Mechanism of Dye Bleaching upon Laser Excitation of Crystal Violet Bound to Bovine Serum Albumin

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The laser induced photobleaching of crystal violet non-covalently bound to bovine serum albumin yields leuco crystal violet and Michler's ketone as reaction products. The first step of the bleaching process is postulated to be an electron or hydrogen atom transfer from the protein to the dye moiety.

With the recent approval of Photodynamic Therapy (PDT) for the treatment of a variety of cancers in Japan, Canada, United States, and several European countries, the technique is attracting a great deal of attention, and has become one of the most encouraging new medical procedures.[1,2] The blooming of PDT is bringing about a rapid growing need for new photosensitizers, and several classes of dyes are presently under intense scrutiny, including triarylmethane dyes (TAM). The development of new, more effective, drugs for PDT requires the understanding of the mechanisms of action of promising dyes, or families of dyes, under conditions that display satisfactory resemblance to those observed *in vivo*. Because TAM dyes are known to efficiently bind to anionic polyelectrolytes in solution,[3] the studies described in this report were performed with crystal violet (CV), a member of the TAM family, non-covalently bound to bovine serum albumin (BSA), used here as a model biological host for the photosensitizer.

As a consequence of fast relaxation processes that occur via rotational motions of its aromatic rings, the fluorescence lifetime of CV is very short in low viscosity media, around 5 picoseconds.[4] Accordingly, CV displays poor photoreactivity in aqueous solution. However, in restricted reaction spaces, such as the microenvironments experienced by small ligands bound to proteins and nucleic acids, rotational relaxation processes may be severely obstructed. For CV and other TAM dyes the loss of rotational degrees of freedom is followed by a remarkable increase in fluorescence lifetime,[5] what generally leads to an enhancement in fluorescence quantum yield and photoreactivity.[3,6]

The binding of CV to BSA (a typical multiple-binding-site carrier)[7] was characterized with the employment of fluorescence spectroscopy (at 20 °C in 0.02 M phosphate buffer pH 7.3). The results presented in Figure 1 show that the increase in CV fluorescence as a function of BSA concentration approaches asymptotically a plateau. This is in agreement with the behavior expected for TAM dyes when the rigidity of the microenvironment that surrounds the dye increases.[5] The BSA binding sites are very efficient in preventing "free rotor" motions in the CV moiety. This decreases the efficiency of radiationless deactivation of CV, a process that involves a low-lying twisted intramolecular charge transfer state,[8] and leads to an improvement in its fluorescence quantum yield.

Figure 2 shows the bleaching of BSA-bound CV upon laser excitation at 532 nm, as measured by the decrease in absorbance at the wavelength of maximum absorption of the BSA-CV complex (595 nm). Under photolysis conditions, essentially all CV molecules are bound to the biopolymer (large [BSA]/[CV] ratio; plateau region of the binding isotherm), therefore experiencing loss of rotational degrees of freedom. The quantum efficiency of bleaching was found to be around three fold higher in nitrogen-purged than in air-equilibrated samples. This is direct evidence for the prevailing involvement of the first excited triplet state of the dye in the bleaching process. Enhanced intersystem crossing (ISC) efficiency for dyes bound to macromolecules, as

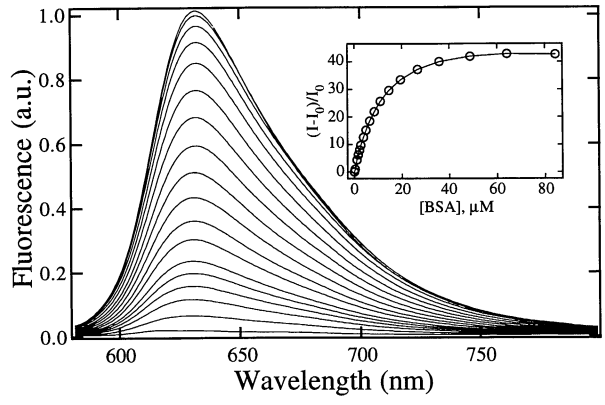


Figure 1. Effect of BSA on the fluorescence of crystal violet. From the bottom, in order of increasing fluorescence at 632 nm, the BSA concentrations were (μM): 0.0, 0.54, 1.08, 1.62, 2.15, 2.69, 3.75, 4.81, 6.39, 8.48, 11.0, 14.6, 19.5, 26.7, 35.8, 48.7, 64.1, 84.4. Inset: Binding isotherm. Excitation at 520 nm; [CV]=10 μM; phosphate buffer 0.02 M pH 7.3; T = 20 °C.

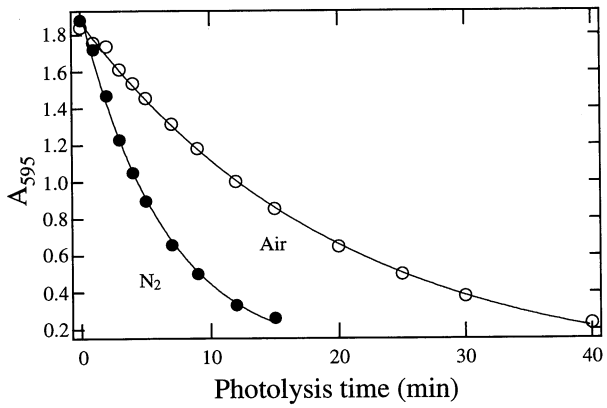


Figure 2. The effect of photolysis time on the maximum absorption of crystal violet bound to BSA (50 μM). Laser excitation at 532 nm (75 mJ/pulse; 10 Hz). Empty circles, air equilibrated sample; solid circles, nitrogen purged sample.

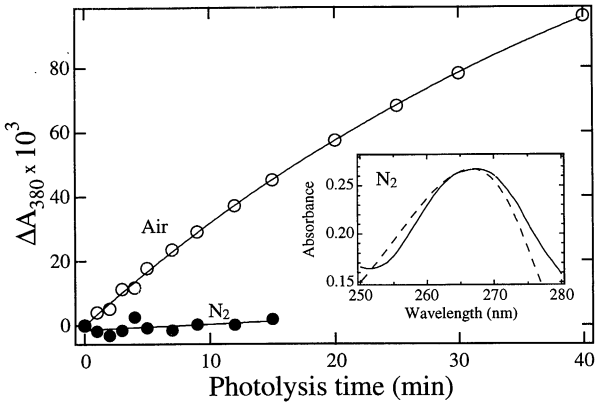


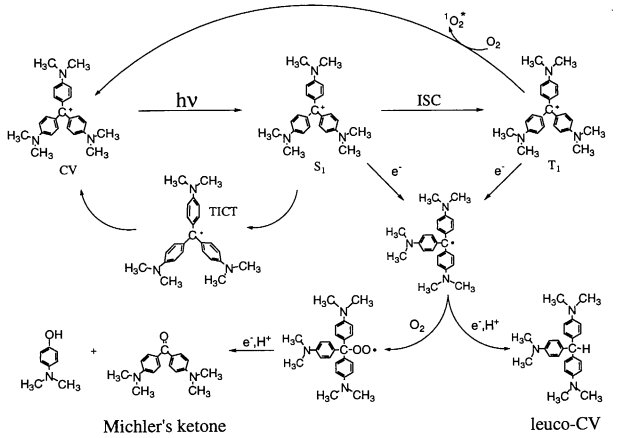
Figure 3. Changes in absorption at 380 nm as a function of photolysis time for the samples shown in Figure 2. The inset shows the differential spectrum obtained after 15 min of photolysis of the nitrogen purged sample (solid line), and the absorption spectrum of leuco crystal violet in acetonitrile (dashed line).

compared to the respective unbound dye, has been observed for a large variety of families of dyes, including triarylmethanes.[3] In the absence of BSA no significant photobleaching was detected.

Figure 3 shows the increase in absorbance in the region of maximum absorption of Michler's ketone (380 nm)[3] as a function of photolysis time. For the nitrogen-purged sample the photoproduct that absorbs in the 380 nm region is not formed in any significant amount. The inset of Figure 3 shows the differential absorption spectrum for the nitrogen-purged sample (250 nm - 280 nm region) after 15 min of photolysis, i.e. the spectrum recorded at t = 15 min minus the spectrum recorded at time zero. The excellent overlap between the differential spectrum and the spectrum of leuco crystal violet clearly indicates the formation of the dye's reduced (leuco) form as a photoproduct. The formation of Michler's ketone and leuco-CV as photolysis products of protein-bound CV was confirmed by extraction of photolyzed samples with ethyl acetate, and characterization of the extracted components by thin-layer chromatography (silica gel; 2:1, hexanes: ethyl acetate).[9]

The results described above can be satisfactorily accommodated by the reaction mechanism depicted in Scheme 1. The reduction of CV violet to its leuco form is a two electron process (formally 2 e^- + H^+ or H + e^-), and consequently under aerobic conditions the intermediate semi-reduced dye radical can react with dissolved molecular oxygen to produce 4-dimethylaminophenol and 4,4'-Bis(dimethylamino) benzophenone (Michler's ketone). The first excited singlet state of CV might be, to some extent, directly involved in the initial electron transfer from BSA to the dye's moiety to form the semi-reduced dye radical (no diffusion needed since the dye is physically attached to BSA), although the CV triplets are expected to play the major role in the H-atom/electron transfer reaction. Under aerobic conditions the singlet oxygen formed by triplet-triplet energy transfer[10] from triplet CV to ground state oxygen might add to the dye molecule to form a thermally-unstable dioxetane intermediate, whose cleavage would also produce Michler's ketone.[11]

The formation of heteroadducts through cross-linking of the



Scheme 1. Routes of deactivation and phototransformation of crystal violet.

semi-reduced dye radical with BSA is another mechanistic possibility presently under investigation. The constellation of groups available at the protein moiety may favor the production of such species. Independently of the bleaching route that is dominant under anaerobic conditions (sequential two-electron abstraction from the protein *or* one-electron abstraction followed by cross-linking), upon laser excitation of the protein-dye complex the photoinduced transformation of the host protein proceeds through a free radical process that does not require molecular oxygen to be initiated. Therefore, CV can be seen as a photosensitizer of potential applicability for the treatment of hypoxic areas of tumors. Based on relative values of quantum efficiency of laser induced photodecomposition of a series of TAM dyes bound to BSA and other model proteins, and to the extent to which the mechanistic details are conserved among the series of dyes, a better comprehension of the relationship between structure and photoreactivity of TAM dyes will be obtained, allowing the development of more effective photosensitizers.

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