Mechanism of photobleaching of Ethyl Violet non-covalently bound to bovine serum albumin

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Upon laser excitation of Ethyl Violet non-covalently bound to bovine serum albumin, leuco-Ethyl Violet and 4,4'-bis(diethylamino)benzophenone are formed as reaction photoproducts

Photodynamic Therapy (PDT)[1] has recently been approved by regulatory agencies of several countries as a new modality of cancer treatment. The current enthusiasm for this general technique has fomented the search for new photosensitizers tailored for specific therapeutical applications.[1b,c] The development of new drugs for PDT requires the comprehension of the mechanisms of action of promising dyes, or families of dyes, under conditions that closely parallel those observed *in vivo*. This report describes a study on the mechanisms of photoreaction of a triarylmethane dye (Ethyl Violet, EV) non-covalently bound to a model biological host (bovine serum albumin, BSA). Triarylmethanes (TAM) are known to efficiently bind to anionic polyelectrolytes in solution,[2] and are among the families of dyes presently under intense evaluation as potential photosensitizers for employment in PDT.

The fluorescence lifetime of triarylmethane dyes in low viscosity environments is typically in the picosecond range as a consequence of fast relaxation processes that occur *via* rotational motions of their aromatic rings.[3] Accordingly, TAM dyes show poor photoreactivity in aqueous solutions. In more viscous media, or in microenvironments that render steric hindrance to rotational relaxation processes, fluorescence and intersystem crossing become more competitive events, and photoreactivity tends to increase.[2c] The effect of BSA on EV fluorescence in 0.01 M phosphate buffer pH 7.3 at 20 °C is shown in Fig. 1. The increase in EV fluorescence as a function of BSA concentration indicates that the BSA binding sites are very effective in preventing 'free rotor' motions in the dye moiety. This reduces the efficiency of radiationless deactivation of EV, a process that involves a low-lying twisted intramolecular charge transfer state (TICT),[3c] and leads to an enhancement in its fluorescence quantum yield from 9.6×10^{-5} (free) to 1.7×10^{-2} (protein-bound), as measured using corrected fluorescence spectra and quinine sulfate[4] as fluorescence standard. The fluorescence decay of EV bound to BSA (a typical multiple-binding-site carrier[5]) follows double exponential functions. The global analysis of three independent determinations yielded fluorescence lifetimes of 392 ± 42 ps and 2.95 ± 0.19 ns (χ^2 = 1.032). These are remarkably high values if compared with the lifetime of EV free in low viscosity media (5 to 10 ps[3b,d]), and suggest the existence of two dominant ground state conformers of EV in the BSA environment (bound to different types of binding sites[5b,c]). Upon photoexcitation, the population of EV molecules displaying conformation closer to that of the TICT state is the one which relaxes faster.[3b] The Scatchard analysis[5b] of our fluorescence data confirmed the existence of two distinct types of BSA binding sites capable of accommodating EV molecules. The amino acid composition of these macromolecular domains may also affect the fluorescence lifetime of bound EV molecules (see below).

The observed increase in EV fluorescence lifetime and quantum yield suggests that the efficiency of intersystem crossing (ϕ_{ISC}) might also undergo expressive increase as a consequence of binding. The bleaching of BSA-bound EV upon laser excitation at 532 nm is shown in Fig. 2. Under photolysis conditions essentially all dye molecules are bound to BSA (plateau region of the binding isotherm; large [protein] : [dye] ratio), therefore experiencing loss of rotational degrees of

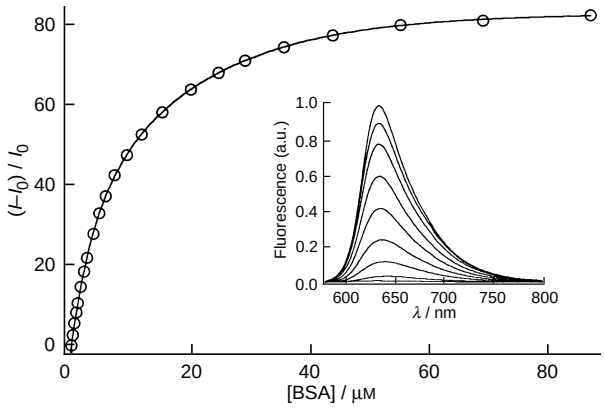


Fig. 1 Effect of BSA concentration on the integrated fluorescence of EV. Inset: representative spectra employed in the construction of the binding isotherm. For visual clarity only one third of the data set is shown. From the bottom, in order of increasing fluorescence intensity at 634 nm, the BSA concentrations were 0.0, 0.27, 1.06, 2.64, 5.76, 11.8, 24.7, 44.0 and 87.4 μM. Excitation at 520 nm; [EV] = 10 μM; phosphate buffer (0.01 M) pH 7.3; T = 20 °C.

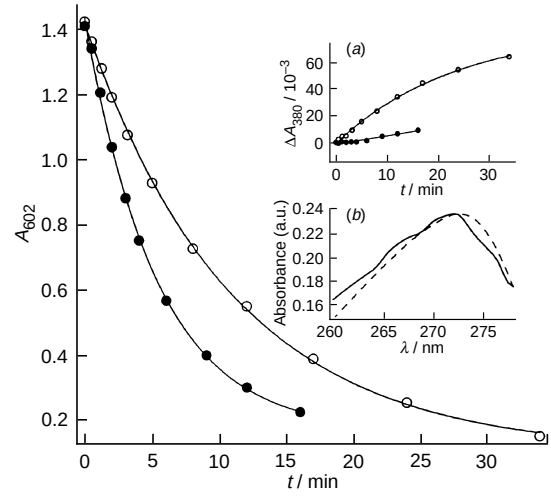
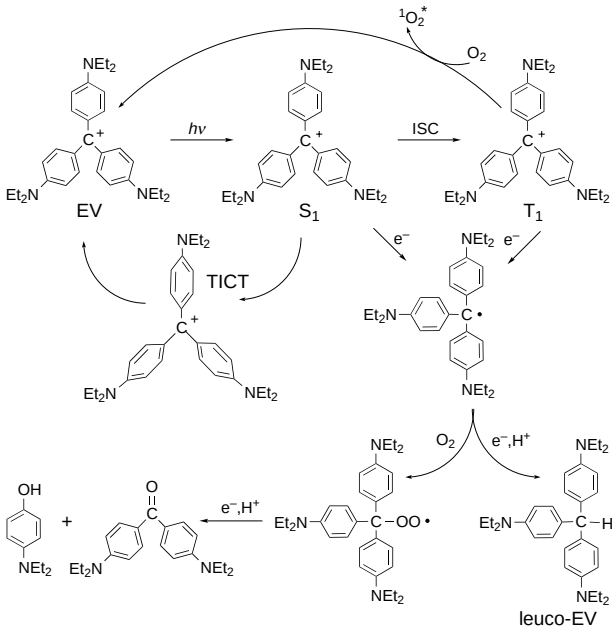


Fig. 2 Effect of photolysis time on the maximum absorption of the BSA–EV complex (602 nm); (●) nitrogen-purged samples and (○) air-equilibrated samples. Excitation at 532 nm, 87 mJ pulse^{-1}, 10 Hz. Inset (*a*): respective changes in absorption at 380 nm, the region in which 4,4-bis(diethylamino)-benzophenone absorbs. Inset (*b*): differential spectrum (—) obtained after 16 min of photolysis of the nitrogen-purged sample, *i.e.* the spectrum recorded at t = 16 min minus the spectrum recorded at t = 0, and the absorption spectrum of leuco-EV in EtOH (---).



Scheme 1

freedom. The quantum efficiency of photobleaching was found to be two fold higher in nitrogen-purged than in air-equilibrated samples. The increased bleaching efficiency in nitrogen-purged as compared to air-equilibrated samples is direct evidence of the involvement of the first excited triplet state of the dye in the bleaching process. The comparison between the photobleaching efficiency of EV free in solution and protein-bound is more prominent. A 13-fold increase is observed as a consequence of binding (air-equilibrated samples). Using Methylene Blue[2c] as a laser flash photolysis standard we have estimated ϕ_{ISC} as 0.03 for BSA-bound EV. For the case of the dye free in solution we were unable to detect any transient signal (ns–μs range) following ns laser excitation at 560 nm.

The analysis of reaction products was performed by extraction of photolysed samples with EtOAc and characterization of the extracted compounds by thin-layer chromatography (silica gel; 2 : 1 hexanes–EtOAc). 4,4′-Bis(diethylamino)benzophenone and leuco-EV were identified as photoproducts. The former was more prominent in air-equilibrated samples, the latter in nitrogen-purged samples. The reaction mechanism shown in Scheme 1 accommodates the results observed. The reductive formation of leuco-EV from EV is a two electron process (formally $2e^- + H^+$ or $H + e^-$), initiated in this case by a photoinduced electron or hydrogen atom transfer from the protein to the dye moiety. 78 out of the 583 BSA amino acid residues are readily susceptible of photosensitized oxidation[2a] (2 Trp, 4 Met, 17 His, 20 Tyr and 35 Cys).[6] After initiation the semi-reduced dye radical can either react with dissolved molecular oxygen to produce 4,4′-bis(diethylamino)benzophenone or accommodate a second electron to form leuco- EV (Scheme 1). The intermediate dye radical can also transfer one electron back to the protein or to molecular oxygen to regenerate EV. Because the electronic excitation of the dye moiety occurs within a ground state protein-dye complex, the first excited singlet state of EV is born physically attached to the protein. Therefore, it is conceivable that not only the dye's triplet, but also the relatively short lived excited singlet populations, engage in the initial electron transfer process that leads to the formation of the semi-reduced dye radical (no diffusion needed for the reaction to occur). The competitive reaction pathways demonstrated by the characterization of reaction products in air-equilibrated and nitrogen-purged samples is also made evident through absorption spectroscopy measurements. Fig. 2 [inset (*a*)] shows that upon laser excitation of BSA-bound EV in nitrogen-purged solution the photoproduct that absorbs in the 380 nm region, 4,4′-bis(diethylamino)benzophenone, is not formed in significant amounts, but the differential absorption spectrum in the 250–280 nm region clearly indicates the formation of the dye's reduced (leuco) form as photoproduct [Fig. 2, inset (*b*)]. Under aerobic conditions a parallel route of formation of 4,4-bis(diethylamino)benzophenone is possible. The singlet oxygen generated by triplet–triplet energy transfer from triplet EV to ground state oxygen might, in principle, add to EV to generate an unstable dioxetane intermediate, whose thermal cleavage would produce 4,4′-bis(diethylamino)benzophenone. This last mechanistic path is, however, unlikely to be playing a significant role here.[7]

Another mechanistic possibility of interest, and presently under investigation, is the formation of heteroadducts through the covalent linkage of the semi-reduced dye radical with BSA. Independently of the bleaching path that is dominant under anaerobic conditions (sequential two-electron abstraction from the protein *or* one-electron abstraction followed by covalent-linking), upon laser excitation of protein-bound EV the photoinduced transformation of the host protein occurs *via* a free radical process that does not require molecular oxygen. This peculiar reaction course is well suited for the treatment of hypoxic or poorly perfused tumour areas. Our preliminary investigations have demonstrated that the photobleaching of Malachite Green and Crystal Violet proceed *via* equivalent paths, suggesting that this novel oxygen-independent mechanism may be rather common when considering the photochemistry of cationic photosensitizers non-covalently bound to biopolymers. Based on the relative values of photodecomposition quantum efficiency for a larger series of TAM dyes bound to BSA and other model proteins, and the extent to which the mechanistic details are conserved among the series, a better understanding of the structure–photoreactivity relationship for TAM dyes will be achieved, allowing the development of more effective photosensitizers.

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Footnote and References

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