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Electrophilic reactivity of cationic triarylmethane dyes towards proteins and protein-related nucleophiles

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Abstract

The adduct forming (bleaching) properties of four cationic triarylmethane dyes (methyl green, MeG^+; malachite green, MG^+; pararosaniline, PR^+; crystal violet, CV^+) were studied at 25 °C, in 100 mM MOPS buffer (pH 8), using simple nitrogen and sulfur nucleophiles (imidazole, 6-aminocaproic acid, 2-mercaptoethanol, reduced glutathione) or proteins (chicken ovalbumin, OA; human serum albumin, HSA; human γ-globulins, IgG) as addends. Among simple nucleophiles, significant adduct formation was observed only with thiols. The apparent dissociation constants (K_d') at pH 8 for the 2-mercaptoethanol adducts of MeG^+, MG^+, PR^+ and CV^+ were 0.034, 0.22, 1.4 and 44 mM, respectively. K_d' values for the glutathione adducts of MeG^+ and MG^+ were 0.027 and 0.21 mM. Methyl and malachite green were the only dyes to be bleached by proteins at moderate concentrations (150 μM). Bleaching was multiphasic, summing contributions from multiple nucleophilic centers. In contrast to the trend in the reactions with simple nucleophiles, MeG^+ was generally more resistant to protein-mediated bleaching than MG^+: OA and HSA contributed 78 and 36%, respectively, to the total color loss in MG^+; the corresponding contributions to the bleaching of MeG^+ were 16 and 15%. With both dyes IgG-mediated bleaching amounted to ca. 30%. It appeared that protein-borne sulfhydryl groups could add to MG^+ but not to MeG^+. The inferior reactivity of MeG^+ towards protein-SH may arise from hindered access of this nucleophile to the central carbon of the TAM^+ nucleus. The exceptional tendency of MG^+ to add protein-SH needs to be accounted for. One possibility is that SH groups, excluded from the central carbon, add to the unsubstituted phenyl ring unique to MG^+.

Keywords: Triarylmethane dyes; Dye-nucleophile adducts; Protein-mediated bleaching

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1. Introduction

Cationic triarylmethane dyes have found widespread use as colorants in industry and as antimicrobial agents [1, 2]. Recent reports indicate that they may further serve as targetable sensitizers in the photodestruction of specific cellular components or cells [3–5]. The current and potential uses of the dyes have led to detailed studies on

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Abbreviations: CAPS, 3-(cyclohexylamino)-1-propanesulfonic acid; CV^+, crystal violet; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); GSH, glutathione, reduced form; HSA, human serum albumin; IgG, gamma globulin (human); 2-ME, 2-mercaptoethanol; MeG^+, methyl green; MG^+, malachite green; MOPS, 3-(N-morpholino)propanesulfonic acid; OA, ovalbumin (chicken); PR^+, pararosaniline; TAM, triarylmethane

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their chemical properties as well as their impact on the bioenvironment. The photochemistry of high-λ irradiation has been addressed in relation both to color stability in industrial products and efficacy in photodynamic therapy [1,5–7]. Nonphotochemical dye-nucleophile addition equilibria have been described [8, 9]. The biohazards of TAM^+ exposure have been surveyed [10]; regulatory measures are in effect concerning the use of some dye species [11].

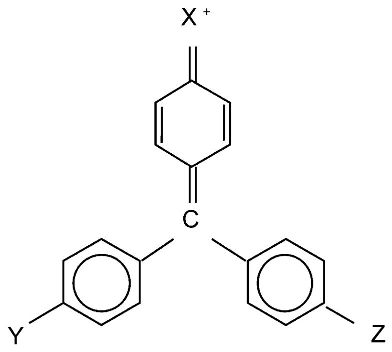
Much of the impact of TAM^+s on biosystems has been ascribed to noncovalent interactions with cellular components (nucleic acids, membranes) and to irreversible redox changes in multiple targets mediated by TAM^+-derived free radicals [1–3,6]. Reversible dye–nucleophile addition equilibria have been invoked only in connection with the inhibitory effect of TAM^+s on glutathione-utilizing enzymes, where substrate depletion by TAM–GSH adduct formation appears to be the principal contributing factor [12]. More recently, we have reported efficient nonphotochemical bleaching of a TAM^+ dye (malachite green) by chicken ovalbumin and human serum albumin [13], showing that dye–protein adducts can also form and suggesting that proteins might be primary, rather than indirect, targets of TAM^+ action under "dark reaction" conditions. The following study further addresses this possibility, focusing on the reactivities of various nitrogen and sulfur nucleophiles and an extended selection of proteins (containing or lacking free SH groups) towards four TAM^+ dyes: Methyl green, malachite green, pararosaniline and crystal violet (Fig. 1). The results indicate that while all four dyes add simple nucleophiles, MG^+ is the only TAM^+ to yield significant levels of dye–protein adducts under biologically relevant conditions.

2. Materials and methods

Pararosaniline acetate was purchased from Aldrich (USA). The remaining TAM^+ dyes, diisopropyl fluorophosphate-inactivated chymotrypsin, papain, chicken ovalbumin, human γ-globulins (Cohn Fraction II,III), globulin- and fatty acid-free human serum albumin were from Sigma (USA). All other chemicals were of the highest purity available and were obtained from Sigma or Merck (Germany). Stock solutions of dye were prepared in methanol.

The reactions were carried out at 25 °C in 100 mM MOPS (pH 8) and/or 100 mM CAPS (pH 10) buffer, containing ca. 9 μM TAM^+ (based on nominal dye content), test nucleophile (μM–mM range, depending on the nucleophile and pH) or test protein (150 μM). The bleaching process was monitored spectrophotometrically at the λ_{max} of the dye being used (635, 620, 540 and 590 nm for MeG^+, MG^+, PR^+ and CV^+, respectively). The reversibility of the bleaching process was tested by acidifying the reaction mixture with glacial acetic acid (final pH 4.5) and recording the recovery of color. With sulfur nucleophiles, reversibility was also tested by depleting the nucleophile with DTNB (added in ca. four fold excess over the initial nucleophile concentration) to shift the dye $\Longleftrightarrow$ adduct equilibrium in the direction of free dye.

Spectrophotometric measurements were made using a Shimadzu 1601PC system equipped with a Peltier unit.



	X/X^+	Y
MeG^+	$N(me)_2$	$N(et)(me)_2$
MG^+	$N(me)_2$	H
PR^+	NH_2	NH_2
CV^+	$N(me)_2$	$N(me)_2$

Fig. 1. Chemical structure of TAM^+ dyes.

3. Results and discussion

3.1. *The reaction of TAM$^+$s with simple nucleophiles*

The progress curves for the bleaching of TAM$^+$s by simple N and S nucleophiles were biphasic (Fig. 2), consistent with a two-stage process (Scheme I) involving rapid equilibration of TAM$^+$ and TAM-nucleophile adduct (Phase I), followed by gradual displacement of the equilibrium towards TAM–OH (Phase II).

Typical progress curves for the recovery of color upon the addition of acetic acid or DTNB are given in Fig. 3. Recovery upon acidification was mono- or biphasic, depending on whether the mixture harbored one or both of the products (TAM–nucleophile and TAM–OH) at the time of acidification. A biphasic color change was observed in thiol-bleached samples chased with DTNB: The initial phase corresponded to the k_{-1} step in Scheme I; the subsequent phase (not shown) reflected the refading of the regenerated TAM$^+$ by solvent addition (k_o).

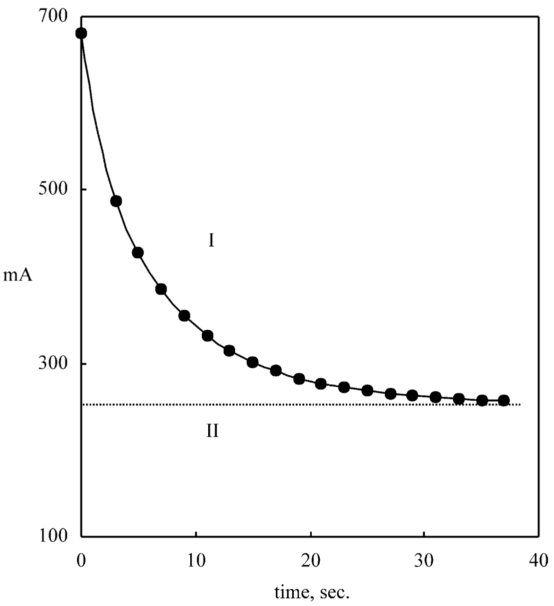


Fig. 2. Progress curve for the reaction of MG$^+$ with 2-ME at pH 8.0. [MG$^+$]~9 μM; [2-ME] = 400 μM; λ = 620 nm.

The relevant constants for the TAM$^+$ $\Longleftrightarrow$ TAM-nucleophile interconversion (k_1, k_{-1} and $K_d = k_{-1} / k_1$; reported below as pH-uncorrected constants, k_1', k_{-1}' and K_d') were obtained by kinetic and/or end-point analyses:

3.2. *Kinetic estimations*

Where possible, the reactions were conducted under pseudo-first-order conditions ([H$^+$] constant; $[\text{nucleophile}]_T >> [\text{TAM}^+]_T$). The rate constant ($k_{eq}'$) for the initial approach to equilibrium (Phase I, Fig. 2) was obtained by employing Eq. (1). [$A_{ii,t}$, extrapolated contribution of Phase II to overall absorbance at time t; $C = \ln\ (A_o - A_{ii,o})$].

$$\ln\left(A_t - A_{ii,t}\right) = C\text{-}\ k'_{eq}t. \qquad (1)$$

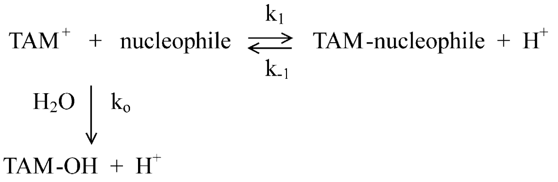
k_1', k_{-1}' and K_d' were then estimated by means of Eq. (2), which relates to

$$k'_{eq} = k_1\left\{K_a / \left(K_a + \left[H^+\right]\right)\right\}[\text{nucleophile}]_T + k_{-1} \times \left[H^+\right] \qquad (2)$$

first-order reversible processes. Plots of k_{eq}' vs $[\text{nucleophile}]_T$ yielded $k_1' = k_1\{K_a / (K_a + [H^+])\}$, $k_{-1}' = k_{-1}[H^+]$ and $K_d' = k_{-1}'/k_1'$. (K_a, acid dissociation constant of the adding nucleophile).

3.2.1. *End-point estimations*

End-point analyses were used primarily to estimate K_d' in fast reactions where k_{eq}' could not be measured. Reactions where $K_d' \sim [\text{TAM}^+]_T$, were analyzed using Eq. (3). Systems monitored under pseudofirst-order conditions ($K_d' >> [\text{TAM}^+]_T$) were analyzed according to Eq. (4).



Scheme I.

$$K'_d = (A_{ii,o}) \times \{[\text{nucleophile}]_T - [\text{TAM}^+]_T[(A_o - A_{ii,o})/A_o]\} \div (A_o - A_{ii,o}) \quad (3)$$

$$A_o/A_{ii,o} = 1 + [\text{nucleophile}]_T/K'_d \quad (4)$$

The method was further used for the purpose of verifying kinetic estimates in thiol addition reactions: In such reactions, k_{-1}' could be calculated independently from the time course of color recovery initiated by the addition of DTNB (Fig. 3). The first-order increase in absorbance was governed by k_{-1}' which, taken together with the end-point estimate for K_d', yielded an end-point estimate for k_1'. The results are given in Table 1.

The kinetic and equilibrium constants showed sulfhydryl groups to be the only nucleophiles with a significant capacity to react with TAM^+s at a biologically relevant pH. The inherently lower reactivity of amines, limited further by protonation, reduced TAM–amine adduct formation to negligible levels at pH < pK_a. The trend in K_d' at pH 8 for the reaction of 2-ME with the four dyes tested ($MeG^+ < MG^+ < PR^+ < CV^+$) parallelled the corresponding trend in K_d' for carbinol formation [9]. A special feature, notable mainly in the reactions of MG^+ with 2-ME and 3-mercaptopropionic acid (Table 1) was the disagreement between the K_d' values estimated by the kinetic and end-point methods. (K_d', end-point ≈0.2–0.4 K_d', kinetic). This feature implicated a sequential mechanism in the bleaching mode: it appeared that the time course of bleaching reflected the rate-limiting formation of a transient primary adduct and that this product subsequently came to equilibrium with a second leucostructure in a fast, internal or solvent-recruiting, process. In keeping with this hypothesis, it was found that the rapid recovery of color caused by DNTB in the MG^+-mercaptoethanol/3-mercaptopropionic acid systems was less than quantitative (54 ± 7%), whereas recovery following acidification (reverting both thiol and hydroxyl adducts) was 96 ± 3%. It is not obvious at this point why the reaction of MeG^+ with 2-ME failed to exhibit similar properties.

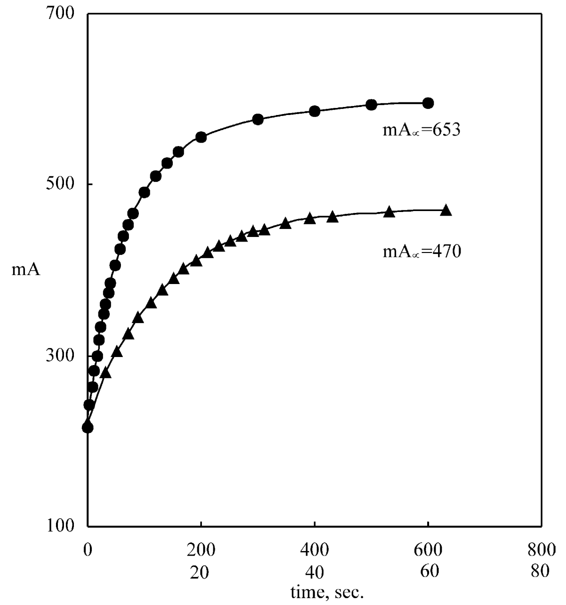


Fig. 3. Progress curves for color recovery in 2-ME-bleached MG^+ samples. (●) Recovery following acidification to pH 4.5 (0–800 sec); (▲) recovery following addition of 1500 μM DTNB at pH 8 (0–80 sec). Conditions in the bleaching phase: $[MG^+] \sim 9$ μM; [2-ME] = 400 μM.

3.3. The reaction of TAM^+s with proteins

The effect of added protein on the time course of bleaching was analyzed as described previously [13], by assuming a process with i parallel, first-order components and fitting the data to Eq. (5).

$$A_t = \sum A_{oi}\exp(-k_i t). \quad (5)$$

The results (Table 2) indicated that significant protein-mediated bleaching occurred only in the case of MG^+ and MeG^+. With MG^+, the faster components of the bleaching process ($k_i \geqslant 0.4$ min^{-1}) apparently related to sulfhydryl content, as they were much less pronounced in the IgG profile. N-nucleophilic side chains most likely contributed to the slower protein-related components ($k_i \geqslant 0.08$ min^{-1}). The tendency of MeG^+ to react with proteins was more limited: whereas OA and HSA contributed 78 and 36%, respectively, to the total color loss in MG$^+$; the corresponding contributions to the bleaching of MeG$^+$ were 16 and 15%. IgG-mediated bleaching, on the other hand, accounted for ca. 30% of the total color loss in both dyes. The relative inertness of MeG$^+$ towards OA and HSA (both SH-containing proteins) contrasted with the reactivity scale observed with 2-ME as the adding nucleophile (Table 1). A simple explanation for such nonreactivity is that the two proteins have a lower affinity for MeG$^+$ than MG$^+$. This argument would be adequate for the nonreactivity of PR$^+$ and CV$^+$, since the unfavorable K_d' values (Table 1) preclude adduct formation between free species in solution at μM concentrations. The inferior reactivity of MeG$^+$ towards OA and HSA, on the other hand, cannot be as readily ascribed to nonbinding: The K_d' at pH 8 for the MeG$^+$-mercaptoethanol reaction is 33 μM; the predicted rate constant for the approach to equilibrium at 150 μM free SH is ~10 min^{-1}. The corresponding values for the MeG$^+$–GSH reaction are 18 μM and ~100 min^{-1}. Based on these values, one would expect that a single reasonably exposed protein-SH

Table 1
Rate and equilibrium constants for TAM–nucleophile adduct formation[a]

Nucleophile	TAM$^+$	pH	k_1' (mM^{-1}min^{-1})	k_{-1}' (min^{-1})	K_d' (mM)
Imidazole	MG$^+$	10	0.0042	0.077	18(22)[b]
6-Aminocaproic acid			0.087	6.1	70(83)
2-Mercaptoethanol			(840)[c]	5.9[c]	(0.0070)
2-Mercaptoethanol	MG$^+$	8	6.0(27)[c]	5.7, 6.0[c]	0.95(0.22)
	MeG$^+$		78(94)[c]	2.6, 3.2[c]	0.033(0.034)
	PR$^+$		–[d]	–	(1.4)
	CV$^+$		–	–	(44)
3-Mercaptopropionicacid	MG$^+$		4.6(7.7)	2.5(1.7)	0.54(0.22)
Glutathione (reduced)	MG$^+$		(130)[c]	28[c]	(0.21)
	MeG$^+$		(610)[c]	11[c]	(0.018)

[a] r^2 in all entries ⩾0.95.
[b] Parenthetic values are "end-point" estimates.
[c] Calculated from the time course of the DTNB-mediated recovery of color.
[d] Reaction too fast to allow kinetic analysis in either the bleaching or the recovery mode.

Table 2
Kinetic components of the protein-mediated bleaching of TAM$^+$ at pH 8[a]

TAM$^+$	Ovalbumin		HSA		IgG	
	A_i / A_o,%	k_i (min^{-1})	A_i / A_o (%)	k_i (min^{-1})	A_i / A_o (%)	k_i (min^{-1})
MG$^+$,[b]	24	fast	22	fast	5.7	fast
	15	0.68	8.7	0.58	7.1	0.47
	39	0.11	5.5	0.080	–	–
	22	0.024[c]	64	0.009	71	0.018
	–	–	–	–	16	slow
MeG$^+$,[d]	6.9	fast	11	fast	14	fast
	2.3	3.9	2.8	2.0	6.4	1.6
	6.6	0.11	10	0.12	13	0.13
	81	0.011	76	0.011	67	0.010

[a] r^2 ⩾0.999 in all entries. Only those proteins and dyes exhibiting protein-mediated bleaching components have been included.
[b] $k_o' = 0.015$ min^{-1}. Data relating to OA have been adapted from [13].
[c] OA-catalyzed solvolytic conversion to MG-OH [13].
[d] $k_o' = 0.010$ min^{-1}.

would suffice to bleach $\geqslant$80% of the dye by a bimolecular process and that the rate would be distinguishable from that of the basal solvolytic conversion to MeG–OH ($k_o' = 0.010$ min^{-1}). The lack of involvement especially of OA (which has four free SH groups [14]) is therefore unexpected and needs to be accounted for. A second possibility is that the cys-SH groups in proteins are more hindered than the cys-SH of GSH, and as such, cannot add to the central carbon of the TAM$^+$ nucleus; protein-mediated bleaching of MeG$^+$ must be due to amino acids (e.g. lysine) having longer-reaching side chains. This argument, in turn, implies that the reactions of MG$^+$ with OA and HSA are atypical and suggests that the more readily accessible unsubstituted phenyl ring, the unique feature of MG$^+$, may serve as a reactive center for the addition of protein-SH, if not other nucleophiles.

To conclude, the results of this study imply that MG$^+$ and MeG$^+$ must be rapidly and quantitatively converted to colorless adducts in vivo, protein-SH groups and/or glutathione (at intracellular concentrations of up to 30 and 8 mM, respectively [15]) being the most likely addends. The less electrophilic TAM$^+$s (PR$^+$, CV$^+$ and related trisubstituted dyes) are essentially inert to nucleophilic addition at physiological pH and are expected to preserve their carbocation structure. Thus the biological effects of MG$^+$ and MeG$^+$ must relate to their thiol adducts; DNA intercalation, membrane insertion and TAM• radical-mediated redox changes appear to be more relevant to cellular damage induced by the less electrophilic species. Inhibition of glutathione-utilizing enzymes by TAM–GSH adducts has already been considered [12]. The high reactivity of MG$^+$ towards sulfhydryl-bearing proteins suggests that proteins which selectively bind and add to the dye are further targets of MG$^+$ action.

Acknowledgements

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