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Spectroscopic and photochemical properties of Malachite Green noncovalently bound to bovine serum albumin

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Abstract

The noncovalent binding of the cationic triarylmethane dye Malachite Green (MG^+) to bovine serum albumin (BSA) was investigated using fluorescence and UV-vis spectroscopy. The results indicate that the BSA binding sites are very effective in hindering fast nonradiative relaxation processes that occur via rotational motion of the aromatic rings of this triarylmethane. As a result, pronounced increases in both fluorescence yield and dye photoreactivity were observed upon protein binding. The 532 nm laser-induced photobleaching of protein-bound MG^+ yields leuco malachite green and 4-dimethylaminobenzophenone as major reaction photoproducts. Based on the nature of these 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.

Keywords: Malachite Green; Triarylmethane dyes; Bovine serum albumin; Noncovalent binding; Dye photobleaching; Laser induced photoinactivation of proteins

1. Introduction

The use of high-intensity pulsed laser irradiation to photoactivate exogenous molecules designed to perform specific tasks in biological systems is a subject of increasing interest. Malachite Green (MG^+) has been successfully used as a photosensitizer in several applications of dye-assisted laser inactivation of enzymes [1]. One of the remarkable strengths of this general technique rests on the strategy of labeling anti-enzyme antibodies—rather the enzymes to be inactivated themselves—with the photosensitizer, since it permits the achievement of a high level of selectivity with regard to the destruction of target biopolymers. By employing this experimental approach, photochemical inactivation of specific membrane proteins was observed in vitro [1,2]. The selective photoinactivation of protein function has great technological significance, since it might permit the achievement of the photonic control of fermentations and other enzymatic processes, and lead to new strategies for employment in photochemotherapy of tumors [3] and photochemical purging of blood and blood products from bacteria, viruses and parasites [4].

MG^+ and other cationic triarylmethane dyes show poor photoreactivity in low viscosity media

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due to fast relaxation processes that occur via rotational motions of their aromatic rings [5,6]. Accordingly, the fluorescence lifetime of triarylmethane dyes is typically in the picosecond range in water and other low viscosity solvents (fluorescence quantum yields $\sim 10^{-5}$) [5–7]. In more viscous media, or in reaction spaces that render steric hindrance to rotational relaxation processes, fluorescence and intersystem crossing become more competitive events, and photoreactivity tends to increase [7–10]. Despite the successful use of MG^+ as a photosensitizer, the mechanisms by which the inactivation of proteins is achieved upon photolysis of protein-MG^+ complexes are not well understood. We report here an investigation of the effect of protein binding on the spectroscopic and photochemical properties of MG^+ using bovine serum albumin as a model target protein.

2. Materials and methods

Malachite Green (MG^+, oxalate salt; Eastman Kodak) was recrystallized from methanol and its purity assessed by thin-layer chromatography (silica gel, methanol-acetic acid 95:5, v:v). Bovine serum albumin (BSA, initial fractionation by cold alcohol precipitation; essentially globulin and fatty acid free) from Sigma, leuco malachite green (MG-H), 4,4'-bis(dimethylamino)benzophenone (Michler's ketone), 4-dimethylaminobenzophenone, and Malachite Green carbinol base from Aldrich were used as supplied. Water was distilled, deionized, and filtered prior to use (Millipore Milli-Q system; resistivity, 18 MΩ cm). The protein binding and laser flash photolysis experiments were carried out at 25°C in 0.06 M phosphate buffer pH 5.8 (sodium salts, from Sigma).

Fluorescence data were obtained on a Timemaster Strobemaster fluorometer from Photon Technology International, Inc. The spectrophotometric studies were performed with a Shimadzu UV-2101PC spectrophotometer. Our laser flash photolysis equipment is similar to a previously described system [11]. The major assembly components are a nanosecond Nd:YAG laser (Continuum, 7010), used as the excitation light source, a 300 W Xenon arc lamp system (Oriel, 66084), which provides the analysis beam, a monochromator (CVI, CM110), a red sensitive photomultiplier tube (Hamamatsu, R446), and a dual channel 600 MHz digital oscilloscope (LeCroy, 9360).

For the dye photobleaching experiments the samples were placed in standard 1 cm (path length) quartz cuvettes at a distance of approximately 10 cm from the light source. The samples were irradiated using the 532 nm line of the Nd:YAG laser operating at a repetition rate of 10 Hz (280 mJ/pulse). The defocused laser beam (circular profile with diameter of about 5 mm) was directed to the center of the quartz cuvette. The temperature controlled cuvette holder allowed continuous magnetic stirring of the samples, and was kept at 5°C to avoid the interference of thermal effects [8] during photolysis. Photobleaching quantum efficiencies were determined considering only the first 5–10% decrease in dye concentration, and using a calibrated solid-state actinometer model PM30VI from Molectron to measure the photolysis energy.

The characterization of reaction photoproducts was performed by extraction of photolysed samples with ethyl acetate and analysis of the extracted compounds by thin-layer chromatography (silica gel; 2:1 hexanes-ethyl acetate) and uv-visible spectroscopy. Typically, a total of 20 ml of 20 μM MG^+ solution bleached to completion in the presence of 80 μM BSA were employed in the analytical experiments. The ethyl acetate was removed from the extracted samples by evaporation at room temperature, and the residues dissolved in 100 μl of ethyl acetate for TLC analysis or, alternatively, in 3 ml of methanol for uv-visible inspection. The molar extinction coefficients employed in the spectroscopic evaluation of reaction yields were the following (in methanol; $\varepsilon_{max} \times 10^{-4}$, M^{-1} cm^{-1}): $\varepsilon_{369} = 3.3$ (Michler's ketone), $\varepsilon_{356} = 2.5$ (4-dimethylaminobenzophenone). In the TLC analysis the photoproducts were detected by the inspection of the chromatogram with ultraviolet light and iodine vapor staining, and characterized on the basis of the R_F values of photolyzed and authentic (standard) samples.

3. Results and discussion

A combination of spectroscopic techniques was employed to characterize the formation of ground-state complexes of MG+ with BSA in 0.06 M phosphate buffer pH 5.8 at 25°C. The use of a relatively low pH in this investigation was aimed at minimizing the interference of the formation of MG^+ carbinol base during data acquisition. At neutral and slightly alkaline (physiological) pHs, the alkaline hydrolysis of MG^+ becomes more competitive, precluding the acquisition of reliable binding information. The protein effect on MG^+ fluorescence is shown in Fig. 1. The increase in MG^+ fluorescence as a function of BSA concentration indicates that the BSA binding sites are very effective in hindering "free rotor" motions in the dye moiety. This decreases the efficiency of radiationless deactivation of the first electronically excited singlet state of MG^+ directly to its ground state, a process mediated by a low-lying twisted intramolecular charge transfer state [5], and leads to a substantial enhancement in the dye's fluorescence quantum yield. Binding experiments performed with two other triarylmethane dyes, Crystal Violet (CV^+) and Ethyl Violet (EV^+) at pH 5.8 and pH 7.3 have demonstrated that the BSA binding isotherms are analogous at both pHs [7]. Indeed, since the flattest region of the BSA titration curve is observed between pH 5 and pH 8, no substantial changes in charge distribution and other structural features within the protein binding sites should result upon changes in pH in the 5.8 to 7.3 range [12].

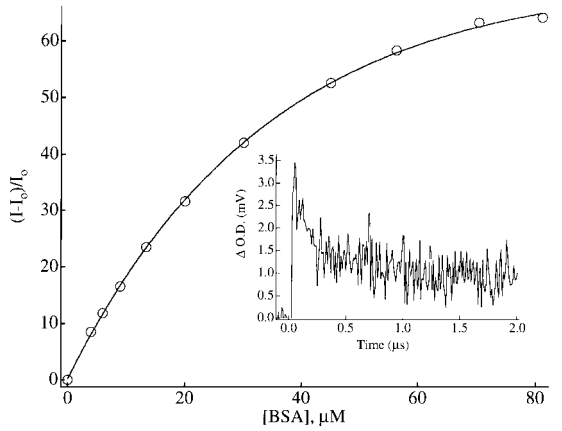


Fig. 1. Effect of BSA concentration on the fluorescence of Malachite Green. Phosphate buffer 60 mM, pH 5.8; $[MG^+]$ = 55 μM; T = 25°C, $\lambda_{exc.}$ = 428 nm. Inset: Transient decay profile (average of 14 acquisitions) for BSA-bound MG^+ in air-equilibrated phosphate buffer. $[MG^+]$ = 20 μM; [BSA] = 80 μM; $\lambda_{exc.}$ = 532 nm; 220 mJ/pulse; transient signal monitored at 470 nm.

Electronic spectroscopy also provided direct evidence of binding. The protein binding leads to a small bathochromic shift in the long-wavelength absorption band of MG^+, and to a decrease in the respective values of the extinction coefficient. The wavelength of maximum MG^+ absorption is 617 nm when free in pure phosphate buffer, and 621 nm when in the presence of 80 μM BSA ($[MG^+]$ = 10 μM). Studies on medium effects on the spectroscopic properties of MG^+ free in solution have demonstrated that only minor shifts to the red region of the spectrum are observed in the dye λ_{max} with decreasing solvent polarity/polarizability (π^* scale [13]). For the hydroxylated solvents water and the (normal) C_1 to C_4 alcohol series, the maximum shift observed was only 6 nm (from water, $\pi^* = 1.06$, to 1-butanol, $\pi^* = 0.47$). This small bathochromic shift indicates that the electronic ground state of MG^+ is only slightly more polar then its first excited singlet state [13–15]. Accordingly, only minor shifts in the long-wavelength absorption band of MG^+ are expected to occur upon its binding to biopolymers, regardless of the polarity of the microenvironment that defines the binding domain. For the sake of comparison, for the intensively solvatochromic dye styryl-7 (a merocyanine-type dye), the bathochromic shift observed in the λ_{max} upon changing the solvent from water to 1-butanol is 65 nm [14].

The effect of protein binding on the intensity of MG^+ absorption is also modest. The presence of 80 μM BSA in 10 μM MG^+ solutions leads to a 5% decrease in the maximum dye absorption as compared to the value obtained for the dye free in phosphate buffer. In view of the small magnitude of the effects of BSA binding on the electronic characteristics of MG^+, fluorescence spectroscopy was identified as a more appropriate technique for the detection and characterization of BSA–MG^+ complexes in aqueous media.

The decrease in efficiency of radiationless relaxation of MG^+ upon protein binding, as indicated by the enhancement in fluorescence quantum yield, suggests that the dye's photoreactivity might also undergo substantial enhancement as a consequence of BSA binding. The fluorescence intensity of BSA-bound MG^+ was not strong enough to allow the measurement of its respective lifetime, but for the cases of BSA complexes of CV^+ and EV^+, we have previously reported that the binding phenomena leads to remarkable enhancements in the respective fluorescence lifetimes (from the picosecond to the nanosecond time domain) [7]. The increase in fluorescence lifetime of triarylmethanes upon protein binding enhances the probability that they would engage in photochemical reactions directly from their singlets (S_1), and also populate more efficiently their respective triplet states (T_1) via intersystem crossing, as compared to the efficiency to which these dyes populate their triplets when free in aqueous media.

Fig. 2 shows the bleaching of BSA-bound MG^+ upon laser excitation at 532 nm, as measured by the decrease in sample absorbance at the λ_{max} of

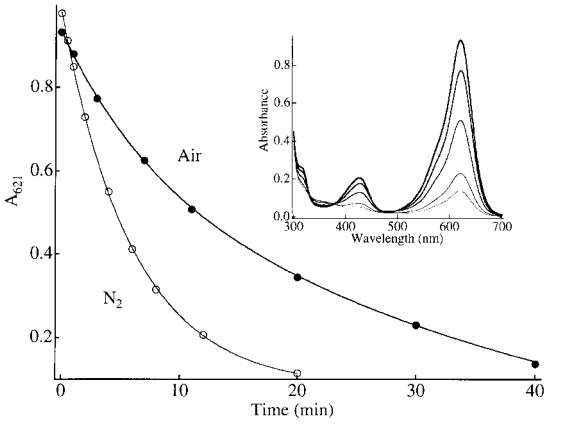
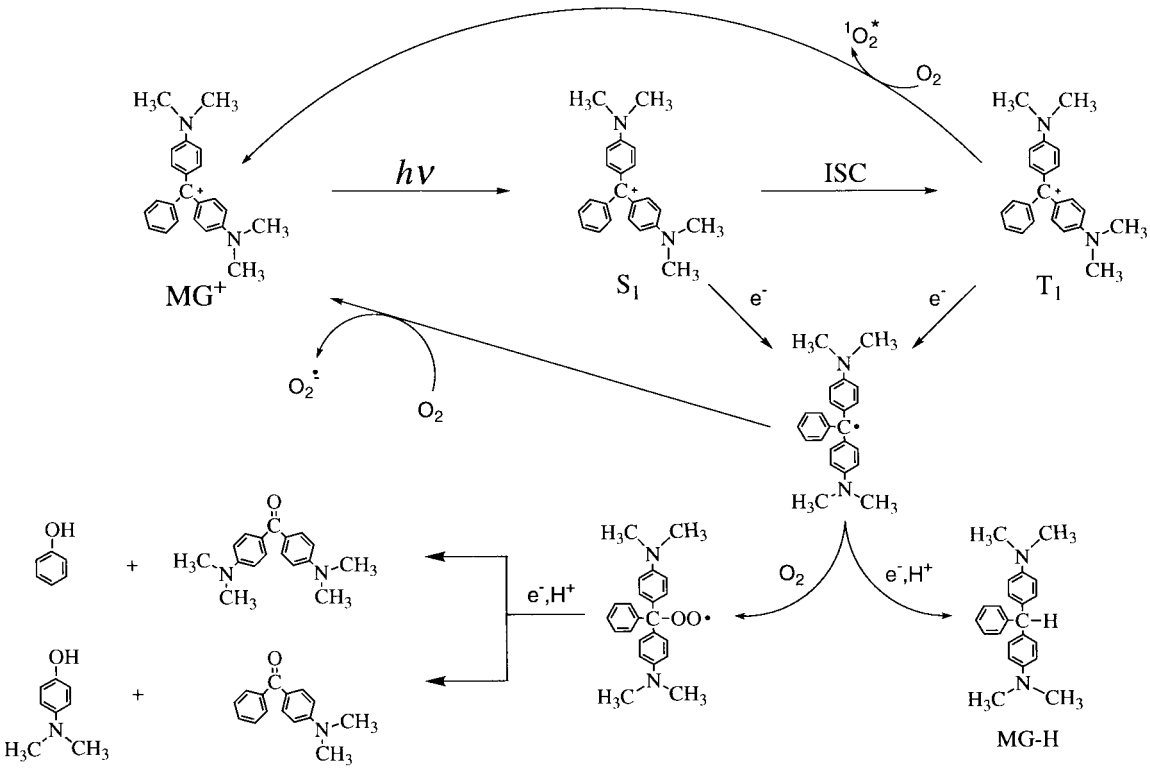


Fig. 2. Effect of photolysis time on the maximum absorption of the BSA-MG^+ complex in phosphate buffer. Solid circles, air-equilibrated samples; empty circles, nitrogen-purged samples. Inset: Effect of photolysis time on the absorption spectra of the BSA-MG^+ complex in air-equilibrated media. From the top (inset), in order of decreasing absorption at 621 nm, the photolysis times were (in minutes): 0, 3, 11, 30, 40. Phosphate buffer 60 mM, pH 5.8; $[MG^+] = 10$ μM; [BSA] = 80 μM; $\lambda_{exc.} = 532$ nm; 10 Hz; 125 mJ/pulse.

the BSA-MG^+ complex. Under photolysis conditions, most dye molecules are noncovalently bound to the protein (plateau region of the binding isotherm, see Fig. 1), thereby experiencing loss of rotational degrees of freedom. In the absence of BSA no significant dye photobleaching was detected. Laser flash photolysis provided additional evidence of the enhancement in MG^+ photoreactivity as a result of protein binding. While no transient signal was detected upon laser excitation of MG^+ free in phosphate buffer, the formation of reaction intermediates was evident by the appearance of transient signals on 532 nm laser excitation of BSA-bound MG^+ (inset of Fig. 1). A more rigorous analysis of the transient decay profile aimed at accessing the relative contribution of triplets and free radicals to the overall population of reaction intermediates was precluded by the extremely weak nature of the signals obtained upon laser flash photolysis of the BSA-MG^+ complex (near the limit of detection of our laser flash photolysis equipment).

The photobleaching efficiency of BSA-bound MG^+ was found to be around two-fold higher in nitrogen-purged (7.8×10^{-5}) than in air-equilibrated samples (3.1×10^{-5}). The higher photobleaching efficiency in nitrogen-purged samples implies that the MG^+ triplet is involved in the bleaching process and/or a semireduced dye radical intermediate ($MG^{\bullet}$) is oxidized, by molecular oxygen, back to the original dye cation with the concomitant formation of superoxide radical. In the former case, the quenching of triplet MG^+ by molecular oxygen, with the simultaneous formation of singlet oxygen, would prevent the triplet from engaging in the initial electron transfer event that leads to dye bleaching (Scheme 1).

Details on the reaction mechanism shown in Scheme 1 were apparent from analytical characterization of the final reaction photoproducts. Analysis of photoproducts was performed by extraction of photolyzed samples with ethyl acetate, and characterization of the extracted compounds by thin-layer chromatography and uv-vis spectroscopy. The formation of a photoproduct that absorbs around 350 nm, identified as 4-dimethylaminobenzophenone, was much more pronounced in air-equilibrated than in nitrogen-purged samples.



Scheme 1. Routes of deactivation and phototransformation of Malachite Green.

Leuco-malachite green was also found in ethyl acetate extracts obtained from air-equilibrated and nitrogen-purged samples. The reduction of MG^+ to its leuco form (MG-H) is a two electron process (formally $2\,e^- + H^+$ or $H\cdot + e^-$), initiated in the BSA-MG^+ complex by an electron or hydrogen atom transfer from the protein to the dye moiety. After initiation, and following the reaction coordinates of the overall bleaching process, the intermediate semireduced dye radical can either react with dissolved molecular oxygen to produce 4-dimethylaminobenzophenone, or receive a second electron to produce the leuco derivative of MG^+. Leuco malachite green can be produced either via sequential two-electron abstraction from the protein (Scheme 1) or via one-electron abstraction followed by the annihilation of two semireduced dye radicals ($MG\cdot + MG^{\bullet} + H^+ \rightarrow MG^+ + MG\text{-}H$).

Because the MG^+ molecule is not symmetric, another benzophenone-type photoproduct, 4,4'-Bis-(dimethylamino)benzophenone (Michler's ketone), should also be formed through the oxygen-dependent route of MG^+ photobleaching (Scheme 1). However, we were unable to detect the presence of Michler's ketone in ethyl acetate extracts obtained from photolyzed samples of BSA-bound MG^+. Under our experimental conditions, the lower detection limit for Michler's ketone by TLC analysis was found to be in the range of 2% of the amount of MG^+ converted into photoproducts, as accessed by a series of calibration experiments carried out with authentic samples of Michler's ketone subjected to the same treatment as the photolyzed samples. The lack of Michler's ketone in the photolyzed extracts was further confirmed by uv-vis spectroscopy. The absorption spectra of the extracted products in methanol showed an absorption maximum in the 350 nm region, in keeping with the predominant formation of 4-dimethylaminobenzophenone. The absorption maximum of Michler's ketone in methanol is at

369 nm, and we did not find any spectroscopic evidence for the formation of this compound as a reaction photoproduct. Therefore, the analysis of the final reaction photoproducts strongly suggests that the intramolecular attack of the MGOO•(-peroxyl radical intermediate (Scheme 1) occurs predominantly at the amino-substituted aromatic rings of Malachite Green to produce 4-dimethylaminobenzophenone.

The primary reaction photoproducts, leuco malachite green and 4-dimethylaminobenzophenone do not fully accumulate in solution over the course of the reaction. The amounts of MG-H and 4-dimethylaminobenzophenone found in solution after prolonged photolysis were in the range of 10% of the respective amount of MG^+ converted into photoproducts. In analogy to what has been previously reported for BSA complexes of CV^+ and EV^+, this fact can be rationalized in terms of the parallel consumption of dye radical intermediates via covalent linkage to BSA to produce the respective protein-dye adducts, and removal of the original photoproducts from solution via secondary reaction mechanisms [7]. Evidence for the formation of protein-dye covalent adducts upon laser photolysis of BSA complexes of CV^+ and EV^+ has been previously obtained using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry [7].

Since in BSA-MG^+ complexes the electronically excited dye moiety is created physically attached to its protein counterpart, it is reasonable to presume that even the short lived singlet populations may, to some extent, engage in the electron transfer event that leads to the formation of the semi-reduced dye radical (no diffusion needed for the reaction to occur). Indeed, the modest effect of oxygen on the photobleaching efficiency of BSA-bound MG^+ suggests that either the contribution of the first excited singlet state of MG^+ is important with regard to the overall mechanism of photobleaching or the respective triplet is partially protected by the protein from oxygen quenching [7,16–18]. In this last case, the reaction rate for the quenching of MG^+ triplet by molecular oxygen in the protein environment would not be high enough to significantly compete with the initial electron transfer event that leads to dye photobleaching. The concept of protection of triplet species against collisional oxygen quenching by biopolymers was originally explored by Cilento in studies on the enzymatic generation of electronically excited triplet species [17,18].

The hypothesis of a significant contribution of the first electronically excited MG^+ singlet to the overall photobleaching of this triarylmethane dye when noncovalently bound to BSA is strengthened by the fact that even under the extreme restraining conditions found in solid matrices at 90 K, MG^+ displays very inefficient and almost undetectable intersystem crossing to its triplet state [19]. Consequently, when bound to BSA or other biopolymers under ordinary biological conditions, the electronic excitation of MG^+ is not expected to produce substantial populations of triplets, albeit the few triplets formed can be expected to become effectively involved in H-atom/electron transfer reactions [7,16].

The low triplet yield of MG^+ implies that this dye is not expected to sensitize singlet oxygen to any significant extent in biological systems, especially when bound to a macromolecular domain that, for topological reasons, offers partial protection against diffusional oxygen quenching. Electron paramagnetic resonance studies carried out with two other triarylmethanes (CV^+ and Victoria Pure Blue BO) have indicated that superoxide radicals are formed upon photolysis of air-equilibrated solutions of these dyes noncovalently bound to macromolecules [20,21], while no evidence was found for the parallel formation of singlet oxygen. Therefore, MG^+ and other triarylmethane dyes can be expected to develop their photodamaging effects towards biopolymers primarily via the classical photosensitization mechanism type I (initiated by superoxide and other oxygen radicals), with very little contribution from the Type II mechanism (initiated by singlet oxygen) [22].

The fact that singlet molecular oxygen itself does not play any significant role in the overall MG^+ photobleaching process is emphasized by the fact that the photobleaching efficiency increases in the absence of oxygen. The apparently typical low efficiency of singlet oxygen formation in aerobic photooxidation processes mediated by

triarylmethane dyes suggest that the alternative route for the formation of 4-dimethylaminobenzophenone on photolysis of BSA-bound MG^+ involving the reaction of a ground state MG^+ cation with singlet oxygen is unlikely to play a meaningful role here. Singlet molecular oxygen might, in principle, add to the MG^+ cation to produce an unstable dioxetane intermediate whose thermal cleavage would lead to the formation of benzophenone-type photoproducts [23].

4. Concluding remarks

For the purpose of the specific inactivation of enzymes and other targeted biopolymers, the ideal photosensitizer should have the following property: upon photon absorption, it should either return to the ground state or react selectively with the target macromolecule through a mechanism that simultaneously leads to the destruction of its own chromophoric system, in order to generate a product transparent to the excitation light. Therefore, after performing its photochemical work, the photosensitizer would be permanently unable to carry out further (non-specific) actions. As a rule, near-infrared absorbing chromophores are especially suitable for such applications, since most proteins, as well as many other biopolymers of interest, do not absorb in the red region of the visible spectrum. Hence, the dye moiety can be selectively excited with high energy laser pulses and induce transformations in the host protein (or in a targeted antigen), with no other photosensitization process simultaneously taking place. This strategy is quite different from that of using singlet oxygen sensitizers, where the photodynamic process is less specific, since the photosensitizer continuously produces the reactive species, and this species can diffuse away from the primary target and lead to generalized oxidative damage. MG^+ displays a behavior close to the "ideal" for more selective photosensitizers, because it strongly absorbs at relatively long wavelengths (maximum around 620 nm), and becomes colorless upon photoreaction with the protein. We have found that other triarylmethane dyes such as CV^+ and EV^+ exhibit similar photosensitization mechanisms when bound to BSA and other proteins, including low density lipoprotein and hexokinase.

Interestingly, the mechanism of formation of the leuco derivatives of MG^+ and other triarylmethanes makes these dyes potential phototherapeutic agents for the treatment of hypoxic areas of tumors, since upon laser excitation the photosensitization can occur through a free radical process that overcomes the need for oxygen to operate, and consequently are not of the classical photodynamic type [22]. The formation of biopolymer-dye covalent adducts and the photoinduced fragmentation of biopolymers represent peculiar oxygen-independent processes well suited for the specific inactivation of biological macromolecules and for employment in photochemotherapy of hypoxic and poorly perfused tumor areas. These reaction routes may be rather common when considering the photochemistry of dyes noncovalently bound to biopolymers, and represent conceptual guidelines for the development of new photosensitizers specifically designed for anaerobic phototherapy [7].

Acknowledgements

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