KINETIC SCHEME FOR Ca^{2+}-ARSENAZO III INTERACTIONS

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SUMMARY: Temperature-jump relaxation kinetic studies show that the complex formation between Ca^{2+} and the metallochromic dye arsenazo III (Ar) is associated with a rapid mode (≤ 10 μs-range) involving both Ca^{2+} and Na^+ of the Na-salt of Ar and a slower mode (~10 ms range) which can be attributed to structural re-arrangements in the 1:2 complex $CaAr_2$. The kinetic data suggest the scheme: $Ca + 2Ar = CaAr + Ar = CaAr_2^* = CaAr_2$. The relatively slow rate-limiting step sets a limit for the use of arsenazo III to study the kinetics of Ca^{2+} processes in cell biology.

A variety of cytoplasmatic reactions involve appreciable changes in the concentration, [Ca], of free intracellular Ca^{2+} ions. The metallochromic dye arsenazo III (Ar) has been found useful for detecting rates of Ca^{2+} release and uptake by numerous biological preparations; for example, by fragmented sarcoplasmic reticulum (1), postsynaptic membrane in frog muscle (2) and in Limulus photoreceptor cells (3). To obtain the actual rate of [Ca] change due to a biological reaction, it is in general necessary to deconvolute the observed dye signal with the rate constants describing the Ca-Ar reactions. Equilibrium binding studies indicate both 1:1 and 1:2 Ca-Ar complexes at cytoplasmic [Ca] values (4,5), and a 2:1 complex when [Ca] > 1 mM (5).

Temperature-jump experiments allow determination of the Ca-Ar reaction rate constants from formal analysis of the dye absorbance relaxation modes. The present study demonstrates that, at cytoplasmic Ca^{2+} and Na^+ concentrations, the overall

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Ca-Ar interaction equilibrates relatively slowly and that the

amplitude of the total optical signal contains significant con-

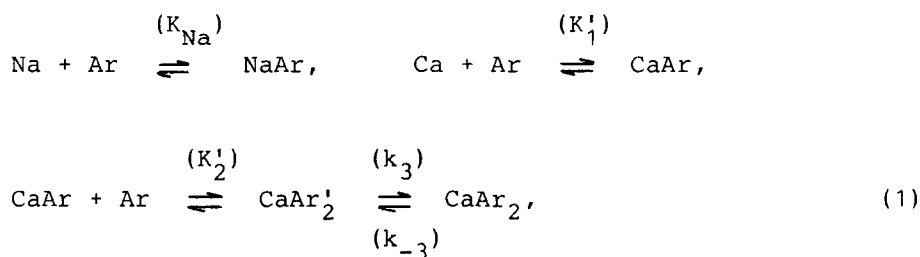tributions from Na^+-Ar complexing.

MATERIALS AND METHODS

Arsenazo III (Aldrich Chemical Co.) was purified according to the method described by Kendrick (6); Ca^{2+} and Na^+ contents of the purified dye and of stock $CaCl_2$ solutions were determined by atomic absorption spectroscopy. The Ca^{2+} content of dye-pH buffer solutions varied between 1.0 and 2.5 μM; the pH buffer used throughout was the Na^+ salt of piperazine-N,N'-bis(2-ethane sulfonic acid)(Pipes buffer), which at pH 7.0 is 50% deprotonated. All vessels, including the temperature-jump measuring cell, were washed with 1 mM EDTA solution to remove any contaminating Ca^{2+} and then rinsed with reflux-distilled water (conductivity of 0.9 μS cm^{-1} at 20° C).
Temperature-jumps were applied to aliquots (1 ml) containing 7-50 μM arsenazo III, 1-100 μM $CaCl_2$ and 30 mM Pipes buffer at pH 7.0; solution temperature was raised from 18 to 21° C. Because of the low salt content, the Joule heating time of the solutions was about 10 μsec. Resulting changes in the optical absorbance at the Ca^{2+}-sensitive wavelength 602 nm were on the order of 0.1%, but could be reliably and reproducibly recorded with a high-resolution chemical relaxation spectrometer (7), registered in a Bryans transient recorder and displayed on a Tektronix dual-beam oscilloscope. The evaluation of relaxation times and amplitudes was done by superimposing simulated relaxation spectra from a calibrated multi-exponential function generator. Calculations for a variety of trial reaction models were carried out with a VAX/VMS computer system.

RESULTS AND DISCUSSION

The overall relaxation signal has a rapid part whose kinetics

are too fast to be reliably resolved (time constants $\tau \leq 10$ μs)

and a slower part with resolvable kinetics associated with time

constants in the 10 ms time range. The fast-mode amplitude, ΔA_F,

reflects at least two reactions: ΔA_F is positive at [Ca]<5μM and

becomes progressively smaller and negative as [Ca] is raised.

This amplitude can thus be attributed to Na^+-Ar interactions at

low [Ca] whereas the rapid Ca^{2+}-Ar interactions dominate the fast

modes at high [Ca]. Because cation-complexed Ar has a higher

absorbance at 602 nm than free dye, it is concluded that Na^+-Ar

complexes are stabilized, and Ca^{2+}-Ar complexes are destabilized,

by a temperature increase, ΔT, from 18 to 21° C.

Calculations using a variety of different reaction models show that the slow mode is due to configurational rearrangements in the $CaAr_2$ complex. Previously reported (static) equilibrium titrations demonstrate that at $[Ca] \leq 100$ µM, Ca^{2+}-Ar complexing involves the reactions Ca+Ar=CaAr and CaAr+Ar=$CaAr_2$ (5). The present kinetic study indicates that this simple model must be extended to a four-step scheme:

$$Na + Ar \overset{(K_{Na})}{\rightleftharpoons} NaAr, \qquad Ca + Ar \overset{(K_1')}{\rightleftharpoons} CaAr,$$

$$CaAr + Ar \overset{(K_2')}{\rightleftharpoons} CaAr_2' \underset{(k_{-3})}{\overset{(k_3)}{\rightleftharpoons}} CaAr_2, \qquad (1)$$

where the steric rearrangement of the 1:2 complexes described by rate constants k_3 and k_{-3} is rate-limiting in the overall complexing of Ar with Na^+ and Ca^{2+}. K_{Na} is determined from Na^+ titrations of Ar in the absence of Ca^{2+} and:

$$K_1' = K_1/(1+K_{Na}^{-1}[Na]), \qquad (2)$$

$$K_2' = K_2(1+k_3/k_{-3})/(1+K_{Na}^{-1}[Na]), \qquad (3)$$

with $k_3/k_{-3} = K_3^{-1}$. Numerical values see Table 1.

The slow-mode time constant, τ_2, is obtained from normal mode analysis of eq.(1) assuming rapid equilibration of all bimolecular steps:

$$\frac{1}{\tau_2} = k_3 \left[\frac{\alpha[Ar]}{K_2' ([Ca]+[Ar]+ K_1') +\alpha[Ar]} \right] + k_{-3} ; \qquad (4)$$

where $\alpha = [CaAr] + 4[Ca] +[Ar]$. The value of K_2' was selected by least square fitting to give optimal consistency with the slope-intercept ratio k_3/k_{-3} in Fig. 1 according to eqs.(3) and (4); this is a discriminatory self-consistency check.
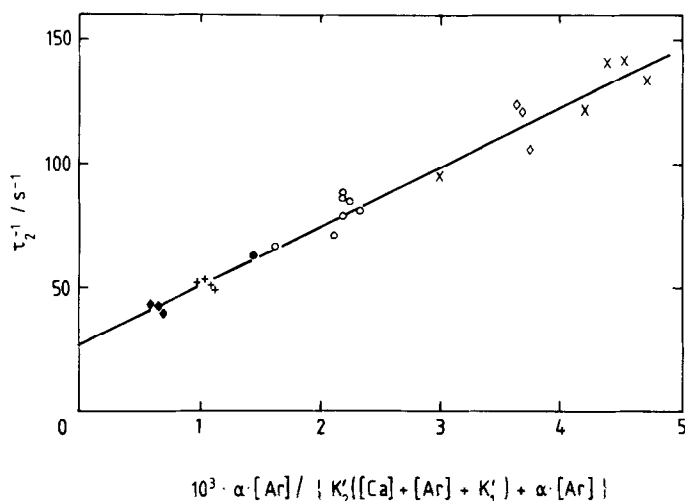
Figure 1: Relaxation rate τ_2^{-1} of the slow normal mode plotted according to eq. (4) of the text; the arsenazo III concentrations are in µM: (♦) 9.3, (+) 15, (●) 20, (o) 29, (◊) 46, (x) 58; 30 mM Pipes, pH 7; 1-100 µM CaCl$_2$, 21° C. The slope yields $k_3 = 2.3 \times 10^4$ s^{-1}, the intercept gives $k_{-3} = 28$ s^{-1}.

Dissociation reaction enthalpies, ΔH, can be calculated from the variation of the fast and total relaxation amplitudes, ΔA_F and ΔA_T, with dye and calcium concentrations. After considerable algebraic manipulation and using the van't Hoff relation $\Delta K/K = \Delta H \cdot \Delta T/(RT^2)$, R is the gas constant and ΔT the temperature increase, the relaxation amplitudes are given by:

$$\Delta A_F = F_{Na}(\Delta K_{Na}/K_{Na}) + F_{1F}(\Delta K_1/K_1) = (F_{Na}\Delta H_{Na} + F_{1F}\Delta H_1)\Delta T/RT^2, \quad (5)$$

$$\Delta A_T = F_{Na}(\Delta K_{Na}/K_{Na}) + F_{1T}(\Delta K_1/K_1) + F_{2T}(\Delta K_3/K_3) \quad (6)$$

$$= (F_{Na}\Delta H_{Na} + F_{1T}\Delta H_1 + F_{2T}\Delta H_3)\Delta T/RT^2$$

As shown in the appendix, the various amplitude factors F_{Na}, F_{1F}, F_{1T} and F_{2T} are complicated functions of reactant concentrations and extinction coefficients ε_{602} at 602 nm. The relative changes $\Delta K/K$ in the equilibrium constants and the ΔH-values are determined from slopes and intercepts in Figures 2 and 3 (see Table 1).
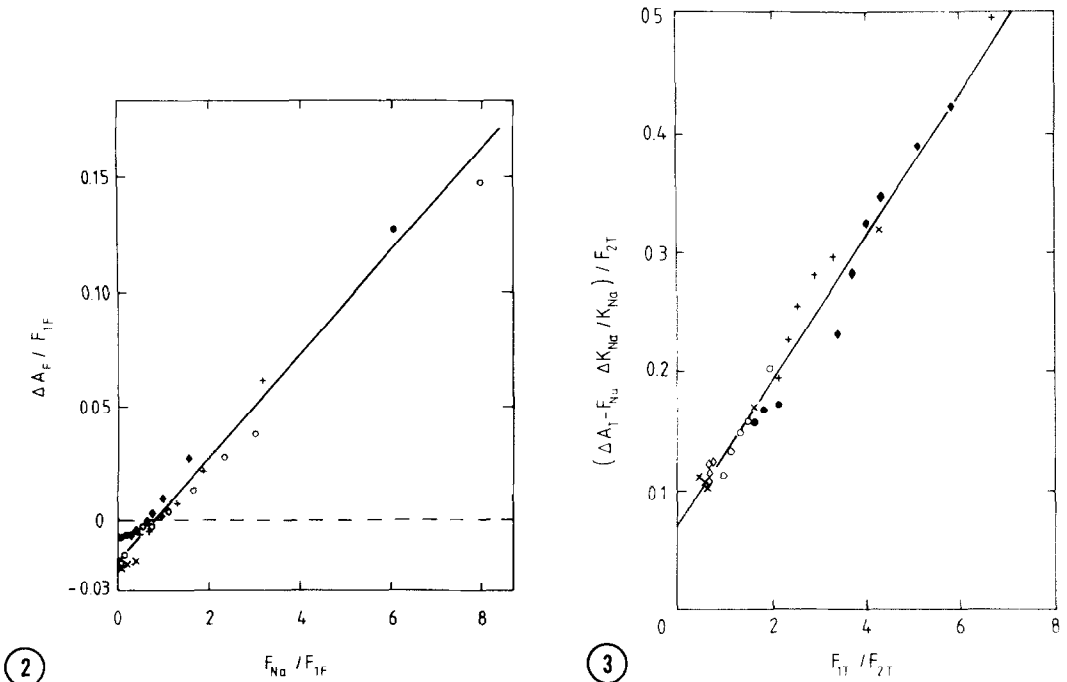
Figure 2: Evaluation of the total amplitude ΔA_F of the fast relaxation mode ($\tau \leqslant 10\mu s$) in terms of the amplitude factors F_{Na} and F_{1F} according to Eq. (5) of the text. The symbols refer to the various arsenazo III concentrations given in the legend to Fig. 1.

Figure 3: Data evaluation according to eq. (6) of the text, yielding $\Delta K_3/K_3$ from intercept (at $F_{1T}/F_{2T}=0$); symbols as in legend to Fig. 1.

In summary, the present study provides a quantitative kinetic reaction model which can be used to calculate biological [Ca] changes when arsenazo III is used as Ca^{2+} indicator. The higher cytoplasmic Na^+ level as well as the presence of Mg^{2+} may, however, alter the Ca^{2+}-Ar complexing parameters compared to those in Table 1. In any case, the present kinetic data are consistent with the previous result (5) that arsenazo III forms 1:1 and 1:2 Ca^{2+}-Ar complexes which coexist also under physiological ionic conditions. The inherent Ca^{2+}-complexing rates of the dye can well approach, or even be slower than the rates of physiological [Ca] changes. This kinetic information must be taken into account when Ca^{2+} release into, or uptake from

Table 1. Optical parameters at 602 nm, apparent equilibrium
constants at ionic strength 0.030 M, pH 7, T=21° C.

Reaction	Equilibrium constant	Dissociation enthalpy (kJ/mol)	Extinction coefficient $(10^4 \, M^{-1} \, cm^{-1})$
Na+Ar=NaAr (fast)	$K_{Na}=2.7 \times 10^{-2} M$	$\Delta H_{Na}=-5.0$	$\varepsilon_{NaAr}=1.14$ $\varepsilon_{Ar}=0.78$
Ca+Ar=CaAr (fast)	$K_1'=5.2 \times 10^{-6} \, M^{(a)}$	$\Delta H_1'=+7.7^{(b)}$	$\varepsilon_{CaAr}=4.4$
CaAr+Ar=CaAr$_2'$ (fast)	$K_2'=1.33 \times 10^{-1} M^{(c)}$	(d)	(d)
CaAr$_2'$=CaAr$_2$ (slow)	$K_3=1.2 \times 10^{-3(e)}$	$\Delta H_3=+16$	$\varepsilon_{CaAr_2}=7.5$

(a) from eq.(2) of the text with $K_1=11 \, \mu M$ (5),

(b) $\Delta H_1' = \left[K_1 \{ K_{Na}^2 + K_{Na} [Na] \} \Delta H_1 - K_1 K_{Na} [Na] \Delta H_{Na} \right] / \left[K_1 \{ K_{Na} + [Na] \}^2 \right]$,

(c) from eq.(3) of the text,

(d) since CaAr$_2'$ is very small, $\Delta H_2'$ and $\varepsilon_{CaAr_2'}$ cannot be determined reliably.

(e) from Fig. 1, intercept: $k_{-3}=28s^{-1}$, slope: $k_3=2.3 \times 10^4 s^{-1}$.

cytoplasm following physiological stimulus is to be measured
with arsenazo III as Ca-indicator.

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APPENDIX

A. Calculation of time constant (eq.4)

Denoting the instantaneous deviation from equilibrium concentration for a reactant c_i by δc_i, for the slow step in scheme (1)

$$\frac{d\ \delta CaAr_2}{dt} = k_3\ \delta CaAr_2' - k_{-3}\ \delta CaAr_2 .$$ (A1)

Because the perturbation of equilibrium is small, differences in mass action relationships can be linearized:

$$[Ca]\ \delta Ar + [Ar]\ \delta Ca = K_1'\ \delta CaAr ,$$ (A2)

$$[CaAr]\ \delta Ar + [Ar]\ \delta CaAr = K_2'\ \delta CaAr_2' .$$ (A3)

Mass balance equations have the form

$$\delta Ca + \delta CaAr + \delta CaAr_2' + \delta CaAr_2 = 0$$ (A4)

$$\delta Ar + \delta CaAr + 2\ \delta CaAr_2' + 2\ \delta CaAr_2 = 0 .$$ (A5)

Equations (A2)-(A5) can be solved for $\delta CaAr_2'$:

$$\delta CaAr_2' = \frac{-[Ar]\ ([CaAr]+4[Ca]+[Ar])}{K_2'([Ca]+[Ar]+K_1')+[Ar]([CaAr]+4[Ca]+[Ar])}\ \delta CaAr_2 ;$$ (A6)

this expression for $\delta CaAr_2'$ reduces eq. (A1) to a single term with the time constant given in eq. (4).

B. Calculation of amplitude coefficients.

The absorbance change due to the temperature-jump has the total magnitude in the fast relaxation region and overall, respectively,

$$\Delta A_F = \epsilon_{Ar}\Delta_F [Ar] + \epsilon_{CaAr}\Delta_F [CaAr] + \epsilon_{CaAr_2'}\Delta_F [CaAr_2'] + \epsilon_{NaAr}\Delta_F [NaAr] ,$$ (B1)

$$\Delta A_T = \epsilon_{Ar}\Delta_T [Ar] + \epsilon_{CaAr}\Delta_T [CaAr] + \epsilon_{CaAr_2'}\Delta_T [CaAr_2'] + \epsilon_{CaAr_2}\Delta_T [CaAr_2] + \epsilon_{NaAr}\Delta_T [NaAr].$$ (B2)

Using the appropriate mass balance and mass action relations, and noting that the slow step can be neglected in ΔA_F, allows eqs. (B1) and (B2) to be written as in eq. (5) and (6) with $\epsilon_{CaAr_2'} \approx \epsilon_{CaAr_2}$,

$$F_{1F} = (\epsilon_{Ar} + \epsilon_{CaAr} - \epsilon_{CaAr_2})X + (\epsilon_{CaAr_2} - 2\epsilon_{CaAr})Y$$ (B3)

$$X = K_1'[CaAr](2[Ar]+K_2')/\{K_2'([Ca]+[Ar]+K_1')+[Ar]([CaAr]+4[Ca]+[Ar])\}$$

$$Y = \{[CaAr]K_1'-([Ca]-K_1')X\}/([Ar]+2K_1').$$

$$F_{Na} = -\epsilon_{NaAr}[Na][Ar]/([Na]+[Ar]+K_{Na})$$ (B4)

$$F_{1T} = K_1'(\epsilon_{Ar} - \epsilon_{CaAr_2}/2)(G+C)$$ (B5)

$$C = \{([Ar]-[CaAr](2[Ca]+[Ar])[CaAr](K_3+1)-W[CaAr]\}/\{W([Ca]+[Ar]+K_1')\}$$

$$W = (K_3+1)[Ar]([CaAr]+4[Ca]+[Ar])+K_2'K_3([Ca Ar]+K_1')$$

$$G = -C+2(K_3+1)\{([Ar]-[CaAr])[CaAr]/W\}$$

$$F_{2T} = K_3(\epsilon_{Ar} - \epsilon_{CaAr_2}/2)(H+D)$$ (B6)

$$D = \{V(K_3+1)(2[Ca]+[Ar])-W[CaAr_2](2[Ca]+[Ar])\}/\{W([Ca]+[Ar]+K_1'\}$$

$$V = [CaAr_2]K_2'([Ca]+[Ar]+K_1')+[CaAr_2][Ar]([CaAr]+4[Ca]+[Ar])$$

$$H = -D+2(K_3+1)(V/W)-2[CaAr_2]$$