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THE KINETICS OF CALCIUM BINDING TO CALMODULIN: QUIN 2 AND ANS STOPPED-FLOW FLUORESCENCE STUDIES

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The rate of calcium dissociation from bovine testis calmodulin was measured by fluorescence stopped-flow using the calcium indicator Quin 2 or the fluorescence probe 8-anilinonaphthalene sulphonate. Two processes are resolved with Quin 2 corresponding to dissociation from the high affinity sites (k_{diss} 2 to 9 s^{-1} for T = 11 to 28°C) and from the low affinity sites (k_{diss} 293 to 550 s^{-1} for T = 11 to 19°C). These rates and the activation parameters as determined for the slow process $\Delta H^{\ddagger}$ = 59 ± 10 $kJ.mol^{-1}$ and $\Delta S^{\ddagger}$ = 30 ± 30 $JK^{-1}.mol^{-1}$ are in good agreement with values determined from the ^{43}Ca NMR exchange rates. These experiments provide confirmation that the calcium induced conformational change cannot be resolved kinetically from the calcium binding or dissociation, and by inference this conformational change is not a rate-limiting process in the function of calmodulin.

Calmodulin is the universal calcium binding regulatory protein in all eukaryotic cells. It has a molecular weight M_r ~ 16,700, contains no tryptophan or cysteine but does contain 1 mol of the unusual amino acid, ε-trimethyllysine. Four potential Ca^{2+} binding sites may be identified in the amino acid sequence of CaM from bovine brain, [1,2]. The Ca^{2+} binding parameters of CaM have been investigated by many workers and although there is a general concensus that four Ca^{2+} ions are bound, the values of the binding constants and the order in which the sites are filled is still the subject of much discussion. The binding of calcium causes changes in tyrosine absorbance and fluorescence and in near and far UV circular dichroism (for review see [3]). Studies by ^{1}H NMR have shown extensive changes in the environment of a large number of residues, associated with both strong and weak Ca binding [4,5] and have led to a specific model for the calcium induced process involving the mobility around a central hydrophobic core of the α-helices comprising the putative calcium binding sites of calmodulin [6].

Forsen and co-workers [7,8] have made extensive use of ^{43}Ca NMR measurements to investigate the Ca^{2+} exchange properties of CaM from bovine

Abbreviations used: CaM, calmodulin; ANS, 8 anilinonaphthalene sulfonate; BAPTA, 1,2 bis (o-aminophenoxyl)ethane N,N,N'N'-tetra-acetic acid; EDTA, ethylene diamine N,N,N'N'-tetra-acetic acid; EGTA, ethylene glycol bis (β-aminoethyl ether)-N,N,N',N'-tetra-acetic acid.

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testis. Their results suggest that the Ca^{2+} binding domains can be grouped into two sites with high and two with low affinity. The calculated Ca^{2+} exchange rate parameters from NMR measurements at 23°C are k_I = 1000 ± 100 s^{-1} (weak sites) and k_{II} = 30 s^{-1} (strong sites). Ikura et al. [5a and b], using ^1H NMR measurements, obtained similar results and concluded that $k_I \geq 600\ s^{-1}$ and $k_{II} \leq 50\ s^{-1}$.

Several stopped-flow kinetic studies of this system have also been performed. Malencik et al. [9] studied the dissociation of Ca^{2+} from the Ca-CaM complex by mixing with EGTA and monitoring changes in tyrosine fluorescence. They were able to measure a single first-order process with k_{diss} equal to 10.4 s^{-1}. In a similar experiment by Schimerlik et al. [10] anthroylcholine was used as a fluorescent probe to monitor the dissociation rate of calcium induced by EGTA. The proposed mechanism is rather complex, with a pronounced anthroylcholine concentration dependence, but they were nevertheless able to extract a value for k_{diss} of 10.1 ± 0.7 s^{-1}. Chau et al. [11] used both tyrosine fluorescence and an indicator system employing BAPTA to obtain a value of k_{diss} of approximately 17.5 s^{-1}. They suggested faster processes could also be present, but they were unable to resolve them. The observation that k_{diss} is independent of the monitoring system employed suggested that the change in CaM conformation which corresponds to the change in tyrosine fluorescence may be correlated with the slow dissociation of Ca^{2+}.

The idea that the conformational change might be slow appears to have originated with Seamon [4] who used ^1H NMR to assign an upper limit of 40 s^{-1} for the conformational transition occurring between the liganded and unliganded states. It is, of course, not necessary to assume that the conformational change is intrinsically slow. If the rate of conformational change is higher than the Ca^{2+} dissociation rate the change in tyrosine fluorescence would still be seen at the rate at which Ca^{2+} directly dissociates.

In order to study the relationship between the rate of the conformational change and the rates of the dissociation of Ca^{2+} from the calmodulin-Ca^{2+} complex, (as inferred from the results obtained by ^{43}Ca NMR) we have now measured the calcium dissociation rate using the indicator Quin 2 (the methoxy-quinoline derivative of BAPTA) as a calcium chelator as well as a fluorescent probe. The measurements have been performed at a range of temperatures in order to clarify both fast and slow Ca^{2+} dissociation rates under stopped-flow conditions, to obtain activation parameters and to investigate further the rate of conformational changes involved in calcium dissociation from calmodulin. We also describe experiments using ANS as a specific indicator of the Ca-CaM complex.

MATERIALS AND METHODS

Calmodulin was purified from bovine testis essentially by the method of Jamieson & Vanaman [12]. The CaM was checked for purity by means of SDS and agarose gel electrophoresis and for calcium content by atomic absorption spectroscopy ($\leq$ 0.15 Ca/CaM). Quin 2 was obtained from Lancaster Synthesis, Morecambe, U.K. The properties of Quin 2 have recently been described by Tsien et al. [13].

All other chemicals were of analytical grade and were obtained from local suppliers. All solutions were prepared in 20 mM Pipes/KOH buffer (pH 7.0) using doubly distilled water and were stored in plastic containers.

Stopped-flow measurements were performed using the system described in detail elsewhere [4]. For the excitation of Quin 2 the Mercury arc line with the wavelength 366 nm was used. The fluorescence was measured at wavelengths greater than 475 nm. At the excitation wavelength used, the Quin 2 fluorescence decreases upon binding Ca^{2+}. For the ANS experiments the same fluorescence excitation and emission wavelengths were used.

Data were collected on a PDP 11/23 computer and analysed by standard non-linear least-squares methods.

RESULTS

It is a necessary requirement in any indicator system that the indicator reactions should be faster than those one wishes to study. Therefore in a preliminary experiment the dissociation rate of Quin 2 from the Ca^{2+} - Quin 2 complex was measured by reacting a mixture of 100 µM Ca^{2+} plus 50 µM Quin 2 with EDTA under stopped flow conditions (Fig. 1). Results showed that k_{off} = 63 (± 2)s^{-1} at 20 mM EDTA and 56 (± 3)s^{-1} at 10 mM EDTA. Assuming that the reaction is a simple bimolecular one and that K_D = 8 x 10^{-8}M [13], we calculate k_{on} = 7.5 x 10^8 $M^{-1}s^{-1}$, i.e. a diffusion controlled process. Therefore, at [Quin 2] = 150 x 10^{-6} M the observed forward rate for calcium chelation under

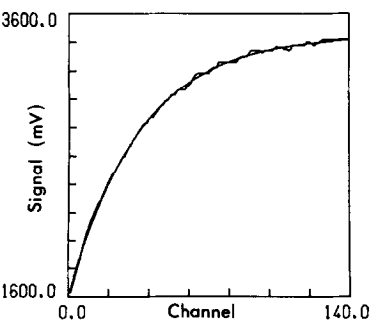

Figure 1. Ca^{2+} dissociation from the Ca^{2+} - Quin 2 complex. Quin 2 plus Ca^{2+} mixed with EDTA (Final concentrations: 25 µM Quin 2, 50 µM Ca^{2+}, 5 mM EDTA). The time scale is 0.5 ms per channel. The line drawn through the curve represents the fit to the data with k_{obs} = 56.6 s^{-1}.

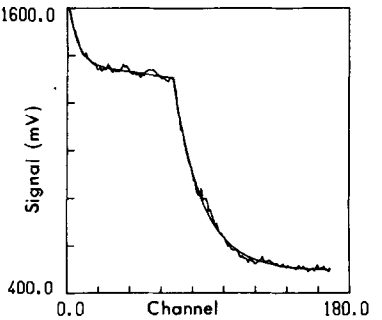

Figure 2. Ca^{2+} dissociation from the Ca-CaM complex. CaM plus Ca^{2+} mixed with Quin 2 (Final concentrations: 6.25 μM CaM, 62.5 μM Ca^{2+}, 100 μM Quin 2). The time scale is 0.5 ms/channel (channels 0-75) and 25 ms/channel (channels 76-end). The line drawn through the curve represents the fit to the data with k_{obs}^{fast} = 356 s^{-1} and k_{obs}^{slow} = 2.19 s^{-1}. The temperature was 11°C.

pseudo first order conditions [Ca^{2+}] ~ 15 μM is given by the equation

$$k_{obs}^{forward} \approx k_{on} [Quin\ 2] + k_{off} \approx 1 \times 10^5\ s^{-1}$$

Consistent with this calculation, in an investigation of the forward reaction with these concentrations of Quin 2 and Ca^{2+}, the reaction was complete within the instrumental deadtime (< 1.2 ms).

Initial experiments on the Quin 2 induced dissociation of Ca^{2+} from the Ca-CaM complex were conducted at 28°C and only a single phase was seen with a k_{obs} value of 9.1 s^{-1}. Further experiments were performed at 19°C and 11°C where two phases were detected (Fig. 2). The fast phase is clearly resolvable and quantifiable at 11°C; at 19°C the rate of the fast phase is such that ~ 60% of the amplitude is lost in the deadtime. The results for the three temperatures are summarized in Table I.

Table I Kinetic parameters for the Quin 2 induced dissociation of Ca^{2+} from the Ca-CaM complex

	SYRINGE 1		SYRINGE 2	rates (± std. dev.) (s^{-1})	
T°C	[CaM]	[Ca]	[Q2]	k_{obs}^{fast}	k_{obs}^{slow}
	μM	μM	μM		
28	12.5	100	200	—	9.1 ± 1.5
19	12.5	50/125	200	550 ± 175	5.3 ± 0.9
11	12.5	50/125	200	293 ± 92	2.1 ± 0.4

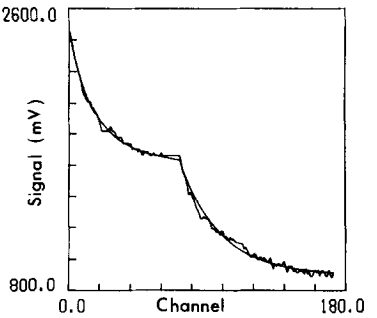

Figure 3. Ca^{2+} dissociation from the Ca - CaM-ANS complex. CaM plus Ca^{2+} plus ANS mixed with EDTA (Final concentrations: 6.25 μM CaM, 100 μM Ca^{2+}, 12.5 μM ANS, 0.5 mM EDTA). The time scale is 0.1 ms/channel (channels 0-75) and 2.5 ms/channel (channels 76-end). The line drawn through the curve represents the fit to the data with k_{obs}^{fast} = 614 s^{-1} and k_{obs}^{slow} = 10.4 s^{-1}. The temperature was 22°C.

An Arrhenius plot of the data for the slow phase yields values of $\Delta H^{\ddagger}_{obs}$ = 59 ± 10 kJ/mol and $\Delta S^{\ddagger}_{obs}$ = 30 ± 30 $JK^{-1}mol^{-1}$. These values are in good agreement with those determined by Forsen et al. [7] from the temperature dependence of the slow exchange rate.

In a similar experiment we studied the EDTA induced dissociation of calcium from the Ca-CaM-ANS ternary complex. ANS binds to CaM in the presence of calcium with enhanced fluorescence intensity [15] so that calcium dissociation results in a reduction of ANS fluorescence. The results with this system were rather similar to those for Quin 2 at high [EDTA] (see Fig. 3). We also attempted to study the forward reaction for this system by reacting a mixture of CaM and ANS ([CaM] = 2.5 to 25 μM; [ANS] = 50 μM) with Ca^{2+} ([$Ca(NO_3)_2$] = 20 μM to 1 mM) but the reaction was complete within the deadtime of the instrument.

DISCUSSION

^{43}Ca NMR studies [7,8] have identified two Ca^{2+} exchange rates for the Ca-CaM complex and these are thought to be associated with exchange at the weak sites (k_I ~ 1000 s^{-1}) and at the strong sites (k_{II} ~ 30 s^{-1}) at 23°C. Earlier stopped-flow studies, on the other hand, have measured only a single dissociation rate with k_{diss} ~ 12 s^{-1}. Care should, however, be exercised when comparing these results since experimental temperatures are not quoted and, as we have shown, the rates are highly temperature dependent. It seems, however, likely that the slow dissociation rate corresponds to the NMR exchange rates for the strong sites. This has been confirmed in the present study by the similarity of rate constants and the activation parameters.

By making stopped-flow measurements at low temperatures we have been able to measure a fast dissociation rate which is similar in magnitude to the fast exchange rate measured by NMR. Although we have not been able to measure activation parameters accurately, it seems most likely that this process does indeed correspond to the calcium dissociation rate from the weak sites.

The present experiments cannot give an absolute value for the rate of the conformational change, since there is no kinetic transient identifiable with the conformation change occurring at a rate different from that of calcium dissociation.

To distinguish formally between these two steps, it is necessary to formulate at least a two-step mechanism:

$$Ca^{2+} + CaM \underset{k_{21}}{\overset{k_{12}}{\rightleftharpoons}} Ca - CaM \underset{k_{32}}{\overset{k_{23}}{\rightleftharpoons}} Ca - CaM^*$$

in which the species Ca - CaM* represents the calcium-induced conformation. Two limiting cases may then be considered:

(1) $k_{32} >> k_{21}$ Under these conditions, any spectroscopic change which monitors the conformational change will necessarily be seen at the rate of calcium dissociation (i.e. k_{21}). Our studies of the rate of formation of the Ca-CaM-ANS complex did not show any slow step, which indicates that k_{23} must be fast ($k_{23} >> 10^3\ s^{-1}$). Also k_{23} must be substantially greater than k_{32} in order to maintain the overall equilibrium in favour of Ca-CaM* this still allows for the necessary inequility, $k_{32} >> k_{21}$ with a value of k_{21} about 10 s^{-1}. From the speed of formation of the Ca-CaM-ANS complex the k_{off} for ANS would be 400-800 s^{-1}. Thus, the rate of the decrease of ANS fluorescence with EDTA appears to follow the rate of Ca^{2+} dissociation. Further interpretation awaits clarification of the multiplicity and possible heterogeneity of ANS binding [16].

(2) $k_{32} << k_{21}$ Under these conditions, the observed rate of calcium dissociation would be limited by the value of k_{32}, i.e. the rate at which Ca-CaM* could be converted to Ca-CaM. Such a slow step would be apparent in relaxation studies carried out at saturating Ca^{2+} but to date there is no experimental evidence to support this interpretation.

These kinetic results indicate that the conformational change of calmodulin upon binding calcium to the strong sites occurs rapidly. The relatively slow interconversion of liganded and non-liganded forms of calmodulin observed in ^{43}Ca-NMR and ^{1}H-NMR is the direct consequence of the slowness of the dissociation of calcium from the high affinity sites. There is no experimental basis for making a distinction in kinetic terms

between conformational change and Ca^{2+} dissociation. Our results are therefore consistent with the idea that the conformational change occurs effectively synchronously with Ca^{2+} binding or dissociation.

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