The binding of Ca^{2+} to actin monomer is monitored by the fluorescence of actin-bound auramine O

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The fluorescence of the cation auramine O was substantially enhanced by the presence of actin monomer. Titrations of this fluorescence enhancement indicated that actin monomer had two auramine O binding sites, each with a dissociation constant of approx. $20\ \mu\text{M}$. Calcium ions had no effect on the number of actin monomer-bound auramine O molecules or on the dissociation constant for that interaction. However, calcium ions increased the maximum change of fluorescence that occurs when actin monomer was fully saturated with auramine O. This effect of calcium was saturable and yielded a Ca^{2+} dissociation constant of $1.6\ \text{mM}$. It was concluded that auramine O bound to sites on actin monomer and independently monitored the binding of Ca^{2+} ion(s) to other site(s) on actin monomer. Further, the magnitude of the Ca^{2+} dissociation constant suggested that this Ca^{2+} -binding site may be representative of the multiple bivalent cation-binding sites on actin monomer which are thought to be directly involved in actin polymerization. However, the exact relationship between these sites remains unclear.

Actin monomer or G-actin, can be induced to polymerize into long double-stranded helical filaments, or F-actin, by physiological ionic strengths and/or millimolar concentrations of bivalent cations, typically Ca^{2+} or Mg^{2+} (Oosawa & Kasai, 1971). G-actin has a single 'high-affinity' bivalent cation-binding site ($K_d \sim 10\ \mu\text{M}$ for Ca^{2+} , and $K_d \sim 30\ \mu\text{M}$ for Mg^{2+} ; Frieden, 1982) which has been the subject of several investigations (Martonosi *et al.*, 1964; Kasai & Oosawa, 1968; Oosawa & Kasai, 1971; Strzelecka-Golaszewska, 1973; Strzelecka-Golaszewska *et al.*, 1978a; Frieden, 1982). These studies generally agree that the binding of Ca^{2+} or Mg^{2+} to this site maintains the potential of actin monomer for self-association.

In addition to this single 'high-affinity' bivalent cation-binding site on G-actin, there are also multiple, poorly defined 'low-affinity' sites (K_d of

the order of magnitude of $1\ \text{mM}$) which, when occupied, are thought to be directly responsible for actin polymerization (Martonosi *et al.*, 1964; Strzelecka-Golaszewska *et al.*, 1978a,b). The binding of auramine O (tetramethyldiaminodiphenyl-ketoimine), a cationic fluorescent compound, to G-actin has been investigated in an attempt to probe the cation-binding sites on G-actin. The experiments described in the present paper characterize the interaction of auramine O with G-actin and the binding of Ca^{2+} ion(s) to the auramine O–G-actin complex.

Experimental procedures

All reagents were of analytical grade, with solutions made from deionized distilled water.

Actin Purification

Rabbit skeletal muscle actin was isolated and purified according to the procedure described by Pardee & Spudich (1982a) with the gel filtration (Sephadex G-150) modification of MacLean-Fletcher & Pollard (1980). G-actin was used within

Abbreviations used: G-buffer, $2\ \text{mM}$ -Tris/HCl (pH 8.0)/ $200\ \mu\text{M}$ -ATP/ $200\ \mu\text{M}$ - CaCl_2 / $1.5\ \text{mM}$ - NaN_3 / $1\ \text{mM}$ -2-mercaptoethanol; C-buffer, $2\ \text{mM}$ -Tris/HCl (pH 8.0)/ $20\ \mu\text{M}$ -ATP/ $20\ \mu\text{M}$ - CaCl_2 / $1.5\ \text{mM}$ - NaN_3 .

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2–3 days of gel filtration, and before all experiments it was centrifuged (3 h, 4°C, 40000 rev./min, type 50 rotor) to remove any actin oligomers. This centrifugation was essential to obtain reproducible rates of actin polymerization. The percentage of non-polymerizable actin was always less than 3% of the total actin content as determined by the method described by Tellam *et al.* (1983). The purified G-actin was in G-buffer; the ATP and Ca^{2+} concentrations were then decreased by dialysis against C-buffer. The stability of G-actin under these conditions was verified by monitoring the intrinsic fluorescence (excitation 280 nm, emission 328 nm) of G-actin according to the procedure described by Lehrer & Kerwar (1972).

The choice of actin concentrations below the critical actin concentration for polymerization at a particular CaCl_2 concentration ensured that only monomeric actin was present in the following experiments. Critical actin concentrations were measured according to the procedure described by Tobacman *et al.* (1983) (results not shown). In addition, auramine O inhibited actin polymerization (result not shown) and thereby facilitated the maintenance of monomeric actin solutions.

Protein determination

Actin concentrations were measured spectrophotometrically by using the value $A_{290}^{1\text{ mg/ml}} = 0.63$ (Houk & Ue, 1974) and an M_r of 42300 (Elzinga *et al.*, 1973). All absorption measurements were made with a Cary 118 spectrophotometer.

Auramine O

This dye was recrystallized according to the procedure described by Conrad *et al.* (1970). A stock solution of auramine O ($\sim 5\text{ mM}$) was made by dissolving the dye in C-buffer followed by filtration through Whatman no. 5 filter paper. Fresh solutions were prepared for each experiment, as a fine precipitate was formed after 3–4 days in solution. The concentration of auramine O was determined spectrophotometrically at 430 nm using a value for the molar absorption coefficient, ϵ , of $4.41 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$ (Conrad *et al.*, 1970). Although this value of ϵ was originally determined in 0.1 M-sodium phosphate buffer at pH 7.4, it was found to also pertain to our C-buffer system.

Fluorescence

Auramine O fluorescence measurements were made in a Perkin-Elmer LS-5 luminescence spectrometer which incorporates a ratio recording system compensating for lamp intensity fluctuations. Emission spectra (excitation at 370 nm, excitation and emission monochromator slits each set at 5 nm) of auramine O were not corrected for variations in detector efficiency with wavelength

nor for concentration-dependent inner filter effects caused by auramine O self-absorption of the incident light.

All fluorescence titrations were carried out at 25°C with excitation at 370 nm (slit width of 10 nm) and emission at 540 nm (slit width of 20 nm). The high absorbance of auramine O in the concentration range of interest (0.3–100 μM) results in a substantial nonlinearity between the fluorescence intensity and auramine O concentration (the primary absorption effect or an inner filter effect). This effect can be minimized by decreasing the absorbance of the incident light by choosing an appropriate excitation wavelength characterized by a low molar absorption coefficient. However, it is also necessary to choose an excitation wavelength corresponding to an isobestic point in the absorption spectra of auramine O in the presence and absence of G-actin. At such an excitation wavelength, self-absorption correction procedures can be applied to the fluorescence of auramine O both in the presence and absence of G-actin. For these reasons the excitation wavelength of 370 nm was chosen (see Fig. 1a). The quantum efficiency of auramine O fluorescence has been reported to be independent of whether excitation occurs at 436 nm or 365 nm, i.e. at the major and minor absorption peaks respectively (Oster & Nishijima, 1956).

The concentration-dependent inner filter effect has been overcome by a semi-empirical correction procedure which corrects the emission intensity for absorbance values as high as 5 (Birdsall *et al.*, 1983). Briefly, observed fluorescence intensities of a range of auramine O concentrations were fitted to eqn. (1) by a nonweighted nonlinear least-squares regression method.

$$F_{\text{obs.}} = (F_{\text{corr.}} + F_{\text{blank}}) \left(\frac{e^{-aL_T d} - e^{-aL_T}}{aL_T(1-d)} \right) \quad (1)$$

and

$$F_{\text{corr.}} = kL_T$$

Where $F_{\text{obs.}}$, $F_{\text{corr.}}$ and F_{blank} are the observed, corrected and blank fluorescence intensities respectively, L_T is the total auramine O concentration and a , d and k are constants of best fit determined from the nonlinear least squares regression analysis. Knowledge of a and d allowed calculation of $F_{\text{corr.}}$ at each auramine O concentration in the absence or presence of G-actin. The auramine O fluorescence increase (ΔF) due to the presence of G-actin was then obtained by subtracting the corrected fluorescence of auramine O alone ($F_{\text{corr.}}^0$) from $F_{\text{corr.}}$ in the presence of G-actin at each auramine O concentration. The dissociation (K_d)

for the G-actin–auramine O interaction was calculated from the abscissa intercept of a plot of ΔF^{-1} versus L_T^{-1} at a constant concentration of G-actin, according to eqn. (2a) (Wang & Edelman, 1971).

$$\frac{1}{\Delta F} = \frac{1}{\Delta F_{\max.}} + \frac{K_d}{\Delta F_{\max.}} \frac{1}{L_T} \quad (2a)$$

$$\frac{\Delta F}{\Delta F_{\max.}} = \frac{L_T}{L_T + K_d} \quad (2b)$$

where $\Delta F_{\max.}$ is the change in fluorescence at infinite auramine O concentration. Alternatively, the magnitudes of K_d and $\Delta F_{\max.}$ were determined directly by nonlinear least-squares regression analysis of values of ΔF at each auramine O concentration by using eqn. (2b). The magnitudes so determined were very similar to, but not identical with, the corresponding values of K_d and $\Delta F_{\max.}$ determined by linear least-squares analysis of data plotted according to eqn. (2a). In general, the magnitudes of dissociation constants quoted in the text have been determined by the nonlinear least-squares method. In eqn. (2), the unbound auramine O concentration has been approximated by the total auramine O concentration as, $L_T \gg [A]$ where $[A]$ is the total actin monomer concentration. Eqn. (2) has been formulated to describe the binding of a ligand, L, to a single class of independent sites on an acceptor, A. Although commenting on the affinity of the ligand for acceptor, eqn. (2) does not allow, by itself, calculation of the stoichiometry (n) of the interaction. To determine n , experiments were performed in which a small auramine O concentration ($0.52 \mu\text{M}$) was titrated with G-actin. The small change in the emission intensity caused by the light scattering of G-actin by itself was subtracted from the corrected fluorescence intensity at each actin concentration. A double inverse plot of this data (ΔF^{-1} versus $G\text{-actin}^{-1}$) allowed determination of the fluorescence change at infinite actin concentration, which was the change observed when all of the auramine O was bound to G-actin. Combination of this knowledge with the $\Delta F_{\max.}$ determined at infinite auramine O concentration and fixed actin concentration allowed calculation of n .

The initial fluorescence intensities obtained in all of these titrations were the average of four to six readings. The final dilution resulting from the addition of auramine O during the titrations was always less than 7% of the initial volume and is insignificant relative to the experimental error observed in duplicate experiments. There was no effect of CaCl_2 , ATP or MgCl_2 (each 2 mM) on the fluorescence of auramine O in the absence of G-actin.

Results

Effect of G-actin on the absorption and fluorescence spectra of auramine O

The absorption and uncorrected fluorescence (excitation at 370 nm) spectra of auramine O in the presence and absence of G-actin in C-buffer are shown in Fig. 1. The overall absorption spectrum

Fig. 1. Absorption and fluorescence emission spectra of auramine O in the presence and absence of G-actin (a) The absorption spectra of auramine O ($19.5 \mu\text{M}$) in the absence (----) and presence (—) of G-actin ($32.5 \mu\text{M}$) in C-buffer with corresponding reference cuvettes containing C-buffer or C-buffer plus G-actin ($32.5 \mu\text{M}$) respectively, were measured at 25°C in a 1 cm pathlength cell. (b) The uncorrected fluorescence emission spectra (excitation 370 nm) of auramine O ($32.1 \mu\text{M}$) were measured in the absence (----) and presence (—) of G-actin ($8.2 \mu\text{M}$) at 25°C in C-buffer. The excitation and emission monochromator slits were each set at 5 nm. The fluorescence spectra remain uncorrected for variations in detector efficiency with wavelength nor for concentration-dependent inner filter effects.

of auramine O in the presence of G-actin (Fig. 1a) is shifted towards longer wavelengths by 2–3 nm with only small changes in the absolute absorption magnitude at all wavelengths. A similar result has been observed for the binding of auramine O to alcohol dehydrogenase (Conrad *et al.*, 1970). Although auramine O has little fluorescence in C-buffer, its fluorescence intensity is substantially enhanced in the presence of G-actin in the same buffer (Fig. 1b). The wavelength of maximum auramine O fluorescence in the presence of G-actin is 506 nm, which is similar to that found for auramine O by itself (503 nm). The concentrations of auramine O that have been used for the recording of these spectra (Figs. 1a and 1b) are sub-saturating with respect to actin (see Fig. 2) but nevertheless highlight changes in these spectra caused by the presence of actin. The perturbations of the absorption and fluorescence spectra of auramine O caused by the presence of G-actin suggest that there is an interaction between this dye and G-actin. Further, the excitation spectrum (emission at 540 nm) of auramine O alone was similar to its visible absorption spectrum, but an additional peak was observed at 283 nm in the presence of G-actin (result not shown). Thus there is a suggestion of fluorescence energy transfer between tryptophan or tyrosine residues in actin and actin-bound auramine O. This observation is a further indication of an interaction between auramine O and G-actin. Auramine O could be dialysed away from G-actin leaving the protein indistinguishable, in its polymerization properties, from G-actin that had not been exposed to the dye (result not shown).

Fig. 2. Fluorescence titration of G-actin with auramine O

The fluorescence of auramine O (excitation, 370 nm; emission, 540 nm) was measured in the absence (■) and presence (●) of G-actin ($2 \mu\text{M}$) as a function of the concentration of auramine O. The buffer (C-buffer) contained $20 \mu\text{M}$ -CaCl₂ and titrations were performed at 25°C (a) The fluorescence intensity (F_{obs}), uncorrected for inner-filter effects; (b) the fluorescence intensity (F_{corr}) after correction for the inner filter effects made according to eqn. (1) as described in the Experimental procedures section; $a = 0.0086 \pm 0.0003$, $d = 5.38 \pm 0.36$ and $k = 2.84 \pm 0.05$; (c) the change in the corrected fluorescence intensity, $\Delta F = F_{\text{corr}} - F_{\text{corr}}^0$, where F_{corr} and F_{corr}^0 are the corrected fluorescence intensities in the presence and absence of G-actin respectively. The curve has been calculated according to eqn. (2b) with $K_d = 21.4 \pm 2.9 \mu\text{M}$ and $\Delta F_{\text{max}} = 335 \pm 14$, as described in the Experimental Procedures section.

Binding of auramine O to G-actin

The change in the uncorrected fluorescence intensity ($F_{\text{obs.}}$) of auramine O in the presence and absence of G-actin as a function of auramine O concentration is shown in Fig. 2(a). The observed fluorescence, both in the presence and absence of G-actin, is substantially decreased at higher auramine O concentrations due to the relatively high absorbance of these solutions at the excitation wavelength (e.g. $50\text{ }\mu\text{M}$ -auramine O has an absorbance of 0.90 at 370nm). The observed fluorescence intensities were corrected for this self-absorption effect (or inner filter effect) by the general semi-empirical correction procedure of Birdsall *et al.* (1983) as described in the Experimental procedures section. The validity of this correction procedure is shown by the direct proportionality between the corrected fluorescence intensity and the auramine O concentration in the absence of G-actin (Fig. 2b). The change in auramine O fluorescence (ΔF) caused by the presence of G-actin ($\Delta F = F_{\text{corr.}} - F_{\text{corr.}}^0$, where $F_{\text{corr.}}$ and $F_{\text{corr.}}^0$ are the corrected fluorescence intensities in the presence and absence of G-actin

respectively) as a function of auramine O concentration is shown in Fig. 2(c). A nonlinear least squares regression analysis of this data according to eqn. (2b) allowed calculation of the curve of best fit with $K_d = 21.4 \pm 2.9\text{ }\mu\text{M}$ and $\Delta F_{\text{max.}} = 335 \pm 14$. The fit of this curve to the data and the linearity of the corresponding double-inverse plot (not shown) indicates that auramine O binds to a single class of sites on G-actin. Experiments performed at different G-actin concentrations gave the same K_d but a $\Delta F_{\text{max.}}$ (i.e. change in fluorescence at infinite auramine O concentration) which was directly proportional to the actin concentration (results not shown). This result demonstrates that the functional saturation of G-actin by auramine O is independent of the G-actin concentration. This is consistent with the view that actin is not self-associating under these conditions. Fig. 3 shows a double-inverse plot of the change of the corrected fluorescence intensity with G-actin concentration, at a fixed auramine O concentration. The ordinate intercept allows calculation of the fluorescence change associated with the complete binding of auramine O to G-actin. The stoichiometry, n , of the interaction was obtained from a combination of this result with the $\Delta F_{\text{max.}}$ obtained from Fig. 2(c). The calculated value of n , 2.2 ± 0.2 at $20\text{ }\mu\text{M}$ - CaCl_2 , indicated that there are approximately two independent auramine O binding sites on G-actin. However, the relative contribution to the total

Fig. 3. Fluorescence titration of auramine O with G-actin

The fluorescence of auramine O ($0.52\text{ }\mu\text{M}$; excitation, 370 nm; emission, 540 nm) was monitored with increasing concentrations of G-actin and corrected for inner-filter effects and for the small increase of the fluorescence signal due to the light scattering of G-actin (see the Experimental procedures section). Extrapolation of this double-inverse plot to infinite G-actin concentration gave the fluorescence change that occurs when all the auramine O was bound. Other experimental conditions were the same as those described in the caption to Fig. 2.

Fig. 4. Effect of CaCl_2 on the binding of auramine O to G-actin

Double-inverse plots of auramine O fluorescence titrations of G-actin ($2.0\text{ }\mu\text{M}$) at $20\text{ }\mu\text{M}$ (●) and $1200\text{ }\mu\text{M}$ (■)- CaCl_2 . The data have been corrected for inner filter effects as described in the Experimental procedures section and plotted according to eqn. (2a). Other experimental conditions were the same as those described in the caption to Fig. 2. The lines that have been drawn are based on linear least-squares regression analyses of the data.

fluorescence intensity by each bound auramine O molecule is unknown.

Effect of CaCl_2 on the binding of auramine O to G-actin

The effect of CaCl_2 on the interaction between G-actin and auramine O is shown in the double-inverse plots of Fig. 4. These linear plots intercept the abscissa at a similar value, indicating that there is no change in the dissociation constant for the G-actin–auramine O complex in the presence of various concentrations of CaCl_2 (see also Table 1). However, ΔF_{max} increased with the increasing CaCl_2 concentration. Corresponding values of n were determined, as described previously, and were also found to be independent of the CaCl_2 concentration (Table 1).

Fig. 5 shows the saturable CaCl_2 concentration dependence of ΔF_{max} , while the inset shows a double-inverse plot of the change of ΔF_{max} with CaCl_2 concentration. Higher concentrations of

CaCl_2 ($> 4\text{ mM}$) could not be used because of the likelihood of actin polymer formation under these conditions. The linearity of this double-inverse plot demonstrates that there is a single class of

Table 1. *Binding parameters for the interaction between auramine O and G-actin*

Values of n (stoichiometry), K_d (dissociation constant) and ΔF_{max} (maximum change in the corrected fluorescence at infinite auramine O concentration) for the G-actin–auramine O interaction were determined at four CaCl_2 concentrations. Values of ΔF at each auramine O concentration were fitted to eqn. (2b) by a nonweighted nonlinear least-squares regression analysis.

$[\text{CaCl}_2]$ (mM)	n	K_d (μM)	ΔF_{max} (relative units)
0.02	2.2 ± 0.2	21.4 ± 2.9	335 ± 14
0.61	2.0 ± 0.4	24.2 ± 3.3	458 ± 21
1.20	2.1 ± 0.4	23.7 ± 1.9	504 ± 28
3.37	2.3 ± 0.5	19.3 ± 3.1	576 ± 33

Fig. 5. *Binding of Ca^{2+} to the G-actin–auramine O complex*

Values of ΔF_{max} , obtained over a range of CaCl_2 concentrations, were determined from experiments such as those described in Fig. 2. The inset shows a double-inverse plot of these data after subtraction from ΔF_{max} at each CaCl_2 concentration of the extrapolated value of ΔF_{max} at zero CaCl_2 concentration. The line drawn through these data is based on a linear least-squares regression analysis.

Ca^{2+} binding site(s) with a dissociation constant of $1.6 \pm 0.4 \text{ mM}$ on the G-actin-(auramine O)₂ complex. The data make no comment on the number of bound Ca^{2+} ion(s). Similar experiments were also performed with MgCl_2 and KCl (each in the presence of $20 \mu\text{M}$ - CaCl_2 which helps prevent the irreversible formation of non-polymerizable actin monomer) with qualitatively similar results to those for CaCl_2 , i.e. these cations (Mg^{2+} and K^+) enhanced the fluorescence of actin-bound auramine O in a saturable manner and yielded dissociation constants of $580 \mu\text{M}$ for Mg^{2+} and 40 mM for K^+ . Interpretation of those data (Mg^{2+} and K^+) is rendered difficult as a consequence of evidence which demonstrates an Mg^{2+} -induced conformational change of actin monomer accompanying the exchange of Mg^{2+} for Ca^{2+} at the 'high-affinity' bivalent cation binding site, and a change in the u.v. absorption spectrum of G-actin in the presence of KCl (Frieden, 1982; Pardee & Spudich, 1982b). Thus, actin monomer, in the presence of Mg^{2+} and Ca^{2+} or K^+ and Ca^{2+} , probably exists in more than one conformational state. In contrast, actin monomer pre-equilibrated with $20 \mu\text{M}$ - Ca^{2+} shows no evidence of conformational changes (i.e. changes in the u.v. or intrinsic fluorescence spectra) concomitant with the addition of higher concentrations of Ca^{2+} unless polymerization occurs (results not shown).

Discussion

Several pieces of evidence indicate that auramine O binds to G-actin; first, the fluorescence of auramine O is enhanced in the presence of G-actin; second, this fluorescence increase, after correction for auramine O self-absorption, is saturable; third, auramine O inhibits actin polymerization (result not shown); and fourth, the fluorescence of auramine O in the presence of G-actin is further enhanced by Ca^{2+} ions. The nature of the interaction between G-actin and auramine O is not clear. Oster & Nishijima (1964) have concluded that the cationic property of auramine O is important in its binding to DNA as another cation, streptomycin, decreases the DNA-enhanced auramine O fluorescence. However, that study did not demonstrate whether the effect of streptomycin was caused by competitive displacement of DNA-bound auramine O or a result of quenching of the bound auramine O fluorescence. The lack of an effect by CaCl_2 on n or K_d for the G-actin-(auramine O)₂ complex indicates that Ca^{2+} ions and auramine O do not compete for sites on G-actin. Although the fluorescence of auramine O is markedly enhanced in the presence of G-actin, the wavelength of maximum fluorescence (λ_{max}) is red-shifted by only 3 nm. The introduction of

auramine O into a nonpolar environment such as butan-1-ol enhances the fluorescence intensity but decreases λ_{max} by about 20 nm (Chen, 1977). In contrast, highly viscous solutions such as 83% glycerol increase λ_{max} by about 7 nm and also substantially enhance the fluorescence intensity compared with water (Chen, 1977). This effect of viscosity is similar to the change in the fluorescence properties of auramine O in the presence of G-actin and has been attributed to the loss of intramolecular quenching modes caused by the restriction of internal rotational motion of this dye (Oster & Nishijima, 1956). Conrad *et al.* (1970) reported that auramine O bound to only one of 17 proteins tested (i.e. liver alcohol dehydrogenase), thereby implying a uniqueness of that interaction. That study also inferred that auramine O by itself was non-fluorescent. However, other studies are able to discern a visible fluorescence of auramine O alone (Chen, 1977; Nishimura, 1971; Fig. 1 of the present paper) and binding of auramine O to serum albumin (Chen, 1977) and now also to G-actin. In view of this it is likely that auramine O may be a useful fluorescent probe of the structure and function of other proteins.

The reason for the increase of fluorescence intensity of G-actin-bound auramine O in the presence of CaCl_2 is not clear. Since this additional fluorescence enhancement must reflect some form of change in the environment surrounding the auramine O molecules, then a corresponding change in K_d or n for the auramine O-G-actin interaction might be expected. The absence of such changes may not be unusual, e.g. the polymerization properties of actin labelled with *N*-(1-pyrene)iodoacetamide are unaffected by that covalent chemical modification even though polymerization is associated with a 26-fold increase of the fluorescence of the attached pyrene group (Tellam & Frieden, 1982). Alternatively, changes in K_d or n for auramine O binding may not be apparent at the relatively low CaCl_2 concentrations used in these experiments, which are necessary to maintain actin in the monomeric state.

The dissociation constant ($1.6 \pm 0.4 \text{ mM}$) for the binding of Ca^{2+} ion(s) to the G-actin-(auramine O)₂ complex is of similar order of magnitude to that describing the binding of various bivalent cations to the multiple 'low-affinity' metal ion binding sites on actin which, when occupied by these metal ions, are thought to be involved in actin polymerization (Martonosi *et al.*, 1964; Strzelecka-Golaszewska *et al.*, 1978a,b; Loscalzo & Reed, 1976). However, those studies have not clearly defined the binding affinity of Ca^{2+} or Mg^{2+} , the physiologically important bivalent cations, to these sites. Indeed, the equilibrium dialysis methods that have been used in some of

these studies are complicated by (a) charge effects at the low ionic strength used in those experiments, (b) the potential for substantial G-actin denaturation during the long periods of incubation (24–48 h) in the absence of ATP (Oosawa & Kasai, 1971) and (c) the possibility of having mixed populations of actin monomer and polymer with differing affinities for metal ions. Thus, the exact relationship between the Ca^{2+} -binding site(s) defined in the present investigation and the 'low-affinity' metal ion binding sites on G-actin is unclear. Indeed, the relationship between these 'low-affinity' sites and the metal ion dependence of the kinetics of actin polymerization has received little attention and requires further investigation.

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