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# Protein-mediated nonphotochemical bleaching of malachite green in aqueous solution

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**Abstract**

The effect of added protein on the nonphotochemical bleaching of malachite green cation ($MG^+$) in aqueous solution (100 mM MOPS, pH 8, or 100 mM CAPS, pH 10; 25 °C) was studied using chicken egg albumin (OA) and human serum albumin (HSA). Bleaching in the absence of protein (carbinol formation) was a pseudofirst-order process with $k_0 = 0.012 \pm 0.002$ and $0.025 \pm 0.001$ $min^{-1}$ at pH 8 and 10, respectively. Progress curves in the presence of OA or HSA were multiphasic; an initially fast decrease in $A_{620}$ was followed by a slower decay which could be resolved into two protein-related, first-order components. Protein concentrations causing 50% reduction in carbinol formation via the solvolytic route were: [OA] = 60 μM (pH 8) and 15 μM (pH 10); [HSA] = > 200 μM (pH 8) and 1.5 μM (pH 10) ($[MG^+]_0 \approx 8.5$ μM). The rate constants for the 2 OA-related components varied with both pH and protein concentration: At pH 8, [OA] = 40–300 μM, $k_1 = 0.26$–$2.4$ and $k_2 = 0.05$–$0.26$ $min^{-1}$; at pH 10, [OA] = 10–150 μM, $k_1 = 1.8$–$12$ and $k_2 = 0.09$ –$2.6$ $min^{-1}$. The $k$ values for HSA did not show a systematic dependence on protein concentration: at pH 8, $k_1 = 0.62 \pm 0.19$ and $k_2 = 0.16 \pm 0.09$ $min^{-1}$; at pH 10, $k_1 = 3.3 \pm 0.50$ and $k_2 = 0.32 \pm 0.10$ $min^{-1}$. The reversibility of the protein-mediated bleaching process was tested by adjusting the pH to 4.5 with glacial acetic acid and monitoring the recovery in $A_{620}$. Recovery in the presence of OA pointed to three leukodye-protein populations, reverting instantly, slowly ($k = 0.003$–$0.006$ $min^{-1}$) or not at all ($k < 10^{-4}$ $min^{-1}$). Recovery in the HSA-bleached samples was instantaneous and nearly quantitative. The results indicate that adduct formation between proteins and $MG^+$ can occur at a significant rate at moderate pH and that some proteins (exemplified by OA in this study) have a capacity to act as irreversible scavengers of the cationic dye. Such involvement of proteins in the nonphotochemical conversion of $MG^+$ to colorless forms may be a limiting factor in biomedical applications which rest on the availability of the cationic species. 

*Keywords:* Malachite green; Protein-mediated bleaching

*Abbreviations:* CAPS, 3-[cyclohexylamino]-1-propanesulfonic acid; HSA, human serum albumin; MG, malachite green; MOPS, 3-[*N*-morpholino]propanesulfonic acid; OA, chicken egg albumin; TAM, triarylmethane

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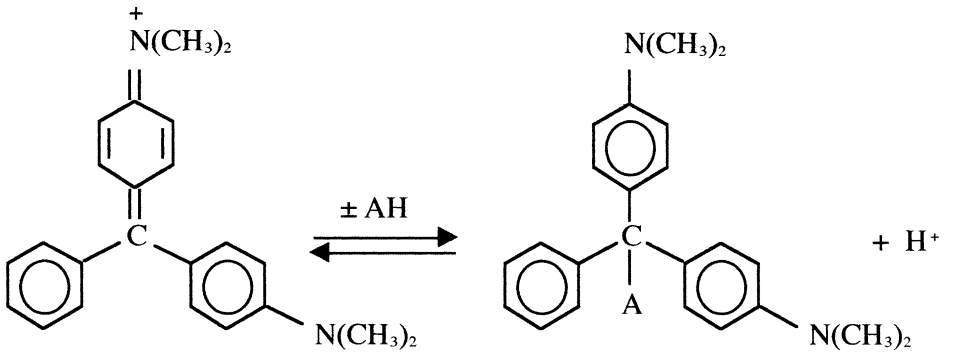


Scheme 1. AH, adding unit (water or a protein-associated nucleophilic center such as $-NH_2$, –OH, –SH).

## 1. Introduction

Cationic triarylmethane dyes such as crystal violet and malachite green have long been used as blood purging agents in preventing transfusion-associated transmission of a number of diseases [1,2]. There has been a recent revival of interest in $TAM^+$s, arising from studies which suggest that these dyes may find further use in the chromophore-assisted laser inactivation (CALI) of tumor cells [3–5]. In this context, attention has been directed to the photochemical properties of $TAM^+$s. It has been shown that high-λ excitation of the chromophore induces radical formation at the central carbon of the $TAM^+$ nucleus, yielding multiple dye products and causing redox damage to proximal biomolecules [6,7]. The "dark" reactions of $TAM^+$s have also been studied. However, the latter type of studies have focused more on the physical organics of dye–nucleophile addition reactions [8,9] than on the chemical impact of the dyes on biomolecules. Reports of immediate biological interest include interactions with the microsomal drug-metabolizing system [10,11] and adduct formation with glutathione, as deduced from the complex nature of the inhibition of DDT dehydrochlorinase and glutathione S-transferase by $MG^+$ and several other $TAM^+$s [12–14]. Information on reactivity towards other biological nucleophiles is unavailable.

The present report aims to elaborate on the electrophilic potential of the $TAM^+$ nucleus, focusing on the tendency of $MG^+$ (frequently the chromophore of choice in CALI) to undergo addition reactions in the multifunctional nucleophilic environment presented by proteins. The balance between the cationic form of the dye and colorless adduct forms (Scheme 1) is an important factor to consider in relation to the efficacy of CALI employing malachite green.

## 2. Materials and methods

Chicken egg albumin (Cat. No. A5503; Lot 19H7002), globulin- and fatty acid-free human serum albumin (Cat. No. A3782; Lot 97H7604) and malachite green hydrochloride were purchased from Sigma Chemical Co., USA. The chromophoric purity of the dye was verified by thin layer chromatography on silica gel (methanol–acetic acid, 95:5 v/v) [11]. Stock dye solutions were prepared in methanol.

The bleaching reactions were carried out at 25 °C in 100 mM MOPS (pH 8) or CAPS (pH 10) buffer containing 0–300 μM protein. The process was initiated by the addition of 20 μl $MG^+$ in methanol (final volume, 3 ml; final dye concentration, ca. 8.5 μM, based on nominal dye content) and monitored through the change in $A_{620}$. The reversibility of the bleaching process was tested by preincubating protein and dye until the protein-mediated reactions were >90% complete, reducing the pH of the medium to 4.5 by the addition of glacial acetic acid and monitoring the recovery in $A_{620}$.

The progress curves were analyzed by nonlinear regression, using Eq. (1) (bleaching mode; $B_i$,

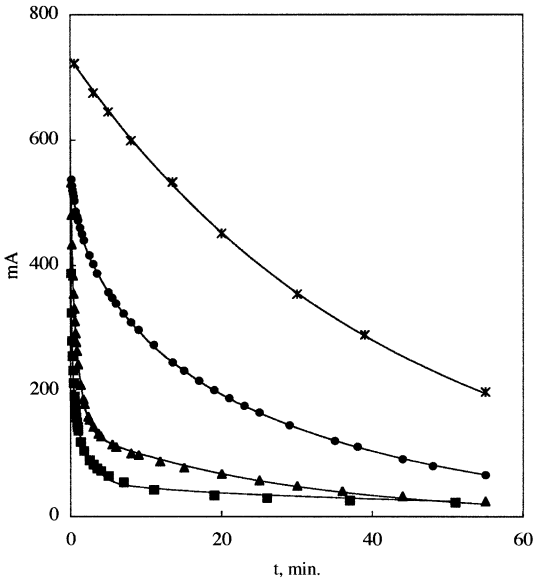


Fig. 1. Progress curves for the control and protein-mediated bleaching of $MG^+$. pH 10: (∗), control; (■), 40 μM HSA; (▲), 40 μM OA. pH 8: (●), 80 μM OA. $[MG^+]_0$, 8.8 μM. The curves are derived from the regression parameters in Table 1.

absorbance of the *i*th component at $t=0$) and Eq. (2) [recovery mode; $R_i=(A_\infty-A_0)_i$].

$$A_t=\Sigma B_i\exp(-k_{bi}t) \quad (1)$$

$$A_\infty-A_t=\Sigma R_i\exp(-k_{ri}t) \quad (2)$$

## 3. Results and discussion

### 3.1. Conversion of $MG^+$ to leukoforms

Typical progress curves for the control and protein-mediated bleaching of $MG^+$ at pH 8 and 10 are shown in Fig. 1. The $MG^+$-to-carbinol conversion in the control reactions was a quantitative, pseudo first-order process, with $k=0.012\pm0.002$ and $0.025\pm0.001$ $min^{-1}$ at pH 8 and pH 10, respectively. Bleaching in the presence of protein, on the other hand, was multiphasic. There was an initial, fast decrease in $A_{620}$, which could not be monitored with the conventional equipment at hand. [While this fast phase could in principle reflect a spectral change upon the binding of dye to protein [15], the behavior of the system in the recovery mode was generally more consistent with a discrete chemical process (see below)]. The remainder of the progress curves could be resolved into three first-order components. The amplitudes ($B_i$) and the rate constants ($k_{bi}$) of the various components are given in Table 1.

### 3.2. OA-mediated bleaching

Amplitude vs [protein] plots for OA-related bleaching at pH 10 are shown in Fig. 2. Overall, $MG^+$ appeared to partition between 4 types of sites, leaving an insignificant level of free dye in solution, even at the lowest protein concentrations used. Three of the sites (represented by $B_{fast}$, $B_1$ and $B_2$) made a direct contribution to the bleaching process; the fourth type of site (represented by $B_3$) bound the dye, but was unable to process it, except to allow solvolytic conversion to the carbinol form. [A possible exception is the $B_3$-type sites in OA-mediated bleaching at pH 8, where the inferior match between the bleaching and recovery modes implicated multiple product formation (cf. Table 2)]. The contribution of the $B_3$ sites to the overall bleaching process showed a steady decrease with increasing protein concentration, pointing to a low-affinity population subject to

competition by the remaining sites. The ratio of $B_{fast}$:$B_1$:$B_2$ remained nearly constant in the low [protein] range. With increasing protein concentration, the contribution of the $B_2$ component was found to decrease. The observed downward trend in $B_2$ most likely reflected an aggregation phenomenon (masking the $B_2$-type sites).

The measurable rate constants for the different components ($k_{b1}$–$k_{b3}$) showed a linear dependence on [OA]. This was unexpected, since the amplitude pattern implicated unimolecular (or, as in $B_3$, solvent-recruiting) reactions at the dye.protein level. As the corresponding rate constants in the $MG^+$–HSA system showed at most a marginal dependence on [HSA], the rate enhancement in the $MG^+$–OA samples was taken to arise from catalytic impurities (or an unidentified cofactor) in the OA preparation.

Table 1
Kinetic components of the protein-mediated bleaching of $MG^+$

| | | | Kinetic component[a] | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Protein | μM | pH | $B_{fast}$ | $B_1$ | $k_{b1}$ | $B_2$ | $k_{b2}$ | $B_3$ | $k_{b3}$ |
| OA | 10 | 10 | 58 | 67 | 1.8 | 170 | 0.50 | 445 | 0.029 |
| | 20 | | 26 | 132 | 5.3 | 288 | 0.74 | 294 | 0.037 |
| | 40 | | 123 | 162 | 7.6 | 317 | 1.04 | 138 | 0.035 |
| | 80 | | 141 | 305 | 6.6 | 240 | 1.59 | 54 | 0.065 |
| | 150 | | 273 | 278 | 12 | 156 | 2.60 | 33 | 0.12 |
| | 40 | 8 | 72 | 81 | 0.26 | 151 | 0.05 | 436 | 0.015 |
| | 80 | | 160 | 65 | 1.10 | 161 | 0.13 | 353 | 0.028 |
| | 150 | | 179 | 109 | 0.68 | 286 | 0.11 | 166 | 0.024 |
| | 300 | | 281 | 69 | 2.44 | 242 | 0.26 | 148 | 0.033 |
| HSA | 1.5 | 10 | 44 | 220 | 2.5 | 201 | 0.17 | 275 | 0.016 |
| | 3 | | 115 | 276 | 3.0 | 148 | 0.24 | 201 | 0.014 |
| | 6 | | 175 | 319 | 3.2 | 115 | 0.35 | 131 | 0.012 |
| | 10 | | 182 | 323 | 5.8 | 142 | 0.54 | 93 | 0.008 |
| | 20 | | 223 | 336 | 4.0 | 119 | 0.39 | 62 | 0.009 |
| | 40 | | 280 | 272 | 4.6 | 139 | 0.46 | 49 | 0.014 |
| | 13 | 8 | 23 | –[b] | – | – | – | 638 | 0.011 |
| | 27 | | 93 | – | – | – | – | 601 | 0.010 |
| | 50 | | 114 | – | – | – | – | 550 | 0.009 |
| | 100 | | 133 | – | – | – | – | 505 | 0.008 |
| | 200 | | 180 | – | – | – | – | 450 | 0.008 |

[a] $B$ in milliabsorbance units; $k$ in units of $min^{-1}$. $r^2 \geqslant 0.999$ (components 1–3). $B_{fast} = A_{dye}–(B_1 + B_2 + B_3)$; $A_{dye}$ (zero-time absorbance of dye in the absence of protein) = 740 ± 25 mA units.

[b] Insignificant contribution: $B_1 \approx B_2 = 44 \pm 27$; $k_{b1} = 0.62 \pm 0.19$; $k_{b2} = 0.16 \pm 0.09$, averaged over all [HSA].

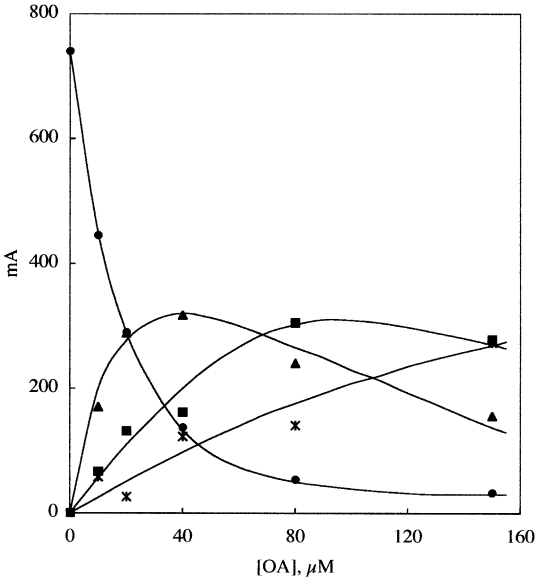


Fig. 2. Variation of amplitudes with protein concentration in the OA-mediated bleaching of $MG^+$ at pH 10. (∗), $B_{fast}$; (■), $B_1$; (▲), $B_2$; (●), $B_3$.

Table 2
Recovery of $A_{620}$ upon acidification of protein-bleached $MG^+$

| Sample | Bleaching pH | Recovery phase | Matching B phase[a] |
|---|---|---|---|
| 40 μM OA | 10 | $R_{fast}$ (316) | $B_2$ (317) |
| | | $R_1$ (67) | $B_3$ (59)[b] |
| | | $R_2$ (145) | $B_{fast}$ (123) |
| | | NR (153) | $B_1$ (162) |
| 80 μM OA | 8 | $R_{fast}$ (99) | $B_1$ (65) |
| | | $R_1$ (259) | $B_3$ (332)[b,c] |
| | | $R_2$ (192) | $B_{fast/2}$ (160/161) |
| | | NR (172) | $B_{2/fast}$ (161/160) |
| 40 μM HSA | 10 | $R_{fast}$ (603) | $B_{fast+1+2}$ (691) |
| | | $R_1$ (44) | – |
| | | $R_2$ (19) | $B_3$ (12)[b] |
| | | NR (34) | – |
| 80 μM HSA | 8 | $R_{fast}$ (60) | $B_{1/2}$ (44) |
| | | $R_1$ (282) | $B_{2/1+3}$ (252) |
| | | $R_2$ | (–) |
| | | NR (100) | $B_{fast}$ (124) |

[a] Cf. Table 1.

[b] The level of the $B_3$ product at the time ($t$) of acidification: $B_{30}–B_{3t} = B_{30}[1–\exp(-k_{b3}t)]$.

[c] Imperfect match. The $B_3$ phase must have yielded multiple products.

At pH 8, a 20–50% reduction was observed in the sum of $B_{fast}$, $B_1$ and $B_2$, caused mainly by the lowered magnitude of $B_1$ (and partly compensated by increased $B_2$ at high [protein]). The $B_{fast}$ vs [protein] profile was essentially the same as at pH 10. The average rate constant decrease (relating to $k_{b1}$ and $k_{b2}$) in the pH 10-to-8 interval was 15-fold.

*HSA-mediated bleaching*

The amplitude vs [protein] patterns in the HSA-related bleaching of $MG^+$ at pH 10 were similar to those for ovalbumin, except for a less significant contribution from $B_2$ and a much tighter dye-protein interaction. (The midpoint of the $B_3$ curve was ca. 15 μM for OA and 1.5 μM for HSA.) Switching to pH 8 reduced the bleaching reaction to an essentially 2-component process represented by $B_{fast}$ and $B_3$ (Table 1).

The rate constants for the $B_1$ and $B_2$ phases were similar to those for the corresponding phases of OA-mediated bleaching, but were independent of [HSA] (except for a slight enhancement in $k_{b2}$ at pH 10) and showed a more moderate dependence on pH (ca. 4-fold decrease over 2 pH units).

### 3.4. Dye recovery at reduced pH

The bleaching reaction was found to be reversible by acidification of the medium to pH 4.5. Typically, an instantaneous increase in $A_{620}$ was followed by a slower, mono- or biphasic approach to an end-point (Fig. 3). Percent recovery varied with the identity of the bleached sample. The amplitudes of the recovery phases and their tentative counterparts in the bleaching mode are given in Table 2. The recovery pattern suggested that the protein-related phases of the bleaching process ($B_{fast}$, $B_1$ and $B_2$) involved colorless adduct formation (protein-MG) rather than protein-catalyzed conversion of $MG^+$ to MG–OH. A significant fraction of the recovery was kinetically distinguishable from the reversion of MG–OH to $MG^+$, with rate constants ranging from immeasurably fast to slow: $k$ (control recovery) $\approx 0.4\ min^{-1}$; $k_{r1}$ (for dye bleached by HSA at pH 10) $= 2\ min^{-1}$; $k_{r2}$ (for dye bleached by OA at pH 8) $= 0.006\ min^{-1}$; $k_{r2}$ (for dye bleached by OA at pH 10) $= 0.003\ min^{-1}$. In addition, there was an irreversible component (NR, $k < 10^{-4}\ min^{-1}$) which corresponded to 5–20% of the initial [$MG^+$], depending on pH and the identity and concentration of the protein present.

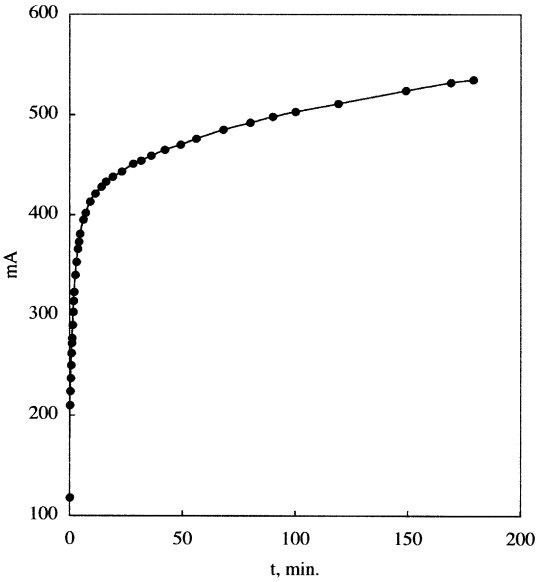


Fig. 3. Time course of dye recovery at pH 4.5. Sample, 8.3 μM MG bleached at pH 8 in the presence of 80 μM OA.

Of the two proteins tested in this study, OA proved to be an efficient bleaching agent for $MG^+$ at near-physiological pH. At pH 8 and 80 μM OA, ca. 20% of the dye was bleached in an instantaneous, protein-mediated process ($B_{fast}$ or $B_2$), which was but slowly reversible at acid pH. An additional 20% was bleached irreversibly ($B_2$ or $B_{fast}$). The correspondence between $R_2$/NR and $B_{fast}$ suggested that, at least in the OA system, the $B_{fast}$ phase reflected a discrete chemical change and not a simple binding process. Under similar conditions, HSA was less effective than OA. However, the trend in $B_{fast}$(= NR) relating to dye bleached by HSA at pH 8 showed that effective bleaching may be achieved at higher concentrations of this protein as well.

The identification of the MG–protein adducts formed requires extensive structural studies. In principle, all three nucleophilic groups cited in Scheme 1 (the side chains of lysine, serine and cysteine residues) could add to $MG^+$; the addition

might further be subject to general base catalysis by neighboring groups (e.g. the side chain of histidine). Compounded by the complexity of the protein microenvironment and its variable impact on acid-base behavior [16,17], the system does not lend itself to kinetic resolution. A tentative assignment relating to the irreversible components of the OA- and HSA-mediated bleaching is that these components reflect a redox process which recruits cysteine sulfhydryls (1 per HSA [18]; 4 per OA [19]) and yields leukomalachite green (MG–H), coupled to the oxidation of –SH to a sulfenic acid (−SOH) or a disulfide. This argument is consistent with the observation that $MG^+$ samples bleached with 2-mercaptoethanol show less than quantitative recovery of color upon acidification.

To conclude, our kinetic results indicate that proteinaceous mixtures with suitably reactive (or abundant) components can scavenge the $MG^+$ cation and interfere with light-induced radical generation by the dye. A further possibility to consider is that adduct formation between $MG^+$ and target proteins could yield inactive (or activated) proteins, adding a radical-independent component to the $TAM^+$ effect. It would be of interest to determine whether or not the $MG^+$-reactivities of OA and HSA are shared by other cellular or extracellular proteins. Preliminary work in this laboratory has shown human plasma to have a high $MG^+$-bleaching capacity at physiological pH, involving plasma components other than HSA. If adduct formation is a common phenomenon, then the strategy of conjugating $MG^+$ to immunoglobulins with the aim of improving target specificity in CALI [5] may prove to be not only a refinement of the method, but an absolute requirement for the preservation of the dye in cationic form.

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