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RAPID KINETIC STUDIES ON CALCIUM INTERACTIONS WITH NATIVE AND FLUORESCENTLY LABELED CALMODULIN

D.A. Malencik, S.R. Anderson, Y. Shalitin and M.I. Schimerlik*

Department of Biochemistry and Biophysics
Oregon State University
Corvallis, Oregon 97331

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SUMMARY: Stopped-flow studies on calcium binding to calmodulin showed that under pseudo first order conditions the reaction was complete within 2.5 milliseconds. The time course for calcium dissociation from the native protein showed a single kinetic phase ($\tau_1^{-1} = 10S^{-1}$) while that from the dansylated derivative revealed a second slower kinetic phase ($\tau_1^{-1} = 10S^{-1}$, $\tau_2^{-1} = 0.31S^{-1}$) that accounted for about one-half of the total fluorescence decrease. Therefore the dansyl derivative of calmodulin may provide a useful tool for studying conformational changes in the protein not reflected by the active site tyrosines.

INTRODUCTION

The calcium binding protein calmodulin has been shown to modulate many of the regulatory effects of this ion at the cellular level (1,2). Equilibrium measurements using circular dichroism (3,4,5), nuclear magnetic resonance (5), tyrosine fluorescence (7), and ultraviolet difference spectroscopy (3,5) have established that the protein undergoes conformation changes upon binding calcium. The first conformational change occurs after the binding of 2 moles of calcium per mole of protein (reflected in the change in tyrosine fluorescence), while the second is completed by the association of the fourth calcium per mole of protein (5). Although all investigators agree on the stoichiometry of four calcium sites per mole of calmodulin, there is disagreement as to whether all four sites are equivalent (6), there are two classes of sites (4), or the binding of calcium is positively cooperative (5) in nature.

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Although the interaction of calmodulin with calcium has been intensively studied using equilibrium methods, there has been no reported studies of the system using rapid kinetic techniques. The purpose of this communication is to present a preliminary report of stopped-flow studies on calcium interactions with native calmodulin as well as a calmodulin covalently labeled with the dansyl fluorophore.

MATERIALS AND METHODS

Kinetic studies were conducted on a Dionex D 137 stopped-flow photometer equipped with a 75 watt xenon lamp interfaced to a Nicolet Model 206 digital storage oscilloscope. Data stored in the scope were fit to either equation (1) or (2) using Marquardt's algorithm as described in Bevington (8) by LSI 11/23 computer.

$$y = A_o + A_1 e^{-t/\tau_1} \quad (1)$$

$$y = A_o + A_1 e^{-t/\tau_1} + A_2 e^{-t/\tau_2} \quad (2)$$

In the above equations, y is the fluorescence intensity at time t and A_o the equilibrium fluorescence intensity. The observed exponential(s) are described by the amplitude(s) A_i and relaxation times τ_i. The data and superimposed theoretical curve were then plotted on a Hewlett-Packard 7225A plotter interfaced to the computer. All values reported are the average of five experiments.

In experiments where tyrosine fluorescence of the native protein was monitored, (excitation at 270 ± 5 mm) a Corning 9-54 cut off filter was used. Dansyl fluorescence was followed (excitation at 330 ± 5 mm) using a Corning 0-52 cutoff filter while the tyrosine fluorescence of the dansylated protein was observed using a narrow band pass filter (±10 nm) centered at 302 nm (Baird Atomic).

Calmodulin was isolated from pork brain by the procedure of Schreiber et al (Biochemistry, in press) and dansylated as described previously (12). Quantitation of the dansylation reaction using a molar absorptivity of 3.3 x 10^3 $M^{-1} \cdot cm^{-1}$ (13) indicates that approximately 1 dansyl group was introduced per calmodulin (Malencik and Anderson, to be published). All experiments were done using a buffer composed of 20 mM MOPS, .15 M KCl, pH 7.2. In experiments where a calcium free preparation was required, the protein and buffer were treated with chelex-100 resin. Atomic absorption indicated that after chelex treatment, the calmodulin contained less than 0.1 mole of calcium per mole of protein and the buffer calcium concentrations were less than 10^{-6}M.

RESULTS

The data in Figure 1A show results obtained when calcium was mixed with the native protein. The lower trace represents the fluorescence of calmodulin versus buffer alone. The second trace at 40 μM final calcium,20 μM

calmodulin shows that about 50% of the total amplitude occurred within the dead time (2.3 msec) of the instrument, while the third trace (150 μM final calcium) shows that under pseudo first-order conditions, equilibrium was attained within the instrumental dead time.

When the protein conformational change following calcium dissociation from the native protein was monitored (Fig. 1B) a single exponential was observed. Fitting the data to equation (1) gave a value of τ_1^{-1} = 10.5 ± 1.5 S^{-1}. Experiments monitoring calcium dissociation from the dansylated protein, however, showed two exponentials (Fig. 2A). The fast phase (A_1/A_t 58 ± 3% τ_1^{-1} = 10.4 ± 1.0 S^{-1}) had the same relaxation time as that found for the native protein, while the slower phase (A_2/A_t = 42 ± 5%) was approximately thirty fold slower (τ_2^{-1} = 3.9 ± 0.5 x 10^{-1} S^{-1}). Neither the rate or the relative amplitude of the two phases depended on EGTA concentration between 0.4 mM and 8 mM EGTA. In order to ascertain whether the behavior of the dansylated protein had been altered by the covalent labeling, the tyrosine fluorescence of the dansyl conjugate was monitored using a narrow band pass filter. The results (Fig. 2B) indicated that the tyrosine fluorescence of the labeled calmodulin still showed a single exponential with approximately the same value for τ_1^{-1} (8.0 ± 1.2 S^{-1}) as that for the native protein.

DISCUSSION

Since the data in Fig. 1 indicated that the kinetics of Ca^{2+} association with calmodulin were too fast to follow, it was only possible to place a lower limit on the observed rate constant for calcium association. In order for the reaction to reach completion within the instrumental dead-time under pseudo first order conditions (150 μM calcium, 20 μM calmodulin) 10 $t_{1/2} \leq$ 2.3 msec. Thus the rate constant for the association step must exceed 3000 S^{-1} (e.g. $\tau^{-1} \geq$ 0.69/0.23 msec). The data for calcium dissociation showed a single exponential (τ^{-1} = 10.4 S^{-1}), indicating that the tyrosine environment(s) appeared to reflect a single conformational change in the protein. This rate constant is similar to that found for reisomerization of troponin C

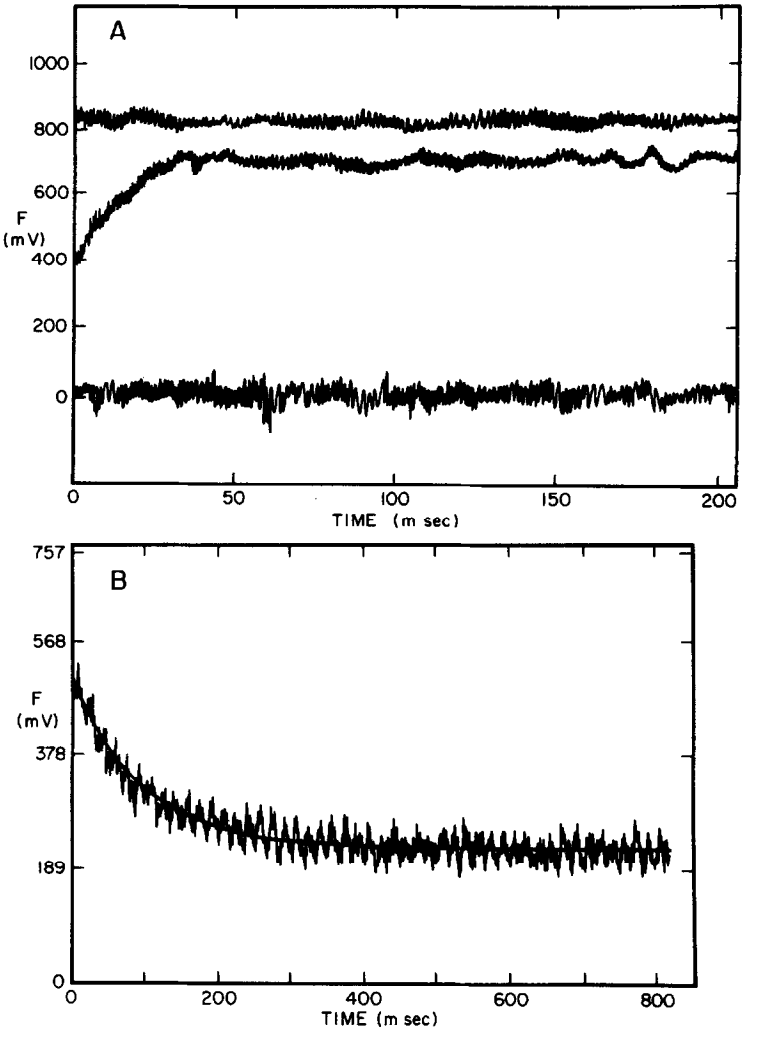

Figure 1: (A) Ca^{2+} binding to Ca^{2+} free calmodulin. Bottom line, Ca^{2+} free calmodulin mixed with Ca^{2+} free buffer; middle curve, final Ca^{2+} concentration, 40 μM; top curve, final Ca^{2+} concentration, 150 μM. The final calmodulin concentration was 20 μM for all three traces. (B) Ca^{2+} dissociation from Ca^{2+}-calmodulin. Calmodulin (final concentration 20 μM) plus Ca^{2+} (final concentration 500 μM) were mixed with buffer plus EGTA (final concentration 2 mM). Line drawn through the curve represents the fit of the data to equation (1) giving values of A_0 = 236 mv, A_1 = 292 mv, τ_1^{-1} = 10.4 s^{-1}.

after calcium dissociation (9,10). The dansylated calmodulin also reacts with calcium within the dead-time of the instrument (data not shown); however, the interesting observation from the dissociation kinetics was that the dansyl group reflects not only the conformational changes observed by tyrosine

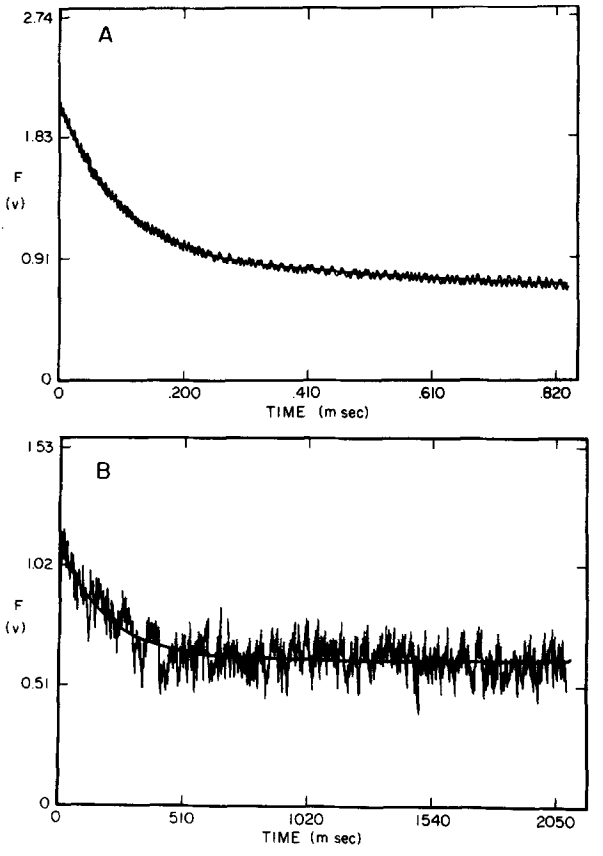

Figure 2: (A) Ca^{2+} dissociation from dansyl calmodulin monitored by dansyl fluorescence. Dansyl calmodulin (final concentation 45 μM) plus Ca^{2+} (final concentration 200 μM) mixed with buffer plus EGTA (final concentration 2 mM). Line drawn through the curve represents the fit of the data to equation (2) giving values of A_0 = 0.0, A_1 = 1.17 v, τ_1^{-1} = 10.4 S^{-1}, A_2 = 0.92 v, τ_2^{-1} = 0.31 S^{-1}. (B) Ca^{2+} dissociation from dansyl calmodulin monitored by tyrosine fluorescence. Dansyl calmodulin (final concentration 46 μM) plus Ca^{2+} (final concentration 300 μM) mixed with buffer plus EGTA (final concentration 2 mM). The curve through the data represents the fit of the data to equation (1) giving values of A_0 = 0.63 v, A_1 = 0.51 v, τ_1^{-1} = 9.3 S^{-1}.

fluorescence, but an additional slower conformational change as well. Since the change in intrinsic tyrosine fluorescence has been attributed to the binding of calcium to the two high affinity sites (7) the dansyl fluorophore may also be reflecting conformational changes that occur when calcium binds to the weaker sites or the exposure of hydrophobic domains in the calcium calmodulin complex (11). The data in Figure 2B indicate that dansylation of

the protein did not affect the behavior of the two native site tyrosine residues, at least with respect to conformational changes upon calcium dissociation. Furthermore, control experiments indicated that the slow phase cannot be attributed to photolysis of the dansyl fluorophore or the effect of EGTA concentration. Therefore, the slow phase was attributed to a slower isomerization in the vicinity of the dansylated lysine group of calmodulin (Malencik and Anderson, in preparation).

In summary, this communication presents the quantitation of the rate of conformational change(s) in native and dansylated calmodulin upon calcium dissociation. Because of the increased informational content revealed by the kinetics of the dansylated protein, it may be useful in the rapid kinetic studies of calmodulin interactions with other modifiers as well as studies of the regulator protein-protein interactions of the calcium calmodulin complex.

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