IN VITRO CALIBRATION OF THE EQUILIBRIUM REACTIONS OF THE METALLOCHROMIC INDICATOR DYE ANTIPYRYLAZO III WITH CALCIUM

S. HOLLINGWORTH, R. W. ALDRICH,* AND S. M. BAYLOR
Department of Physiology, University of Pennsylvania, Philadelphia, Pennsylvania 19104-6085; and the
**Department of Neurobiology, Stanford University, Stanford, California 94305*

ABSTRACT The equilibrium reactions of the metallochromic indicator dye Antipyrylazo III with calcium at physiological ionic strength have been investigated spectrophotometrically. Dye absorbance as a function of wavelength was measured at various total dye and calcium concentrations. Analysis of the absorbance spectra indicated that at pH 6.9 at least three calcium:dye complexes form, with 1:1, 1:2, and possibly 2:2 stoichiometries. The dissociation constant and the changes in dye extinction coefficients on formation of the 1:2 complex, the main complex which forms when Antipyrylazo III is used to study cytoplasmic calcium transients, have been characterized.

INTRODUCTION

The metallochromic indicator dye Antipyrylazo III (Ap III), first used in biology by Scarpa et al. (1978), has been widely employed to study cytoplasmic calcium (Ca) transients in frog skeletal muscle fibers (Kovacs et al., 1979, 1983*a*; Palade and Vergara, 1981, 1982; Baylor, et al., 1982*b*; Kovacs and Szucs, 1983; Kovacs et al., 1983*b*; Baylor et al., 1983, 1985*a*, *b*; Quinta-Ferreira et al., 1984; Melzer et al., 1984; Irving et al., 1985; Rios et al., 1985; Maylie et al., 1987*b*). The dye-related absorbance signals, which can be readily measured following electrical stimulation once Ap III has been introduced into the myoplasm, arise in response to the myoplasmic Ca transient predominantly from the formation of a 1:2, Ca:Ap III, complex (Rios and Schneider, 1981; Kovacs et al., 1983). Calibrations of the reaction of Ap III with Ca, required for the quantitative interpretation of muscle absorbance signals, have been reported previously (Palade and Vergara, 1981; Rios and Schneider, 1981). However, a compelling analysis, based on measurements over a wide range of wavelengths and reactant concentrations, has not yet appeared. In particular, although the calibrations of Rios and Schneider correctly relate changes in the absorbance of the dye to buffered concentrations of free Ca, our own preliminary calibrations indicated that the values reported for the extinction coefficient changes and the dissociation constant of the 1:2 complex might involve compensating errors, both being underestimated by factors of about two. The error introduced in the quantitative interpretation of muscle Ca transients by underestimating the dye's extinction coefficient would be particularly important at high intracellular dye concentrations (Kovacs et al., 1983; Baylor et al., 1983), when Ap III may buffer a significant fraction of the myoplasmic calcium released during activity. The main aim, therefore, of the in vitro calibrations presented here was to measure the extinction coefficients and dissociation constant associated with the formation of the 1:2 complex.

The calibrations were carried out by measuring the absorbance spectra of solutions with various total dye and total calcium concentrations and by analyzing the spectra under assumptions about the equilibrium Ca:Ap III reaction. Complete spectra between 400 and 850 nm, rather than absorbance changes at a few wavelengths, were measured and analzyed, so that the contribution of Ca:Ap III complexes other than the 1:2 complex (Rios and Schneider, 1981; Palade and Vergara, 1981) could be better evaluated.

At pH 6.9, three Ca:Ap III complexes were identified having 1:1, 1:2, and possibly 2:2 stoichiometries. A fourth complex, likely due to the formation of divalent-free dimers of Ap III (Baylor et al., 1986), also influences the measured spectra and must be taken into account in the analysis.

At 720 nm, a wavelength frequently used to monitor the myoplasmic Ca transient, the change in extinction coefficient on forming the 1:2 complex (referred to a single dye molecule) is estimated to be 1.50×10^4 M^{-1} cm^{-1}. This value is about 1.8 times larger than that reported by Rios and Schneider (1981), who extrapolated from calibrations over a smaller range of Ca concentrations in which they assumed that only the 1:2 complex formed. The value obtained for the dissociation constant of the 1:2 complex at 20°C in 140 mM KCl and pH 6.9 was 35,100 μM^2. However, since intracellular binding of Ap III (Irving et al., 1985; Baylor et al., 1985*a*, 1986; Maylie et al., 1987*b*) may alter the effective dissociation constants of Ca:dye complexes, this value may not be appropriate for Ap III

within the myoplasm. A preliminary report of these findings has been presented (Hollingworth et al., 1986).

METHODS

Ap III absorbance spectra were measured between 400 nm and 850 nm in 2- or 5-nm increments using an ultraviolet/visible spectrophotometer (LKB Instruments, Inc., Gaithersburg, MD) interfaced to a PDP 11/73 computer (Digital Equipment Corp., Maynard, MA).

Solutions

Ap III (ICN K & K Laboratories Inc., Plainview, NY) was dissolved to the required concentration in a solution containing 140 mM KCl, 10 mM K_2 piperazine-N,N'-bis[2-ethane-sulfonic acid] (PIPES) as pH buffer and 10 μM EGTA. The EGTA served to bind contaminating calcium, the concentration of which was estimated to be between 5 and 10 μM, and possibly other metal ions. Additions of calcium were made to the dye solutions from a certified 1-M stock of $CaCl_2$ (BDH Chemicals Ltd, Poole, England) to give the required total calcium concentrations. All solutions were then retitrated to pH 6.90 (or in some cases, pH 4.0) before absorbance measurements were made. For the calibrations at pH 4.00, 10 mM K-acetate replaced PIPES as the pH buffer, and all spectra were corrected for a 10-μM calcium contamination since EGTA does not bind calcium strongly at this low pH. For calibrations in which the free calcium ion concentration, denoted [Ca], was buffered with 10 mM HEDTA (N-hydroxy-ethylethylenediaminetriacetic acid, potassium salt), the concentration of KCl was reduced to 100 mM. The apparent dissociation constant of HEDTA was calculated from the affinity constants for calcium ion binding and the various protonation reactions given in Martell and Smith (1974). The value obtained was 6.68 μM at 20°C for 0.1 M ionic strength, pH 6.90. During all measurements, the temperature within the cuvette housing was maintained at 20°C by placing the spectrophotometer in a refrigerator.

Analysis

Ap III absorbance spectra were measured at various concentrations of total dye, $[D]_T$, and total calcium, $[Ca]_T$. At each $[D]_T$, Ca-difference spectra were obtained from the absolute absorbance measurements by subtracting a spectrum measured in the absence of calcium. The Ca-difference spectra were analyzed under various assumptions about the reaction of calcium and Ap III at equilibrium. In order to obtain properties of the 1:2 Ca:Ap III complex (CaD_2), two additional complexes had to be considered: 1:1 Ca:Ap III (CaD), and divalent-free dimers of Ap III (D_2). Analytic solutions were derived to the equations describing the equilibria of the reaction schemes considered, namely, formation of CaD and CaD_2 or formation of D_2 and CaD_2. These solutions were fitted to the difference spectra by the method of least squares, to obtain best estimates of the dissociation constants of the complexes and of the changes in extinction coefficient at each wavelength, λ. Thus, for the scheme involving CaD and CaD_2, difference spectra were fitted with the equation

$$\Delta E(\lambda) = \Delta\epsilon_1(\lambda)\, f_1 + \Delta\epsilon_2(\lambda)\, f_2. \quad (1)$$

$\Delta E(\lambda)$ is the normalized absorbance change $\Delta A(\lambda)/([D]_T\, \ell)$, where ℓ is the pathlength of the cuvette. $\Delta E(\lambda)$ is, therefore, $\Delta A(\lambda)$ expressed in molar extinction units. The constants $\Delta\epsilon_1(\lambda)$ and $\Delta\epsilon_2(\lambda)$ are the changes in extinction coefficient on forming the 1:1 and 1:2 complexes, respectively. They are related to the absolute extinction coefficients of free dye, $\epsilon_D(\lambda)$, and of complexed dye, $\epsilon_{CaD}(\lambda)$ or $\epsilon_{CaD_2}(\lambda)$, by

$$\Delta\epsilon_1(\lambda) = \epsilon_{CaD}(\lambda) - \epsilon_D(\lambda) \quad (2)$$

for the 1:1 complex, and by

$$\Delta\epsilon_2(\lambda) = \epsilon_{CaD_2}(\lambda)/2 - \epsilon_D(\lambda) \quad (3)$$

for the 1:2 complex. Thus, $\Delta\epsilon_2(\lambda)$ calculated by Eq. 3 is per free dye molecule reacted. Its value must be doubled for comparison with the changes in extinction coefficient per CaD_2 complex formed, $\epsilon_{CaD_2}(\lambda) - 2\epsilon_D(\lambda)$, as calculated by Rios and Schneider (1981) and Palade and Vergara (1981).

In Eq. 1, f_1 and f_2 are the fractions of $[D]_T$ bound in the 1:1 and 1:2 forms, respectively. They depend on the experimental values of $[D]_T$ and $[Ca]_T$, and on the dissociation constants of the 1:1 and 1:2 complexes, K_1 and K_2. These latter values were adjusted during the fitting procedure to obtain the best fit. Thus, the fractions f_1 and f_2, corresponding to the combinations of $[Ca]_T$ and $[D]_T$ for each of the difference spectra to be fitted, were calculated for initially chosen values of K_1 and K_2. The analytic solutions to the reaction equilibria derived in the Appendix were used for these calculations (Eqs. A6 and A8 for the CaD and CaD_2 scheme). At each λ, $\Delta\epsilon_1(\lambda)$ and $\Delta\epsilon_2(\lambda)$ were then obtained by a linear least squares fit of Eq. 1 to the combined set of $\Delta E(\lambda)$ measurements. The squared deviations from the fits at each λ were then summed over all λ. New values of K_1 and K_2 were chosen and the process repeated until the best estimates of K_1 and K_2 were obtained. The best estimates were considered to be those which minimized the squared deviations summed over all λ.

RESULTS

If a Ca:dye reaction has as product a single stoichiometric Ca:dye complex, then the absorbance changes which occur when Ca is added to a dye solution will have the same spectral shape for all $[D]_T$ and $[Ca]_T$. For a given $[D]_T$ the amplitude of the absorbance change at any wavelength will increase monotonically with $[Ca]_T$ towards a saturating level, which corresponds to all of the dye having changed from the Ca-free to the Ca-bound form. The spectra plotted in Fig. 1 show clearly that at pH 6.9 Ap III does not form a single product in its reaction with Ca. Absolute spectra measured with 40 μM Ap III at increasing $[Ca]_T$ are plotted in Fig. 1 *A*, and the Ca-difference spectra obtained by subtracting the zero-Ca spectrum from the other spectra in Fig. 1 *A* are plotted in Fig. 1*B*. The Ca-difference spectra do not have the same shape, indicating that more than one Ca-Ap III complex must form. For instance, the isosbestic points (λ's for which $\Delta A(\lambda) = 0$) in Fig. 1 *B* occur at different wavelengths for different $[Ca]_T$, while at 720 nm, the absorbance change at first increases with $[Ca]_T$ but decreases again at higher $[Ca]_T$. Similar evidence for the formation of multiple Ca:Ap III complexes has been reported previously (Palade and Vergara, 1981; Rios and Schneider, 1981) and indicates that calibrations of the Ca:Ap III reaction must consider reaction schemes involving more than one Ca:Ap III complex. Alternatively, $[D]_T$ and $[Ca]_T$ might be restricted to ranges for which the formation of a single product can be assumed (cf. Rios and Schneider, 1981).

Analysis of Ca:Ap III Complexation at Low $[D]_T$

Baylor et al. (1986) have shown that in the absence of divalent cations the in vitro spectra of Ap III depend on $[D]_T$ in a manner suggesting that Ap III forms divalent-free dimers with a dissociation constant of about 4 mM. At

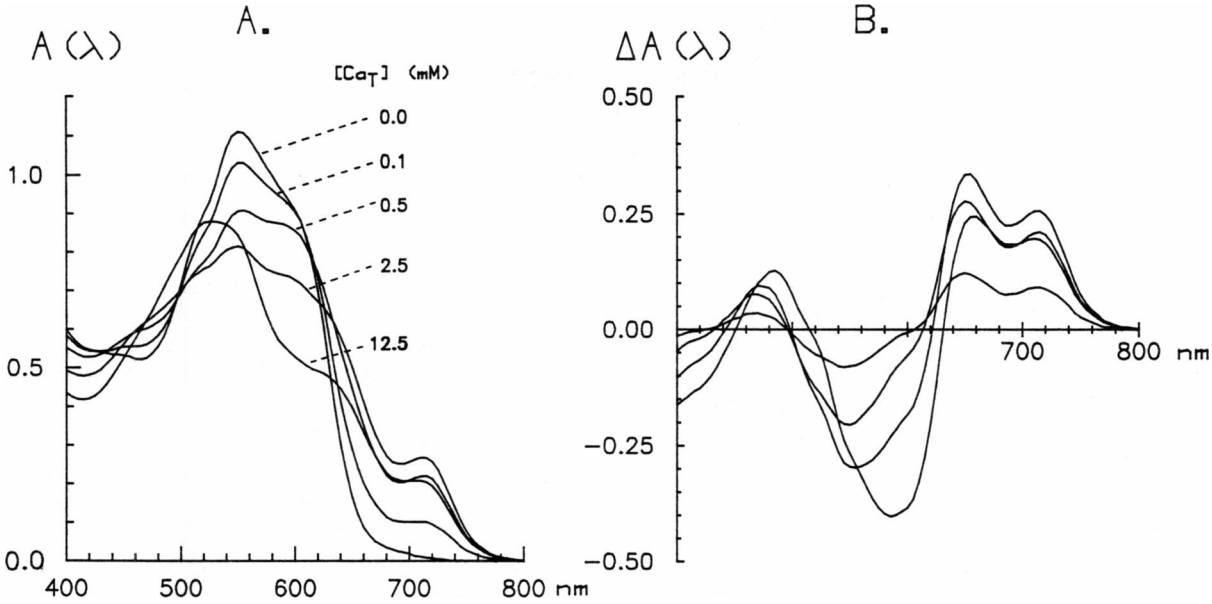

FIGURE 1 Absorbance spectra measured with 40 μM Ap III. (*A*) Absolute spectra measured at the $[Ca]_T$ indicated in millimolars. 40 μM Ap III in a 1.0 cm cuvette. (*B*) Ca-difference spectra obtained by subtracting the spectrum for $[Ca]_T$ = 0.0 mM from the other spectra in (*A*). pH 6.90, 20°C.

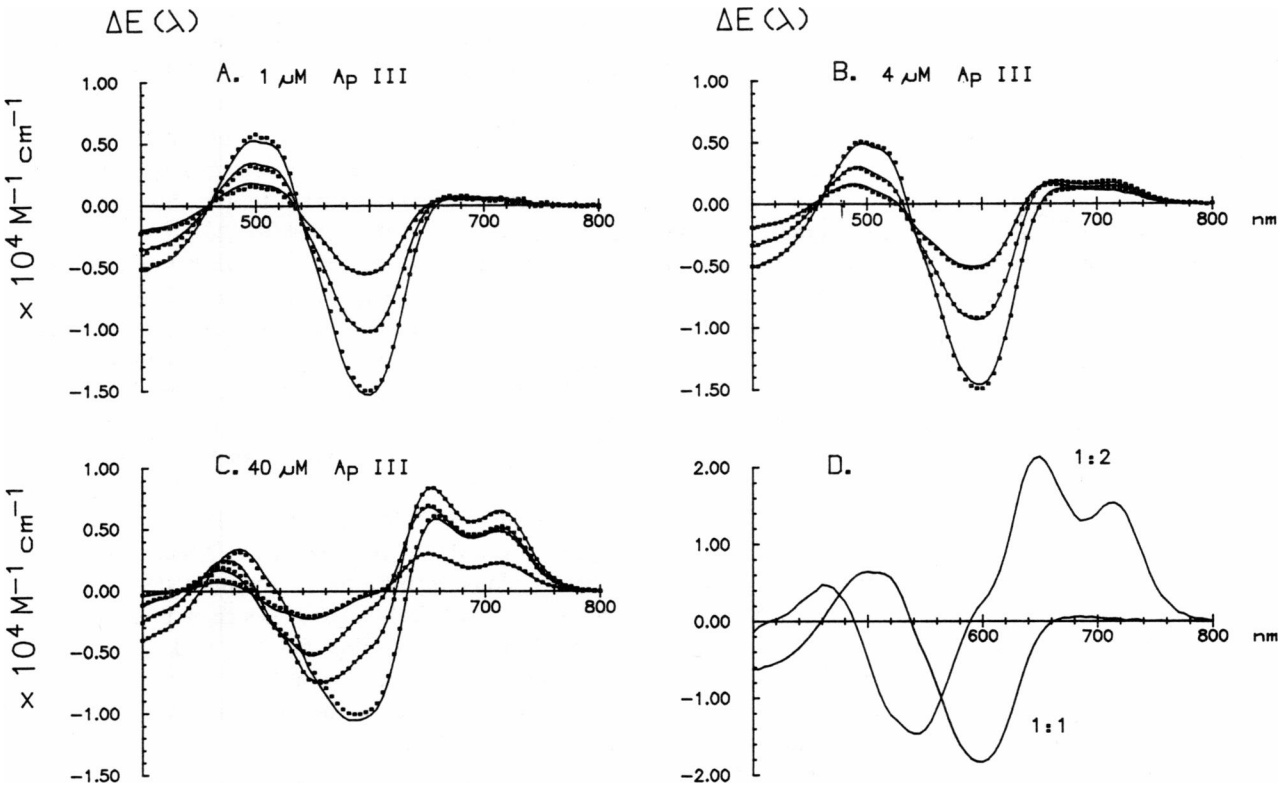

FIGURE 2 Ca–Ap III complexation at low $[D]_T$. (*A*), (*B*), and (*C*) (filled squares): Ca-difference spectra measured at $[D]_T$ of 1, 4, and 40 μM. Spectra are plotted in extinction units $\Delta E(\lambda) = \Delta A(\lambda)/([D]_T \ell)$ where ℓ is the cuvette pathlength. In (*A*) and (*B*), ℓ = 10 cm and $[Ca]_T$ = 1, 3, and 12.5 mM. Data in (*C*) are replotted from Fig. 1 *B*. Curves: calculated $\Delta E(\lambda)$ from the least squares best fit model of a scheme involving the formation of CaD and CaD_2 (see text for details). The spectrum for $[D]_T$ of 40 μM and $[Ca]_T$ of 12.5 mM was not included in the fitting due to possible interference from additional Ca–Ap III complexes (Fig. 4). The best fit value of K_1 was 2.2 mM and of K_2 was 35,100 μM^2. (*D*) Extinction spectra of the 1:1 and 1:2 complexes, $\Delta\epsilon_1(\lambda)$ and $\Delta\epsilon_2(\lambda)$, obtained from the least squares fit. pH 6.90, 20°C.

high dye concentrations, the presence of dimers will alter spectral shapes (Baylor et al., 1986) and, by reducing the concentration of monomers, will increase the apparent dissociation constant of Ca:Ap III complexes. The initial calibrations were therefore carried out at low $[D]_T$ ($\leq$40 μM) where dimer formation is negligible.

Fig. 2, *A, B,* and *C* (*squares*) show Ca-difference spectra obtained at three $[D]_T$ for increasing $[Ca]_T$. At each $[D]_T$ there is some evidence for multiple complex formation; more strikingly, the shapes of the curves at 1 μM and 40 μM $[D]_T$ are different. Those at 40 μM, particularly for small $[Ca]_T$, resemble spectra measured at relatively high $[D]_T$ and low [Ca] (Palade and Vergara, 1981; Baylor et al., 1982*b*), conditions under which the principal complex formed is CaD_2 (Palade and Vergara, 1981; Rios and Schneider, 1981). In contrast, the spectra at 1 μM Ap III more closely resemble magnesium–Ap III difference spectra (Baylor et al., 1982*b,* 1986) for which the principal complex is thought to be 1:1 with a dissociation constant of 6.7 mM (Rios and Schneider, 1981). Thus, a likely explanation for the shapes of the spectra in Fig. 2 is that Ap III can form both CaD and CaD_2 complexes. The curves in Fig. 2, *A, B,* and *C* are least squares best-fits of this two-complex scheme to the data. Fitting was carried out using Eq. 1 as described in Methods, and the fractions of $[D]_T$ complexed in the 1:1 and 1:2 forms were calculated by Eqs. A6 and A8 (see Appendix). The good agreement between measured and fitted spectra in Fig. 2 strongly suggests that at low $[D]_T$ Ap III forms two principal complexes with calcium and that these complexes have 1:1 and 1:2 Ca:Ap III stoichiometry.

The curves in Fig. 2 *D* are the best estimates of the changes in extinction coefficient, $\Delta\epsilon_1(\lambda)$ and $\Delta\epsilon_2(\lambda)$, which occur on formation of the two complexes. The spectrum of the 1:1 complex, with a peak decrease in absorbance close to 600 nm ($\Delta\epsilon_1[600] = -1.83 \times 10^4$ M^{-1} cm^{-1}) and negligible change in absorbance above 720 nm, has a shape closely similar to that measured previously for changes in pH or magnesium (Baylor et al., 1982*b,* 1986). (However, the isobestic wavelength observed at 538 nm for the 1:1 Ca:Ap III complex in Fig. 2 *D* is somewhat less than the 550 nm value previously observed for changes in pH or magnesium). The shape of the 1:2 spectrum in Fig. 2 *D* is similar to those published previously (Palade and Vergara, 1981; Baylor et al., 1982*b*). The value of $\Delta\epsilon_2$ estimated per dye molecule is 1.50×10^4 M^{-1} cm^{-1} at 720 nm, 2.20×10^4 M^{-1} cm^{-1} at 650 nm, and -1.41×10^4 M^{-1} cm^{-1} at 550 nm. The value of 1.50×10^4 M^{-1} per dye molecule (or 3.0×10^4 M^{-1} cm^{-1} per CaD_2 complex) obtained for $\Delta\epsilon_2$ at 720 nm, a wavelength where interference from absorbance changes due to the 1:1 complex should be minimal, is about 1.8 times larger than that previously reported by Rios and Schneider (1981).

In Fig. 2 the fitted values of the dissociation constant of the 1:1 and 1:2 complexes are 2.2 mM and 35,100 μM^2, respectively. Surprisingly, the value for the 1:2 complex is similar to that measured by Rios and Schneider (1981) and not larger by a factor of 1.8, i.e., in the same proportion as $\Delta\epsilon_2$ (720), as would have been expected if the difference between the calibrations lay only in the interpretation of the absorbance data. In fact, the measured absorbance changes at a given [Ca] are different. For instance, $\Delta A(720)$ measured by Rios and Schneider for $[D]_T$ of 50 μM and [Ca] of 100 μM is about 0.078 (their Fig. 1) whereas $\Delta A(720)$ estimated for the same $[D]_T$ and [Ca] from the fit to the data in Fig. 2 of this paper is 0.133, about 1.7 times larger. The source of this difference appears to be the pH buffers used: 10 mM PIPES in this study and 17.7 mM mono-tris[hydroxymethyl]aminomethane maleate (Tris-maleate) by Rios and Schneider.

Fig. 3 *A* compares Ca-difference spectra measured with PIPES (dotted curve) and Tris-maleate (full curve) as pH buffer. In each case $[D]_T$ was 50 μM and $[Ca]_T$ was 110 μM to give [Ca] close to 100 μM. The two curves have different shapes, probably because there is a larger relative contribution from the 1:1 complex when Tris-maleate is present. At 720 nm, where absorbance changes due to the 1:1 complex should be minimal, $\Delta A(720)$ in Tris-maleate is 0.079, closely similar to the value measured by Rios and Schneider. In comparison, $\Delta A(720)$ measured in PIPES is 0.136, about 1.7 times larger than that measured in Tris-maleate. Tris-maleate does not appear to buffer a substantial fraction of the added Ca (Sillen and Martell, 1964; Fischer et al., 1979), a finding which we also confirmed. The zero-Ca spectrum of Ap III is, however, altered in the presence of Tris-maleate, suggesting an interaction between Tris-maleate and Ap III. The spectrum in Fig. 3 *B* is the difference spectrum obtained by subtracting the zero-Ca spectrum measured with PIPES from that measured with Tris-maleate. The shape of this spectrum is similar but not identical to that measured previously for the formation of divalent-free dimers of Ap III (Baylor et al., 1986). No consistent evidence for a similar change in the zero-Ca spectrum could be found when spectra measured with PIPES were compared to spectra measured with 10 mM EGTA alone as pH buffer. (With 10 mM PIPES as pH buffer, EGTA concentrations between 20 μM and 10 mM had no effect on the zero-Ca spectra). Thus the most likely explanation for the reduced amplitude of $\Delta A(720)$ in the presence of Tris-maleate is an interaction between Tris-maleate and Ap III which increases the apparent dissociation constant of the 1:2 Ca:Ap III complex to about 1.7 times its value when measured with PIPES.

Calibrations at pH 4.0

In order to obtain a direct measurement of $\Delta\epsilon_2(\lambda)$, a large fraction of Ap III must be driven into the 1:2 form (f_2 approaching 1.0). This is difficult to attain at pH 6.9 because increasing $[Ca]_T$ reduces [D] and, since the ratio f_1/f_2 is proportional to $1/[D]$, favors the formation of 1:1 complex. The similarity of the 1:1 Ca:Ap III difference

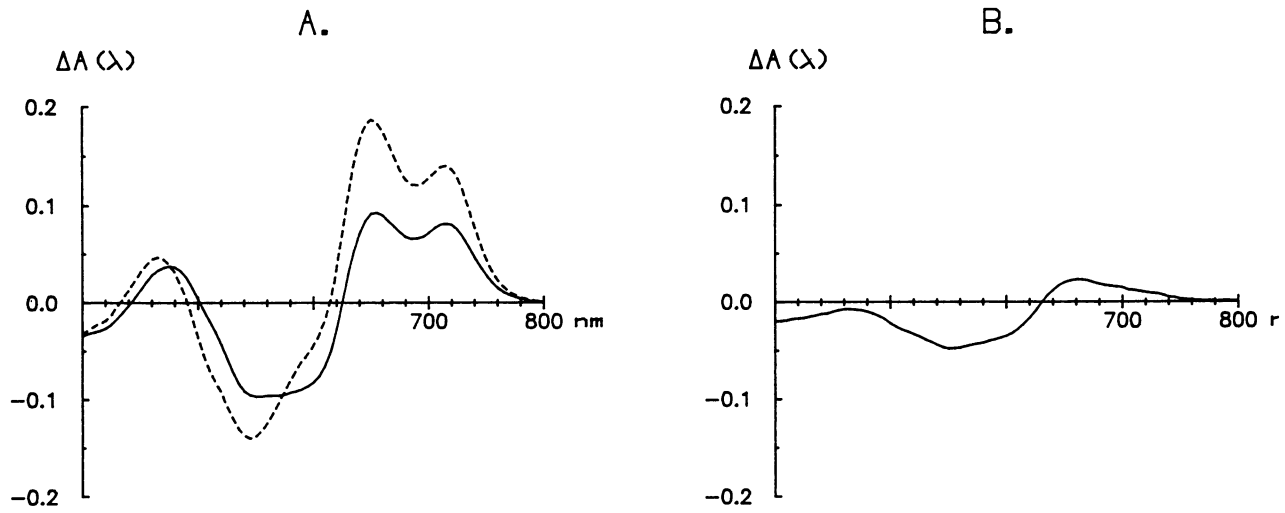

FIGURE 3 Comparison of Ap III-difference spectra measured with PIPES or Tris-maleate as pH buffer. (*A*) Ca-difference spectra measured at 50 μM $[D]_T$ and 110 μM $[Ca]_T$ with 10 mM PIPES (dotted curve) or 17.7 mM Tris-maleate (solid curve) as pH buffer. (*B*) Divalent-free difference spectrum obtained by subtracting the zero-Ca spectrum measured in 10 mM PIPES from that measured in 17.7 mM TRIS-maleate ($[D]_T$ = 50 μM). pH 6.9, 20°C.

spectrum to the Ap III difference spectra for changes in pH or magnesium ion concentration suggests that calcium, magnesium and hydrogen ions might compete for the same 1:1 binding site. If so, the apparent dissociation constants of the 1:1 complexes of Ap III with calcium and magnesium would depend on the hydrogen ion concentration, increasing with decreasing pH. For example, a strong dependence of dissociation constant on pH has been reported previously for the 1:1 complexes of magnesium with Arsenazo I and Arsenazo III (Baylor et al., 1982*a*).

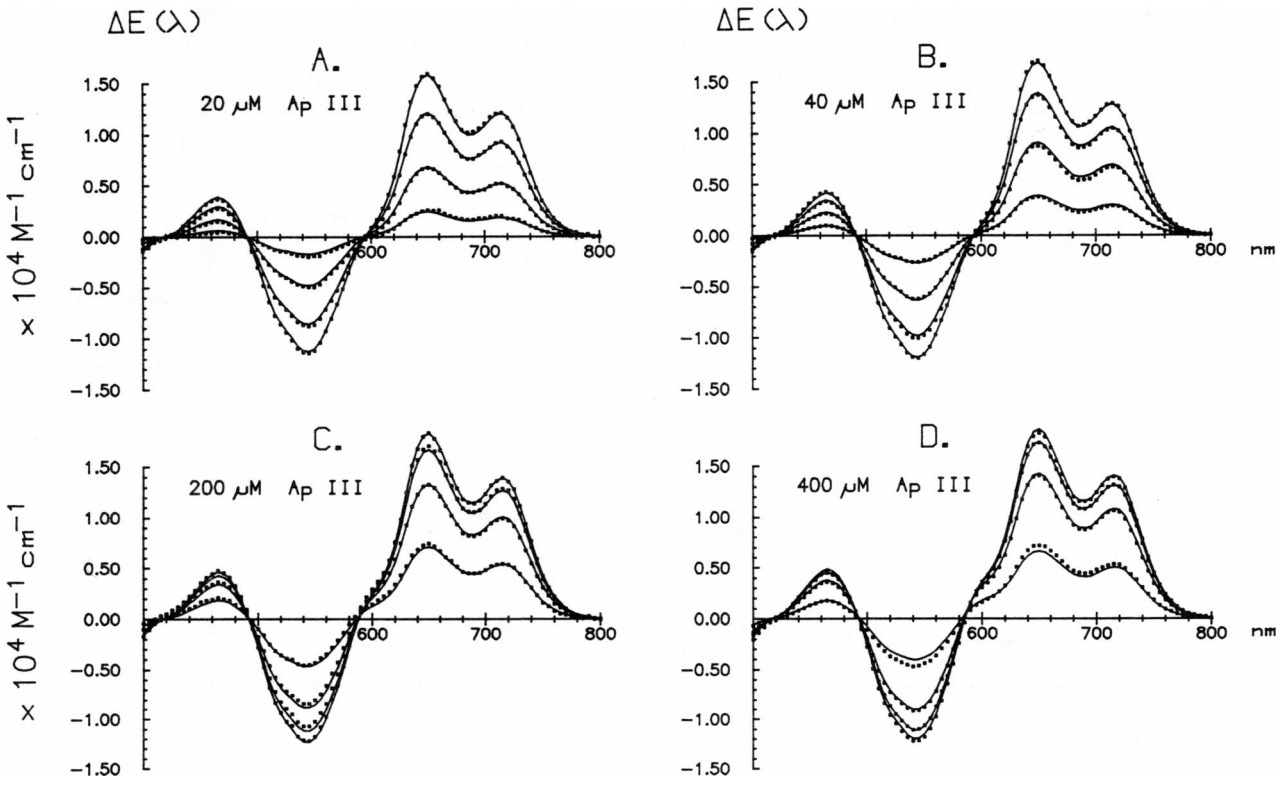

FIGURE 4 Ca–Ap III complexation at pH 4.0. (filled squares) Ca-difference spectra measured at $[D]_T$ of 20, 40, 200, and 400 μM. In (*A*) and (*B*) ℓ = 1.0 cm; in (*C*) and in (*D*), ℓ = 0.1 cm. For all $[D]_T$, $[Ca]_T$ = 0.1, 0.5, 2.5 and 12.5 mM. (solid curves) Calculated spectra from the least squares best fit of a scheme involving the formation of D_2 and CaD_2. The best fit value of K_2 was 25,400 μM^2, and of K_d was 1.9 mM. $\Delta\epsilon_2(720)$ was 1.46 × 10^4 M^{-1} cm^{-1}. pH 4.0, 20°C.

Thus, formation of the 1:1 Ca:Ap III complex might be suppressed at low pH. Preliminary calibrations indicated that this was indeed the case and that at pH 4.0 a large fraction of Ap III could be driven into the 1:2 form by increasing $[Ca]_T$. Furthermore, since changes in pH produce only small changes in absorbance at 720 nm, $\Delta\epsilon_2(720)$ measured at pH 4.0 should be close to the value which would be measured at pH 6.9.

Calibrations were carried out therefore at pH 4.0, and Fig. 4 shows Ca-difference spectra (filled squares) measured at four values of $[D]_T$. The shapes of the difference spectra at each $[D]_T$ are closely similar, resembling that of the 1:2 complex in Fig. 2 *D*. There is little evidence in the shapes for a contribution from the 1:1 complex, and furthermore, the amplitudes of the absorbance changes increase monotonically with increasing $[Ca]_T$. Thus, the data of Fig. 4 appear to be consistent with the formation of a single, CaD_2 complex. There are, however, slight differences between the spectra in panels *C* and *D* and those in *A* and *B*, which were measured at a 10-fold lower $[D]_T$. For instance, the isobestic wavelength which is between 590 nm and 600 nm at low $[D]_T$ is between 580 nm and 590 nm at the higher $[D]_T$. Examination of the zero-Ca spectra at pH 4.0 (data not shown) demonstrated the existence of divalent-free dimers at this pH, similar to those observed at pH 6.9 (Baylor et al., 1986) and a likely explanation for the difference between the low and high $[D]_T$ spectra in Fig. 4 is the influence of divalent-free dimers. The spectra at pH 4.0 were fitted, therefore, with an equation of the same form as Eq. 1 but involving the formation of D_2 and CaD_2 complexes. The fractions of $[D]_T$ complexed as D_2 and CaD_2, f_d and f_2 respectively, were evaluated according to Eqs. A12 and A13 (see Appendix). The least squares best fit is plotted as curves in Fig. 4, and gives an excellent description of the measured data. The fitted value of $\Delta\epsilon_2(720)$, $1.46 \times 10^4\ M^{-1}\ cm^{-1}$ per dye molecule, is in close agreement with the value $1.50 \times 10^4\ M^{-1}\ cm^{-1}$ estimated from the fit at pH 6.9 (Fig. 2).

The 720-nm absorbance changes at pH 6.9 and pH 4.0 are compared in Fig. 5, where they are plotted against $[Ca]_T$. Data points are plotted as squares and the curves are the calculated $\Delta E(720)$ from the best fit models of Figs. 2 and 4. The contrast between the two sets of calibrations is clearly seen for $[D]_T$ of 40 μM. At pH 4.0, $\Delta E(720)$ rises monotonically towards the predicted saturating change ($\Delta\epsilon_2(720) = 1.46 \times 10^4\ M^{-1}\ cm^{-1}$), while at pH 6.9, $\Delta E(720)$ is maximal at less than half the predicted value, for $[Ca]_T$ of about 2 mM. $\Delta E(720)$ decreases at higher $[Ca]_T$ where formation of the 1:1 complex, which has almost no absorbance at 720 nm, is favored. Also included at pH 6.9 are the measured (open squares) and predicted (dotted curve) changes for $[D]_T$ of 400 μM. The predicted curve is calculated from the model of Fig. 2, which gave an excellent fit to the pH 6.9 data for $[D]_T$ between 1 and 40 μM. However, at $[D]_T$ of 400 μM the measured absorbance changes are significantly smaller than predicted. A small reduction in $\Delta E(720)$ for low $[Ca]_T$ is expected, due to the presence of divalent-free dimers at the higher $[D]_T$ (see Fig. 6), but this cannot explain the large discrepancies apparent in Fig. 5. A more probable explanation is that at least one additional Ca:Ap III complex forms at high $[Ca]_T$ and high $[D]_T$, for which a likely stoichiometry is therefore Ca_2D_2 (cf. Palade and Vergara, 1981, 1983). The absorbance changes at $[D]_T$

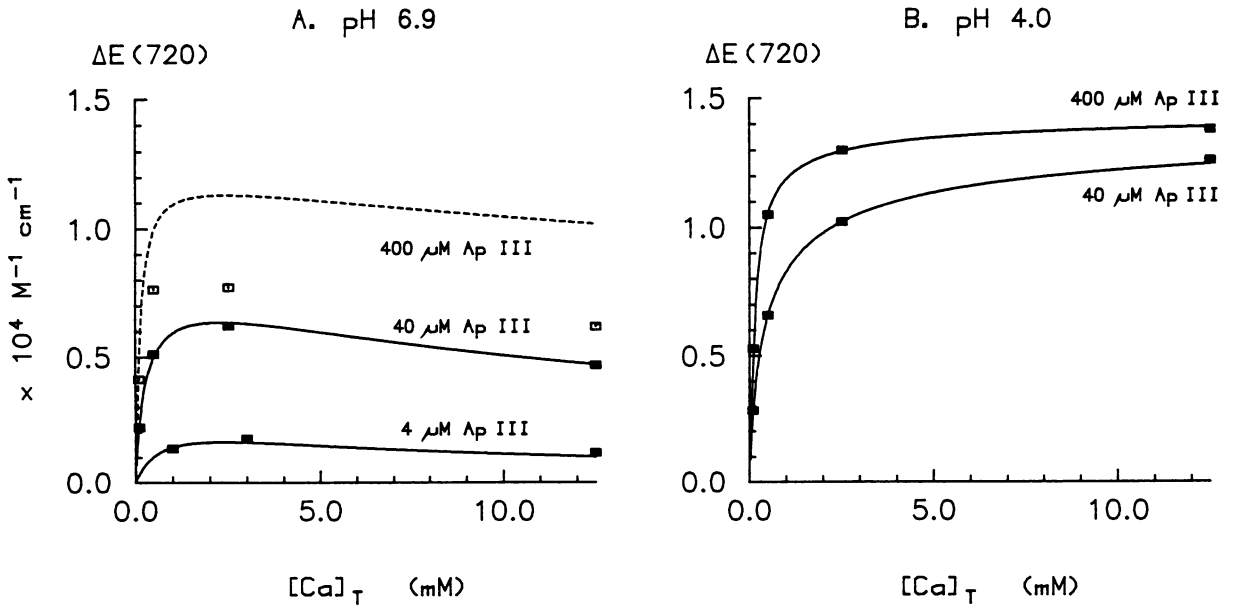

FIGURE 5 Comparison of calibrations at pH 6.90 and pH 4.0. (filled squares) $\Delta E(720)$ measured at pH 6.90 (*A*) for $[D]_T$ of 4 and 40 μM and at pH 4.0 (*B*) for $[D]_T$ of 40 and 400 μM plotted against $[Ca]_T$. (solid curves) $\Delta E(720)$ calculated from the best fit models of Fig. 2 *A* and Fig. 4 *B*. Open squares and dotted curve in (*A*) are $\Delta E(720)$ measured and calculated (model of Fig. 2) for $[D]_T$ of 400 μM at pH 6.9.

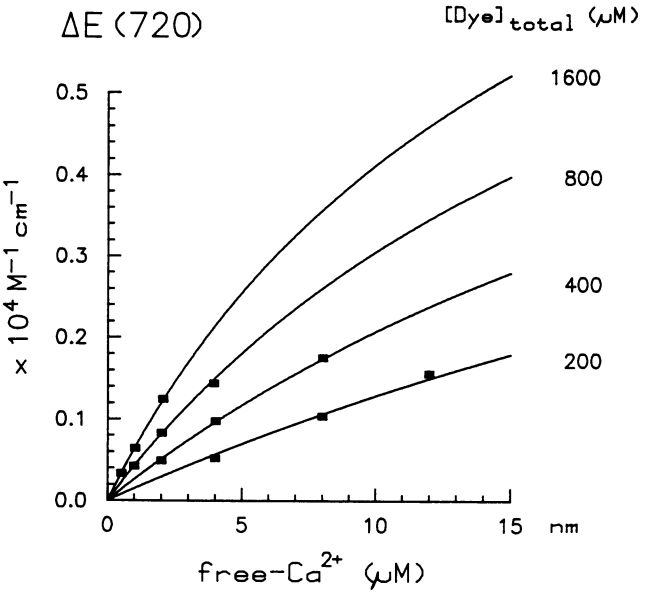

FIGURE 6 Ap III complexation at high $[D]_T$. (filled squares) $\Delta E(720)$ plotted against free-Ca concentration at the indicated $[D]_T$. Free [Ca] buffered using 10 mM HEDTA (see Methods for further details). (*Curves*): $\Delta E(720)$ calculated from the least squares best fit of a scheme involving the formation of D_2 and CaD_2. The best fit value of K_2 was 34,300 μM^2 and of K_d was 5.4 mM. $\Delta\epsilon_2(720)$ was fixed to be 1.50×10^4 M^{-1} cm^{-1} (Fig. 2). pH 6.90, 20°C.

equal to 400 μM were not investigated further, however, because the properties of the 1:2 complex, which is the main focus of these calibrations, appear to be well estimated at low $[D]_T$.

Fig. 5 also illustrates the probable reason for the lower value of $\Delta\epsilon_2(720)$ estimated by Rios and Schneider (1981), who restricted their calibrations to free-Ca concentrations less than 1 mM. They assumed that only CaD_2 complex formed in this range, and extrapolated from the measurements to obtain $\Delta\epsilon_2(720)$. Formation of the 1:1 complex (and also of the additional complex at higher $[D]_T$, however, reduces $\Delta E(720)$, even at free-Ca concentrations of less than 1 mM. This leads to an apparent saturation and, therefore, to an underestimate of the extrapolated value of the maximum absorbance change. For example, if our pH 6.9 data at $[D]_T$ of 40 and 400 μM and for $[Ca]_T$ of less than 1 mM (four points in Fig. 5 *A*) are fitted under the assumption that only the 1:2 complex forms, a lower value for $\Delta\epsilon_2(720)$, 1.05×10^4 M^{-1} cm^{-1} per dye molecule, is obtained. This value is much closer to that measured by Rios and Schneider.

Calibrations at High D_T and Low [Ca]

In order that Ap III signals monitoring the myoplasmic Ca transient may be well resolved, dye concentrations of 0.5–1 mM are often used. Under these conditions, CaD_2 is the main complex which forms. The apparent dissociation constant of the complex will, however, be modified by the presence of divalent-free dimers. In order to examine this effect, calibrations were carried out at a range of $[D]_T$ (0.2–1.6 mM) using the Ca-buffer HEDTA to set free [Ca] in the low micro-Molar range. The results of these calibrations are shown in Fig. 6 in which the values of $\Delta E(720)$ for each $[D]_T$ (filled squares) are plotted against free [Ca].

Since divalent-free dimers do not absorb appreciably above 700 nm (Baylor et al., 1986) and because there will be little contamination from the 1:1 complex at the low [Ca] and high $[D]_T$ used, the absorbance changes were fitted with Eq. 4,

$$\Delta E(\lambda) = \Delta\epsilon_2(\lambda)\, f_2, \tag{4}$$

in which λ was restricted to the range 700–750 nm. For each λ in this range $\Delta\epsilon_2(\lambda)$ was fixed to the value obtained at pH 6.9 and low $[D]_T$ (Fig. 2 *D*), and f_2 was calculated under the assumption that only CaD_2 and D_2 formed, according to Eqs. A15, A17, and A18. The curves in Fig. 6 are calculated from a single fit to all of the data to obtain the least squares best estimates of the dissociation constants of CaD_2 and D_2, namely, K_2 and K_d respectively. The best-fit value of K_2, 34,300 μM^2, is in good agreement with that obtained at low $[D]_T$, 35,100 μM^2. The best fit value of K_d was 5.2 mM, in reasonable agreement with the value of 4.0 mM given by Baylor et al. (1986), which was estimated from fits to changes in the zero-Ca absorbance spectra. These calibrations at high $[D]_T$, conditions similar to those employed within a muscle fiber, confirm that under our in vitro conditions K_2 is probably close to 35,000 μM^2. However, they also indicate that the influence of divalent-free dimer formation must be considered. The effect of dimerization can be expressed in terms of an apparent dissociation constant of the 1:2 complex, $K_2^{apparent}$, defined as

$$K_2^{apparent} = [Ca]\,([D]_T - 2\,[CaD_2])^2/[CaD_2]. \tag{5}$$

It then follows that for the case when the free magnesium ion concentration, [Mg], is zero, $K_2^{apparent}$ is given by

$$K_2^{apparent} = K_2\,(1 + [D]/(K_d/2))^2. \tag{6}$$

In the presence of magnesium the analogous relationship is given by

$$K_2^{apparent} = K_2\,(1 + [D]/(K_d/2) + [Mg]/K_{Mg})^2. \tag{7}$$

K_{Mg}, the dissociation constant for the 1:1 Mg:Ap III complex, has been previously estimated by Rios and Schneider (1981) to be 6.7 mM.

DISCUSSION

Comparison with Previous Calibrations

The results presented here demonstrate that at pH 6.9 Ap III has a complicated reaction with Ca. Two Ca:Ap III complexes, with CaD and CaD_2 stoichiometries, are required to explain the calibrations at low dye concentra-

tions ($[D]_T \leq 40$ μM, Fig. 2) and at least one additional Ca:dye complex is required to explain calibrations at higher $[D]_T$ (Fig. 5 *A*). The properties of this third complex were not investigated, but a likely possibility is that the complex has Ca_2D_2 stoichiometry.

The change in extinction coefficient of the 1:2 complex, $\Delta\epsilon_2(720)$, estimated from the fit at low $[D]_T$ was 1.50×10^4 M^{-1} cm^{-1}. This value was confirmed in calibrations: (*a*) at pH 4.0, where formation of the 1:1 complex was suppressed and a large fraction of the dye was driven into the 1:2 form (Fig. 4), and (*b*) at high $[D]_T$ and low free-Ca (Fig. 6) provided that the formation of divalent-free dimers was also considered. This estimate of $\Delta\epsilon_2(720)$ is about 1.8 times larger than that previously reported by Rios and Schneider (1981) and 1.5 times larger than that proposed in a preliminary report by Palade and Vergara (1981). Rios and Schneider measured absorbance changes at 720 and 550 nm for a limited range of $[D]_T$ (50–700 μM) and [Ca] (1 μM to 0.8 mM) and fitted this data under the assumption that only the 1:2 complex formed. However, the 1:1 complex does form in this limited range and reduces the measured absorbance changes with increasing [Ca], thus leading to an underestimate of $\Delta\epsilon_2(\lambda)$ in the 1:2 fits. Formation of the third Ca:Ap III complex at high $[D]_T$ will further contribute to this interference. The properties of the additional Ca:Ap III complexes could be more readily identified in the present study in which complete spectra, rather than absorbance changes at two specific wavelengths, were measured.

Palade and Vergara (1981) first proposed the three-complex scheme (CaD, CaD_2 and Ca_2D_2) for Ap III. They fitted this scheme at three wavelengths to data obtained from a wide range of $[Ca]_T$ at two values of $[D]_T$ (500 and 12.5 μM). However, they considered that there were too many parameters to characterize each complex accurately and viewed the fitted values as preliminary. It is not surprising, therefore, that their value of $\Delta\epsilon_2(710)$ (1.02×10^4 M^{-1} cm^{-1}) differs from that reported here (1.53×10^4 M^{-1} cm^{-1}, Fig. 2 *D*), particularly when one considers that the large value which they reported for $\Delta\epsilon_2(590)$ (2.37×10^4 M^{-1} cm^{-1}) indicates that they were attributing 1:1 absorbance changes to the 1:2 complex.

An initially suprising finding of this study was that the value estimated for the dissociation constant of the 1:2 complex (35,100 μM^2) is close to that measured by Rios and Schneider (1981) and not larger in the same proportion as $\Delta\epsilon_2(720)$, as would have been expected if the difference between the calibrations lay only in the interpretation of the absorbance data. In fact, the measured absorbance changes at a given [Ca] are different. The source of this difference appears to be an interaction between Ap III and the Tris-maleate buffer used by Rios and Schneider, which reduces the apparent dissociation constant of the 1:2 complex. Best and Abramcheck (1985) have reported previously that caffeine interacts with Arsenazo III to form a 1:1 complex with a dissociation constant of about 20 mM. They also observed an interaction between caffeine and Ap III, but the reaction was not studied in detail.

Relation to Intracellular Measurements

One serious problem with all of the indicator dyes so far used in skeletal muscle is that they appear to bind to intracellular constituents (Beeler et al., 1980; Baylor et al., 1982*a,b,* 1985 *a,b,* 1986; Irving et al., 1985; Maylie et al., 1987*a,b,c;* Hollingworth and Baylor, manuscript in preparation). In the case of Ap III, ~⅔–¾ of the dye is probably bound within the myoplasm. It is important, therefore, to consider the extent to which dye properties measured in ionic calibrating solutions are appropriate for interpreting absorbance signals measured from the dye within the myoplasm.

In many instances, the binding of dye appears to result in a small shift (5–10 nm) in the absorbance spectra to longer wavelengths (Baylor et al., 1986). As a result, some alteration in the extinction coefficients of dye within the myoplasm compared to ionic solutions in cuvette must occur. However, these spectral effects of binding are not large and the peak values of cuvette extinction coefficients can probably be applied to intracellular dye signals with little error.

In contrast, the effects of dye binding on the on- and off-rates of the reaction of Ca with the dyes and of the dissociation constants of Ca:dye complexes do not appear to be small. Baylor et al. (1982*a*) concluded from the wide variation in free-magnesium concentration (0.3–6.1 mM), estimated with three different indicator dyes (Dichlorphosphonazo III, Arsenazo III, and Arsenazo I), that at least two of the dyes behave quite differently in myoplasm compared with cuvette. The most likely cause of this variation was thought to be the effect of binding on the dissociation constants of the dyes for magnesium. Similarly, a wide variation has been reported for the amplitude of the myoplasmic Ca-transient in cut fibers, which is 2–3 μM when calibrated with Ap III (Irving et al., 1985; Maylie et al., 1987*b*) but approximately 20 μM when calibrated with tetramethylmurexide (TMX), the least bound of any indicator dye so far used (bound fraction apparently 27%—Maylie et al., 1985, 1987*c*). One possible explanation for this discrepancy is that the bound fraction of Ap III can no longer react normally with calcium (Irving et al., 1985; Maylie et al., 1987*b*). Measurements using two of the tetracarboxylate indicator dyes, Azol (Tsein, 1983) and Fura2 (Grynkiewicz et al., 1985), have indicated that intracellular binding appears to slow the off-rates of both of these dyes in myoplasm compared to cuvette by factors of about eight and four respectively (Baylor et al., 1985*b;* Hollingworth and Baylor, manuscript in preparation). Estimates of the on-rates of the two

depend on the calibration of [Ca], which is uncertain, but they appear to be reduced by a factor of at least 6–7 and possibly by as much as 20–70. It is likely, therefore, that the dissociation constants of the tetracarboxylate dyes in myoplasm also differ from those measured in cuvette. In general, in the presence of intracellular binding of dye, the use of dissociation constants obtained from in vitro calibrations in ionic solutions to analyze intracellular dye signals must be approached with considerable caution.

Although the calibration of myoplasmic Ap III signals in terms of changes in [Ca] is uncertain, Ap III remains one of the most promising intracellular Ca-indicators currently available. Importantly, the waveform of the Ap III absorbance signals measured at 650 or 720 nm appears to closely follow changes in myoplasmic [Ca], although probably with a small kinetic delay of between 1 and 2 ms (Baylor et al., 1985*b;* Maylie et al., 1987*c*). TMX also responds with a rapid signal from the myoplasm. However, about 13% of TMX appears to be bound with Ca at rest (attributed to entry into a high [Ca] environment) and, following stimulation, the Ca:dye signal undershoots the baseline after its initial rise (Maylie et al., 1985, 1987*c*). Thus, in addition to changes in myoplasmic [Ca], TMX appears to respond to a second process, possibly reflecting changes of [Ca] within the sarcoplasmic reticulum. Ca-related signals obtained from the myoplasm with Arsenazo III, another widely used indicator dye, are slower than those measured with Ap III (Palade and Vergara, 1982; Baylor et al., 1983), probably as a result of the slower formation of a second Ca:dye complex with a time constant of about 10 ms (Quinta-Ferreira et al., 1984). The two tetracarboxylate dyes which have been tested in skeletal muscle, Azo1 and Fura2, also give signals which are significantly slower than those measured with Ap III, with approximate time constants of 7 and 40 ms, respectively (Baylor et al., 1985; Hollingworth and Baylor, manuscript in preparation).

The absorbance signal from the 1:2 Ca:Ap III complex is, therefore, probably one of the most reliable indicators of the time course of changes in myoplasmic [Ca]. The extinction coefficients reported in this paper allow the measured absorbance signal to be calibrated in terms of the changes in the concentration of CaD_2. The accurate calibration of this signal in terms of the underlying change in [Ca] must, however, await a better understanding of how intracellular binding alters the apparent value of K_2 in myoplasm.

APPENDIX

The purpose of this appendix is to derive solutions to the equations describing the equilibria of the Ca–Ap III reaction schemes considered in the body of the paper.

Three equilibrium complexes are considered: 1:1 and 1:2 Ca:Ap III complexes (CaD and CaD_2), and divalent-free Ap III dimers (D_2). The equilibrium concentrations of these complexes are given by

$$f_1 K_1 D_T = Ca\ D \tag{A1}$$

$$f_2 K_2 D_T = 2\ Ca\ D^2 \tag{A2}$$

and

$$f_d K_d D_T = 2\ D^2. \tag{A3}$$

Ca and D are the concentrations of free-calcium and free-dye. f_1, f_2, and f_d are the fractions of total dye concentration, D_T, bound as CaD, CaD_2 and D_2 respectively, and K_1, K_2 and K_d are the dissociation constants of the three complexes. For simplicity, concentration brackets and valences have been omitted in all equations.

Formation of CaD and CaD_2

The concentrations of free-dye, D, and free-calcium, Ca, are given by

$$D = D_T(1 - f_1 - f_2) \tag{A4}$$

and

$$Ca = Ca_T - D_T(f_1 + f_2/2) \tag{A5}$$

where Ca_T is the total Ca concentration. From Eqs. A1, A2, and A4:

$$f_2 = \frac{(1 - f_1)\, f_1}{K + f_1} \tag{A6}$$

where

$$K = K_2/(2\ K_1\ D_T). \tag{A7}$$

Eqs. A1, A4, A5, and A6 can be combined to give

$$f_1^3 + \frac{1}{1 - K'}\left[\frac{Ca_T K_2}{2\ D_T K_1^2} - \frac{K_2^2}{4\ D_T K_1^3} + \frac{K_2}{K_1\ D_T}\right] f_1^2 + \frac{1}{1 - K'} \cdot \left[-\frac{Ca_T K_2}{2\ D_T K_1^2} + \frac{K_2^2}{4\ D_T K_1^3} + \frac{K_2}{4 K_1^2} + \frac{Ca_T K_2^2}{4 D_T^2 K_1^3} + \frac{K_2^2}{4 K_1^2 D_T^2}\right] f_1 - \frac{1}{1 - K'}\left[\frac{Ca_T K_2^2}{4 D_T^2 K_1^3}\right] = 0 \tag{A8}$$

where

$$1/(1 - K') = 4 K_1^2/(4 K_1^2 - K_2). \tag{A9}$$

Eqs. A8 and A6 can, therefore, be solved to give the fractions f_1 and f_2.

Formation of CaD_2 and D_2

Here Ca and D are given by

$$Ca = Ca_T - f_2\ D_T/2 \tag{A10}$$

and

$$D = D_T\ (1 - f_2 - f_d). \tag{A11}$$

From Eqs. A2, A3, and A10,

$$f_d = \frac{2\ f_2\ K_2/K_d}{2\ Ca_T - f_2\ D_T}, \tag{A12}$$

which, combined with Eqs. A2, A10, and A11, gives

$$f_2^4 - \left[2 + \frac{4\,Ca_T}{D_T} + \frac{4\,K_2}{K_d\,D_T}\right] f_2^3 + \left[\frac{K_2}{D_T^2} + 1 + \frac{8\,Ca_T}{D_T} + \frac{4\,Ca_T^2}{D_T^2} + \frac{4\,K_2}{D_T K_d} + \frac{8\,Ca_T K_2}{D_T^2 K_d} + \frac{4\,K_2^2}{D_T^2 K_d^2}\right] f_2^2 - \left[\frac{2\,Ca_T K_2}{D_T^3} + \frac{4\,Ca_T}{D_T} + \frac{8\,Ca_T^2}{D_T^2} + \frac{4\,Ca_T K_2}{D_T^2 K_d}\right] f_2 + \frac{4\,Ca_T^2}{D_T^2} = 0. \quad (A13)$$

Eqs. A13 and A12 can, therefore, be solved for f_2 and f_d.

Formation of CaD_2 and D_2 with Buffered Free-calcium

Here the analysis follows that of Rios and Schneider (1981) but allows for the formation of D_2 in addition to CaD_2. At a given Ca concentration, Eqs. A2 and A3 give

$$f_d = K_2\,f_2/(K_d\,Ca) \quad (A14)$$

and from Eqs. A2, A12, and A14,

$$f_2^2\,(1 + K_2/[K_d\,Ca])^2 - f_2\,(2\,[1 + K_2/(K_d\,Ca)] + K_2/[2\,Ca\,D_T]) + 1 = 0 \quad (A15).$$

Ca is obtained from the buffer equilibrium using Ca_T modified for Ca bound to the dye, i.e., by definition

$$Ca'_T = Ca_T - f_2\,D_T/2. \quad (A16)$$

Thus,

$$Ca = 0.5\,[(Ca'_T - K_B - B_T) + ([Ca'_T - K_B - B_T]^2 + 4\,K_B\,Ca'_T)]^{0.5} \quad (A17)$$

which is Eq. 3 of Rios and Schneider (1981). K_B is the apparent dissociation constant of the buffer for calcium and B_T, the total buffer concentration. Dye-bound Ca was estimated following Rios and Schneider from the measured absorbance change $\Delta A(\lambda)$ and the estimated value of $\Delta\epsilon_2(\lambda)$, since fitting was restricted to $\lambda \geq 700$ nm where only CaD_2 contributes to the absorbance changes. Thus,

$$Ca'_T = Ca_T - \Delta A(\lambda)/(2\,\ell\,\Delta\epsilon_2(\lambda)) \quad (A18)$$

where ℓ is the cuvette pathlength. f_2 was calculated, therefore, by solving Eq. A15 with Ca calculated from Eqs. A17 and A18.

Where the solutions to reaction schemes involve polynomials of third or fourth degree, the polynomials were solved according to the method of van der Waerden (1953).

We are grateful to Drs. W. K. Chandler, E. Rios, and M. F. Schneider for helpful discussions, as well as to Dr. Chandler, in whose laboratory preliminary calibration measurements were made.

Financial support was provided by the U. S. National Institutes of Health (grant NS-17620 to Dr. Baylor and the Muscular Dystrophy Association (fellowship to Dr. Hollingworth).

Received for publication 15 September 1986 and in final form 24 November 1986.

REFERENCES

Baylor, S. M., W. K. Chandler, and M. W. Marshall. 1982*a*. Optical measurement of intracellular pH and magnesium in frog skeletal muscle fibres. *J. Physiol. (Lond.)*. 331:105–137.

Baylor, S. M., W. K. Chandler, and M. W. Marshall. 1982*b*. Use of metallochromic dyes to measure changes in myoplasmic calcium during activity in frog skeletal muscle fibres. *J. Physiol. (Lond.)*. 331:139–177.

Baylor, S. M., S. Hollingworth, C. S. Hui, and M. E. Quinta-Ferreira. 1985*b*. Calcium transients from intact frog skeletal muscle fibres simultaneously injected with Antipyrylazo III and Azol. *J. Physiol. (Lond.)*. 365:70P.

Baylor, S. M., S. Hollingworth, C. S. Hui, and M. E. Quinta-Ferreira. 1986. Properties of the metallochromic dyes Arsenazo III, Antipyrylazo III and Azo 1 in frog skeletal muscle fibres at rest. *J. Physiol. (Lond.)*. 377:89–141.

Baylor, S. M., M. E. Quinta-Ferreira, and C. S. Hui. 1983. Comparison of isotropic calcium signals from intact frog muscle fibers injected with Arsenazo III or Antipyrylazo III. *Biophys. J.* 44:107–112.

Baylor, S. M., M. E. Quinta-Ferreira, and C. S. Hui. 1985*a*. Isotropic components of Antipyrylazo III signals from frog skeletal muscle fibers. *In* Calcium in Biological Systems. R. P. Rubin, G. Weiss, and J. W. Putney, Jr., editors. Plenum Press, New York. 339–349.

Beeler, T. J., A. Schibeci, and A. Martonosi. 1980. The binding of Arsenazo III to cell components. *Biochim. Biophys. Acta.* 629:317–327.

Best, P. M., and C. W. Abramcheck. 1985. The metallochromic indicator dye Arsenazo III forms 1:1 complexes with caffeine. *Biochim. Biophys. Acta.* 838:179–182.

Fischer, B. E., U. K. Haring, R. Tribolet, and H. Sigel. 1979. Metal ion/buffer interactions. Stability of binary and ternary complexes containing 2-amino-2(hydroxymethyl)-1,3-propanediol (Tris) and adenosine 5′ triphosphate (ATP). *Eur. J. Biochem.* 94:523–530.

Grynkiewicz, G., M. Poenie, and R. Y. Tsien. 1985. A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J. Biol. Chem.* 260:3440–3450.

Hollingworth, S., R. W. Aldrich, and S. M. Baylor. 1986. In vitro calibration of the metallochromic indicator dye Antipyrylazo III (Ap III). *Biophys. J.* 49(2, Pt. 2):457*a*. (Abstr.)

Hollingworth, S., and S. M. Baylor. 1986. Calcium transients in frog skeletal muscle fibers injected with Azol, a tetracarboxylate Ca^{2+} indicator. *In* Optical Methods in Cell Physiology. P. DeWeer and B. M. Salzberg, editors. J. Wiley and Sons, New York. 261–283.

Irving, M., W. K. Chandler, J. Maylie, and N. L. Sizto. 1985. Antipyrylazo III calcium transients in cut frog twitch fibers. *Biophys. J.* 47:350*a*.

Kovacs, L., E. Rios, and M. F. Schneider. 1979. Calcium transients and intramembrane charge movement in skeletal muscle fibres. *Nature (Lond.)*. 279:391–396.

Kovacs, L., E. Rios, and M. F. Schneider. 1983*a*. Measurement and modification of free calcium transients in frog skeletal muscle fibres by a metallochromic indicator dye. *J. Physiol. (Lond.)*. 343:161–196.

Kovacs, L., and G. Szucs. 1983. Effect of caffeine on intramembrane charge movement and calcium transients in cut skeletal muscle fibres of the frog. *J. Physiol. (Lond.)*. 341:559–578.

Kovacs, L., R. A. Schumperli, and G. Szucs. 1983*b*. Comparison of birefringence signals and calcium transients in voltage clamped cut skeletal muscle fibres of the frog. *J. Physiol. (Lond.)*. 341:579–593.

Martell, A. E., and R. M. Smith. 1974. Critical Stability Constants. Vol. I. Plenum Publishing Corp., New York.

Maylie, J., G. Boyarsky, W. K. Chandler, M. Irving, and N. L. Sizto. 1985. Optical studies on cut muscle fibers using the calcium indicator tetramethylmurexide. *Biophys. J.* 47:351*a*. (Abstr.)

Maylie, J., M. Irving, N. L. Sizto, and W. K. Chandler. 1987*a*. Comparison of Arsenazo III optical signals in intact and cut frog twitch fibers. *J. Gen. Physiol.* In press.

Maylie, J., M. Irving, N. L. Sizto, and W. K. Chandler. 1987*b*. Calcium signals recorded from cut frog twitch fibers containing Antipyrylazo III. *J. Gen. Physiol.* In press.

Maylie, J., M. Irving, N. L. Sizto, G. Boyarsky, and W. K. Chandler. 1987*c*. Calcium signals recorded from cut frog twitch fibers containing tetramethylmurexide. *J. Gen. Physiol.* In press.

Meltzer, W., E. Rios, and M. F. Schneider. 1984. Time course of calcium release and removal in skeletal muscle fibers. *Biophys. J.* 45:637–641.

Palade, P., and J. Vergara. 1981. Detection of Ca^{++} with optical methods. *In* Regulation of Muscle Contraction: Excitation-Contraction Coupling. A. D. Grinnell and Mary A. B. Brazier, editors. Academic Press, Inc., New York. 143–158.

Palade, P., and J. Vergara. 1982. Arsenazo III and antipyrylazo III calcium transients in single skeletal muscle fibers. *J. Gen. Physiol.* 79:679–707.

Palade, P., and J. Vergara. 1983. Stoichiometries of Arsenazo III–Ca complexes. *Biophys. J.* 43:355–369.

Quinta-Ferreira, M. E., S. M. Baylor, and C. S. Hui. 1984. Antipyrylazo III (Ap III) and Arsenazo III (Az III) calcium transients from frog skeletal muscle fibers simultaneously injected with both dyes. *Biophys. J.* 45:47*a*. (Abstr.)

Rios, E., and M. F. Schneider. 1981. Stoichiometry of the reactions of calcium with the metallochromic indicator dyes Antipyrylazo III and Arsenazo III. *Biophys. J.* 36:607–621.

Rios, E., E. Stefani, G. Brum, and J. Goldman. 1985. Extracellular Ca modifies Ca release from the sarcoplasmic reticulum (SR) in skeletal muscle fibers. *Biophys. J.* 47:353*a*. (Abstr.)

Scarpa, A., F. J. Brinley, and G. Dubyak. 1978. Antipyrylazo III, a "middle range" Ca^{2+} metallochromic indicator. *Biochemistry.* 17:1378–1386.

Sillen, L. G., and A. E. Martell. 1964. Stability constants of metal ion complexes. Special Publication No. 17. Chemical Society, London.

Tsien, R. Y. 1983. Intracellular measurements of ion activities. *Annu. Rev. Biophys. Bioeng.* 12:91–116.

Van der Waerden, B. L. 1953. Modern Algebra. Frederick Ungar Publishing Co., Inc., New York. 177–183.