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Journal of Photochemistry and Photobiology A: Chemistry 114 (1998) 201–207

Journal of Photochemistry and Photobiology A:Chemistry

Photophysical studies on the interaction of two water-soluble porphyrins with bovine serum albumin. Effects upon the porphyrin triplet state characteristics

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Received 27 November 1997; received in revised form 7 January 1998; accepted 9 January 1998

Abstract

The interaction of two metal-free water soluble porphyrins (PPh): *meso*-tetrakis (*p*-sulfo-natophenyl)porphyrin ($TPPS_4$) and *meso*-tetrakis(4-*N*-methyl-pyridiniumyl)porphyrin (TMPyP), with bovine serum albumin (BSA) was investigated in the pH range from 4.0 to 8.5 using the flash-photolysis technique in comparison with results obtained by optical absorption, fluorescence and resonance light scattering. It was found that in the presence of BSA, $TPPS_4$ can exist in aqueous solutions as free monomers, aggregates and/or monomers bound to a single BSA molecule and monomers inside the BSA aggregates, while TMPyP does not form aggregates at binding. Binding to BSA transforms the profile of the triplet decay curve from monoexponential to biexponential form with the component lifetimes and relative amplitudes depending on binding characteristics. The triplet lifetime of a bound porphyrin monomer is longer than that of a free one. The aggregation of $TPPS_4$ observed at $[TPPS_4]/[BSA] > 1$ reduces the T–T absorption since the lifetimes of the aggregate excited states are very short and/or quantum yield of the aggregate triplet state is very low. The porphyrin binding to BSA reduces the quenching constants of the porphyrin triplet states by molecular oxygen due to obstacles produced by binding. This effect is especially pronounced for the porphyrin molecules located inside BSA aggregates formed around the porphyrin molecules in excess BSA.

Keywords: Water-soluble porphyrins; Bovine serum albumin; Flash-photolysis; Triplet state; Binding; Aggregates

1. Introduction

The application of porphyrins and porphyrin-like compounds (termed below as porphyrins) in medicine has increased significantly over the last decade due to the interesting properties of natural and synthetic porphyrins and their derivatives, especially due to their efficacy in photodynamic therapy (PDT) of cancer [1,2], psoriasis, atheromatous plaque, viral and bacterial infections including HIV [3,4], and blood substitutes (see review on Ref. [5] and references therein).

The porphyrin efficacy in PDT is attributed to singlet oxygen production due to energy transfer from the porphyrin triplet state to the oxygen ground triplet state, and thus depends on porphyrin triplet state characteristics such as lifetime and quantum yield [1,2]. The interaction of porphyrin molecules with biological structures, such as membranes, macromolecules, etc., can change the characteristics of their excited states [6–8].

The phenomenon of aggregation plays a significant role in photophysical behavior of porphyrins. In particular, the formation of aggregates changes their absorption spectra [9–12], quantum yield, lifetimes of singlet and triplet states and hence the production of the singlet molecular oxygen [13–16]. On the other hand, many biological structures either exist as self-assembling forms or are able to form aggregates and superaggregates, albumins in particular [17]. Thus, it seems to be reasonable to study the influence of aggregation on the interaction of biological structures with porphyrins.

In our previous studies using the optical absorption, fluorescence and resonance light scattering (RLS) spectroscopies, we have demonstrated the influence of aggregation on spectral and fluorescence properties of metal-free water soluble *meso*-tetrakis(*p*-sulfonatophenyl)porphyrin ($TPPS_4$) and *meso*-tetrakis(4-*N*-methyl-pyridiniumyl)porphyrin (TMPyP) (PPh, Fig. 1) upon their binding to bovine serum

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1010-6030/98/$19.00
PII S1010-6030(98)00216-0

albumin (BSA) [18,19]. In the present paper, we report on the studies of the $TPPS_4$ and TMPyP interaction with BSA using the flash-photolysis technique keeping in mind its possible influence on their triplet state characteristics. The data presented are compared with the optical absorption, fluorescence and RLS results.

2. Materials and methods

$TPPS_4$ and TMPyP were obtained from Midcentury Chemicals, and bovine serum albumin (BSA) from Sigma. All solutions were prepared in Milli-Q quality water. In all experiments we used a fresh BSA stock solution in water. Experiments were made at 24 ± 1°C.

The BSA and PPh concentrations were controlled spectrophotometrically using $\varepsilon^{280} = 4.55 \times 10^4\ M^{-1}\ cm^{-1}$, $\varepsilon^{425} = 2.26 \times 10^5\ M^{-1}\ cm^{-1}$ and $\varepsilon^{425} = 2.26 \times 10^5\ M^{-1}\ cm^{-1}$ for BSA, TMPyP and $TPPS_4$, respectively. The absorbance was measured on a Hitachi U-2000 spectrophotometer. The absorbance of solutions at 532 nm was always less than 0.3.

The titration of PPh solutions in function of the BSA concentration was performed at fixed pHs: at pH 4.0 and 8.5 for $TPPS_4$, and at pH 5.0 and 8.5 for TMPyP. Measurements were made in 0.02 M phosphate buffer. pH values were controlled by a Corning-130 pH-meter with an Ingold glass Ag/AgCl semimicro combination electrode.

The triplet state was produced by short light pulses of the second harmonics (532 nm) of Nd:YAG laser SL400 Spectron Laser System (pulse duration 10 ns) or by the xenon pulse lamp on the flash-photolysis equipment described in Ref. [20]. The triplet state decay profiles were monitored by the triplet–triplet ($T_n \leftarrow T_1$) absorption. The experiments were made in a 1-cm quartz cuvette (laser excitation) or in a 20-cm quartz cuvette (pulse lamp excitation) using a standard registration system connected with a PC-compatible computer.

The samples were deoxygenated by bubbling nitrogen through the solution, and for comparison, some samples were deoxygenated by subjecting to a vacuum pump. The saturation of solutions by oxygen was made by bubbling oxygen through the solution at the atmospheric pressure for 20 min. The intermediate oxygen concentrations were obtained by mixing deoxygenated solutions at intermediate oxygen pressures.

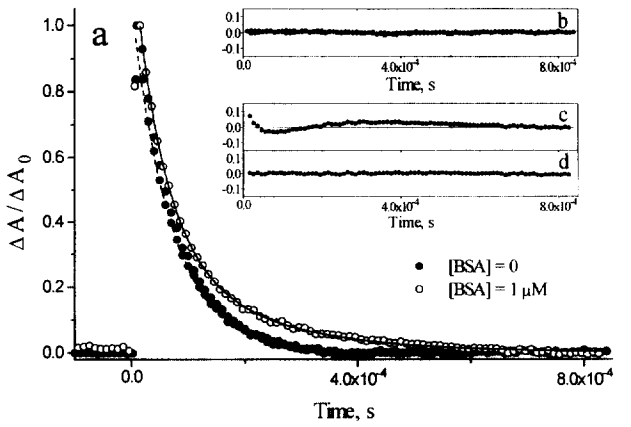


Fig. 1. (a) Normalized decay profiles of the $TPPS_4$ triplet state in deoxygenated solutions at pH 4.0 monitored at 470 nm; [BSA] = 0 (•••) and 1 μM (○ ○ ○), [$TPPS_4$] = 50 μM. Residuals of these decay profiles at [BSA] = 0 (b) and 1 μM (c) for a single-exponential fits and at [BSA] = 1 μM for a dual-exponential fit (d).

3. Results

The light pulse induces the transition of porphyrin molecules in solution into the triplet state. This changes the optical absorption of solution due to the difference between the singlet–singlet and triplet–triplet molar absorption coefficients (ε_{S-S} and ε_{T-T}, respectively)

$$\Delta A = (\varepsilon_{T-T} - \varepsilon_{S-S})[PPh(T_1)^*]$$

where $[PPh(T_1)^*]$ is the concentration of the porphyrin in the triplet state.

The addition of BSA changes the absorption spectra of PPh solutions; hence, the excitation effect should also vary due to different absorbance at the excitation wavelength. Therefore, we have to correct the ΔA according to the expression

$$\Delta A = \Delta A_{exp}{}^* A_{BSA}/A_0 \quad (1)$$

where ΔA_{exp} is an experimental value, A_0 and A_{BSA} are the absorbance values at the excitation wavelength (532 nm) in the absence of BSA and at a certain BSA concentration, respectively.

The ΔA can also differ due to changes of ε_{T-T} due to the BSA addition. Therefore, we compare the dependence of ΔA on [BSA] at two different wavelengths: at 700 nm, where the ε_{T-T} is practically independent of [BSA], and at 470 nm, where ε_{T-T} is much higher than that at 700 nm.

3.1. Homogeneous aqueous solutions

The decay profiles of triplet states (TDP) for both porphyrins in the absence of BSA appears pure monoexponential under any experimental conditions (oxygen presence or absence and different pHs)

$$\Delta A = \Delta A_0 \times \exp(-t/\tau)$$

where ΔA is the current triplet–triplet absorbance, ΔA_0 is that immediately after the light pulse, $\tau = 1/k$ is the triplet lifetime (k is a corresponding decay constant) and t is the time after the pulse (Fig. 1). This demonstrates that the contribution of bimolecular quenching processes between triplets, such as T–T annihilation, is low under conditions used. The decay constant in general should be written as

$$k = k_0 + \Sigma k_{qi}[Q_i]$$

where k_0 is the decay constant of a monomolecular process, k_{qi} is the bimolecular constant of the triplet quenching by the quencher Q_i and $[Q_i]$ is the concentration of this quencher. In particular, it can be molecular oxygen.

3.2. Solutions in the presence of BSA

3.2.1. The dependence of ΔA_0 on the BSA concentration

The ΔA_0 value, corrected according to Eq. (1), depends on the BSA concentration. The character of this dependence is the same at 700 nm and 470 nm in all cases.

For $TPPS_4$ at pH 4.0 the ΔA_0 at the BSA addition at first decreases about 3 times, reaches the minimum at a certain BSA concentration ($[BSA]_{tm}$), and increases again at higher [BSA] (Fig. 2b). At pH 8.5, the effect is similar, the reduction of ΔA_0 being less than that at pH 4.0 (Fig. 2c). For TMPyP, the ΔA_0 value does not practically change at the BSA addition at both pHs used (Fig. 2d).

3.2.2. The TDP in deoxygenated solutions

For $TPPS_4$ in deoxygenated solutions at pH 4.0 in the presence of BSA the TDP appears biexponential (Fig. 1) up to $[BSA]_{tm}$.

$$\Delta A = \Delta A_{01}\exp(-t/\tau_f) + \Delta A_{02}\exp(-t/\tau_b) \quad (2)$$

The change of the total ΔA_0 at the BSA addition makes it unjustified to analyze absolute values of the component amplitudes ΔA_{01} and ΔA_{02}; hence, we will compare only their relative contributions

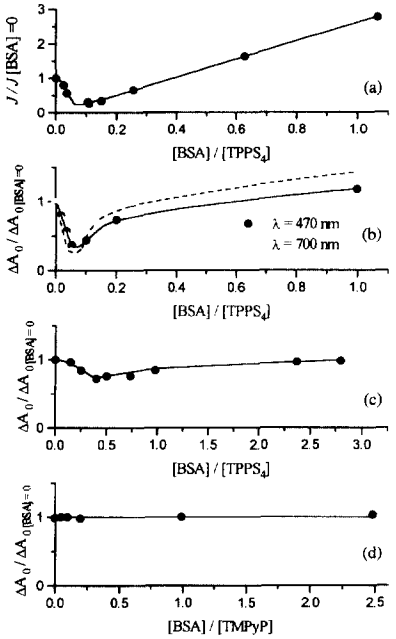


Fig. 2. Integral fluorescence intensity of $TPPS_4$ at pH 4.0 (a); amplitudes of the triplet decay profile of $TPPS_4$ at pH 4.0 (b) and pH 8.5 (c) and TMPyP at pH 8.5 (d), monitored at $\lambda = 470$ nm (●●●) and 700 nm (○○○). All data are presented as a function of the [BSA] / [PPh] ratio and normalized to those at [BSA] = 0.

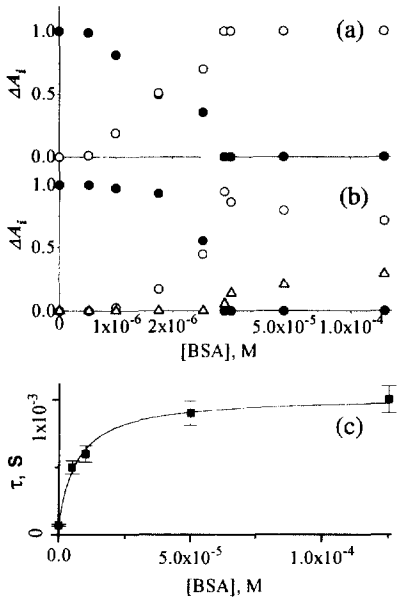


Fig. 3. Relative amplitudes of components with lifetimes τ_1 (●●●), τ_2 (○○○) and τ_3 (△△△) (see Table 1) for the $TPPS_4$ triplet decay profile at pH 4.0 and $[O_2] = 0$ (a) and 260 μM (b) as a function of [BSA]; (c) the [BSA] dependence of τ_2 for the $TPPS_4$ triplet decay at pH 4.0 and $[O_2] = 0$ (■■■). The line is a fit by Eq. (3) with $K' = 1.48 \times 10^5\ M^{-1}$ and $\tau_b = 1.02$ ms.

Table 1
The lifetimes of the components of the porphyrin triplet decay profiles in the presence of BSA in the oxygen presence and absence

Component lifetimes (s)	$TPPS_4$		TMPyP	
	pH 4.0	pH 8.5	pH 5.0	pH 8.5
Deoxygenated solutions, $[O_2] = 0$				
τ_1	5.4×10^{-5}	3.2×10^{-4}	8.5×10^{-5}	2.3×10^{-5}
τ_2	–	3.2×10^{-3}	2.4×10^{-4}	1.1×10^{-4}
Air saturated solutions, $[O_2] = 0.26$ mM				
τ_1	2.0×10^{-6}	2.3×10^{-6}	2.0×10^{-6}	2.0×10^{-6}
τ_2	1.8×10^{-5}	2.3×10^{-5}	3.4×10^{-6}	5.2×10^{-6}
τ_3	6.6×10^{-5}	–	–	3.3×10^{-4}

$$\Delta A_{0i} = \Delta A_{0i} / \sum_{i=1}^{n} \Delta A_{0i}$$

where i is the component number and n is the total number of components.

The relative contribution of the first ('short-lived') component ΔA_{01} decreases with the [BSA] increase, while ΔA_{02} of the second ('long-lived') one increases (Fig. 3a). At $[BSA] > [BSA]_{tm}$ the 'short-lived' component disappears and the TDP becomes monoexponential again. The lifetime of the 'short-lived' component is constant (Table 1) and that of the 'long-lived' one increases with the [BSA] increase (Fig. 3c).

In all other cases ($TPPS_4$ at pH 8.5, TMPyP at pH 5.0 and TMPyP at pH 8.5), the BSA addition also transforms the TDP from monoexponential to biexponential form; however, in these cases both lifetimes appear independent of [BSA]

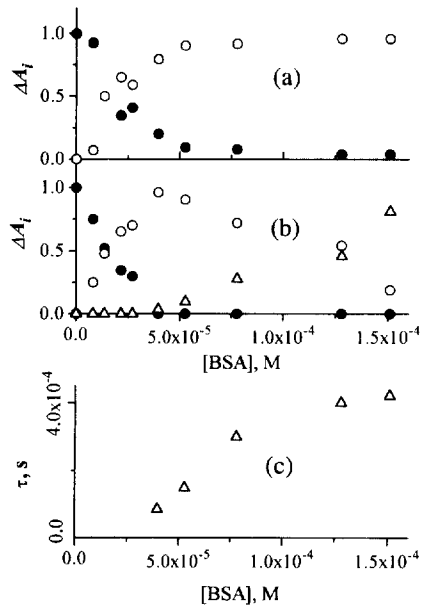


Fig. 4. Relative amplitudes of components with lifetimes τ_1 (●●●), τ_2 (○○○) and τ_3 (△△△) (see Table 1) for the $TPPS_4$ triplet decay profile at pH 8.5 and $[O_2]=0$ (a) and 260 μM (b) as a function of [BSA]; (c) the [BSA] dependence of τ_3 for the $TPPS_4$ triplet decay at pH 8.5 and $[O_2]=260$ μM.

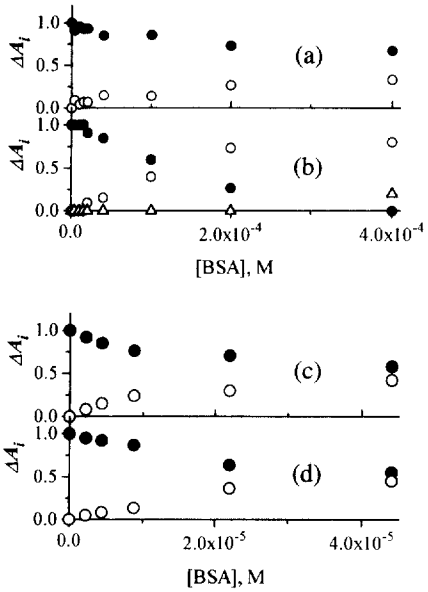


Fig. 5. Relative amplitudes of components with lifetimes τ_1 (●●●), τ_2 (○○○) and τ_3 (△△△) (see Table 1) for the TMPyP triplet decay profile as a function of [BSA]: $[O_2]=0$ (a) and 260 μM (b) at pH 8.5, (c) and (d) the same $[O_2]$ at pH 5.0.

(Table 1), while the relative contribution of the 'short-lived' component ΔA_{01} decreases, and that of the 'long-lived' one ΔA_{02} increases with the [BSA] increase (Figs. 4a, 5a and 5c).

Thus, we can notice the presence of at least two different PPh species in solutions in the presence of BSA, which can be associated with free PPh molecules and the ones bound with BSA.

3.2.3. Changes of the triplet state decay profile by molecular oxygen

The oxygen reduces the triplet lifetimes of all the TDP components. Moreover, in the presence of both BSA and oxygen, three TDP components can be observed.

Thus, for $TPPS_4$ at pH 4.0 in air-saturated solutions, three different components of the triplet decay are really observed, with lifetimes independent of [BSA] (Table 1) and their relative contributions changing with [BSA]. When $[BSA] < [BSA]_{tm}$ two components with constant lifetimes $\tau_1 = 2.0$ μs and with $\tau_2 = 18.0$ μs are present in the solution. The relative contribution of the first one, ΔA_{01} decreases with the [BSA] increase in the range $0 < [BSA] < [BSA]_{tm}$, and at $[BSA] = [BSA]_{tm}$ it disappears. The relative contribution of the second one, ΔA_{02} increases with the [BSA] increase in the same [BSA] range. However, at $[BSA] > [BSA]_{tm}$ a new component appears with $\tau_3 = 66.0$ μs, whose relative contribution ΔA_{03} increases with the [BSA] increase while ΔA_{02} begins to decrease (Fig. 3b).

For $TPPS_4$ and TMPyP at pH 8.5, three triplet decay components are also observed in air-saturated solutions. The dependence of their relative contributions on [BSA] are similar to that of $TPPS_4$ at pH 4.0 (Figs. 4b and 5b). For $TPPS_4$, τ_1 and τ_2 are independent of [BSA] (Table 1), while τ_3 increases with the [BSA] increase (Fig. 4c). For TMPyP, all three lifetimes are independent of [BSA] (Table 1).

For TMPyP at pH 5.0 in air-saturated solutions, the TDP is biexponential in the presence of BSA in the [BSA] range studied and the component lifetimes being independent of [BSA] (Table 1). The relative contribution of the first component decreases, and that of the second one increases with the [BSA] increase (Fig. 5d).

The data make it possible to assume that at least three PPh species exist in solutions in the presence of BSA with different rates of quenching of their triplet states.

The values of bimolecular quenching constants k_q determined from the k dependence on the oxygen concentration (Fig. 6)

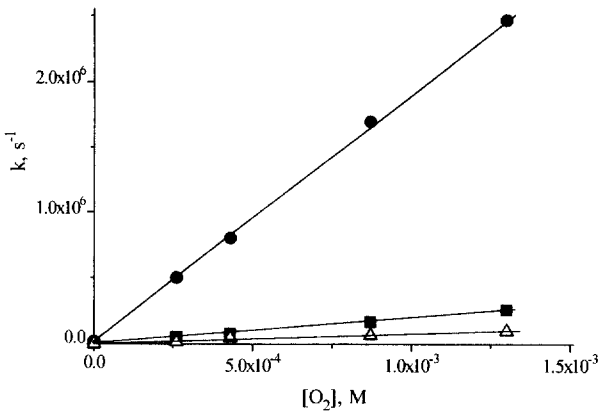


Fig. 6. Plots of the determination of the triplet quenching constants by O_2 (k_q) for the $TPPS_4$ triplet at pH 4.0, calculated as $1/\tau = k = k_0 + k_q \times [O_2]$: for τ_1 (●●●), for τ_2 (■■■) and for τ_3 (△△△).

Table 2
Quenching constants by molecular oxygen ($k_q \times 10^{-8}$ M^{-1} s^{-1}) of the triplet states of the free porphyrin (k_{q1}), porphyrin bound with the BSA monomer (k_{q2}) and porphyrin located inside the BSA aggregates and the binding constants with BSA (K, M^{-1}) of the porphyrins in the ground state

	$[TPPS_4] = 50.0$ μM		$[TMPyP] = 45.0$ μM	
	pH 4.5	pH 8.5	pH 5.0	pH 8.5
k_{q1}	19.0	17.0	19.0	18.0
k_{q2}	2.1	3.1	11.0	7.0
k_{q3}	0.55	0.09	–	0.93
K	1.5×10^8	3.2×10^6	7.3×10^5	1.8×10^6

$$k = k_0 + k_q \times [O_2]$$

for all TDP components are presented in Table 2.

The k_q values for the triplet in the absence of BSA, as well as for the first TDP components in the presence of BSA are practically equal for both $TPPS_4$ and TMPyP at any pHs ($\approx 1.8 \times 10^9$ M^{-1} s^{-1}). Since this is a diffusion-controlled process, we believe this value to be diffusion limited for the reactions between PPh and molecular oxygen in homogeneous aqueous solutions. The k_q values for second components, observed at intermediate BSA concentrations, are lower than the first ones, sometimes 10-fold ($TPPS_4$, pH 4.0), and the values for the third ones (high BSA concentrations) are even lower.

4. Discussion

Due to the presence of nitrogen atoms in their structures, $TPPS_4$ and TMPyP can exist in solutions in states of various protonation. The pK value of the $TPPS_4$ first protonation is about 5.2 [21], so at pH 4.0 it is in a mono- or biprotonated form (since pK_1 and pK_2 are very close) and its total charge is 3^- or 2^-, at pH 8.5 it is deprotonated and total charge is 4^-. TMPyP is deprotonated in the pH range from 1.0 to 12.0 and its total charge is 4^+ at both used pHs [21]. On the other hand, the pH changes induce rearrangements of the BSA molecule configuration. Indeed, at pH > 5.0, BSA transforms into a very compact form, which is conserved up to pH 7.0 [17]. Close to pH 8.0, there occur new structural transformations of the BSA molecule [22]. These charge and structural changes make the porphyrin binding constants to BSA dependent on the porphyrin type and pH (Table 2 [18]).

Recently [18,19], we have demonstrated that for $TPPS_4$, the binding to BSA stimulates the aggregation of the porphyrin molecules on the surface of BSA; thus, there should exist an equilibrium of several porphyrin forms in the solution in the presence of BSA:

$$\mathrm{BSA} + \mathrm{PPh} \overset{\gamma_1}{\rightleftarrows} (\mathrm{PPh}...\mathrm{BSA}) + \mathrm{PPh} \overset{\gamma_2}{\rightleftarrows} (2\mathrm{PPh}...\mathrm{BSA}) + \mathrm{PPh} \overset{\gamma_3}{\rightleftarrows} \ldots \overset{\gamma_{k-1}}{\rightleftarrows} ((k-1)\mathrm{PPh}...\mathrm{BSA}) + \mathrm{PPh} \overset{\gamma_k}{\rightleftarrows} \ldots \overset{\gamma_{m-1}}{\rightleftarrows} ((m-1)\mathrm{PPh}...\mathrm{BSA}) + \mathrm{PPh} \overset{\gamma_m}{\rightleftarrows} (m\mathrm{PPh}...\mathrm{BSA})$$

or in a simplified form

$$\mathrm{BSA} + \mathrm{PPh} \overset{\gamma_1}{\rightleftarrows} (\mathrm{PPh}...\mathrm{BSA}) + \mathrm{PPh} \overset{\gamma_{aggr}}{\rightleftarrows} \mathrm{Aggr}$$

The total porphyrin concentration in the solution $[PPh]_0$ is:

$$[PPh]_0 = [PPh_f] + [Aggr] + [PPh_b]$$

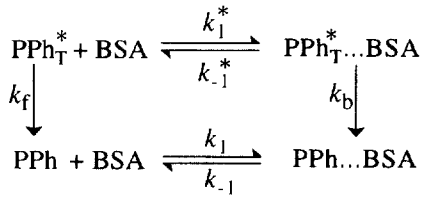
where $[PPh_f]$ is the free porphyrin monomer concentration, $[Aggr] = \sum_{k=2}^{m} k[k\mathrm{PPh}...\mathrm{BSA}]$ is the total concentration of aggregated porphyrins with the aggregation numbers from 2 to a maximum m, and $[PPh_b] = [PPh...BSA]$ is the concentration of the bound porphyrin monomer. The [Aggr] at first increases with the BSA concentration increase, reaches a maximum value at a certain BSA/$TPPS_4$ concentration ratio (ACM) and decreases at higher BSA concentrations in favor of the bound porphyrin monomer, so that at the BSA excess, only this porphyrin form exists in the solution. The porphyrin aggregation changes its absorption and fluorescence spectra in the solution, its total fluorescence and the resonance scattered light intensity. The averaged $TPPS_4$ aggregation number $\langle k \rangle$, calculated from the fluorescence data, is 10 ± 2 at pH 4.0 and 3 ± 1 at pH 8.5 [18]. The maximum content of porphyrin aggregates as defined from the optical absorption spectra, maximum of the scattered light intensity and the minimum of the total fluorescence intensity are observed at the same [BSA]/[$TPPS_4$] ratio as the minimum of ΔA_0 (Fig. 2a,b; [18,19]). We should suppose that the decrease of both the T–T absorbance and the fluorescence intensity, is due to the $TPPS_4$ aggregation. This correlates with the facts that for $TPPS_4$ at pH 8.5, where $\langle k \rangle$ is lower than at pH 4.0, the effect of reduction of ΔA_0 is also smaller and for TMPyP, which does not aggregate at binding to BSA, ΔA_0 is independent of [BSA] (Fig. 2b,c,d).

The effect of reduction of the fluorescence intensity and ΔA_0 at the aggregation could be explained in terms of a rapid quenching of the excited singlet directly to the ground state and/or enhanced intersystem crossing to the triplet followed by the quenching of the triplet [23]. The last process reduces considerably the lifetimes of the triplet states, and within the instrumental time resolution used, we cannot observe the T–T absorption of aggregates. Thus, under our conditions we can observe the total T–T absorption just of free and bound PPhs monomers.

The presence of these two forms in solutions allows to expect the transformation of monoexponential triplet decay profile into biexponential form by the BSA addition

$$\Delta A = \Delta A_{0f} \times \exp(-t/\tau_f) + \Delta A_{0b} \times \exp(-t/\tau_b)$$

The basic equilibrium between the free and bound monomers at excitation can be presented as



Here $k_f = 1/\tau_f$, $k_b = 1/\tau_b$ and k_1, k_{-1}, k_1^* and k_{-1}^* are the constants of binding and dissociation in ground and excited (triplet) states, respectively. The corresponding constants of equilibrium are $K = k_1/k_{-1}$ and $K^* = k_1^*/k_{-1}^*$. The ratio $\Delta A_{0f}/\Delta A_{0b}$ is determined by the equilibrium between the free and bound porphyrin forms.

The detailed analysis of this diagram [6,23] shows that τ_f and τ_b should be only arbitrarily corresponded to the lifetimes of the free and bound porphyrin monomers, since the dynamic exchange between these two porphyrin forms should change both τ_f and τ_b.

Three possible situations can be considered.

(1) The BSA–porphyrin complex lifetime is comparable with that of its triplet excited state ($k_{-1}^* \cong k_{b\,max}$). The exchange should be effective during the lifetime of excitation and thus τ_f and τ_b should depend on the equilibrium characteristics as [23]

$$\tau_b = (\tau_{f0} + \tau_{b\,max} K'[\mathrm{BSA}])/(1 + K'[\mathrm{BSA}]) \quad (3)$$

where τ_{f0} is the triplet lifetime without BSA, $\tau_{b\,max} = 1/k_{b\,max}$ is the limit of that at a high BSA excess and $K' = (\tau_{f0}/\tau_{b\,max})\,K^*$.

(2) The complex is relatively stable as compared with the lifetime of its excited state ($k_{-1}^* < k_{b\,max}$). In this case τ_f depends on [BSA] as

$$1/\tau_f = 1/\tau_{f0} + k_1^* \times [\mathrm{BSA}] \quad (4)$$

while $\tau_b = \tau_{b\,max}$ is independent of [BSA].

(3) The complex is relatively unstable ($k_{-1}^* \gg k_{b\,max}$) and $\tau_f = \tau_{f0}$ and

$$1/\tau_b = k_{-1}^*(1 + K^*[\mathrm{BSA}]) \quad (5)$$

The dependence of τ_b on [BSA] for the $TPPS_4$ deoxygenated solutions at pH 4.0 demonstrates the case (1) ($k_{-1}^* \cong k_{b\,max}$). The calculation in accordance with Eq. (3) (Fig. 3c) gives the values $(2.2 \pm 0.2) \times 10^6$ M^{-1} and $(1.0 \pm 0.1) \times 10^{-3}$ s^{-1} for K^* and $\tau_{b\,max}$, respectively. The K^* value is about 70-fold less than the binding constant in the ground state $K = 1.5 \times 10^8$ M^{-1} (Table 2).

The binding constants for $TPPS_4$ at pH 8.5, TMPyP at pH 5.0 and TMPyP at pH 8.5 are lower than that for $TPPS_4$ at pH 4.0 already for PPhs in the ground states (Table 2). Therefore we should expect $k_{-1}^* \gg k_{b\,max}$ (the situation (3)). Indeed, in all these cases, $\tau_f = \tau_{f0}$ and is independent of [BSA]. However, τ_b is also independent of [BSA], which is contrary to Eq. (4). This fact should be explained by the assumption that the K^* values are lower than those for ground states and in the [BSA] range used ([BSA] $< 1.5 * 10^{-4}$ M) the values $K^* \times [\mathrm{BSA}] < 1$. Therefore, the dependence of τ_b on [BSA] is negligible. This assumption seems reasonable since excitation reduces the binding constants.

The presence of oxygen reduces the lifetimes of free and bound triplets due to the quenching and gives $k_{-1}^* < k_{f0}, k_b$ (situation (2)). In this case τ_b should be independent of [BSA], which is really observed. However, τ_f seems independent of [BSA] also. We can explain this fact taking into account that the values of the constants of the complex formation in the excited states (k_1^*) should be much lower than their diffusion limit ($\approx 2.0 * 10^9$ M^{-1} s^{-1}) and at [BSA] $< 1.5 \times 10^{-4}$ M we have $k_1^* \times [\mathrm{BSA}] < 3 \times 10^5$ s^{-1} which is less than $1/\tau_{f0}$. For example, for $TPPS_4$ at pH 4.0, we have $1/\tau_{f0} = 5 \times 10^5$ s^{-1}. Therefore, the dependence of τ_{f0} on [BSA] (Eq. (5)) should be very weak in this [BSA] range.

Binding to BSA reduces the quenching constants of PPh triplets by the molecular oxygen. The reduction is larger for higher binding constants (Tables 1 and 2). The reason for this reduction should lie in the obstacles for contact of oxygen molecules with bound PPh.

Recently [18], we have also demonstrated the formation of BSA aggregates around a porphyrin molecule at the protein excess. We observe no specific influence of the BSA aggregation on the spectral and fluorescence characteristics of porphyrins. It is reasonable to suppose that porphyrin molecules can locate inside these BSA aggregates both in a free and a bound form. Thus, in the absence of oxygen, we should expect no difference in the behaviour of porphyrin triplets inside and outside the BSA aggregates. Hence, it should be supposed that this aggregation produces obstacles for the contact of porphyrin and oxygen molecules that are much stronger than a simple binding of PPh to BSA; therefore, the quenching constants of PPh triplets located inside the BSA aggregates should be lower than those located outside. Thus, the third longest triplet component (τ_3) observed only in the presence of oxygen should be explained in terms of the decay of triplets located inside the BSA aggregates and weaker quenched by oxygen. This effect should be stronger at higher BSA aggregation numbers and lower mobility of reagents inside the aggregates. The aggregation number is higher at pH 8.5 as compared with pH 4.0 or pH 5.0 [18]. On the other hand, the PPh mobility should be lower at a higher complex stability (higher binding constant), which in its turn depends on pH. Therefore, we do not observe the third triplet component for TMPyP at pH 5.0, since in this case, the BSA aggregation number and the binding constant of the PPh to BSA are lower than those under other conditions. The highest effect was observed for $TPPS_4$ at pH 8.5 (Table 2).

5. Conclusion

Based on data presented above, we can conclude that in the presence of BSA, $TPPS_4$ can exist in aqueous solutions in different forms, namely: (1) free porphyrin monomers; (2) porphyrin aggregates bound to a single BSA molecule;

(3) porphyrin monomers bound to a single BSA molecule; and (4) porphyrin monomers inside the BSA aggregate.

The relative contents of these forms depend on the porphyrin and albumin structures and the charge states, which determine the values of binding constants, and on their concentrations.

At $[BSA] < [TPPS_4]$, the first form is dominant; its concentration decreases in favor of the second one with the [BSA] increase. At further [BSA] increase, the $TPPS_4$ aggregates decompose and at $[BSA] \geq [TPPS_4]$, the porphyrin-bound monomers dominate in solutions. The BSA aggregates are formed at $[BSA] \gg [TPPS_4]$. The $TPPS_4$ aggregation number is higher for the monoprotonated state (≈ 10 at pH 4.0) than for nonprotonated one (≈ 3 at pH 8.5).

Contrary to this, TMPyP does not form aggregates at the binding to BSA and exists in solutions only as monomers (free or bound to single BSA molecule or BSA aggregate).

The characteristics of triplet states of PPh bound forms differ from those of the free ones. The lifetime of excitation of the bound aggregates is very short and/or the quantum yield of the aggregate triplet state is very low, which is manifested in the reduction of the T–T absorption at PPh aggregation. Contrary to this, the lifetime of the triplet of the bound porphyrin monomer is longer than that of the free porphyrin monomer. The porphyrin binding to BSA reduces the probability of the porphyrin triplet quenching by the molecular oxygen since the binding makes the contact between the reagents more difficult. This effect is extremely high for the porphyrin molecules located inside the BSA aggregates.

These effects should be taken into account at possible porphyrin medical applications.

Acknowledgements

The authors are indebted to CNPq, FINEP and FAPESP Brazilian agencies for partial financial support. I.E. Borissevitch is recipient of a visiting grant from CNP_q. We are grateful to Prof. G. Borissevitch and Prof. M. Tabak for useful discussion and to Prof. M.G. Neumann for the help in the flash-photolysis experiments.

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