The efficiency of malachite green, free and protein bound, as a photon-to-heat converter

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ABSTRACT Dye assisted laser inactivation of proteins has been found to be a methodology that can achieve high selectivity. Despite the fact that the methodology is successful, knowledge of the detailed inactivation mechanism would allow full optimization of this technique. Here, pulsed-laser photoacoustic calorimetry is used to study the photophysical properties, principally the heat release behavior, of protein bound malachite green. We found that when bound to bovine serum albumin the dye is a good photon-to-heat converter, but ~2.6% of the absorbed photon energy (λ_{exc} = 624 nm) is not released as heat in less than 10 μs. This observation suggests that a mechanism other than simple heat-induced inactivation may be the principle process; a long lived excited triplet state of malachite green (or species derived from it) is postulated to play a major role.

INTRODUCTION

The selective degradation of biopolymers is a goal that has been a topic of interest for many researchers. One strategy recently developed for chromophore-assisted laser inactivation (CALI) is to attach malachite green to a biopolymer in vitro or in vivo, and then to subject the system to intense laser irradiation in the red portion of the visible spectrum (Jay, 1988). Malachite green was chosen because as a free species in water it is known to efficiently absorb 620 nm irradiation (a wavelength not absorbed by many biopolymers) and it has a short lived (ps), nonfluorescent excited state. The inactivation strategy is thus to label a biopolymer with several malachite green chromophores (perhaps as many as 10 dye molecules per biopolymer) and then expose the labeled biopolymer to a series of intense, nanoseconds-wide laser pulses. The malachite green chromophores ideally would absorb a photon and then rapidly and nonradiatively relax to the ground-state, releasing all the photon energy as heat. For each laser flash, a number of such cycles could occur for each biopolymer, resulting in a considerable deposition of heat into a small volume in a short period of time (i.e., the time of the laser pulse). The local temperature rise could be quite substantial, enough to denature (or otherwise inactivate) the biopolymer while the equilibrium temperature rise would be much smaller. Such an overall strategy can easily be envisioned as having many strengths; for example, a variety of mechanisms for specificity can be achieved including labeling an appropriate antibody with the malachite green rather than labeling the biopolymer to be inactivated itself (Jay, 1988).

Several successful applications of the CALI methodology have been demonstrated (Jay, 1988; Jay and Keshishian, 1990). However, while it is clear that the strategy is successful, there are a number of questions concerning the mechanism by which inactivation is achieved, questions that must be answered in order to optimize and fully utilize the CALI approach. It is appropriate to consider whether the inactivation of biopolymer function is brought about by a local temperature rise induced by photon absorption or whether alternative physical or chemical mechanisms for inactivation are operative. In this paper, we examine the photophysical properties, particularly the heat-generation properties, of malachite green and bovine serum albumin covalently labeled with malachite green, both under single photon absorption conditions. In part, we wished to determine if malachite green is the perfect photon-to-heat converter, yielding 100% of the photon energy as heat. We also experimentally address the question of whether or not proteins covalently labeled with malachite green undergo volume changes after absorption of a single red photon. To directly measure and monitor heat generation and photoinitiated volume changes we use photoacoustic calorimetry.

Photoacoustic calorimetry (PAC), as implemented for these studies, involves the low power, pulsed irradiation of an optically thin aqueous solution and the monitoring of the acoustic wave that results from the nonradiative release of absorbed photon energy. This relaxation results in a local temperature rise that causes an expansion of the irradiated volume which in turn creates a pressure (acoustic) wave that is detected. Thus, PAC is both related to and complementary to absorption and fluorescence spectroscopic methods; it requires absorption of a photon, but then detects the portion of the

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photon energy released as heat, rather than detecting photon emission. As such, PAC is the ideal technique to investigate the fundamental mechanism of the CALI procedure.

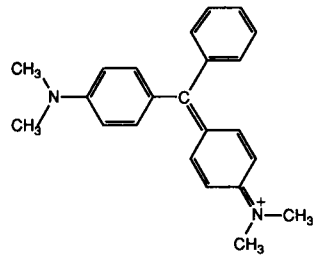
EXPERIMENTAL PROCEDURES

Photoacoustic measurements. Our photoacoustic calorimeter is similar to those that have been described previously (Rothberg et al., 1983; Burkey et al., 1986). For the bulk of these experiments, photoexcitation at 624 nm (λ_{exc} = 624 nm; equivalent photon energy of 45.8 kcal mol^{-1}) was achieved by the output of a nitrogen laser (λ_{exc} = 337.1 nm) pumped dye laser (600 ps) operated with sulforhodamine B dye (Eastman Kodak Co., Rochester, NY). While several control experiments were carried out with 337.1 nm excitation (equivalent photon energy of 84.8 kcal mol^{-1}) the work discussed herein refers to 624 nm excitation unless otherwise explicitly stated. The output of the laser was directed sequentially through an optical trigger (to signal the start of the data collection period), an energy attenuator (a cuvette variously filled with solutions prepared from copper chloride and acetonitrile with transmittances of 100 to 40%), a computer-controlled shutter (to ensure irradiation only occurred when data collection was desired), a 3-mm aperture (typical) to select only a portion of the much larger beam profile, and a beam-splitter (to direct ~40% of the photolysis beam on a second photoacoustic cell containing a solution prepared from copper chloride and acetonitrile, the signal of which was used as a relative measure of photolysis energy, and onto the sample of interest). Absolute photolysis energies on the sample cuvette ranged from ~5 to 20 μJ per pulse.

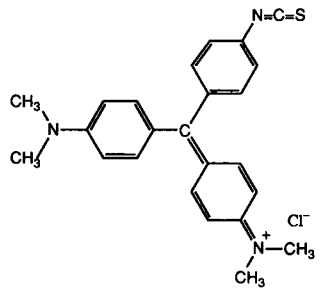
Solutions of the aqueous samples of interest are placed in a standard 1-cm pathlength quartz cuvette that is contained within a brass, thermostated cuvette holder. The acoustic wave is detected and converted into a voltage wave by a custom-built, piezoelectric-based transducer (~500 kHz) (Patel and Tam, 1981) pressed against a greased face (perpendicular to the light path) of the cuvette and held in place by the cuvette holder. The voltage wave, which is linearly proportional in amplitude to the pressure wave, is amplified (60–70 dB) and digitized (8-bit DAC, ±512 mV range, 10 ns/channel; 1024 channels) for each laser shot. Typically, data from 75 to 100 laser shots, collected at 1 Hz, are averaged to yield one waveform that equals one data point. The photoacoustic signal that is used to measure the relative energy of each laser pulse is handled in a similar fashion (8-bit DAC, ±512 mV range, 50 ns/channels; 512 channels).

Absorbance and fluorescence measurements. All absorbance measurements were obtained on a Hitachi (Danbury, CT) model U-2000 spectrophotometer. The fluorescence spectra were recorded on a Perkin-Elmer Corp. (Norwalk, CT) model LS-50 fluorimeter. The fluorescence results were obtained under conditions similar to those used in the PAC experiments.

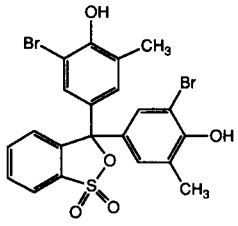
Materials. NaP_i buffer, 0.1 M, pH 5.8 and 7.4, prepared from NaH_2PO_4 and Na_2HPO_4 obtained from Sigma Chemical Co. (St. Louis, MO) and Dulbecco's phosphate buffered saline (D-PBS, pH 7.0; Gibco BRL Life Technologies Inc., Gaithersburg, MD) were used as solvents. Malachite green cation (MG) for use as the free dye was obtained from Eastman Kodak Co. as the oxalate salt and used as received. Bromocresol green (BC-G; Sigma Chemical Co.) and bromocresol purple (BC-P; Eastman Kodak Co.) were used as received. The malachite green isothiocyanate derivative used to label the protein was obtained from Molecular Probes Inc. (Eugene, OR) as the chloride salt. The structures of all dyes are shown in Fig. 1. Malachite green labeled samples of bovine serum albumin (MG-BSA) were prepared as previously described (Jay, 1988). Assuming identical extinction coefficients for the free dye and the dye bound to the protein, we estimate approximately five dye molecules are bound to the average protein molecule. Any protein that does not itself absorb at the excitation wavelength would be a good test protein; BSA was chosen because it is readily available, easily handled, extensively studied, and has been used as a test protein in CALI experiments.



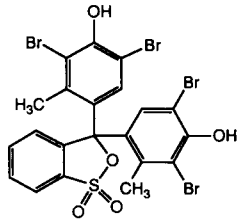
Malachite Green (MG)



Malachite Green Isothiocyanate chloride



Bromocresol Purple (BC-P)



Bromocresol Green (BC-G)

FIGURE 1 Structures of the dyes used in this work.

Data analysis. All data were analyzed according to the basic equation of photoacoustic calorimetry (Eq. 1). In Eq. 1, S is a measure of the amplitude of the photoacoustic signal and is

$$S = K' \frac{\alpha \, MW}{C_p \, \rho} f_h E_p (1 - 10^{-A}), \quad (1)$$

obtained by integrating the first negative excursion of the acoustic wave as shown in Fig. 2 (*upper inset*); K' is an empirically determined experimental sensitivity constant, α is the adiabatic coefficient of thermal expansion of the solvent, MW is molecular weight of the solvent, C_p is the molar heat capacity of the solvent, ρ is the density of the solvent, f_h is the fraction of excitation energy released as heat, E_p is the energy of the laser pulse incident on the sample, and A is the absorbance of the sample at the excitation wavelength (i.e., $1-10^{-A}$ is fraction of the incident light that is absorbed by the sample). Eq. 1 is similar to that described previously (Grabowski et al., 1984; Westrick et al., 1987; Herman and Goodman, 1989).

RESULTS

MG and MG-BSA are photolytically stable. We initially addressed the photolytic stability of the molecules of interest under our pH and light intensity conditions. To test a compound for photolytic stability, we photolyzed it under typical concentration and buffer conditions for a longer period of time using higher intensity pulses than

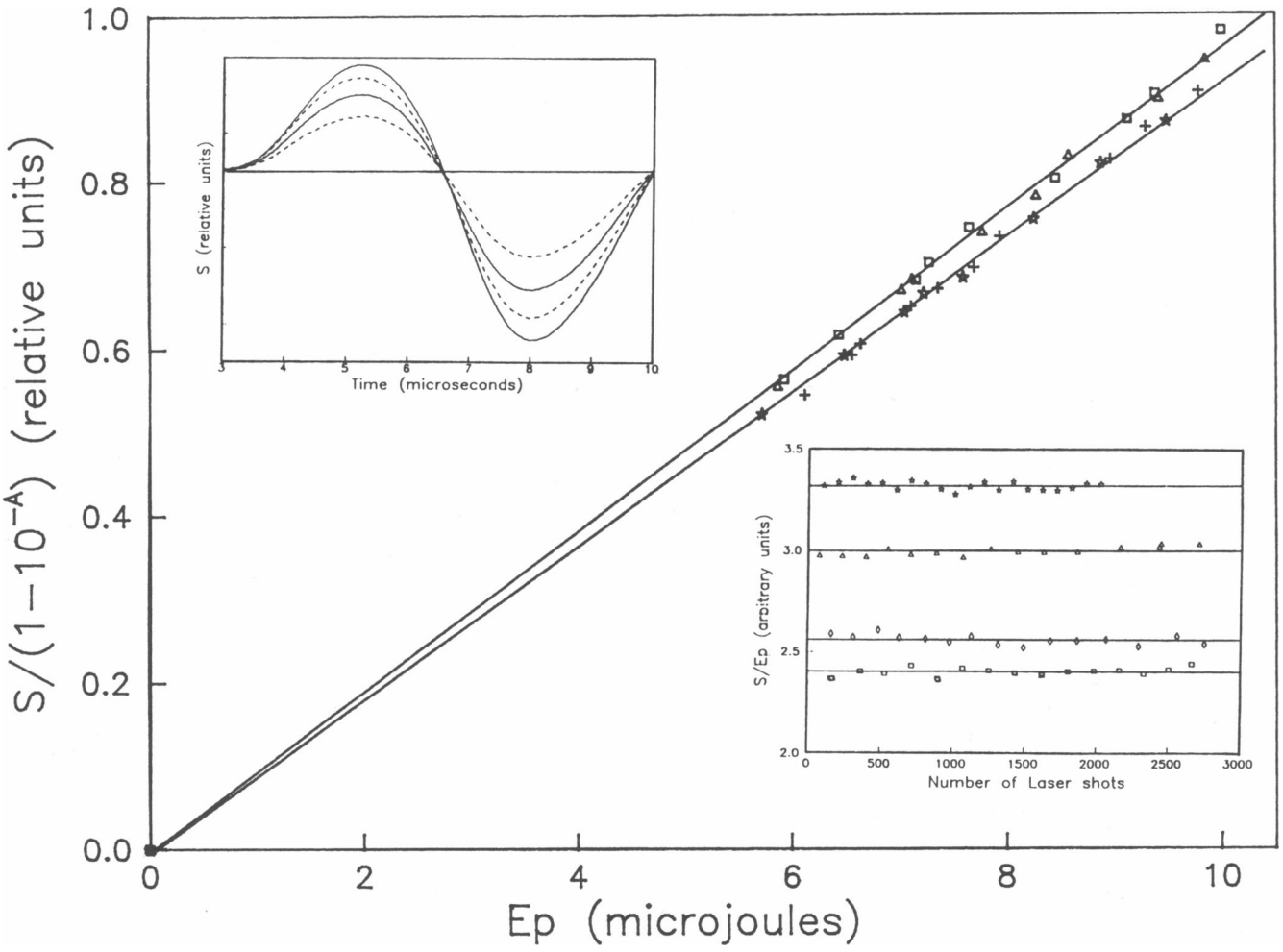


FIGURE 2 A typical data set collected to determine the relative efficiency of conversion of photon energy into heat by several samples of MG (□, A_{624} = 0.394) (Δ, A_{624} = 0.391) and MG-BSA (+, A_{624} = 0.404) (*, A_{624} = 0.390) solutions. These data were collected in aqueous 0.1 M NaP_i buffer at pH 5.8 using 624 nm light as the excitation source. The results of this one data set defines the f_h(MG-BSA) to be 0.96 of f_h(MG). The upper insert shows 7 μs of typical waveforms (MG, ——) (MG-BSA, ----) recorded and the portions integrated to define the photoacoustic signal (S) of each point in the linear plots. The lower inset demonstrates the photolytic stability of MG (Δ, □) and MG-BSA (*, ◇) at pH 7.0 (*, 18 μJ/pulse) (□, 12 μJ/pulse) and pH 5.8 (Δ, 11 μJ/pulse) (◇, 14 μJ/pulse). Note that these photolytic stability experiments were carried out under conditions where the total number of photons absorbed was far greater than the absorbed in a typical f_h determination.

that used for the bulk of data collection. We found that MG at pH 5.8 and λ_{exc} = 337.1 nm (data not shown) or λ_{exc} = 624 nm (Δ) (Fig. 2, *lower inset*), or that MG at pH 7.0 and λ_{exc} = 624 nm (□) (Fig. 2, *lower inset*) showed no significant change in the observed photoacoustic signal with irradiation time. Likewise, prolonged irradiation of MG-BSA at pH 5.8 (◇) (Fig. 2, *lower inset*) or pH 7.0 (*) (Fig. 2, *lower inset*) and λ_{exc} = 624 nm effected no changes in the observed PAC signal. Stability on prolonged photolysis at the higher laser intensities was also found to be independent of the concentration of the MG-BSA (1 to 3 μM) at pH 7.0 (data not shown).

MG and MG-BSA have fast heat-release time constants. For all of the systems examined herein, we experimentally found that all heat-releasing events after photon absorption occurred rapidly with respect to the response time of our transducer; hence time-resolved treatment of these photoacoustic data is not necessary (Rudzki et al., 1985; Hung and Grabowski, 1991). Specifically, the time-dependent nature of the observed photoacoustic signals are indistinguishable for MG and MG-BSA, both at pH 5.8 (e.g., *upper inset* of Fig. 2) and at pH 7.0 (data not shown) for λ_{exc} = 624 nm. In addition, these waves are identical in shape to those observed for copper chloride or copper sulfate in our buffer systems; these copper salts have been used previously to determine the response time of a detection system (Braslavsky and Heihoff, 1989). Confirmation of this conclusion that time-resolved treatment is not necessary was obtained by using the time-resolved data analysis method and finding only one heat-deposition event (see Discussion).

MG and MG-BSA undergo no volume change after single photon absorption. The observed photoacoustic signal is the direct result of a volume change (heat-induced expansion) upon photostimulation (Westrick et al., 1987; Herman and Goodman, 1989). The volume change arising from a given amount of energy deposited in a solution is a function of the solvent expansion parameter X_S, where $X_S = (\alpha\ MW/C_p\ \rho)$ as shown in Eq. 1. While three of the four physical parameters that define X_S are temperature dependent, the temperature dependence of X_S for pure water is essentially controlled by the temperature dependence of α which ranges from $3.03 \times 10^{-4}\ K^{-1}$ at 30.0°C to $0\ K^{-1}$ at 3.98°C to $-0.68 \times 10^{-4}\ K^{-1}$ at 0°C (Riddick et al., 1986). Therefore the observed PAC signal of a solute in pure water, for a photoinitiated event that is solely due to the conversion of photon energy into heat, should scale directly with X_S and, importantly, will go to 0 at 3.98°C; we have observed this behavior a number of times with appropriate systems (e.g., 8-anilinonaphthalene sulfonate or dansyl amide). Conversely, any contributions to the observed PAC signal from a "volume of reaction" (i.e., a volume change due to a chemical process or to changes in protein conformation) should remain constant over a small temperature range (0–30°C; Westrick et al., 1987). For a photon-initiated process that includes a volume of reaction (ΔV_{rxn}), the observed PAC signal will be the sum of the volume change due to heat deposition and volume change due to reaction, as a consequence of Eq. 1 expands to Eq. 2.

$$S = K' \frac{\alpha\ MW}{C_p\ \rho} f_h E_p (1-10^{-A}) + K'\ \Delta V_{rxn} \frac{E_p}{Lh\nu} (1-10^{-A}). \quad (2)$$

Thus, Peters and co-workers (Westrick et al., 1987, 1990; Peters and Snyder, 1988; Westrick and Peters, 1990; Marr and Peters, 1991) and others (Herman and Goodman, 1989; Goodman and Herman, 1989) have used the temperature dependence of the solvent expansion parameter to determine both the reaction enthalpy and reaction volume for photoinitiated events in aqueous systems.

Data that compares the temperature dependence of the photoacoustic signals for MG and MG-BSA at several temperatures between 30.1 and 1.4°C are shown in Fig. 3. The behavior observed for both compounds are indistinguishable, with the waves becoming flat (Fig. 3, *dashed line*) at the same temperature in both cases, indicating that MG and MG-BSA have the same reaction volume. Likewise, comparing the observed PAC signal as a function of temperature for MG and MG-BSA in D-PBS buffer at pH 7.0 shows both species to have identical behavior (data not shown). Because MG absorbs a photon and returns to the ground state in an infinitely fast process compared with our detection time constant, it must have a reaction volume of zero. Because MG-BSA behaves in an identical fashion to MG, in these variable temperature experiments, it also must have an immeasurable reaction volume.

Incidentally, when the amplitudes of the waves shown in Fig. 3 are plotted versus T, they scale closely but not exactly with the known X_S values for pure water (plot not shown); the difference is due to the presence of 0.1 M buffer. We have shown in other experiments that the observed photoacoustic signal for a compound that has no reaction volume upon photostimulation tracks directly with X_S for pure water and up to 10 mM buffer. However, as the buffer concentration is increased beyond 10 mM, the observed photoacoustic signal as a function of temperature shows an increasingly positive deviation (especially at lower temperatures) from the known X_S for pure water.

f_h of standards. As a consequence of the extremely high efficiency of the excited singlet state (S_1) deactivation process mediated by internal rotations of the phenyl rings, malachite green cation (as well as similar species formed from other triphenylmethane dyes) exhibits

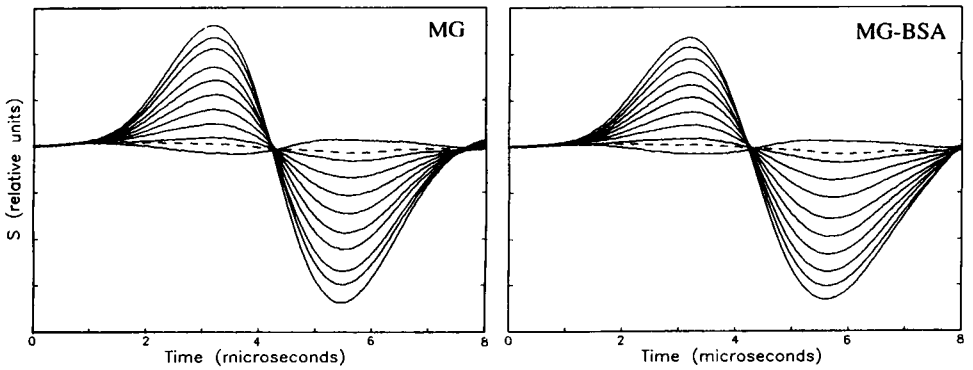


FIGURE 3 The effect of temperature on the observed photoacoustic signal for MG (*left*) and MG-BSA (*right*) in 0.1 M NaP_i at pH 5.8 for 624 nm excitation. In both cases the waveforms as a function of temperature are (in order according to decreasing magnitude of the wave at 3 μs; °C): 30.1, 27.0, 24.0, 20.0, 17.0, 13.7, 10.3, 7.5, 4.5, 3.2, and 1.4. For both compounds, the observed waves become essentially flat (*dashed line*) at the same temperature.

essentially no fluorescence ($\Phi_f \approx 10^{-4}$) in low viscosity liquid solutions (Forster and Hoffman, 1971; Vogel and Rettig, 1985), and has a lifetime of 2–3 ps (Ippen et al., 1976; Robl and Seilmeier, 1988). In addition, intersystem crossing from S_1 to T_1 of MG cannot effectively compete with the rapid internal conversion process. Therefore, for all practical purposes, the malachite green cation in aqueous solution will convert all absorbed photon energy into heat with a deposition time constant of 2–3 ps; MG can therefore serve as a calibration standard with $f_h = 1.0$ (Mardelli and Olmsted, 1977) in the pH ranges where it is present in appreciable concentration ($\sim 1.8 <$ pH $< \sim 13$; Weast, 1985). Likewise, BC-P will be a useful standard at pH > 6.8 while BC-G will be in the useful form at pH > 5.6 (Dean, 1973; Weast, 1985). An additional attractive feature of the triphenylmethane dyes for use as PAC standards is that they have relatively large extinction coefficients for a large portion of the UV-vis spectrum.

Several control experiments confirmed that the various dye molecules used as standards are well behaved in the pH range in which they are used. At pH 5.8, MG and BC-G have indistinguishable heat-release behavior; f_h(BC-G):f_h(MG) = 0.992 ± 0.009 (average of two experiments, each experiment being composed of two or more independent samples of each of BC-G and MG). At pH 7.4, f_h(BC-P):f_h(BC-G) = 0.998 (one experiment). From these data, and other qualitative experiments (not shown), we conclude that the f_h of the ionic forms of the three dye molecules are indistinguishable from 1.0. This conclusion is based on the knowledge that the same form of BC-G is being photoexcited at the two slightly different pH values and the assumption that the photophysical properties are not perturbed by the small change in pH. However, these same experiments (as well as others) make it very clear that it is inappropriate to compare, in a quantitative manner, photoacoustic signals taken in similar buffer solutions but at sufficiently different pH values without the appropriate corrections. For example, naively comparing the photoacoustic signals for MG at pH 5.8 to BC-P at pH 7.4, both in 0.10 M NaP_i buffer, give the apparent ratio f_h(MG, pH 5.8): f_h(BC-P, pH 7.4) = 0.948 ± 0.016 (average of three independent experiments); this ratio is a meaningless number since the K' of Eq. 1, which depends upon the acoustic propagation properties of the medium as well as other factors, cannot be expected to be constant for these two different solutions where ionic strength in particular is not held constant. We are aware that the observed photoacoustic signal in aqueous solution is dependent on a variety of factors that must be held constant (or explicitly treated) for quantitative comparisons. All our quantitative experiments account for these factors: quantitative comparisons are only made between data taken in the same buffer/pH; temperature is always controlled even when it is not varied; all other instrumental parameters (e.g., distance between the position of the laser beam in the cell to the detector, cell geometry, etc.) are held constant.

f_h of MG-BSA. At pH 5.8, the f_h(MG-BSA) = 0.973 ± 0.021 from the average of two independent experiments, one of which is shown in Fig. 2. At pH 7.0, f_h(MG-BSA) = 0.975 ± 0.004, also the average of two independent experiments. In each of these four experiments, the f_h(MG-BSA) was measured relative to that of f_h(MG) which is believed to be 1.0 (see above). Therefore, we also believe that in the pH range 5.8–7.0, f_h(MG-BSA) = 0.974 ± 0.013 (average of four experiments), or that 2.6% of the absorbed photon energy does not appear as heat within 50 ns of photon absorption. Because these f_h

determinations all involved measuring MG-BSA against MG, it is unambiguously clear that the f_h of MG-BSA is smaller than the f_h of MG.

Fluorescence quantum yield of MG-BSA is three to four times that of MG. In aqueous solution, at pH 5.8, the fluorescence quantum yields of both MG and MG-BSA are very low (λ_{exc} = 445 nm); Φ_f(MG-BSA) $\approx 10^{-3}$ to 10^{-4}, and this value is approximately three to four times higher than Φ_f(MG). The observed fluorescence spectra of MG-BSA (shown in Fig. 4) is practically indistinguishable in shape from that observed for MG (not shown). Normalization of the maximum in emission intensity to the maximum for the peak in the red region of the absorption spectra (Fig. 4) allows us to estimate the singlet energy of MG-BSA as ~45 kcal mol^{-1}.

DISCUSSION

From the information presented above, it is clear that malachite green as a free cation in aqueous solution excited at 624 nm releases all the absorbed photon energy as heat; f_h(MG) = 1.0. Likewise the bromocresol green and bromocresol purple ions also release all absorbed photon energy as heat when excited at 624 nm. In contrast the absolute amount of heat released for excitation of malachite green-labeled bovine serum albumin is less than that for free malachite green; f_h(MG-BSA) = 0.974 ± 0.013.

Four independent measurements of the heat-release properties of MG-BSA at pH 5.8 or 7.0, compared with MG, clearly show that a small portion of the absorbed photon energy is not being released as heat within 50 ns of photon absorption. Our best estimate indicates that 2.6% of the available photon energy is being dissipated by the dye-labeled protein by some other mechanism than fast internal conversion/vibrational relaxation. These data refer to single photon absorption conditions; in none of our experiments did we find any evidence of multiple photon processes for either the free dye or the dye-labeled protein (for example, we did not see deviation from linearity for high photolysis energies in plots of the type shown in Fig. 2; Song and Endicott, 1991). As might be anticipated, these results for MG and MG-BSA are independent of whether the pH of the solution is 5.8 or 7.0 (and the buffer identity); we expect them to be identical for an even wider pH range. What happens to the rest of the absorbed 624-nm photon energy that is not released as heat within 50 ns? We have carried out several experiments to address this issue.

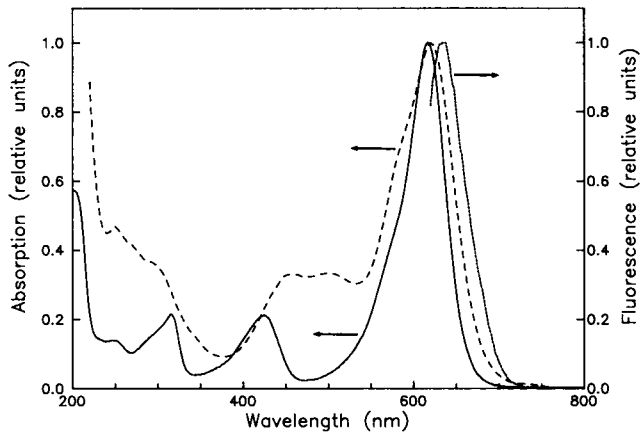


FIGURE 4. Absorption spectra (*left ordinate*) of the free dye (MG, ——) and the dye-labeled protein (MG-BSA, ------) in 0.1 M NaP_i at pH 5.8. Also shown (*right ordinate*) is the very weak fluorescence spectrum (·····; λ_{exc} = 445 nm) obtained for the dye-labeled protein. A fluorescence spectrum for the free dye was obtained that was practically indistinguishable in shape from that shown for the dye-labeled protein.

First, we examined the fluorescence properties of MG and MG-BSA. A search of the extensive literature on malachite green led us to expect very weak fluorescence from either the free dye or the dye-labeled protein. Our fluorescence measurements confirmed these expectations, the fluorescence of these species, excited at 445 nm, is very weak. Using wide slit widths, we were able to record the fluorescence spectra of MG and MG-BSA, and found that the spectra are practically identical in shape but that the fluorescence efficiency of MG-BSA is about three to four times that of MG; therefore Φ_f(MG-BSA) $\leq 10^{-3}$. Does this weak fluorescence account for the energy not detected by the photoacoustic calorimeter? Assuming the largest fluorescence quantum yield consistent with the data (10^{-3}) and an energy of ~44 kcal mol^{-1} emitted by an average fluorescing molecule (as obtained from the observed fluorescence spectrum), we estimate that fluorescence accounts for $\leq$0.1% of the available photon energy. Therefore, for MG-BSA, an energy dissipation mechanism other than fluorescence must account for the 2.6% energy discrepancy.

Another possible mechanism for loss of absorbed photon energy by MG-BSA is conversion of a portion of the energy into chemical potential energy by changing the conformation of the protein; this type of behavior has been extensively investigated using the PAC technique by Peters and co-workers (Westrick et al, 1987, 1990; Peters and Snyder, 1988; Westrick and Peters, 1990; Marr and Peters, 1991). The temperature dependence of the observed photoacoustic signal (Fig. 3) should reveal any volume changes that would accompany protein conformational changes. The data from several experiments indicate that under our low energy photolysis conditions, the dye-labeled protein behaves

indistinguishably from the free dye with regard to any photoinitiated reaction volume. Therefore, if the 2.6% of the photon energy is being consumed to produce a new protein conformation, a volume change associated with such a hypothetical conformational change is not detected by this approach; reaction volumes as small as 2 ml mol^{-1} can be detected by the PAC technique.

Oxygen quenching of the excited singlet state (i.e., energy transfer processes) does not play any role in our experimental probes of MG and MG-BSA. Oxygen concentration, under air-saturated conditions, should be on the order of 2×10^{-4} M (Murov, 1973). If the lifetime of the first excited singlet state of MG-BSA were 15 ps (i.e., five times the known lifetime of the free dye), one would need a oxygen quenching rate constant of $\sim 1 \times 10^{13}$ M^{-1} s^{-1} (Stern-Volmer formalism; Gilbert and Baggott, 1991) in order to achieve a 3% reduction in the excited state concentration within the timescale of our detector. Obviously, such a rate constant for this bimolecular process is physically meaningless; in addition, such energy transfer is forbidden by spin selection rules (Quina, 1982). Therefore, energy transfer from the singlet excited state to oxygen can not play a role in accounting for the 2.6% energy discrepancy observed by PAC.

Intersystem crossing (ISC) to a long lived triplet excited state may account for the 2.6% energy discrepancy. Enhanced quantum efficiency for intersystem crossing (Φ_{ISC}) has been noted before for a dye bound to a macromolecule as compared with the unbound dye (Bellin and Yankus, 1968; Jones et al., 1991). Our observations of a slightly increased fluorescence efficiency and the decreased heat yield for MG-BSA (compared with MG) is consistent with the suggestion of a decreased internal conversion rate constant. The formation of a small percentage of a long lived triplet for MG-BSA is consistent with all observations including a slow heat-deposition event not being observed (vide infra). The reaction of the triplet (the lifetime of ^{3}MG in ethylene glycol-water mixture is ~ 0.1 s at 77 K; Leaver, 1974) with oxygen to generate low concentration of a dye-peroxy species or singlet oxygen, either of which could then react with the protein, is a possibility and has been considered before for protein and amino acid photooxidation (Bellin and Yankus, 1968). Alternatively, the triplet may decay by a pathway involving electron transfer.

In an attempt to address the possibility of any slow (50 ns $< \tau <$ 10 μs) energy release component present for MG-BSA, we deconvoluted selected sets of our MG-BSA data against MG using time-resolution software (Hung and Grabowski, 1991) which is similar to that described previously (Rudzki et al., 1985). The results of the deconvolution indicate that there is only a single, fast ($\tau <$ 50 ns) heat-deposition detectable by our PAC. Model deconvolutions suggest that for noise-free data, we could not detect a heat deposition with a lifetime on the order of 1 μs if less than ~ 2 to 3% of the excitation energy is being deposited as heat for this event. If the 2.6% energy discrepancy noted above corresponds to production of a long lived triplet state, and if such a triplet is quenched by oxygen with a rate constant of 5×10^9 M^{-1} s^{-1}, we would expect the lifetime of the triplet to be ~ 1 μs. Likewise, since the decay of $O_2(^1\Delta_g)$ should occur with a 2–7 μs lifetime (Monroe, 1985), it is likely that neither event, if occurring, will be detected by our apparatus because each will involve considerably less heat deposition than that needed to be observed.

CONCLUSION

We have examined the heat-release properties of malachite green-labeled bovine serum albumin in buffered aqueous solution at pH 5.8 and 7.0, and found that for MG-BSA, 2.6% of the absorbed photon energy at an excitation wavelength of 624 nm is not released as detectable heat within 10 μs of being absorbed, while for MG all the absorbed photon energy is released as heat within 50 ns. While the observed fluorescence efficiency of MG-BSA was found to be about three to four times that of MG, MG-BSA is still too weakly fluorescent to account for the energy discrepancy. Under our low energy, single-photon photolysis conditions, we could find no evidence of photodegradation. No detectable protein conformational change was found either: MG and MG-BSA exhibited identical photoacoustic signal temperature dependence, implying that MG-BSA undergoes a conformational change of less than 2 ml mol^{-1} if any. The PAC data was examined to see if a slow heat deposition was occurring; no evidence was found for a significant heat deposition with a lifetime of 50 ns to 10 μs. Thus, it appears that 2.6% of the excitation energy for 624 nm (single-photon) photolysis of MG-BSA (but not MG) is converted to a long lived state; the first excited triplet state of malachite green and its subsequent reaction products are possibilities. It is clear from these studies that malachite green even when bound to a protein is in fact a good photon-to-heat converter, but not a perfect one since a small fraction of the photon energy of the protein bound dye is not recovered as prompt heat. Under the conditions of CALI (multiple photon absorption by any given MG moiety), the fraction of the photon energy not recovered as heat may be used to build up some concentration of relatively long lived reactive intermediates; these species may in fact cause chemical damage that inactivates the targeted protein.

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