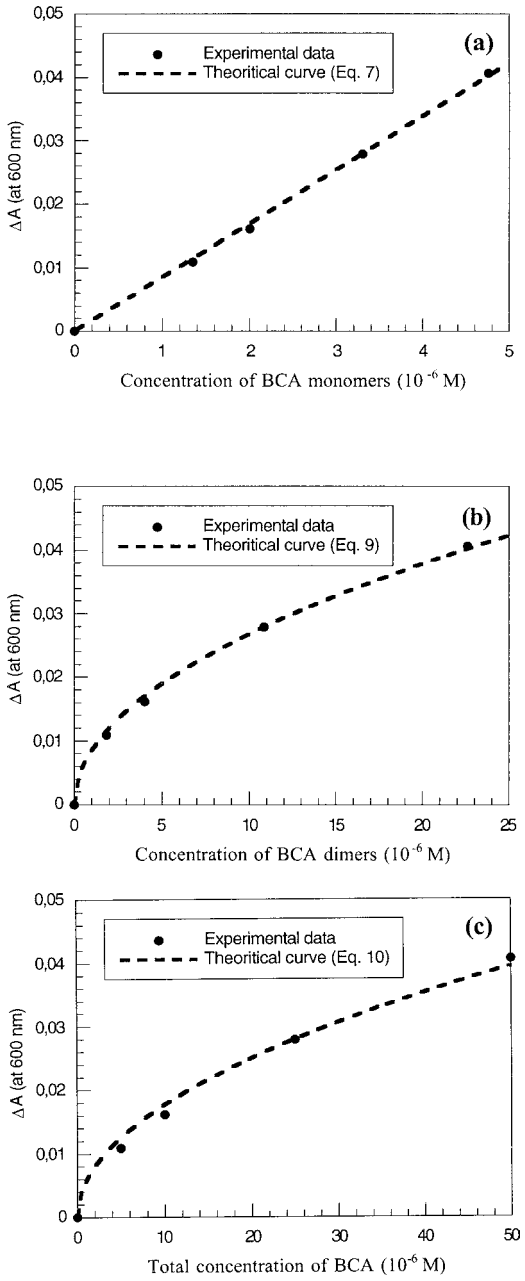

Figure 5. Differential absorption measured at the end of the laser pulse at 600 nm because of triplet population on laser excitation of BCA in deaerated phosphate buffer against the monomer concentration of BCA (a), the concentration of BCA dimers (b) and the total concentration of BCA (c). The laser fluence at 354.7 nm was of 11 mJ/cm^2.

ΔA on C_d or C_t may be determined. The dimerization constant K_d is defined by the following equation:

$$K_d = \frac{C_d}{C_m^2} \tag{8}$$

Using Eqs. 7 and 8,

$$\Delta A = \beta(\varepsilon_T - \varepsilon_m) l \sqrt{\frac{C_d}{K_d}} \tag{9}$$

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

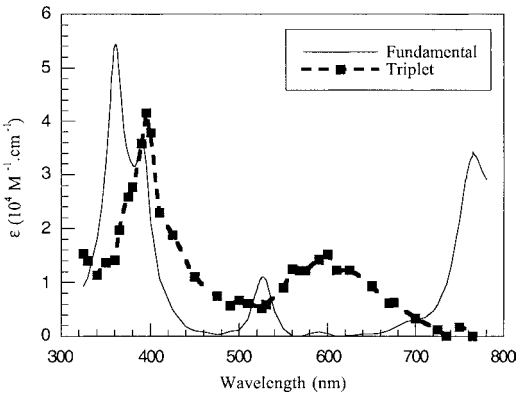

Figure 6. Ground-state (—) and calculated triplet-state (■) absorption spectra of BCA monomers in phosphate buffer pH 7.0.

$$\Delta A = \beta(\varepsilon_T - \varepsilon_m) l \left(\frac{\sqrt{1 + 8K_d C_t} - 1}{4K_d} \right) \tag{10}$$

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 ΔA variations, the expressions of ΔA are then given by Eq. 11 and Eq. 12, respectively.

$$\Delta A = \beta(\varepsilon_T - \varepsilon_d) C_d l \tag{11}$$

$$\Delta A = \beta(\varepsilon_T - \varepsilon) C_t l \tag{12}$$

where ε_d is the extinction coefficient of the dimers and ε 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, ΔA must vary linearly with C_d or with C_t. Examination of Fig. 5b,c reveals no linear correlation between ΔA 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 ($\varepsilon \sim 15\,600\ M^{-1}\ cm^{-1}$). ϕ_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 ϕ_T^{BCA} is particularly weak, whereas ϕ_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 ϕ_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 5×10^{-6} to 5×10^{-5} M), because of the high dimerization constant, the concentration of monomers varies in solution from 1.3×10^{-6} to 4.8×10^{-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 ϕ_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. $^1O_2$, 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 $^1O_2$ production (Table 2). Indeed, because the dye triplet state is the precursor of singlet-oxygen formation, $\phi(^1O_2)$ has a high value in solvents where BCA is monomeric, and $\phi(^1O_2)$ is weak in media where BCA is aggregated.

Acknowledgements—These experiments were possible thanks to grants from the Belgian National Fund for Scientific Research (FNRS) and the French National Center for Scientific Research (CNRS).

REFERENCES

1. Beems, E. M., T. M. A. R. Dubbelman, J. Lugtenburg, J. A. Van Best, M. F. M. A. Smeets and J. P. Boegheim (1987) Photosensitizing properties of bacteriochlorophyllin *a* and bacteriochlorin *a,* two derivatives of bacteriochlorophyll *a*. *Photochem. Photobiol.* **46**, 639–643.
2. Schuitmaker, J. J., J. A. Van Best, J. L. van Delft, T. M. A. R. Dubbelman, J. A. Oosterhuis and D. De Wolff-Rouendaal (1990) Bacteriochlorin *a,* a new photosensitizer in photodynamic therapy: *in vivo* results. *Investig. Ophthalmol. Vis. Sci.* **31**, 1444–1450.
3. van Leengoed, H. L. L. M., J. J. Schuitmaker, N. Van der Veen, T. M. A. R. Dubbelman and W. M. Star (1991) Fluorescence and photodynamic effects of bacteriochlorin *a* observed *in vivo* in "sandwich" observation chambers. *Br. J. Cancer* **67**, 467–471.
4. Schuitmaker, J. J., H. L. L. M. van Leengoed, N. Van der Veen, T. M. A. R. Dubbelman and W. M. Star (1993) Laser-induced in vivo fluorescence of bacteriochlorin *a:* preliminary results. *Lasers Med. Sci.* **8**, 39–42.
5. Hoebeke, M., H. J. Schuitmaker, L. E. Jannink, T. M. A. R. Dubbelman, A. Jakobs and A. Van de Vorst (1997) Electron spin resonance evidence of the generation of superoxide anion, hydroxyl radical and singlet oxygen during the photohemolysis of human erythrocytes with bacteriochlorin *a*. *Photochem. Photobiol.* **66**, 502–508.
6. Hoebeke, M. and X. Damoiseau (2002) Determination of bacteriochlorin a singlet oxygen quantum yield: a comparative study in phosphate buffer and aqueous dispersion of dimiristoyl-L-*a*-phosphatidylcholine liposomes. *Photochem. Photobiol. Sci.* **1**, 283–287.
7. Damoiseau, X., H. J. Schuitmaker, J. W. M. Lagerberg and M. Hoebeke (2000) Increase of the photosensitizing efficiency of the bacteriochlorin *a* by liposome-incorporation. *J. Photochem. Photobiol. B: Biol.* **60**, 50–60.
8. Hoebeke, M., X. Damoiseau, H. J. Schuitmaker and A. Van de Vorst (1999) Fluorescence, absorption and electron spin resonance study of bacteriochlorin *a* incorporation into membrane models. *Biochem. Biophys. Acta* **1420**, 73–85.
9. Omata, T. and N. Murata (1983) Preparation of chlorophyll *a,* chlorophyll *b* and bacteriochlorophyll *a* by column chromatography with DEAE Sepharose CL-6B and Sepharose CL-6B. *Plant Cell Physiol.* **25**, 1093.
10. Oster, G., B. Broyde and J. S. Bellin (1964) Spectral properties of chlorophyllin *a*. *J. Am. Chem. Soc.* **5**, 1309–1313.
11. Mayer, L., M. Hope and P. Cullis (1986) Vesicles of variable sizes produced by rapid extrusion procedure. *Biochem. Biophys. Acta* **858**, 161–168.
12. Al Rabaa, A. R., F. Tfibel, F. Mérola, P. Pernot and M. P. Fontaine-Aupart (1999) Spectroscopic and photophysical study of an anthryl probe: DNA binding and chiral recognition. *J. Chem. Soc., Perkin Trans.* **2**, 341–351.
13. Fontaine-Aupart, M. P., E. Renault, L. Brian, J. F. Delouis and M. Gardès-Albert (1995) Triplet excited-state characterization and determination of the photoionozation mechanism of the antitumoral drug pazelliptine. *J. Photochem. Photobiol. A: Chem.* **90**, 95–102.
14. Bensasson, R. V., E. J. Land and T. G. Truscott (1993) *Excited States and Free Radicals in Biology and Medicine*. Oxford University Press, Oxford.
15. Bensasson, R. V., E. J. Land and T. G. Truscott (1983) *Flash Photolysis and Pulse Radiolysis: Contributions to the Chemistry of Biology and Medicine*. Pergamon Press, Exeter.
16. Bhattacharyya, B. and J. Wolff (1984) Immobilization-dependent fluorescence of colchicine. *J. Biol. Chem.* **249**, 11836–11843.
17. Likhtenshtein, G. I. (1976) *Spin Labeling Methods in Molecular Biology*. Wiley-Interscience, New York.
18. Brault, D. (1990) Physical chemistry of porphyrins and their interactions with membranes: the importance of pH. *J. Photochem. Photobiol. B: Biol.* **6**, 79–86.
19. Balny, C., S. S. Brody and G. Hui Bon Hoa (1969) Absorption and fluorescence spectra of chlorophyll-a in polar solvents as a function of temperature. *Photochem. Photobiol.* **9**, 445–454.
20. Balteau, E., X. Damoiseau and M. Hoebeke (2002) Singlet oxygen involvement in bacteriochlorin *a* photosensitrization and photobleaching mechanisms. (In press)
21. Lampre, I., D. Markovitsi, A. Sharonov and M. Veber (1998) Triarylpyrylium salts: dynamics of the monomer-dimer equilibrium via a triplet absorption study. *Chem. Phys. Lett.* **293**, 423–428.