Journal of Photochemistry and Photobiology B: Biology 141 (2014) 275–282

Contents lists available at ScienceDirect

Journal of Photochemistry and Photobiology B: Biology

journal homepage: www.elsevier.com/locate/jphotobiol

Effect of dye localization and self-interactions on the photosensitized generation of singlet oxygen by rose bengal bound to bovine serum albumin

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ARTICLE INFO

Article history:
Received 26 July 2014
Received in revised form 20 September 2014
Accepted 23 September 2014
Available online 5 October 2014

ABSTRACT

The spectroscopic and photophysical properties of rose bengal (RB) encased in bovine serum albumin (BSA) have been examined to evaluate the photosensitized generation of singlet molecular oxygen ($^1O_2$). The results show that RB photophysical and photosensitizing properties are highly modulated by the average number of dye molecules per protein (n). At $n \ll 1$, the dye molecule is tightly located into the hydrophobic nanocavity site I of the BSA molecule with a binding constant $K_b = 0.15 \pm 0.01\ \mu M^{-1}$. The interaction with surrounding amino acids induces heterogeneous decay of both singlet and triplet excited states of RB and partially reduce its triplet quantum yield as compared with that in buffer solution. However, despite of the diffusive barrier imposed by the protein nanocavity to $^3O_2$, the quenching of $^3RB^*$:BSA generates $^1O_2$ with quantum yield $\Phi_\Delta = 0.35 \pm 0.05$. In turns, the intraprotein generated $^1O_2$ is able to diffuse through the bulk solution, where is dynamically quenched by BSA itself with an overall quenching rate constant of $7.3 \times 10^8\ M^{-1}\ s^{-1}$. However, at $n > 1$, nonspecific binding of up to ≈6 RB molecules per BSA is produced, allowing efficient static quenching of excited states of RB preventing photosensitization of $^1O_2$. These results provide useful information for development of dye-protein adducts suitable for using as potential intracellular photosensitizers.

1. Introduction

Photosensitized generation of singlet molecular oxygen ($^1O_2$) has very relevant implications in photochemistry and photobiology with several uses in different fields, such as wastewater treatment, fine chemical synthesis, and photodynamic therapy (PDT) [1–3]. In the latter case, current increasing interest is focused in the development of efficient hybrid photosensitizer systems composed by a sensitizer molecule encased either in biocompatible self-assembled structures or in biomolecules [4–6]. Recently, with the design and development of genetically-encodable fluorescent proteins, such as green fluorescent protein (GFP) family, it has been shown that they were also able to photogenerate $^1O_2$, with potential use in antimicrobial PDT applications [7]. However, most of the GFP variants showed very low quantum yield of $^1O_2$ generation, *e.g.* $\Phi_\Delta < 0.004$, because of the intrinsic low efficiency of the GFP chromophore [8]. On the contrary, it has been formerly reported that blue-light excitation of a genetically encoded flavoprotein bearing flavin mononucleotide FMN produced $^1O_2$ with quantum yield value similar to that for the free flavin ($\Phi_\Delta \approx 0.5$), giving reason to name this flavoprotein as mini-Singlet-Oxygen-Generator (miniSOG) [9]. However, later photophysical characterization studies of miniSOG demonstrated that the photosensitized efficiency of $^1O_2$ is much lower, e.g. $\Phi_\Delta \approx 0.03$, due to the role of the peptide backbone to enhance electron-transfer (Type I) in detriment of energy-transfer (Type II) mechanism [10,11].

On the other hand, as a different strategy to obtain efficient biocompatible $^1O_2$-photosensitizer systems, we have characterized the photophysical and photochemical properties of well-known Type II artificial sensitizers such as zinc phthalocyanine (ZnPc) [6], rose bengal (RB) [12], and methylene blue (MB) [13], non-covalently bound to both bovine and human serum albumins (BSA and HSA), considering the large concentration of these proteins in blood and their function in the transport and delivery of small drugs and bioactive molecules [14]. However, despite of the moderate efficiency of generation of $^1O_2$ of these supramolecular photosensitizer assemblies, the photophysical and photosensitizing properties of the dye-albumin adducts were mainly dependent on the type of binding-site in the albumin, molecular oxygen

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http://dx.doi.org/10.1016/j.jphotobiol.2014.09.014
1011-1344/

accessibility to the dye binding location, and also of the average number of sensitizer molecules per protein (n), being each adduct system practically a particular case of study. Therefore, the results indicate that the photoinduced generation of $^1O_2$ by sensitizers encased in proteins cavities is a rather complex issue, where all or some of the above mentioned factors can induce different effects depending on the nature of the guest sensitizer and the host protein.

For a deeper understanding of the role of these different effects on the photosensitizing properties of sensitizer-protein adducts, in this work we studied the system formed by rose bengal (RB) encased in bovine serum albumin (BSA), under concentration conditions where the protein/dye molar ratio was varied more than two-order of magnitude to analyze separately the contribution of the self-interaction and compartmentalization processes of the bound dye. The xanthene dye RB was chosen as suitable sensitizer because besides of its large quantum yield of generation of $^1O_2$ in aqueous media ($\Phi_\Delta = 0.76$) [15], their absorption and emission spectra are very sensitive to both specific and global environmental effects [15–18]. The results reported here expand those observed in our previous study of the system RB-HSA [12], demonstrating how both photophysical and photosensitizing properties of the dye-protein adduct are modulated by n, as consequence of dye self-interaction and compartmentalization effects. All together, the present results provide useful information on the role of supramolecular interactions between the dye and the albumin and also about the fate of $^1O_2$ in this type of systems, which can be applied for the further development of non-covalent sensitizer-protein adducts with suitable efficiency of $^1O_2$ generation.

2. Experimental section

2.1. Materials

Rose bengal (RB), chemically 4,5,6,7-tetrachloro 2′,4′,5′,7′-tetraiodo-fluorescein di sodium salt, bovine serum albumin (BSA, ⩾98% free lipids), Trizma® base (tris[hydroxymethyl]-aminomethane, electrophoresis reagent minimum 99.9%) and deuterium oxide and chloride, 99.9 atom %D, were purchased from Sigma–Aldrich Argentina (Buenos Aires, Argentina). Hydrochloric acid (HCl, 36%) was from Merck Argentina (Buenos Aires, Argentina). All experiments were performed at 25 °C in 20 mM Tris–HCl pH 7.4 employing triply distilled water, except for experiments of near-infrared luminescence of singlet molecular oxygen, $^1O_2$, for which D_2O (pD 7.4 adjusted with DCl) was used as solvent to improve the signal detection. Compressed ultrapure argon (99.99%) was purchased from Indura SRL (S.M. de Tucumán, Argentina).

2.2. Steady-state spectroscopic measurements

UV–Vis absorption spectra were registered using either a Hewlett Packard 8453 (Palo Alto, CA, USA) or an OceanOptics USB2000 (Dunedin, FL, USA) UV–Vis spectrophotometer. Fluorescence emission measurements were done with a Hitachi F-2500 (Kyoto, Japan) spectrofluorometer, equipped with a red-extended R-928 photomultiplier, and using excitation and emission slits with 5 nm of bandwidth. The fluorescence quantum yield (Φ_F) of RB in BSA solutions was determined by comparison of the integrated fluorescence intensity using as reference the emission of dye in buffer solution ($\Phi_F = 0.018$) [19]; keeping constant the absorbance value at the excitation wavelength (525 nm). Steady-state fluorescence anisotropy r of the RB was determined with a classical L-format and calculated as described elsewhere [20,21].

The steady-state quenching of the intrinsic fluorescence of BSA by addition of RB was studied by excitation at 295 nm. Since RB absorbs light both at the excitation and emission spectral regions of BSA, the observed emission spectra were corrected by the primary and secondary inner filter effects as described before [20,21].

2.3. Time-resolved spectroscopic measurements

Fluorescence emission decays of RB were obtained with a time-correlated single photon counting lifetime spectrofluorometer (Tempro-01 of Horiba Jobin Yvon, Glasgow, UK), using as excitation source an 1 MHz pulsed Nanoled® emitting at 560(±15) nm from Horiba. In order to avoid scattering from the excitation source in the collected dye emission, an interference filter at 543(±10) nm (PIL-1, Schott, Jena, Germany) was placed before the sample holder. The fluorescence emission decays of BSA were collected at 340 nm after excitation with an 1 MHz pulsed Nanoled® emitting at 277(±11) nm. In all cases, the fluorescence emission was monitored through an f/4 monochromator with output slit of 12 nm of bandwidth. The fluorescence intensity decays were fitted with the Fluorescence Decay Analysis Software DAS6® of Horiba Jobin Yvon by deconvolution of the pulse function using the multi-exponential model function,

$$I(t) = \sum_{i=1}^{j} \alpha_i \exp(-t/\tau_{F,i}) \quad (1)$$

where j is the number of single exponential decays, $\tau_{F,i}$ and α_i are the relative fluorescence lifetime intensity amplitude at $t = 0$ of each decay, respectively. In the case of $j > 1$, the average lifetime ($\tau_{F,av}$) was calculated by the following equation, with f_i as the fractional contribution of each decay time to the steady-state intensity.

$$\tau_{F,av} = \sum_{i=1}^{j} f_i \tau_{F,i} \text{ with } f_i = \alpha_i \tau_{F,i} / \sum_{i=1}^{j} \alpha_i \tau_{F,i} \quad (2)$$

Transient absorption spectra of RB were recorded with the m-LFP 112 laser-flash photolysis system of Luzchem Research Inc. (Ottawa, Canada), using as excitation source the second harmonic beam (532 nm, 7 ns fwhm, ~5 mJ/pulse) from a Nd-YAG laser Minilite II of Continuum Inc. (Santa Clara, CA, USA). To minimize degradation of the sample, up to 10-single laser shots were averaged. The transient decays were fitted using a single or double exponential function with the following equation

$$\Delta A_\lambda(t) = \Delta A_\infty + \sum_{i=2} \Delta A_{0,i} \exp(-t/\tau_{T,i}) \quad (3)$$

where $\Delta A_{0,1}$ and $\Delta A_{0,2}$ are the initial difference absorbance associated with each transient of lifetime $\tau_{T,1}$ and $\tau_{T,2}$, respectively, and ΔA_∞ is the ending difference absorbance value. In this case, the average lifetime $\tau_{T,av}$ of the transient species was calculated as:

$$\tau_{T,av} = \frac{\sum_{i=2} \Delta A_{0,i} \tau_{T,i}}{\sum_{i=2} \Delta A_{0,i}} \quad (4)$$

Triplet (Φ_T) and bleaching (Φ_b) quantum yields of RB in BSA solutions were calculated by comparison of the respective transient absorption changes at $t = 0$ (ΔA_0) at each maximum divided by the ground state absorption of the solution at the laser excitation wavelength with that observed for the dye alone in buffer solution, assuming the same extinction coefficient of the transient species without and with protein [12,13].

Transient luminescence detection of singlet molecular oxygen $^1O_2$ at 1270 nm, produced by photosensitization of RB after laser excitation at 532 nm, was performed with a Ge photodiode J16TE2-66G from Teledyne Judson Technology (Montgomeryville, PA, USA). The initial spike in the luminescent signal produced by spurious fluorescence of the dye or scattered laser light was subtracted with the residual signal observed for a RB solution in D_2O