MYOPLASMIC BINDING OF FURA-2 INVESTIGATED BY STEADY-STATE FLUORESCENCE AND ABSORBANCE MEASUREMENTS

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ABSTRACT Binding of the fluorescent Ca^{2+} indicator dye fura-2 by intracellular constituents has been investigated by steady-state optical measurements. Fura-2's (*a*) fluorescence intensity, (*b*) fluorescence emission anisotropy, (*c*) fluorescence emission spectrum, and (*d*) absorbance spectra were measured in glass capillary tubes containing solutions of purified myoplasmic proteins; properties *b* and *c* were also measured in frog skeletal muscle fibers microinjected with fura-2. The results indicate that more than half, and possibly as much as 85%, of fura-2 molecules in myoplasm are in a protein-bound form, and that the binding changes many properties of the dye. For example, in vitro characterization of the Ca^{2+}-dye reaction indicates that when fura-2 is bound to aldolase (a large and abundant myoplasmic protein), the dissociation constant of the dye for Ca^{2+} is three- to fourfold larger than that measured in the absence of protein. The problems raised by intracellular binding of fura-2 to cytoplasmic proteins may well apply to cells other than skeletal muscle fibers.

INTRODUCTION

The fluorescent Ca^{2+} indicator dye fura-2 (Grynkiewicz et al., 1985) has been widely used to measure the intracellular free calcium ion concentration, $[Ca^{2+}]$, in various cell types. $[Ca^{2+}]$ estimated from the fura-2 fluorescence signal usually depends on the assumption that the dye behaves in the same way in the intracellular environment as in calibrating salt solutions. This assumption may not, however, be correct. For example, evidence from intact and cut muscle fibers suggests that many Ca^{2+} indicator dyes, including arsenazo III, antipyrylazo III, dichlorophosphonazo III, azo-1, and tetramethylmurexide (Baylor et al., 1982, 1986; Maylie et al., 1987*a*, *b*, *c*) are bound within the myoplasm and that this binding can alter both spectral and kinetic properties of the dyes. Dye binding can also be demonstrated in vitro; for example, there are reports in the literature that several dyes, including fluorescein (Laurence, 1952), phenol red (Rodkey, 1961), and arsenazo III (Beeler et al., 1980), bind to soluble proteins and as a result change their absorbance spectra and/or fluorescence properties.

Recent evidence suggests that fura-2, like other dyes, may also bind to intracellular constituents. Fura-2 diffuses in the myoplasm of frog skeletal muscle fibers with a diffusion constant about threefold smaller than expected on the basis of its molecular weight (Baylor and Hollingworth, 1988). This slow diffusion suggests that a majority of the fura-2 molecules are bound to relatively immobile myoplasmic constituents (cf. Maylie et al., 1987*a*, *b*, *c*), possibly soluble proteins (cf. Beeler et al., 1980). To further investigate fura-2 binding in muscle, we have measured and compared a number of fura-2 properties both in myoplasm (in vivo) and under various in vitro conditions. For the in vivo measurements, single frog skeletal muscle fibers were pressure-injected with fura-2. In vitro fura-2 measurements were carried out in salt solutions at different viscosities, both in the absence and presence of the three most abundant (wt/vol) soluble myoplasmic proteins: aldolase, glyceraldehyde phosphate dehydrogenase, and creatine kinase (Ottaway and Mowbray, 1977). The in vitro properties measured included fura-2's dissociation constant (K_D) for Ca^{2+}, its absorbance spectrum, its fluorescence intensity, its fluorescence emission spectrum, and its fluorescence emission anisotropy.

The fluorescence emission anisotropy of a fluorophor provides a particularly useful way to establish whether the molecule is bound (Laurence, 1952). This parameter is related to the rotational mobility of the fluorophor. If the fluorophor is bound to large and relatively immobile proteins, its rotational mobility is expected to decrease and, consequently, its anisotropy is expected to increase. The anisotropy measurements indicate that a majority of fura-2 is bound in myoplasm. This method of estimating intracellular binding of a fluorescent indicator dye may be readily adapted to other preparations.

A preliminary report of this study has been presented (Olson et al., 1988).

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METHODS

In Vitro Measurements

In vitro measurements were made at 20°C in quartz capillary tubes filled with fura-2 solution, sealed by Vaseline at each end, and placed on an optical bench apparatus in the same position as were the muscle fibers used for the in vivo measurements (cf. Baylor and Hollingworth, 1988). Capillary internal diameter, measured on the apparatus, was 217 μm ($\pm$3.1 μm SD; n = 42). Fluorescence measurements were usually made from the 300-μm diam field of view of the collecting objective (Leitz No. 1569109; 32×; N. A. 0.60), but sometimes a rectangular mask (200 × 75 μm) was placed to restrict the field of illumination. There were no significant differences in results obtained with the two types of illumination. In all experiments incident light from a tungsten-halogen lamp was passed through an interference filter (Omega Optical, Inc., Brattleboro, VT) of 420-nm peak transmittance and a 30-nm half-band width in order to excite the dye to fluoresce. For measurements of fluorescence intensity, a secondary filter (495-nm cut-on filter; Schott Glass Technologies Inc., Duryea, PA) was placed between the collecting lens and the photodiode to block the excitation wavelength and permit collection of fluorescent light of wavelengths longer than 495 nm. For the emission spectrum measurements, the secondary filter was replaced with one of a series of interference filters (Omega Optical Inc.) having a 10-nm half-band pass and a peak transmittance which ranged from 480 to 670 nm in 10-nm increments. In the case of all measurements the fura-2–related fluorescence intensity was obtained by subtraction of the non–fura-2 component evaluated by making analogous measurements from capillaries filled with identical solutions without dye. The non–fura-2 component, which was attributable to an overlap in band-pass between the primary and secondary filters, was generally <10% of the fura-2 component.

For the measurements with muscle proteins, correction for protein absorbance was made for both excitation and emission wavelengths by means of Eq. 5′ of Baylor et al. (1981). In general, these corrections resulted in small changes to the raw data. For example, total fluorescence intensity was corrected by 3.1% in a 79 mg/ml protein mixture (aldolase + creatine kinase + glyceraldehyde-3-phosphate dehydrogenase) and by 2.2% in a 55 mg/ml solution containing aldolase alone. The emission spectrum was also corrected at each wavelength, for example, 3.3% at 480 nm, 2.8% at 580 nm, and 2.6% at 670 nm in 79 mg/ml proteins. No correction was made, however, for either the spectral characteristics of the light source and photodetector or the transmittance properties of the secondary filters. Thus, some details of the results, for example, the exact shape of the fluorescence emission spectra (cf. Fig. 7), are apparatus-dependent.

The 420-nm excitation wavelength was used throughout the study primarily because the light intensity from the tungsten-halogen bulb in use on the apparatus decreased steeply at wavelengths below 400 nm, where the measurements therefore became unreliable. There were, however, some advantages in using a 420-nm excitation beam. First, photobleaching of the dye (cf. Becker and Fay, 1987) was negligible; only a 5% decrease of fluorescence intensity could be observed even after 30 min of continuous illumination of a capillary filled with 0.5 mM fura-2 solution. Second, no autofluorescence of muscle fibers (Baylor and Hollingworth, 1988) or protein solutions was detected with 420-nm illumination; this permitted a more straightforward assessment of dye properties in vivo and in vitro. Third, at 420 nm, absorbance of the Ca^{2+}–fura-2 complex is only 1–2% of Ca^{2+}-free fura-2 (cf. Fig. 9 *A*), and correspondingly, the fluorescence from the Ca^{2+}–fura-2 complex is negligible (1–2% of the fluorescence of Ca^{2+}-free fura-2; cf. Fig. 8 *A*). Thus, the fluorescence measurements reported in this paper give direct information about the Ca^{2+}-free form of fura-2.

Fluorescence Emission Anisotropy Measurements

Anisotropy measurements were made by placing in the light path two linear polarizers which passed light polarized either parallel (0°) or perpendicular (90°) to the long axis of the capillaries. The 420-nm excitation light passed through the first polarizer, oriented at either 0° (cf. Fig. 1) or 90°, before illuminating the sample. The fluorescent light was detected after passing through the second polarizer, alternately oriented parallel and perpendicular to the first polarizer.

Anisotropy ($\underline{A}$) is defined by the equation (cf. Cantor and Schimmel, 1980):

$$\underline{A} = \frac{F_{\parallel} - F_{\perp}}{F_{\parallel} + 2F_{\perp}}, \qquad (1)$$

where $F_{\parallel}$ and $F_{\perp}$ are the dye-related fluorescence intensities measured with the second polarizer oriented parallel and perpendicular, respectively, to the first polarizer.

The value of $\underline{A}$ was measured in all experiments using both 0° and 90° incident light. If the dye molecules are randomly oriented within the sample, the value of $\underline{A}$ measured using either orientation of the first polarizer (0° or 90°) should be the same (as was, in fact, observed). In general, the value of $\underline{A}$ must lie between zero (if the molecular rotation of the dye is extremely fast compared with the dye's fluorescence lifetime) and the limiting anisotropy, $\underline{A}_0$ (if the dye's rotation is extremely slow compared with its fluorescence lifetime). Depending on the angle α between the absorption dipole and the emission dipole of the molecule, $\underline{A}_0$ can theoretically take a value between -0.2 (α = 90°) and $+0.4$ (α = 0°). In general, α is a function of the excitation wavelength (Chen and Bowman, 1965).

To evaluate the accuracy of the apparatus used in this study, $\underline{A}$ of rhodamine B (5 μM) was measured in a solution containing 95% glycerol and 5% water (10°C). An interference filter of 540-nm peak transmittance (30-nm half-band width) was used to set the excitation wavelength, and a 590- or 610-nm cut-on filter was used as a secondary filter, to permit collection of fluorescent light of wavelengths longer than 590 or 610 nm, respectively. The $\underline{A}$ value obtained was 0.366 ($\pm$0.003 SEM; n = 4), which was in good agreement with the published value of 0.364 obtained under the same conditions (Chen and Bowman, 1965).

Fluorescence Intensity Measurements

Fluorescence intensities reported in this study were obtained in one of two ways. "Unpolarized" fluorescence, denoted F, was measured in the absence of polarizers, a procedure that gave the largest values of sample fluorescence and therefore signal-to-noise ratio. A second measure, denoted $\underline{F}$ and referred to as "total" fluorescence (cf. Cantor and Schimmel, 1980), was obtained in the presence of the first and second polarizers (cf. Fig. 1) and calculated as $F_{\parallel} + 2F_{\perp}$. For this purpose, the separate values of total fluorescence intensity obtained with 0° and 90° polarized excitation light were averaged. Total fluorescence (rather than unpolarized fluorescence) is required in the equations derived in the Appendix and was used in connection with the data and analysis in Fig. 8 *B*. Unless specified otherwise, fluorescence intensity as used in the remainder of the text refers to unpolarized fluorescence intensity.

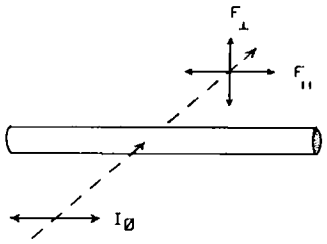


FIGURE 1 Schematic drawing of the experimental method for measuring fluorescence emission anisotropy. Samples (muscle fibers or glass capillaries) were illuminated by a 420-nm excitation light beam (dotted line in front of the sample) which passed through a 0° (I_0) or 90° (I_{90}) linear polarizer. Fluorescent light of wavelengths longer than 495 nm (dotted line in back of sample) was collected and measured after passing through a second polarizer oriented parallel ($F_{\parallel}$) or perpendicular ($F_{\perp}$) to the first polarizer.

Absorbance Spectral Measurements

In vitro dye absorbance spectra were measured as previously described (Hollingworth et al., 1987) in a UV/visible spectrophotometer (model 4050; LKB Instruments, Inc., Gaithersburg, MD).

Chemicals

KCl, $MgCl_2$, PIPES, sucrose, glycerol, and KOH were all reagent grade. Fura-2 (penta-potassium salt) was purchased from Molecular Probes Inc. (Eugene, OR; Lot Nos. 7D and 8A) and used under the assumption that each vial labeled "1 mg" contained exactly 1 mg of fura-2. There may, however, be some variation in content between vials and lots. For example, measurement of dye concentration by means of absorbance at 362 nm (cf. Baylor and Hollingworth, 1988) on five different occasions gave values that were 77% (±10% SD) of the nominal value. For this reason only fura-2 from the same vial was used within any given experiment.

EGTA (Sigma Chemical Co., St. Louis, MO; Lot No. 126F-5618) was used for controlling free Ca^{2+} concentration. A concentrated stock solution of EGTA (400 mM, pH adjusted to 7.0 by KOH) was made under the assumption that the EGTA purity was 97%, as indicated by Sigma Chemical Co. The stock solution of $CaCl_2$ (100 mM) was made up from a certified 1 M $CaCl_2$ solution (BDH Chemicals Ltd., Poole, England). The same stock solutions of $CaCl_2$ and EGTA were used throughout the study.

Proteins

Aldolase (ALD; 160,000 mol wt; Lot Nos. 37F-9545, 77F-9565, 86F-9545), creatine kinase (CK; 82,600 mol wt; Lot No. 57F-9605), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 140,000 mol wt; Lot No. 46F-9725) were obtained from Sigma Chemical Co. and were dialyzed against a large volume of 10 mM PIPES solution (pH adjusted to 7.0 by KOH) to remove Sigma's buffer salts (primarily Na_3citrate). Dialysis of proteins was performed for 20–24 h at 4°C using a cellulose dialysis tubing (Sigma Chemical Co.) which retained proteins with molecular weight greater than 12,000. Protein concentration of the dialyzed solution was measured with a Coomassie Blue G-250 reagent solution (Pierce Chemical Co., Rockford, IL) and BSA as a standard. The loss of protein concentration during dialysis was 0–7%. Dialyzed protein was stored frozen at −20°C and was used within 3 d.

Solutions

The composition of the standard buffer solution was (in mM): 110 KCl, 10 EGTA, 10 PIPES (pH adjusted to 7.0 by KOH). Calculated ionic strengths of the solutions were 0.16 M (without fura-2), 0.17 M (with 0.5–1.0 mM fura-2), and 0.19 M (with 2.0 mM fura-2). These values are close to the range of 0.14–0.18 M estimated for the ionic strength of the myoplasmic solution of frog muscle (cf. Godt and Maughan, 1988). In some in vitro experiments, ionic strength was changed in the range 0.06–0.3 M, simply by changing KCl concentration.

Ca^{2+}-EGTA Solutions

Solutions with variable free Ca^{2+} concentrations ($[Ca^{2+}]$) were set by Ca^{2+}-EGTA solutions, whose composition was basically the same as the standard buffer solutions, except for minor modifications. The composition of these solutions was (in mM): 73–90 KCl, 10 EGTA, 10 PIPES, 0.5 fura-2, 1.5–2.0 $MgCl_2$. Ionic strength was set to 0.15 M by adjustment of the KCl concentration. Free Ca^{2+} concentration was calculated under the assumption that the apparent dissociation constant for the Ca^{2+}-EGTA reaction at pH 7.0 was 393 nM (cf. Martell and Smith, 1974). Since a relatively high fura-2 concentration, 0.5 mM, was used, the Ca^{2+} bound by fura-2 was taken into account in the calculation of $[Ca^{2+}]$; for this calculation an apparent dissociation constant of 135 nM was initially assumed (Grynkiewicz et al., 1985). Free Mg^{2+} concentration ($[Mg^{2+}]$) was set to 1.5 mM, under the assumption that the apparent dissociation constant for the Mg^{2+}-EGTA reaction was 34.8 mM (Martell and Smith, 1974) and that of the Mg^{2+}–fura-2 reaction was 9.8 mM (Grynkiewicz et al., 1985).

Solutions of the required free $[Ca^{2+}]$'s were prepared by mixing a buffer solution containing both 10.57 mM $CaCl_2$ and 10 mM EGTA (Ca^{2+}-EGTA solution) and a solution containing only 10 mM EGTA (EGTA solution) in the appropriate proportion (cf. Horiuti, 1988).

Since large pH changes may cause denaturation of proteins, the pH of the Ca^{2+}-EGTA and EGTA solutions was roughly adjusted to 6.8–6.9 by KOH before addition of proteins and fura-2. Final adjustment to pH 7.00 ± 0.01 was made using a pH electrode and meter (model PHM 84; Radiometer A/S, Copenhagen, Denmark) calibrated with a pH standard (7.00 ± 0.01, S1326, Radiometer A/S). The fluorescence intensity of 0.5 mM fura-2 was measured in solutions of various $[Ca^{2+}]$, and results were least-squares fitted to theoretical curves of Ca^{2+}-dye binding with 1:1 stoichiometry to give the best-fitted K_D ($K_{D,obs}$) satisfying

$$K_{D,obs} = \frac{F - F_{min}}{F_{max} - F}[Ca^{2+}]. \quad (2)$$

In Eq. 2 F_{max}, F_{min}, and F are fluorescence intensities measured in 0 $[Ca^{2+}]$ (EGTA solution), saturating $[Ca^{2+}]$, and in an intermediate $[Ca^{2+}]$, respectively. Because the Ca^{2+}-free and Ca^{2+}-bound forms of fura-2 have different values of absorbance at 420-nm excitation, the observed fluorescence intensities theoretically require a small correction in order for Eq. 2 to strictly apply. It may be calculated, however (see Baylor et al., 1981, Eq. 5), that the largest error in the vertical positioning of the points in Fig. 8 *A* is 1% of the maximum fluorescence. This error was therefore ignored in the K_D fittings in Fig. 8 *A*. The value of $K_{D,obs}$ thus obtained could be expressed in terms of an apparent dissociation constant for Ca^{2+} in the absence of Mg^{2+} ($K_{D,Ca}$) and for Mg^{2+} in the absence of Ca^{2+} ($K_{D,Mg}$), according to

$$K_{D,obs} = K_{D,Ca}(1 + [Mg^{2+}]/K_{D,Mg}). \quad (3)$$

By means of Eq. 3, $K_{D,Ca}$ could be calculated from a given value of $K_{D,Mg}$ value (see Results) and a given $[Mg^{2+}]$ (= 1.5 mM). Since $K_{D,Ca}$ of fura-2 in various conditions was unknown, it was initially assumed to be 135 nM (Grynkiewicz et al., 1985) in order to estimate free $[Ca^{2+}]$'s. When the best-fitted value of $K_{D,obs}$, and thus $K_{D,Ca}$, was obtained by least-squares fitting to the experimental results, $[Ca^{2+}]$ of each solution was recalculated by means of the new value of $K_{D,Ca}$, and the best-fitted $K_{D,obs}$ was again obtained. This procedure for correction of the estimate of $K_{D,Ca}$ of fura-2 was repeated until the change in best-fitted $K_{D,obs}$ in each step become small (<0.01 pCa unit). In the presence of 55 mg/ml aldolase ($K_{D,Ca}$ approximately 600 nM), the corrected value of $K_{D,Ca}$ obtained by this procedure was 7% higher than the initial estimate.

Viscosity

The viscosity of the various protein solutions was measured at 20°C using an Ostwald-type viscometer (Cannon Instrument Co., State College, PA) calibrated by distilled water. The precision of the viscometer was checked at 20°C using standard sucrose or glycerol solutions (Weast and Astle, 1978) and the maximum error was estimated to be 0.1 centipoise (cP) in the viscosity range of 1.0–3.2 cP.

The suggested theoretical equation for the viscosity of a macromolecular solution is (Höber et al., 1945):

$$\frac{(\eta - \eta_o)}{\eta_o} = kVC, \quad (4)$$

where η and η_0 are, respectively, viscosities of solution and solvent (1.0 cP for water), V is partial specific volume of the macromolecule, C is macromolecular concentration (mg/ml), and k is a function of molecular shape. Eq. 4 indicates that solution viscosity is a linear function of protein concentration, if k and V are constant. Although k and V probably differ with each protein, measured viscosity values of several protein solutions

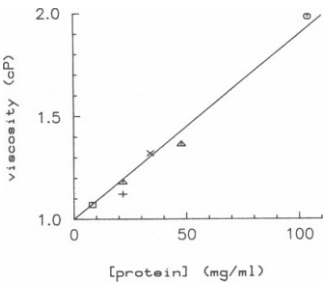


FIGURE 2 Viscosity of protein-containing solutions measured in vitro (20°C). Proteins were dissolved in the standard buffer solution: , parvalbumin; +, GAPDH; X, CK; △, ALD; ○, mixture of ALD (48 mg/ml) + CK (34 mg/ml) +GAPDH (22 mg/ml). Solid line is the least-squares fit of a straight line to the experimental data.

were reasonably well fitted by a linear relation with slope (kV) of 0.00896 (cP · ml · mg^{-1}) (Fig. 2). Therefore, the viscosity η (cP) of the other protein solutions in this study was estimated using this best-fitted line:

$$\eta = 0.00896\ C + 1.0. \quad (5)$$

For example, the viscosity of a 79 mg/ml protein (ALD + CK + GAPDH) solution is expected to be 1.71 cP. The anisotropy and fluorescence intensity of fura-2 measured in this solution (cf. Fig. 4) were therefore compared with those obtained in a solution without proteins containing 540 mM sucrose (viscosity 1.74 cP) in order to keep constant any effects due to viscosity per se. At intermediate protein concentrations, the sucrose concentration was linearly reduced in order to maintain viscosity constant (to within ±0.05 cP).

In Vivo Measurements

Single twitch muscle fibers dissected from semitendinosus or iliofibularis muscles of *Rana temporaria* were mounted on the optical bench apparatus and were stretched to long sarcomere length (3.5–4.2 μm). The Ringer's solution bathing the fibers contained (in mM): 120 NaCl, 2.5 KCl, 11.8 $CaCl_2$, and 5 PIPES (pH adjusted to 7.1 by NaOH) at a temperature of 20°C. Membrane impermeant fura-2 (penta-potassium salt) was pressure injected into myoplasm from a dye-filled micropipette. Use of the fura-2 "free acid" avoids the complications caused by partial hydrolysis of the ester (Malgaroli et al., 1987) and probably also avoids sequestration of the dye into nonmyoplasmic compartments (Baylor and Hollingworth, 1988; cf. Almers and Neher, 1985). After dye injection, a narrow length of fiber (90–300 μm) was illuminated with 420-nm light for measurement of dye fluorescence. The dye-related fluorescence intensity was obtained by subtraction of the nonfluorescent component, measured after removal of the fiber from the light path. Since fura-2 diffuses slowly away from the injection site, it was possible to make measurements of the dye's fluorescence emission anisotropy in resting fibers as a function of dye concentration simply by making fluorescence measurements in the presence of polarizers at different distances from the site of dye injection. Myoplasmic fura-2 concentration in resting muscle fibers was estimated as described in Baylor and Hollingworth (1988).

It was also of interest to measure the anisotropy of the fura-2 that reacted with Ca^{2+} in response to a single action potential. For these measurements, changes in dye fluorescence as a function of time t, $\Delta F(t)$, were measured in the presence of the first and second polarizers. $\Delta F_\parallel(t)$ and $\Delta F_\perp(t)$ (the components of $\Delta F(t)$ measured with the second polarizer oriented parallel and perpendicular, respectively, to the first polarizer) were measured at both 0° and 90° orientations of the first polarizer. In both cases (cf. Fig. 10) the anisotropy of the reacting dye was calculated as $(\Delta F_\parallel - \Delta F_\perp)/(\Delta F_\parallel + 2\Delta F_\perp)$ and is referred to as $\Delta\underline{A}$. Again, it should be noted that because 420-nm excitation light was used, the ΔF signals were in all cases negative, representing the disappearance of Ca^{2+}-free fura-2 during activity. No correction for nonfluorescence components was required in the ΔF measurements, as analogous changes measured in the absence of dye were negligible.

RESULTS

Anisotropy Values In Vivo and In Vitro

The in vivo anisotropy ($\underline{A}$) of fura-2 injected into single muscle fibers was 0.272 (±0.003, SEM; n = 10 from four fibers), a value markedly higher than $\underline{A}$ of 0.109 (±0.001, SEM; n = 7) measured in a salt solution of 1.0 cP viscosity in capillary tubes (in vitro). As will be seen below, this large difference indicates that fura-2 in the myoplasmic environment has a significantly reduced rotational mobility as compared with that in a simple salt solution.

The $\underline{A}$ values obtained both in vivo and in vitro were independent of the polarization of the excitation beam (0° or 90°), as expected if the fura-2 molecules were randomly oriented in myoplasm and in capillaries. For example, in the in vivo measurements with 0° excitation light, $\underline{A}$ was 0.275 (±0.005, SEM) compared with the value 0.269 (±0.006, SEM) obtained using 90° light. This finding is consistent with the observations of Baylor and Hollingworth (1988) who found no evidence for dichroism of the fura-2 absorbance change in skeletal muscle fibers in response to electrical stimulation. (Baylor and Hollingworth were not able to assess the possibility of dichroism of fura-2 in the resting state because of interference from the relatively larger fiber intrinsic absorbance at 420 nm.) It therefore seems likely that the large fraction (0.60–0.65) of bound fura-2 estimated to exist in myoplasm (Baylor and Hollingworth, 1988) involves binding of dye to soluble proteins rather than to oriented structures such as the myofilaments or the sarcoplasmic reticulum.

In all in vitro measurements, the value of $\underline{A}$ obtained with 0° polarized light was essentially identical to that obtained with 90° polarized light. The in vitro values of $\underline{A}$ given in the remainder of Results were obtained as the average of these two polarized values.

Viscosity Effects on Anisotropy

The significant increase in $\underline{A}$ in myoplasm compared with a 1.0 cP salt solution might be explained simply as an effect of myoplasmic viscosity, since an increased viscosity should decrease the rotational mobility of the dye molecules (and therefore increase anisotropy). To investigate the contribution of viscosity, $\underline{A}$ of fura-2 was measured in vitro, while the viscosity of the solution was changed by addition of either sucrose or glycerol (Fig. 3 *A*). Although there was some difference in $\underline{A}$ as a function of viscosity when set with sucrose compared with glycerol, the results indicate that a value of ~6–8 cP corresponds to an $\underline{A}$ of 0.27, the value observed for fura-2 in vivo. Although the precise value of myoplasmic viscosity is uncertain, its upper limit predicted from the work of Kushmerick and Podolsky (1969) and Maylie et al. (1987*a*,*b*) is 2–2.5 cP, based on measurements of the myoplasmic diffusivities of several substances of molecular weight less than 800. Therefore,

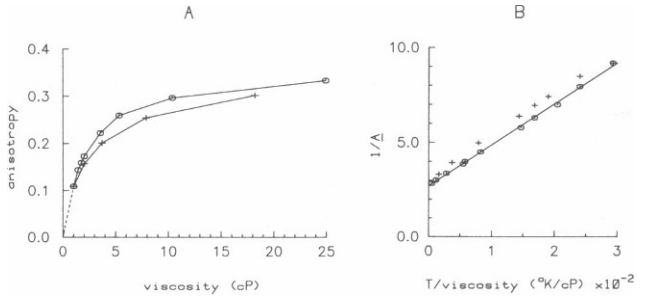


FIGURE 3 (*A*) Fluorescence anisotropy of fura-2 (ordinate) measured in vitro as a function of viscosity (abscissa) (20°C). Appropriate amounts of sucrose (O) or glycerol (+) were added to the standard buffer solution to achieve the indicated viscosity (in cP). Fura-2 concentration was usually 0.5 mM, but for a few points, higher concentrations (up to 2.0 mM) were used. Anisotropy was extrapolated to 0 viscosity, where an anisotropy value of 0 is expected (*dotted line*). Excitation wavelength was 420 ± 15 nm. (*B*) Perrin plots of experimental data shown in *A* plus a few additional points which, for clarity, have not been shown in *A*. Abscissa shows absolute temperature (constant at 293°K) divided by viscosity. Ordinate is the inverse of anisotropy (O for sucrose, + for glycerol). Solid line is the best fit of the sucrose data (O) by linear least-squares fitting (slope = 2.154×10^{-2} cP·°K^{-1}; y-intercept = 2.717).

myoplasmic viscosity alone cannot totally explain the large value of $\underline{A}$ observed in vivo.

Fig. 3 *B* shows Perrin plots of $\underline{A}$ (cf. Cantor and Schimmel, 1980) obtained in sucrose and glycerol solutions at constant temperature (20°C = 293°K). If the solvent has no effect on fura-2 molecules other than through viscosity, a linear relationship should result. The data in Fig. 3 *B* obtained with sucrose are better fitted by a straight line than those obtained with glycerol. Deviation from linearity in the latter solution might be due to glycerol's having some solvent effect on the dye. Hence, in the remaining experiments the required viscosities were set using sucrose.

The intercept at T/viscosity = 0 of the least-squares fitted line to the sucrose data (Fig. 3 *B*) gave a limiting anisotropy ($\underline{A}_0$) of 0.37. This value indicates that there is a small angle, ~13°, between the absorption and emission dipoles of fura-2 at 420-nm excitation (see, e.g., Cantor and Schimmel, 1980). Thus, with 420-nm excitation, $\underline{A}$ of fura-2 varies between 0 and 0.37, depending on the dye's molecular mobility and fluorescence lifetime.

Fluorescence intensity (not shown) was also found to increase with increasing viscosity, set either by sucrose or glycerol additions, as reported by Poenie et al. (1986). Compared with measurements in a 1-cP solution, fluorescence intensity increased by 27% and 21% in a 2.0-cP solution set by sucrose and glycerol, respectively, and increased by 60% in a 6.2-cP solution set by sucrose.

Anisotropy and Fluorescence Intensity in the Presence of Proteins

Since the observed $\underline{A}$ value for fura-2 in myoplasm could not be explained solely by viscosity, we then explored the possibility that the binding of dye to molecules of large molecular weight might contribute to the elevated value of $\underline{A}$. As mentioned above, it is more likely that fura-2 binds to soluble myoplasmic constituents than to oriented structural components. Thus, in vitro measurements of $\underline{A}$ and fluorescence intensity of fura-2 were made in the presence of soluble proteins.

Since little information is available concerning the quantities of soluble proteins in frog myoplasm, three soluble proteins (ALD, CK, and GAPDH; see Methods), which are reported to be present in relatively large amounts (weight per unit volume) in the myoplasm of rabbit white muscle (cf. Ottaway and Mowbray, 1977), were chosen to construct a model solution to test for fura-2 binding to myoplasmic proteins. According to Ottaway and Mowbray (1977), rabbit white muscle contains (in mg/g muscle) 32 ALD, 23 CK, 14.6 GAPDH, and 55 total soluble proteins. Since the sum of the three concentrations exceeds the total amount of soluble proteins reported, it follows that some fraction of these proteins is not soluble (Ottaway and Mowbray, 1977; Arnold and Pette, 1968, 1970). Although there is uncertainty concerning the nonsoluble fraction, the ratio of the three protein concentrations was kept the same as in Ottaway and Mowbray (1977) for most of our in vitro measurements, namely, by weight: 46% ALD, 33% CK, 21% GAPDH. It may be noted that a total soluble protein concentration of 55 mg/g muscle corresponds to 95 mg/ml myoplasmic water (which excludes the volume occupied by the sarcoplasmic reticulum, mitochondria, and solid matter), if a conversion factor of 0.58 ml of myoplasmic water per gram muscle is used (Baylor et al., 1983). Thus, while the relative amounts of the three proteins mentioned above were kept constant, anisotropy and fluorescence intensity were measured in vitro as total protein concentration was varied from 0 to 79 mg/ml (Fig. 4). For these measurements, the viscosity of the solutions was kept constant at ~1.7 cP by appropriate alteration of the quantity of sucrose present.

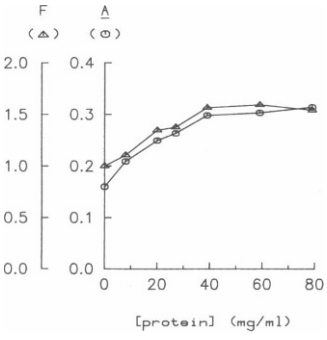


FIGURE 4 Anisotropy $\underline{A}$ (O) and fluorescence intensity F (△) of fura-2 in the presence of proteins (ALD + CK + GAPDH) measured in vitro (20°C). Dialyzed proteins were added to the standard buffer solution, up to 79 mg/ml, while the ratio of protein concentrations was kept constant (see text). Sucrose concentration was decreased proportionally from 540 mM (0 protein solution) to 0 mM (79 mg/ml protein solution) to keep viscosity approximately constant at 1.7 cP. Fluorescence intensity was normalized to that obtained without proteins. Fura-2 concentration was 0.5 mM throughout. Excitation wavelength was 420 ± 15 nm.

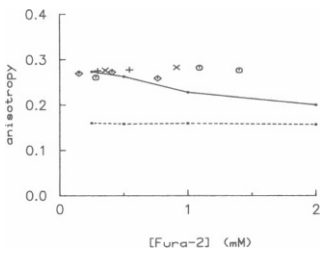


FIGURE 5 Anisotropy in various fura-2 concentrations in vitro and in vivo (20°C). Anisotropy was measured as a function of Ca^{2+}-free fura-2 concentration in vivo (four single fibers; +, X, ♦, O) and in capillaries (in vitro) containing no protein (---●---) or 27 mg/ml ALD + CK + GAPDH (—●—). For the in vitro measurements, fura-2 was added to the standard buffer solution without compensation for an expected small change in ionic strength. Viscosity was kept approximately constant at 1.7 cP with sucrose. Fiber Nos., 042387.1*a*, 042387.1*b*, 021988.1, 022088.1. Excitation wavelength was 420 ± 15 nm.

As shown in Fig. 4, both $\underline{A}$ and fluorescence intensity increased as protein concentration increased and approached saturating values at the higher protein concentrations. The changes in $\underline{A}$ and fluorescence intensity probably reflect the binding of fura-2 to proteins, with binding reducing the molecular mobility of the dye and, in some way (see below), enhancing its fluorescence intensity. In 60–80 mg/ml proteins, the absolute value of $\underline{A}$ was increased to ~0.31, a value much higher than that observed in the absence of proteins (0.16 at 1.7 cP), whereas fluorescence intensity was increased by ~60%. Significant increases in $\underline{A}$ were also observed when each of the three proteins was examined individually, thus indicative of a significant binding of fura-2 to each protein. Fura-2 binding to aldolase alone is examined in more detail in Figs. 8 and 9.

At 27 mg/ml protein, the increase of fluorescence intensity observed in Fig. 4 was ~40%, which, from the saturating level of the fluorescence intensity increase, suggests that about two-thirds of the fura-2 molecules were in a protein bound form (cf. Eq. A10 in the Appendix). Since $\underline{A}$ was 0.26 in the 27 mg/ml protein mixture, which is close to $\underline{A}$ of 0.27 obtained in myoplasm, this protein concentration was used in many of the following in vitro experiments. Although $\underline{A}$ was not measured in vitro at 95 mg/ml protein (the estimated protein concentration in the myoplasm of rabbit muscle), the data in Fig. 4 suggest that this value of $\underline{A}$ would be close to 0.31. Possible reasons for the observed in vivo value for frog myoplasm being less than 0.31 will be considered in the Discussion.

Fig. 5 shows $\underline{A}$ measured as a function of fura-2 concentration, [fura-2], in vitro (*lines*), and in vivo (*individual symbols*). In the absence of proteins the in vitro measurements (*dashed line*) show no significant change of $\underline{A}$ (0.16) up to 2 mM [fura-2]. The in vitro measurements with 27 mg/ml protein, however, show a clear dependence of $\underline{A}$ on [fura-2], as would be expected if fura-2 binding sites became saturated at the higher values of [fura-2]. In contrast, $\underline{A}$ in vivo showed no clear dependence on [fura-2]. Thus, there was some discrepancy between $\underline{A}$ measured in vitro with proteins and $\underline{A}$ measured in vivo, particularly at [fura-2] above 1 mM. This result indicates that the protein model solution used in this study did not perfectly mimic the intracellular environment; this is perhaps not surprising, given the complexity of the myoplasmic composition (Ottaway and Mowbray, 1977; Godt and Maughan, 1988). The direction of the discrepancy in Fig. 5 suggests that there is a significantly larger pool of fura-2 binding sites in frog myoplasm compared with those available in the in vitro model solution.

Effects of Ionic Strength on Fura-2 Binding to Proteins

Since fura-2 molecules are mostly pentavalent anions at pH 7.0, dye binding to proteins might vary strongly with ionic strength. When the ionic strength of in vitro solutions was increased from 63 to 300 mM simply by increasing the concentration of KCl, both $\underline{A}$ and fluorescence intensity of fura-2 showed a marked decrease in the presence of the 27 mg/ml protein mixture (Fig. 6 *A*), while no change was observed in the absence of proteins (Fig. 6 *B*). (A qualitatively similar effect on $\underline{A}$ was also observed when ionic strength was increased using Na_3citrate [not shown].) These results suggest that electrostatic interactions play a significant role in determining the binding of fura-2 to protein.

It should be noted in Fig. 6 *A* that, in 300 mM ionic strength and with 27 mg/ml proteins, $\underline{A}$ was still significantly higher than that observed without proteins (0.21 vs. 0.16), thus indicative of some binding of fura-2 to proteins in this solution. On the other hand, fluorescence intensity was about the same as that observed without proteins (1.01 vs. 1.00). This result indicates that the changes in $\underline{A}$ and fluorescence intensity of fura-2 with protein binding do not always correlate perfectly with each other. This, in turn, suggests the existence of more than one bound state (cf. Appendix).

Does EGTA Compete with Fura-2 Binding to Proteins?

Since our standard buffer solution contained 10 mM EGTA and most of the EGTA molecules are divalent anions at pH 7.0 with a Ca^{2+} binding pocket similar to that of fura-2, it might be possible that EGTA molecules would bind to proteins and consequently compete with fura-2 molecules for the binding sites. It has, for example, been reported that EGTA binds to some proteins, including NAD-specific isocitrate dehydrogenase (Aogaichi et al., 1980), bovine α-lactalbumin (Kronman and Bratcher, 1983), and parvalbumin (Parello et al., 1979). It was also reported that BAPTA (1,2-bis [O-aminophenoxy] ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid) binds to various Ca^{2+} binding proteins, such as trypsin, calmodulin, parvalbumin, and serum albumin, and that this binding is antagonized by EDTA (Chiancone et al., 1986). To clarify the possibility mentioned above, $\underline{A}$ and fluorescence intensity of fura-2

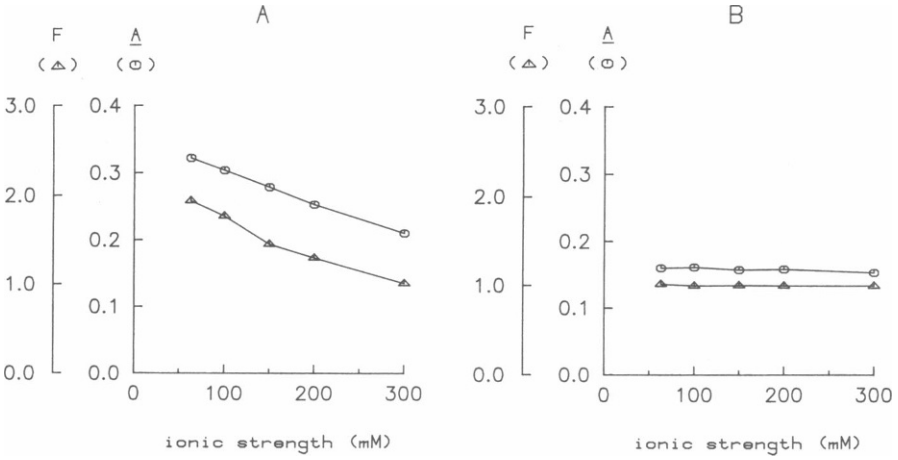


FIGURE 6 Effect of ionic strength of fura-2 anisotropy $\underline{A}$ (○) and fluorescence intensity F(△) measured in vitro either in the presence (*A*) or absence (*B*) of 27 mg/ml ALD + CK + GAPDH (20°C). Ionic strength was altered by adjustment of the KCl concentration in the buffer solution (5.5–243 mM). Fura-2 concentration was kept constant at 0.5 mM and viscosity approximately constant at 1.7 cP with sucrose. Fluorescence intensity was normalized to that obtained in 150 mM ionic strength without proteins. Excitation wavelength was 420 ± 15 nm.

were measured in vitro in 10, 1, and 0 mM EGTA in the presence of the 27 mg/ml protein mixture. KCl concentration was varied from 110 mM (at 10 mM EGTA) to 140 mM (at 0 mM EGTA) to keep ionic strength constant at 0.17 M, while [fura-2] (0.5 mM), viscosity (~1.8 cP by addition of 410 mM sucrose) and pH (7.0) were kept constant. Even in the solution without EGTA, $\underline{A}$ showed essentially the same value, 0.26, as that in 10 mM EGTA, while fluorescence intensity decreased only slightly, by 5% (which was within experimental error and may be partly due to a small Ca^{2+} contamination in the 0 EGTA solution). A similar experiment was also carried out with 26 mg/ml aldolase instead of the protein mixture (constant ionic strength, 0.15 M by 93–123 mM KCl; viscosity, ~1.5 cP by addition of 330 mM sucrose; [fura-2], 0.5 mM; pH, 7.0). Again, $\underline{A}$ showed no significant change with EGTA concentration between 0 and 10 mM, while fluorescence intensity was slightly smaller, by 8%, in 0 EGTA compared with 10 mM EGTA solution. The small decrease of fluorescence intensity observed in the absence of EGTA (if real) is again reasonably attributed to a small Ca^{2+} contamination. Thus, there was no evidence of competition between fura-2 and EGTA molecules for the protein binding sites.

Fluorescence Emission Spectrum

In vitro fluorescence emission spectra of fura-2 measured in the absence and in the presence of 79 mg/ml proteins (ALD + CK + GAPDH) are shown in Fig. 7 *A*. This high protein concentration was chosen because the great majority of fura-2 molecules are likely to be in the protein-bound form in 79 mg/ml proteins (cf. Fig. 4 where $\underline{A}$ and the fluorescence intensity change are near saturation at the high protein concentration). Thus the emission spectrum measured in 79 mg/ml protein should be very similar to that of protein-bound fura-2. In the absence of protein, the emission spectrum showed a broad profile, with a peak near 520–530 nm (Fig. 7, *A* and *B, open symbols*). On the other hand, in 79 mg/ml protein, fluorescence intensity increased at every wavelength measured (480–670 nm in 10-nm steps) with an approximately 10-nm shift to shorter wavelengths observed at the peak of the spectrum (Fig. 7 *A, solid symbols*). A spectral shift due to protein binding is more clearly seen in Fig. 7 *B*, in which the spectra shown in Fig. 7 *A* have been normalized to the peak fluorescence for each data set. Fluorescence intensities of the normalized spectrum in 79 mg/ml proteins (Fig. 7 *B, solid symbols*) are consistently higher at shorter wavelengths (480–510 nm) and lower at longer wavelengths (530–670 nm) than those measured without proteins (*open symbols*); thus, protein binding causes a "blue shift" of the fura-2 spectral emission profile by roughly 10 nm. A very similar spectral shift was also observed in 55 mg/ml aldolase alone (not shown). The "blue shift" could also be observed when ionic strength was decreased from 300 to 63 mM in the presence of the 27 mg/ml protein mixture (cf. experiment of Fig. 6 *A*, spectrum not shown), a finding consistent with the previous conclusion that there is an increase in the binding of fura-2 to proteins at low ionic strength.

In the absence of proteins, factors such as viscosity (1.0–6.2 cP), pH (7.0–7.4), free Mg^{2+} concentration (0–1.5 mM), and ionic strength (0.1–0.17 M by KCl; 0.39 M by KCl + Na_3 citrate) produced no detectable shift in the emission spectrum.

The normalized emission spectrum of fura-2 in myoplasm is also shown in Fig. 7 *B* (*crosses*). The striking similarity between the in vivo spectrum and that obtained in vitro with 79 mg/ml proteins supports the interpretation that a significant fraction of fura-2 in myoplasm was bound to proteins. If it is assumed that the spectrum of myoplasmic fura-2 in Fig. 7 *B* reflects a linear combination of the two in vitro spectra in Fig. 7 *A* (protein-bound fura-2 and protein-free fura-2), a best fit of the myoplasmic spectral shape can be obtained using 85% of the former plus 15% of the latter. Two other spectra from myoplasm (not shown) were best fitted by 92 or 80% of the former and 8 or 20% of the latter, respectively. These results suggest that 80–90% of the fura-2 in myoplasm may be

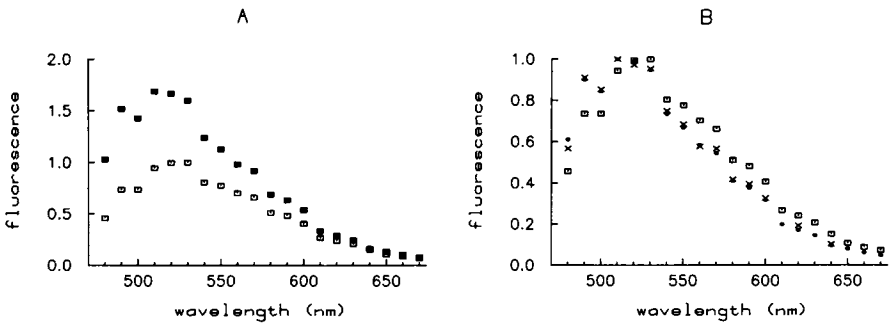


FIGURE 7 Fura-2 fluorescence emission intensity (ordinate) as a function of wavelength (abscissa) obtained in vitro and in vivo (20°C). (*A*) In vitro fluorescence emission spectra of 0.5 mM fura-2 obtained with 79 mg/ml ALD + CK + GAPDH (■) and without proteins (□) in the standard buffer solution (viscosity of 1.7 cP). Fluorescence intensity was normalized to that obtained at 530 nm without proteins. (*B*) Normalized fluorescence emission spectra from *A* (with 79 mg/ml ALD + CK + GAPDH [●] and without proteins [□]), and in vivo (X, fiber No., 022088.1) at a fura-2 concentration of 0.64 mM. Each data set has been normalized to the peak fluorescence for that data set. Excitation wavelength was 420 ± 15 nm.

protein-bound, with no more than 10–20% being freely soluble.

Effect of Protein Binding on Fura-2's Dissociation Constant for Ca^{2+}

An important question related to the use of fura-2 as an intracellular Ca^{2+} indicator is whether the dissociation constant of fura-2 for Ca^{2+} ($K_{D,Ca}$) is altered by the binding of dye to protein. It was difficult to reliably measure K_D in the presence of the mixture of ALD, CK, and GAPDH because a protein precipitate formed in high [Ca^{2+}]. Very little precipitate was observed with aldolase alone, however, and K_D measurements were therefore done only with aldolase, at concentrations of 27 and 55 mg/ml. The latter concentration corresponds to 32 mg/g whole muscle, the reported amount of aldolase in rabbit white muscle (Ottaway and Mowbray, 1977). As shown in Fig. 8 *A* fluorescence intensity decreased with increasing free [Ca^{2+}], and only a slight fluorescence (1–2% of maximum fluorescence; see Methods) was observed in saturating [Ca^{2+}] (~100 μM).

Fluorescence data obtained in the various [Ca^{2+}]'s were generally well fitted by a binding curve of 1:1 stoichiometry (Fig. 8 *A*), but some scatter in the data was observed at high [Ca^{2+}]'s (log [Ca^{2+}] > −5.5). Since it is difficult to exactly control [Ca^{2+}] in the range much higher than the K_D of the Ca^{2+}-chelator, data obtained at ~10 μM free [Ca^{2+}] and above were omitted from the K_D fitting. In the absence of aldolase, a $K_{D,obs}$ of 188 nM was obtained (left-most curve, Fig. 8 *A*). $K_{D,Ca}$ calculated given a $K_{D,Mg}$ of 9.8 mM (Grynkiewicz et al., 1985) was 162 nM (see Eq. 3 in Methods), which is in close agreement with the 161-nM value obtained from kinetic measurements by Kao and Tsien (1988), and the 135-nM value reported by Grynkiewicz et al. (1985) from steady-state titrations. A slightly higher value of $K_{D,obs}$, 212 nM (not shown) was measured when viscosity was increased to 2.0 cP by 655 mM sucrose. For comparison with the K_D measurements in the presence of aldolase (viscosity 1.3–1.5 cP), the average value of the two measurements of $K_{D,obs}$ made at 1.0 and 2.0 cP in the absence of proteins was used, 200 nM.

In the presence of either 27 or 55 mg/ml aldolase, the whole titration curve shifted towards higher [Ca^{2+}]'s, with $K_{D,obs}$ being 428 or 687 nM, respectively (middle and right-most curves, Fig. 8 *A*). These results indicate that aldolase alters fura-2's effective dissociation constant for Ca^{2+} (see also Discussion).

Minimal Scheme for Fura-2's Reaction with Aldolase and Ca^{2+}

To estimate the degree of binding of fura-2 to aldolase in the conditions of Fig. 8 *A*, $\underline{A}$ and total fluorescence intensity were measured as a function of aldolase concentration, Fig. *8 B* (cf. Fig. 4). For these measurements the solution conditions were similar to those for the $K_{D,obs}$ measurement, except for the absence of Mg^{2+} (ionic strength 0.15 M, 94 mM KCl, 10 mM EGTA, 10 mM PIPES, pH 7.0, viscosity kept approximately constant at 1.5 cP by sucrose). The results indicate that $\underline{A}$ and total fluorescence intensity were nearly saturated in 55 mg/ml aldolase (also next paragraph), so that the 687-nM value of $K_{D,obs}$ (Fig. 8 *A*) primarily reflects that of aldolase-bound fura-2.

The fluorescence intensity data in Fig. 8 *B* were analyzed by least-squares fitting with three adjustable parameters: the dissociation constant of the fura-2-aldolase complex; the number of fura-2 binding sites per aldolase molecule; and the fluorescence intensity of aldolase-bound fura-2 relative to that of non-aldolase-bound fura-2 (cf. assumption *a* in the Appendix). Best-fitted parameters were 66 μM for the dissociation constant, 3.0 binding sites per molecule, and a 1.8-fold increase of fura-2 total fluorescence for protein-bound fura-2 (cf. upper curve in Fig. 8 *B*). According to the fit, 70 or 90%, respectively, of fura-2 molecules were in the aldolase-bound form in 27 or 55 mg/ml aldolase. A best-fit analysis of the $\underline{A}$ data in Fig. 8 *B* (cf. the Appendix) was also carried out based on Eq. A11 (Appendix) and the best-fitted parameters obtained above. For this analysis, maximum $\underline{A}$ ($\underline{A}(2)$ in the Appendix) was treated as an adjustable parameter. The theoretical $\underline{A}$ values (lower curve in Fig. 8 *B*), calculated using the best-fitted $\underline{A}(2)$ of 0.330 and an $\underline{A}(1)$ of 0.143 (from the Perrin plot, Fig. 3 *B*), show reasonable agreement with the

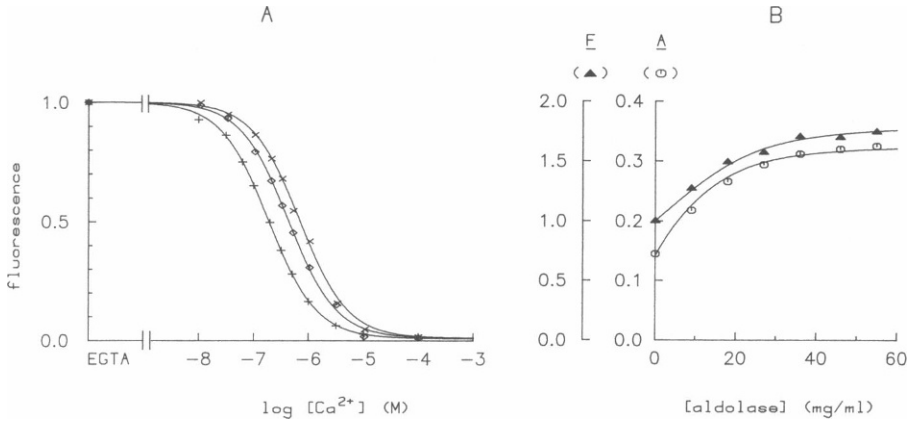
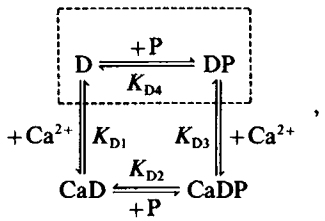


FIGURE 8 (*A*) Effect of aldolase on the dissociation constant of fura-2 for Ca^{2+}. In vitro fura-2 fluorescence intensity was measured without polarizers as a function of free [Ca^{2+}] at 0 mg/ml (+), 27 mg/ml (◇), and 55 mg/ml (X) aldolase. Ionic strength 0.15 M, 1.5 mM free [Mg^{2+}], pH 7.0 (20°C). No sucrose was added to compensate solution viscosity. Solid lines indicate the least-squares fit of the experimental data by a binding curve of 1:1 (Ca^{2+}/fura-2) stoichiometry. Fitted dissociation constants were 188 nM (*left-most curve*), 428 nM (*middle curve*), and 687 nM (*right-most curve*). Fura-2 concentration was 0.5 mM throughout. Excitation wavelength was 420 ± 15 nm. (*B*) Effects of aldolase concentration on anisotropy $\underline{A}$ (○) and total fluorescence intensity $\underline{F}$ (▲; proportional to $F_{\parallel} + 2F_{\perp}$) of fura-2 measured in vitro (20°C, ionic strength 0.15 M, 0 mM [Mg^{2+}], pH 7.0; viscosity, 1.5 cP). $\underline{F}$ was normalized to that obtained without aldolase. The curves represent a least-squares fit to the data sets by means of the theory described in the Appendix (see text for fitted constants). Fura-2 concentration calculated from its absorbance at 362 nm was 0.45 mM. The effect of aldolase on normalized fluorescence measured without polarizers (data not shown; cf. Fig. 4) was very similar to that for $\underline{F}$; this normalized fluorescence increased with aldolase concentration to a value of 1.93 at 55 mg/ml.

experimental values in Fig. 8 *B*. The combined data sets in Fig. 8 *B* (*circles* and *triangles*) are thus well fitted by these parameter selections. It should be pointed out, however, that other choices of the four adjustable parameters also gave acceptable fits to the data. The parameter that was least well determined in the fitting was the value of the fura-2–aldolase dissociation constant, for which values between 40 and 110 μM still gave reasonable fits to the data.

The minimal reaction scheme of dye binding with Ca^{2+} and/or aldolase is



where D, P, DP, CaD, and CaDP represent free dye, free aldolase, aldolase-bound dye, Ca^{2+}-bound dye, and Ca^{2+}-aldolase-bound dye, respectively. K_{D1}, K_{D2}, K_{D3}, and K_{D4} denote the dissociation constants of the reactions as indicated above. The dashed box in the reaction scheme denotes Ca^{2+}-free fura-2, the form of the dye that produces essentially all of the fluorescence measured using the 420-nm excitation beam. The ability of protein-bound fura-2 to directly bind Ca^{2+} (as opposed to a competitive occlusion by protein of fura-2's metal-binding site) is indicated by three lines of evidence. First, in 55 mg/ml aldolase, the fit in Fig. 8 *B* indicated that 90% of fura-2 was in the protein-bound form. Thus, if protein-bound fura-2 was not able to bind Ca^{2+}, a 10-fold shift in $K_{D,obs}$ would have been expected, rather than the 3.4-fold shift that was measured. Second, as mentioned earlier, EGTA, which has a metal-binding site similar in structure to that of fura-2, did not appear to competitively antagonize the binding of fura-2 to proteins. This result suggests that the Ca^{2+}-chelating pocket of fura-2 is not directly involved in the fura-2–protein interaction. Third, in the presence of CK and saturating [Ca^{2+}], fura-2 has a markedly prolonged rotational correlation time (T. Wensel, personal communication), consistent with the existence of a protein-bound form of Ca^{2+}–fura-2.

At equilibrium, the following relationship applies:

$$K_{D1} \cdot K_{D2} = K_{D3} \cdot K_{D4}. \quad (6)$$

If K_{D3} is three to four times higher than K_{D1}, as suggested by the experimental result obtained at 55 mg/ml aldolase (Fig. 8 *A*), Eq. 6 predicts that Ca^{2+}-bound dye binds to aldolase with three to four times lower affinity than Ca^{2+}-free dye. Thus the fraction of Ca^{2+}–fura-2 that is bound to aldolase, f_{CaD} (= [CaDP]/([CaD] + [CaDP])), is always smaller than the fraction of dye bound to aldolase in the Ca^{2+}-free pool, f_D (= [DP]/([D] + [DP])). For example, as calculated above, f_D was 0.70 and 0.90 in 27 and 55 mg/ml aldolase, respectively, at zero [Ca^{2+}]. Thus, in saturating [Ca^{2+}], f_{CaD} should be 0.50 and 0.72, respectively, if $K_{D2} = 4 \times K_{D4}$.

It also follows that [P] and therefore f_D should increase as a function of increasing [Ca^{2+}]. An increase in [P] in turn would cause an increase in $\underline{A}$ measured from Ca^{2+}-free fura-2 (cf. Eq. A11 in the Appendix). If it is assumed that K_{D1} is 190 nM (Fig. 8 *A*), that K_{D3} is four times higher than K_{D1}, and that the best fit parameters for fura-2–aldolase binding in 0 [Ca^{2+}] apply (K_{D4} = 66 μM, three binding sites per aldolase molecule), the increases in f_D and $\underline{A}$ associated with an increase of [Ca^{2+}] from 0 to 1 μM can be calculated to be 0.07 (from 0.70 to 0.77) and 0.009 (from 0.295 to 0.304), respectively, in the presence of 27

mg/ml aldolase (= 170 μM). Therefore, the relation between fluorescence intensity and $[Ca^{2+}]$ in the presence of 27 mg/ml aldolase (cf. Fig. 8 *A*) should be slightly displaced from a theoretical binding curve which corresponds to 1:1 stoichiometry. However, the degree of displacement of the data points from a 1:1 binding curve is expected to be within ±0.6% of maximum fluorescence intensity from the calculation above; this small displacement would not be detectable in the experimental data in Fig. 8 *A* and has been ignored in the fitting. In 55 mg/ml aldolase, the changes in f_D and $\underline{A}$ are entirely negligible (no larger than 0.01 and 0.001, respectively).

Alteration in Fura-2's Absorbance Spectrum with Protein Binding

Since the increase of fluorescence intensity of fura-2 observed in the presence of proteins (Fig. 4, Fig. 7 *A*, Fig. 8 *B*) might be due, at least in part, to an increase of fura-2's absorbance in the protein-bound state, dye absorbance spectra were measured in the absence and presence of protein (55 mg/ml aldolase), both in 0 $[Ca^{2+}]$ and in saturating $[Ca^{2+}]$ solutions. Only aldolase was used as the model protein for this experiment, because of the precipitation problem mentioned above for the protein mixture when in high $[Ca^{2+}]$.

As shown in Fig. 9 *A*, aldolase (55 mg/ml) shifted the absorbance spectrum of Ca^{2+}-free fura-2 to longer wavelengths ("red shift"), with essentially no change in peak absorbance. On average, the spectral shift was ~5–6 nm. Because of this red shift, the average increase in fura-2 absorbance for wavelengths between 405 and 435 nm (the band pass of the 420-nm excitation filter used for the fluorescence measurements) was 40–50%. Thus, if fluorescence intensity is directly proportional to dye absorbance, approximately half of the increase in fluorescence intensity seen in Fig. 8 *B* at high aldolase concentration is attributable to the absorbance increase; the remaining increase may be due to an increase in fura-2's fluorescence lifetime (see Discussion).

In contrast, no alteration in absorbance spectrum was observed for fura-2 in the presence of aldolase at saturating levels of free $[Ca^{2+}]$ (Fig. 9 *A*). As noted above, the estimated fraction of fura-2 bound to aldolase in the saturating $[Ca^{2+}]$ condition was ~0.7, somewhat less than the 0.9 value for the protein-bound fraction in the zero $[Ca^{2+}]$ measurements. Nevertheless, this degree of binding is sufficient to indicate that the absorbance spectrum of Ca^{2+}-bound fura-2 was not significantly changed when bound to aldolase.

As shown in Fig. 9 *B*, the change in fura-2 absorbance associated with Ca^{2+} binding has a slightly modified shape in the presence of 55 mg/ml aldolase. The principal modification at wavelengths longer than 380 nm is a 5–6 nm red shift.

Anisotropy Measurements during Fiber Activity

As previously described (Baylor and Hollingworth, 1988), a large decrease in fura-2's fluorescence intensity was readily detected from single muscle fibers microinjected with fura-2 and stimulated electrically. After a single action potential, the peak change in fluorescence, ΔF, considered as a fraction of the resting fluorescence, F, was between −0.6 and −0.8 for fibers that contained fura-2 concentrations <0.2 mM. This fractional change corresponds to the conversion of a similar fraction of Ca^{2+}-free fura-2 to Ca^{2+}-bound fura-2 during activity. As discussed earlier, it seems likely that fura-2 in myoplasm exists in multiple populations (two or more) with different values of anisotropy, for example, protein-free and protein-bound fura-2, with smaller and larger anisotropies, respectively. Moreover, it is possible that these different dye populations bind Ca^{2+} during muscle activity with different reaction rates. For example, fura-2 not bound to molecules of large molecular weight might react with the relatively rapid kinetic constants reported for fura-2 in salt solutions (Kao and Tsien, 1988), whereas the dye bound to proteins might react with significantly slower rates. The overall somewhat

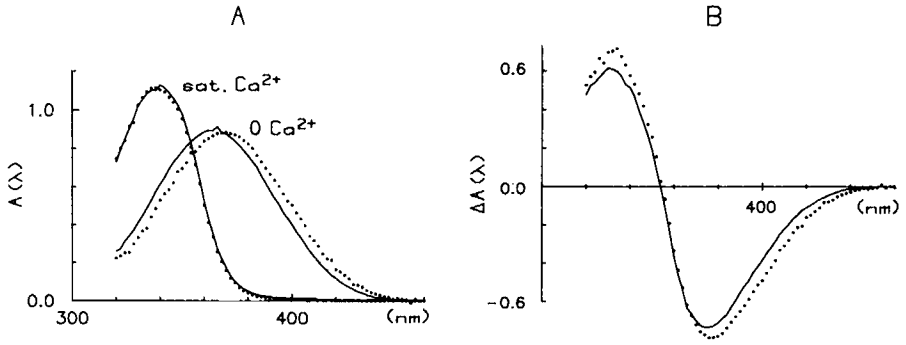


FIGURE 9 In vitro fura-2 absorbance (ordinate) as a function of wavelength (abscissa) measured in the absence and presence of aldolase (55 mg/ml). (*A*) Absolute spectra measured in a 1-mm cuvette containing 0.32 mM fura-2 plus the following (in mM): "O Ca^{2+}" (93 KCl, 10 EGTA, 10 PIPES, no added Ca^{2+}); "sat Ca^{2+}" (75 KCl, 10 EGTA, 10 PIPES, 11 $CaCl_2$). Continuous data are spectra measured without aldolase; dotted data are spectra measured with aldolase. Temperature, 21°–24°C; pH 7.0. (*B*) Fura-2 "Ca^{2+}-difference" spectra from part *A* obtained as the "sat Ca^{2+}" spectra minus the "O Ca^{2+}" spectra (*continuous data*, without aldolase; *dotted data*, with aldolase).

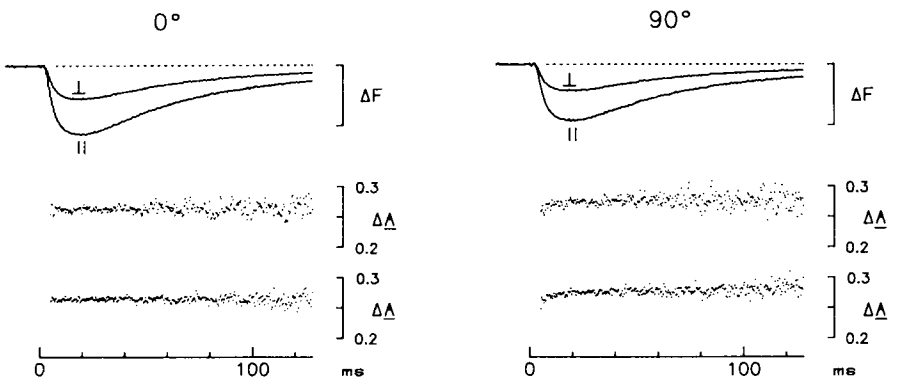


FIGURE 10 (*Upper*) Changes in fluorescence (ΔF) of fura-2 as a function of polarized light in a single muscle fiber stimulated at 0 time to give a single action potential. All traces were obtained using a 420 ± 15 nm excitation beam, polarized either parallel (0°; left-hand side) or perpendicular (90°; right-hand side) to the fiber axis. The ΔF traces labeled ∥ and ⊥ were obtained with the second polarizer oriented parallel and perpendicular, respectively, to the first polarizer. The middle traces, labeled $\Delta\underline{A}$, were obtained as linear combinations of the ΔF traces shown above, namely, $\Delta\underline{A} = (\Delta F_{\parallel} - \Delta F_{\perp}) / (\Delta F_{\parallel} + 2\Delta F_{\perp})$. The lowermost traces, also labeled $\Delta\underline{A}$, show the average of four such traces obtained in an identical fashion as the middle traces from four sets of ΔF measurements during the experiment. The display of the $\Delta\underline{A}$ traces begins at the earliest time in the rising phase of the ΔF traces such that the $\Delta\underline{A}$ values were reliable. (Before the displayed time, the $\Delta\underline{A}$ values were unreliable because they represent ratios of small numbers.) The ΔF calibration bar corresponds to 50 mV (arbitrary fluorescence units). Fura-2 fluorescence intensities before stimulation were: $F_{\parallel}$ = 73.3 mV, $F_{\perp}$ = 35.2 mV (0° excitation) and $F_{\parallel}$ = 61.1 mV, $F_{\perp}$ = 28.5 mV (90° excitation), and corresponding anisotropy values were 0.265 (0° excitation) and 0.276 (90° excitation). The light source itself (a tungsten-halogen bulb) emitted ~25% more 420-nm light polarized in the 0° compared with the 90° direction. The concentration of Ca^{2+}-free fura-2 in the resting state was estimated to be 135 μM. Peak values of ΔF were: $\Delta F_{\parallel}$ = −57.0 mV, $\Delta F_{\perp}$ = −27.6 mV (0° excitation) and $\Delta F_{\parallel}$ = −46.0 mV, $\Delta F_{\perp}$ = −21.5 mV (90° excitation). Fiber No., 042288.1; temperature, 16°C; fiber diameter, 92 μm; sarcomere spacing, 3.8 μm. Upper traces were taken at the site of dye injection, 32–35 min after injection. Each $\Delta F_{\parallel}$ trace is a single sweep, whereas each $\Delta F_{\perp}$ trace is the average of two sweeps taken just before and just after the corresponding $\Delta F_{\parallel}$.

slow rates observed in muscle fibers (Baylor and Hollingworth, 1988) would presumably represent a kinetic average over the various dye populations. It was therefore of interest to see if changes in fura-2 anisotropy could be detected during muscle activity.

Fig. 10 (*upper*) shows original records of changes in fura-2 fluorescence intensity (ΔF) in response to a single action potential, on the left for 0° polarized excitation light and on the right for 90° light. Within each pair, the traces labeled ∥ and ⊥ are the ΔF traces measured with the second polarizer oriented parallel and perpendicular to the first polarizer, respectively. As expected, a large fractional decrease in fluorescence was seen in each of the traces ($\Delta F/F \simeq -0.75$; see legend of Fig. 10), with all traces having very similar time courses. As shown in the lower traces ($\Delta\underline{A}$) in Fig. 10, there was little or no change with time in the anisotropy of the dye fluorescence which disappeared on complexation with Ca^{2+}. The average value of $\Delta\underline{A}$ during activity was 0.262 for 0° excitation light and 0.275 for 90° light. These values are not significantly different from the resting values of $\underline{A}$ observed in this fiber, 0.265 and 0.276 respectively (see legend of Fig. 10).

Similar results were seen in five other fibers, with the $\Delta\underline{A}$ traces showing no consistent change as a function of time. (The small increase in $\Delta\underline{A}$ seen at early time with 90° polarized light was not a consistent feature of the measurement in other fibers.) For the six experiments combined, the average values of $\Delta\underline{A}$ were 0.259 (± 0.003, SEM) for 0° light and 0.270 (± 0.005, SEM) for 90° light. These values are not significantly different from each other ($P >$ 0.05, Student's t-test); nor is the average of the 0° and 90° $\Delta\underline{A}$ values, 0.265 (± 0.003), significantly different from the average value of $\underline{A}$ reported in the first section of Results for fura-2's anisotropy in fibers in the resting state, 0.272 (± 0.003).

Thus, the measurements during fiber activity revealed no evidence for the existence of two or more populations of fura-2 having significantly different values for both anisotropy and Ca^{2+} fura-2 reaction kinetics. The existence of such populations is not, however, ruled out by the experimental results. For example, if the exchange of Ca^{2+}-free fura-2 among such populations was rapid in comparison with the time course of the Ca^{2+}–fura-2 transients shown in Fig. 10, no significant change in the $\Delta\underline{A}$ traces with time would be expected. This follows since the anisotropy change in this case would represent an equilibrium average of the anisotropies of the underlying populations of Ca^{2+}-free fura-2. The fact that the average value of $\Delta\underline{A}$ during activity was the same as the value of $\underline{A}$ prior to activity is consistent with the conclusion reached previously (Baylor and Hollingworth, 1988) that essentially all of the Ca^{2+}-free fura-2 in myoplasm is available to react with Ca^{2+}, with the fraction actually reacting determined by the amplitude of the underlying free Ca^{2+} transient.

DISCUSSION

Fura-2 Binding to Myoplasmic Proteins

In vitro measurements indicate that soluble myoplasmic proteins (ALD, CK, GAPDH) from rabbit muscle increase fura-2's anisotropy and fluorescence intensity in a concentration-dependent manner (Figs. 4, 8 *B*). An increase in $\underline{A}$ is consistent with the binding of fura-2 to these large molecular weight proteins (ALD, 160 kD; CK, 82.6 kD; GAPDH, 140 kD; Altman and Dittmer, 1972),

and a consequent reduction in the dye's rotational mobility. In addition, an increase in steady-state fluorescence intensity would be explained if protein-bound fura-2 had a red-shifted absorbance spectrum (as observed in Fig. 9 *A*) and/or a longer fluorescence lifetime (cf. Cantor and Schimmel, 1980). In a pulsed laser experiment with nanosecond time resolution, T. Wensel (personal communication) found that in the presence of CK, fura-2 had an increase in its average fluorescence lifetime, from ~1.0 to ~2.1 ns. Thus both absorbance and lifetime effects would appear to contribute to the increase in fluorescence intensity observed in our experiments. It should be noted that an increase in lifetime by itself would result in a reduction in $\underline{A}$. Thus, the observed large increase in $\underline{A}$ with protein binding is explained by the large decrease in rotational mobility expected when fura-2 associates with molecules of 130–250 times larger molecular weight.

The values of $\underline{A}$ observed in frog myoplasm, 0.27, was closely approximated in vitro by a protein mixture of 27 mg/ml ALD + CK + GAPDH, a concentration much lower than the concentration of soluble myoplasmic proteins reported in rabbit white muscle (95 mg/ml). One possible explanation for this difference is that there are other fura-2 binding sites in myoplasm which compete with those on the soluble proteins. Small molecules such as amino acids, phosphocreatine, creatine, and carnosine, which are reported to be abundant in frog skeletal muscle (Godt and Maughan, 1988), might bind fura-2 molecules while producing little change in $\underline{A}$ (or in the apparent diffusion constant of fura-2) because of their small molecular weights. Alternatively, it is also possible that frog myoplasm might contain a significantly smaller quantity of soluble protein (e.g., only 30% of rabbit muscle). The lack of a dependence of $\underline{A}$ on [fura-2], up to 1.4 mM in myoplasm (Fig. 5), supports the former possibility.

The in vivo fraction of Ca^{2+}-free fura-2 bound to soluble myoplasmic constituents was estimated to be ~⅔ from the combined $\underline{A}$ and fluorescence intensity measurements in the presence of ALD + CK + GAPDH (Fig. 4) or 80–90% from a comparison of spectral emission data (Fig. 7). These estimates, together with the nearly threefold slower diffusion constant observed in myoplasm compared with that expected from fura-2's molecular size (Baylor and Hollingworth, 1988), strongly support the conclusion that a majority of fura-2 molecules (60–85%) is bound in frog myoplasm.

Change of the Ca^{2+}–Fura-2 Dissociation Constant Due to Protein Binding

The value of $K_{D,obs}$ of fura-2 for Ca^{2+} was found to be 2.1-fold and 3.4-fold larger in the presence of 27 and 55 mg/ml aldolase, respectively; in these conditions, the estimated protein-bound fractions of Ca^{2+}-free fura-2 were ~70 and 90%, respectively. A similar apparent increase in $K_{D,obs}$ might be explained if a large fraction of EGTA molecules were bound to aldolase and bound EGTA had a lower K_D for Ca^{2+}. However, this seems unlikely. First, there was no evidence of any competition between EGTA and fura-2 for binding to aldolase (26 mg/ml), at least in terms of the measurements of $\underline{A}$ and fluorescence intensity. Second, the EGTA concentration (10 mM) was much higher than the aldolase concentration (340 μM), so that the fraction of aldolase-bound EGTA should have been small. For example, even if all aldolase molecules bound three EGTA molecules and aldolase-bound EGTA (~1 mM) had an infinitely high affinity for Ca^{2+}, this degree of binding would have caused a large error at low free $[Ca^{2+}]$'s (e.g., a 47% error at 100 nM $[Ca^{2+}]$) but a much smaller error at higher $[Ca^{2+}]$'s (e.g., a 16% error at 1 μM $[Ca^{2+}]$). The estimated shift in fitted K_D introduced by these errors would be ~−0.15 pCa units. However, the presence of such an error would probably have been recognized in this worst-case situation, since the data would no longer have been well-fitted by a theoretical 1:1 binding curve.

An analogous argument indicates that possible errors introduced by Ca^{2+} binding to aldolase are probably also negligible. Additionally, aldolase does not require divalent cations as a cofactor for its enzyme activity; even CK, a known divalent cation (Mg^{2+} or Ca^{2+})-activated enzyme, requires 10^{-4} M concentrations of divalent cations for its half activation (Watts, 1973).

The value of $K_{D,obs}$ of aldolase-bound fura-2 thus turned out to be ~690 nM or slightly higher. If $K_{D,Mg}$ of fura-2 is assumed to be unchanged, $K_{D,Ca}$ was increased 3.4-fold by binding to aldolase. On the other hand, if $K_{D,Mg}$ is assumed to be changed in proportion to $K_{D,Ca}$, the increase of $K_{D,Ca}$ (and $K_{D,Mg}$) is estimated at 3.8-fold. Since this is a nearly maximum effect of aldolase, $K_{D,Ca}$ of fura-2 in myoplasm might be changed by less than this amount (if other myoplasmic fura-2 binding sites had a similar effect on K_D), since the protein binding of fura-2 in vivo is estimated to be 60–85%. Although the effective dissociation constant of fura-2 for Ca^{2+} in myoplasm will remain uncertain until the properties of fura-2 bound to other binding sites are clarified, its value is probably not the same as, and is quite likely higher than, that obtained in a salt solution lacking myoplasmic constituents of larger molecular weight (cf. Baylor and Hollingworth, 1988).

Alterations in Fura-2 Absorbance Spectra with Protein Binding

A 5–6-nm "red shift" was detected in the in vitro absorbance spectrum of Ca^{2+}-free fura-2 in the presence of 55 mg/ml aldolase (Fig. 9). It has not yet been determined whether Ca^{2+}-free fura-2 in frog myoplasm also has a red-shifted absorbance spectrum. We think that an in vivo red shift, if present, is unlikely to be as large as the 5–6-nm shift observed in Fig. 9 *A*, because Baylor and Hollingworth (1988) did not detect a spectral shift in fura-2's absorbance signal during activity. However, a red shift of 2–3 nm might not have been resolved in their data,

measured for wavelengths between 380 and 460 nm. If so, the 420-nm extinction coefficient used to calibrate their fura-2 signals would have produced a 16–25% overestimate in the amount of Ca^{2+}–fura-2 complex formed during activity, as well as in the concentration of Ca^{2+}-free fura-2 in fibers at rest. Thus, a similar overestimate (16–25%) might apply to the abscissa in Fig. 5 for the in vivo measurements. Resolution of this uncertainty will require more complete absorbance spectral measurements in vivo. It should be noted, however, that even with a 25% error in the in vivo [fura-2] estimated in Fig. 5, the in vivo anisotropy values still show less dependence on fura-2 concentration than do the in vitro measurements.

Combined Effects of Errors in the Estimation of Free $[Ca^{2+}]$

As presented in Results, the binding of fura-2 free-acid to intracellular proteins alters many properties of the dye relevant to an accurate estimate of cytoplasmic free $[Ca^{2+}]$. In this regard it may be useful to consider the likely effects of these alterations on the estimation of $[Ca^{2+}]$ by the most commonly employed calibration procedure, which is based on measurement of the ratio, R, of fluorescence intensity obtained with a 340-nm excitation beam to that obtained with a 380-nm beam:

$$[Ca^{2+}] = K_D\left(\frac{R - R_{min}}{R_{max} - R}\right)\left(\frac{S_{f2}}{S_{b2}}\right) \quad (7)$$

(Eq. 5 of Grynkiewicz et al., 1985). In this equation K_D represents the effective dissociation constant of fura-2 for Ca^{2+}, whereas R_{min} and R_{max} represent values of R obtained at 0 and saturating $[Ca^{2+}]$ levels, respectively. S_{f2} and S_{b2} are constants which, when multiplied by the fura-2 concentration, determine the fluorescence levels that would be measured with 380-nm excitation if all the dye were in, respectively, the Ca^{2+}-free or Ca^{2+}-bound state. R_{min}, R_{max}, S_{f2}, and S_{b2} are apparatus-dependent quantities.

From Eq. 7 it is clear that any alteration in K_D introduces a proportional error in $[Ca^{2+}]$. For example, if the degree of intracellular binding and consequent alteration of K_D are similar to that estimated above for frog myoplasm, the use of K_D measured from an in vitro calibration solution lacking cytoplasmic protein would, by itself, lead to a two- to threefold underestimate of the actual free $[Ca^{2+}]$ level. Second, a protein-induced shift in the absorbance spectrum of Ca^{2+}-free fura-2 but not Ca^{2+}-bound fura-2 (cf. Fig. 9 *A*) would likely lead to a further underestimate of the actual free $[Ca^{2+}]$, due to the influence of the second term in Eq. 7, $(R-R_{min})/(R_{max}-R)$. Under the assumption that fluorescence intensity excited at wavelength λ is proportional to absorbance at wavelength λ, the value of R_{min} (which depends on Ca^{2+}-free but not Ca^{2+}-bound fura-2; cf. Grynkiewicz et al., 1985) measured in the usual in vitro conditions would be higher than appropriate for the cytoplasm; in contrast, that of R_{max} (which depends on Ca^{2+}-bound but not Ca^{2+}-free fura-2) would be correct. Thus, the $(R-R_{min})/(R_{max}-R)$ term would be erroneously small, with the amplitude of the error varying with the level of $[Ca^{2+}]$ being estimated. It should be noted that for small levels of $[Ca^{2+}]$, errors in this term might even lead to an estimate of a "negative" intracellular level of free $[Ca^{2+}]$ (cf. Klein et al., 1988). Third, the last term in Eq. 7, if based on the usual in vitro measurements, would also lead to an additional, although apparently minor, underestimate of $[Ca^{2+}]$. This conclusion also follows from the spectra in Fig. 9 *A*, since S_{f2} would likely be smaller and S_{b2} unchanged in the in vitro compared with the in vivo situation. (Note that at 380 nm, protein binding leads to a small increase in absorbance of Ca^{2+}-free fura-2 whereas the absorbance of Ca^{2+}-bound fura-2 is apparently unchanged.) Finally, additional, less-easily characterized effects on the $[Ca^{2+}]$ calibration may occur due to changes in fluorescence intensity that result from shifts in the fluorescence emission spectrum (Fig. 7) and increases in fluorescence lifetime (see above).

The net result of these effects is likely to vary with preparation and calibration conditions, but cumulative errors as large as 10-fold would appear to be within the realm of possibility. In this regard it is interesting to note that the peak amplitude of the $[Ca^{2+}]$ transient observed in cut frog skeletal muscle fibers in response to action potential stimulation is ~10-fold higher when calibrated from the tetramethyl-murexide absorbance transient (Maylie et al., 1987*c*) than when calibrated by the antipyrylazo III absorbance transient (Maylie et al., 1987*b*). Since the simultaneously calibrated amplitudes of the antipyrylazo III and fura-2 $[Ca^{2+}]$ transients from frog skeletal muscle have a very similar peak value (after correction for the kinetic delay of fura-2; Klein et al., 1988; Baylor and Hollingworth, 1988), it follows that the fura-2 calibration of $\Delta[Ca^{2+}]$ is about an order of magnitude smaller than that obtained with tetramethyl-murexide. The latter dye has the smallest bound fraction (0.3; Maylie et al., 1987*a–c*) among five Ca^{2+}-indicator dyes used to date in intact and cut frog skeletal muscle fibers and might therefore have the least error associated with its calibration. In conclusion, given the present state of knowledge of the properties of indicator dyes within the cytoplasmic environment, considerable quantitative uncertainty would appear to apply to most calibrations of $[Ca^{2+}]$.

Comparisons with Measurements from Other Laboratories

Several results concerning the possible binding of fura-2 to intracellular constituents have been reported previously from other laboratories. Williams et al. (1985) were the first to report measurements of fura-2's fluorescence emission anisotropy, both from in vitro salt solutions and from the cytoplasm of single amphibian smooth muscle cells loaded by means of fura-2-AM. No significant difference

was found between the two fura-2 anisotropy values ($\underline{A}$ = 0.08 ± 0.01 in the cytoplasm and $\underline{A}$ = 0.05 ± 0.01 in vitro). This finding stands in surprising contrast to the results of the present paper obtained from amphibian skeletal muscle. (A less striking difference between the two studies concerns the somewhat different absolute level reported for the in vitro results obtained at a viscosity of 1 cP and in the absence of muscle constituents: $\underline{A}$ = 0.05 in Williams et al. and $\underline{A}$ = 0.11 in this paper.) The large difference in the in vivo values cannot be attributed to a large difference in fura-2's limiting anisotropy ($\underline{A}_o$) in the two measurement conditions ($\underline{A}_o \geq 0.34$ at λ = 380 nm in Williams et al.; $\underline{A}_o$ = 0.37 at λ = 420 nm in this paper). Rather, the surprisingly small value of $\underline{A}$ measured from the cytoplasm of smooth muscle would appear to require the presence of a relatively low viscosity solution (near 1 cP) as well as the lack of an association of nearly all of the dye with muscle constituents of large molecular weight. If correct, the difference between the smooth muscle results and our results indicates that the degree of binding of fura-2 may vary widely with preparation and/or experimental conditions. Alternatively, if completely or partially esterified fura-2 did not bind significantly to large molecular weight constituents of myoplasm, the small anisotropy value in smooth muscle might indicate the lack of complete hydrolysis to the free-acid form of the dye at the time of the anisotropy measurements.

Timmerman and Ashley (1986) concluded that fura-2 was not significantly bound in barnacle myoplasm, since its diffusion constant was reduced approximately 10-fold in comparison with that measured in free solution, a reduction similar to that observed with glycine, a molecule which they considered unlikely to be bound (cf. Stephenson et al., 1981). However, an alternative interpretation suggested by the measurements in this paper and in Baylor and Hollingworth (1988) is that both fura-2 and glycine were immobilized to a large extent, because of myoplasmic binding. In this regard, it would be of interest to know the viscosity of barnacle myoplasm and whether the fluorescence emission spectrum of fura-2 in barnacle muscle has a spectral shift of the type detected in Fig. 7 (attributed by us to the presence of a large fraction of protein-bound fura-2).

Klein et al. (1988) detected several discrepancies in the properties of fura-2 in the myoplasm of cut frog skeletal muscle fibers in comparison with those measured in in vitro calibration solutions lacking muscle components. As pointed out by these authors, the discrepancies might reasonably be attributed to the presence of bound fura-2 molecules in myoplasm. However, their most direct measurement of dye binding—related to the degree to which the concentration of dye which diffused to the center of the fiber exceeded that of the endpool solution (cf. Maylie et al., 1987*a–c*)—did not reveal a significant bound fraction of the dye. An explanation suggested to explain the various results was that fura-2 might in fact undergo significant binding while simultaneously being excluded from the water space within the myofilament lattice (cf. Timmerman and Ashley, 1986). In this regard, it would be of interest to characterize fura-2's fluorescence emission spectrum and anisotropy level in this preparation. It might also be of interest to evaluate dye concentration at the fiber center by a simultaneous monitoring of fura-2's absorbance and fluorescence changes during activity (cf. Baylor and Hollingworth, 1988).

CONCLUSION

Fura-2 binding to soluble myoplasmic proteins has been demonstrated in vitro. This binding appears to alter many properties of the dye directly related to the estimate of free Ca^{2+} concentration under intracellular conditions, such as the dye's fluorescence intensity, fluorescence emission spectrum, absorbance spectrum (and therefore, probably, the fluorescence excitation spectrum), and dissociation constant for Ca^{2+}. In vivo measurements also point to the presence of a large bound fraction of fura-2 in myoplasm, with corresponding alterations in the dye's fluorescence emission anisotropy and fluorescence emission spectrum (this study), as well as the Ca^{2+}–fura-2 reaction kinetics, the dye's diffusion constant and probably the dye's dissociation constant for Ca^{2+} (Baylor and Hollingworth, 1988). These alterations make it difficult to reliably estimate the time course and amplitude of the myoplasmic free Ca^{2+} concentration from measurements with fura-2 alone. Since the three soluble proteins (ALD, CK, GAPDH) used in this study are commonly distributed in many cell types, similar binding problems likely apply to fura-2 in the cytoplasm of cells other than skeletal muscle. The technical approaches used here and elsewhere (e.g., Laurence, 1952; Rodkey, 1961; Beeler et al., 1980; Baylor et al., 1982, 1986; Chaillet and Boron, 1985; Maylie et al., 1987*a,b*) should be helpful in characterizing dye binding in other preparations.

APPENDIX

This Appendix derives equations which relate total fluorescence intensity and fluorescence anisotropy ($\underline{A}$) to the fraction of protein-bound fura-2. The general approach is similar to that given by Laurence (1952) who derived analogous equations in terms of the related parameter, fluorescence polarization.

To obtain general forms of the equations, a mixture of n different populations of the dye is initially considered. If $\underline{F}(i)$ denotes total fluorescence intensity emitted from the ith population, and $F_\parallel(i)$ and $F_\perp(i)$ denote, respectively, components of the fluorescence measured parallel and perpendicular to a polarized excitation beam (see Methods), then

$$\underline{F}(i) = k \cdot [\underline{F}_\parallel(i) + 2F_\perp(i)] \quad (1 \leq i \leq n), \qquad \text{(A1)}$$

where k is a constant (see, e.g., Cantor and Schimmel, 1980). In the absence of interactive events between the fluorescent dye populations (quenching, energy transfer, etc.), the total fluorescence intensity emitted from the mixture, $\underline{F}$, can be expressed as the sum of that emitted from each population:

$$\underline{F} = \sum_i \underline{F}(i). \qquad \text{(A2)}$$

Similar relations apply for $F_\parallel$ and $F_\perp$, respectively:

$$F_\parallel = \sum_i F_\parallel(i) \tag{A3}$$

$$F_\perp = \sum_i F_\perp(i) \tag{A4}$$

Each $\underline{F}(i)$ can also be expressed as

$$\underline{F}(i) = C \cdot r(i) \cdot f(i), \tag{A5}$$

where C is the total molar concentration of the dye in the mixture, $f(i)$ is the mole fraction of the dye in the ith population ($\Sigma_i f(i) = 1$), and $r(i)$ is fluorescence intensity per unit molar concentration of the dye in the ith population.

Given Eq A5, Eq. A2 can then be rearranged to obtain

$$\underline{F} = C \cdot \sum_i [r(i) \cdot f(i)]. \tag{A6}$$

The anisotropy of the dye in the ith population ($\underline{A}(i)$) and of the mixture ($\underline{A}$) are defined as

$$\underline{A}(i) = \frac{F_\parallel(i) - F_\perp(i)}{F_\parallel(i) + 2F_\perp(i)} \tag{A7}$$

$$\underline{A} = \frac{F_\parallel - F_\perp}{F_\parallel + 2F_\perp} \tag{A8}$$

Given Eqs. A1–A7, Eq. A8 can be rewritten, after substitution and rearrangement, as

$$\underline{A} = \frac{1}{\sum_i [r(i) \cdot f(i)]} \sum_i [r(i) \cdot f(i) \cdot \underline{A}(i)]. \tag{A9}$$

As considered in the body of the paper, the number of dye populations can be reduced to two—(*a*) protein-free fura-2 and (*b*) protein-bound fura-2—if either of the following assumptions apply: (*a*) all binding sites on proteins bind dye molecules independently, with 1:1 stiochiometry and a single dissociation constant, but with protein-bound dye possibly having different anisotropy and total fluorescence intensity values; (*b*) all protein-bound dye molecules are identical in terms of anisotropy and total fluorescence values, but possibly have different values of dissociation constant.

Then, $\underline{F}$ and $\underline{A}$ of the mixture satisfy

$$\underline{F} = C \cdot [r(1) \cdot f(1) + r(2) \cdot f(2)] \tag{A10}$$

$$\underline{A} = \frac{r(1) \cdot f(1) \cdot \underline{A}(1) + r(2) \cdot f(2) \cdot \underline{A}(2)}{r(1) \cdot f(1) + r(2) \cdot f(2)}, \tag{A11}$$

where $f(1) + f(2) = 1$. In the case of assumption *a*, each protein-bound dye state occurs as a fixed fraction of all such states (i.e., for any $i \geq 2$, $f(i)/\Sigma_{j\neq 1} f(j)$ is a constant). Thus $r(2)$ in Eqs. A10 and A11 may be interpreted as the average of all $r(i)$ from these states (weighted according to the constant factors, $f(i)/\Sigma_{j\neq 1} f(j)$), whereas $\underline{A}(2)$ is the average of all $\underline{A}(i)$ from these states (weighted according to the constant factors, $r(i) \cdot f(i)/\Sigma_{j\neq 1} f(j)$). In the case of assumption *b*, $r(2)$ and $\underline{A}(2)$ are simply the values common to all protein-bound dye states.

$\underline{A}(1)$, $\underline{A}(2)$, $r(1)$, $r(2)$ can be experimentally determined by measuring $\underline{A}$ and $\underline{F}$ when all fura-2 molecules are in the protein-free form ($\underline{A} = \underline{A}(1)$, $\underline{F} = C \cdot r(1)$) and in the protein-bound form ($\underline{A} = \underline{A}(2)$, $\underline{F} = C \cdot r(2)$) (cf. Fig. 8 *B* for 0 protein and saturating protein, respectively). Note that, in general, $\underline{A}$, in contrast to $\underline{F}$, is a nonlinear function of the protein-bound fraction, $f(2)$, except in the case that fluorescence intensity is unchanged when dye binds to proteins ($r(1) = r(2)$).

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