Kinetic Studies Show That Ca^{2+} and Tb^{3+} Have Different Binding Preferences toward the Four Ca^{2+}-Binding Sites of Calmodulin†

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ABSTRACT: The stepwise addition of Tb^{3+} to calmodulin yields a large tyrosine-sensitized Tb^{3+} luminescence enhancement as the third and fourth ions bind to the protein [Wang, C.-L. A., Aquaron, R. R., Leavis, P. C., & Gergely, J. (1982) *Eur. J. Biochem. 124*, 7–12]. Since the only tyrosine residues in calmodulin are located within binding sites III and IV, these results suggest that Tb^{3+} binds first to sites I and II. Recent NMR studies have provided evidence that Ca^{2+}, on the other hand, binds preferentially to sites III and IV. Kinetic studies using a stopped-flow apparatus also show that the preferential binding of Ca^{2+} and lanthanide ions is different. Upon rapid mixing of 2Ca–calmodulin with two Tb^{3+} ions, there was a small and rapid tyrosine fluorescence change, but no Tb^{3+} luminescence was observed, indicating that Tb^{3+} binds to sites I and II but not sites III and IV. When two Tb^{3+} ions are mixed with 2Dy–calmodulin, Tb^{3+} luminescence rises rapidly as Tb^{3+} binds to the empty sites III and IV, followed by a more gradual decrease ($k = 0.4\ s^{-1}$) as the ions redistribute themselves over the four sites. These results indicate that (i) both Tb^{3+} and Dy^{3+} prefer binding to sites I and II of calmodulin and (ii) the binding of Tb^{3+} to calmodulin is not impeded by the presence of two Ca^{2+} ions initially bound to the protein. Thus, the Ca^{2+} and lanthanide ions must exhibit opposite preferences for the four sites of calmodulin: sites III and IV are the high-affinity sites for Ca^{2+}, whereas Tb^{3+} and Dy^{3+} prefer sites I and II.

Calmodulin has four Ca^{2+}-binding domains, numbered I through IV starting from the N-terminus (Vanaman et al., 1977). There have been numerous studies of Ca^{2+} binding to calmodulin [for reviews, see Wolff & Brostrom (1979), Klee et al. (1980), and Cheung (1980)]. Although some analyses of Ca^{2+}-binding data lead to models of four intrinsically equal, but interacting sites (Crouch & Klee, 1980; Burger et al., 1984), most reports agree that there are two classes of binding sites whose affinities are sufficiently different so that some of them (the higher affinity sites) can be nearly saturated with metal ions while the rest (the lower affinity sites) remain unoccupied. In order to clarify the assignment of the four sites to the two classes differing in affinity, we (Wang et al., 1982a) and others (Kilhoffer et al., 1980; Wallace et al., 1982) employed the trivalent lanthanide ions Tb^{3+} and Eu^{3+}, which have been extensively used as calcium analogues with other metal-binding proteins (Martin & Richardson, 1979). The value of these lanthanides as probes of metal-binding sites in proteins arises from the facts that (i) they bind with high affinity to Ca^{2+}-binding sites owing to similarities with Ca^{2+} in their ionic radii and coordination chemistries, and (ii) Tb^{3+}, in particular, can act as an acceptor of fluorescence energy if it binds close (within ~0.5 nm) to an intrinsic donor fluorophore in the protein (Luk, 1971). In calmodulin, Tyr-99 and Tyr-138, which are located within binding sites III and IV, respectively, may act as energy donors, and one would expect a large luminescence enhancement upon Tb^{3+} binding to these sites.

When Tb^{3+} is added to calmodulin in a stoichiometric titration, Tb^{3+} luminescence appears (upon excitation at 280 nm) only when more than two ions are added to the protein. Since energy transfer requires close proximity between the donor groups and the bound ions, it follows that the first two Tb^{3+} ions bind to sites I and II, which contain no donor fluorophores, and the third and fourth ions bind to sites III and IV (Wang et al., 1982a). Other experiments in which both Tb^{3+} binding and Ca^{2+} binding to calmodulin were monitored by changes in intrinsic (Tyr) fluorescence and circular dichroism, as well as competitive titrations of 2Tb–calmodulin with Ca^{2+}, appeared consistent with the view that Ca^{2+} too was more strongly bound to sites I and II.

This interpretation has recently been questioned in light of recent ^{1}H (Ikura et al., 1983a,b), ^{43}Ca (Forsén et al., 1983), and ^{113}Cd NMR (Andersson et al., 1983) studies on calmodulin and its proteolytic fragments which provide evidence that the first two Cd^{2+} (and Ca^{2+}) ions bind to sites III and IV. This would imply that Ca^{2+} and Tb^{3+} exhibit different preferences for the four sites. Accordingly, we have reexamined this possibility by carrying out new studies employing a stopped-flow apparatus to measure the kinetics of Tb^{3+} or Ca^{2+} binding to calmodulin in the absence and presence of the other ion. These kinetic studies indicate that the binding of Tb^{3+} to calmodulin is not impeded by the presence of two Ca^{2+} ions already bound to the protein, as it is in the case of troponin C (TnC)[1] where the two ions are known to compete for the same sites. Thus, the two kinds of metal ions must exhibit different preferences for the four sites of calmodulin: Ca^{2+} binds first to sites III and IV whereas Tb^{3+} prefers sites I and II.

Materials and Methods

Calmodulin was prepared from calf brain on the basis of the methods of Watterson et al. (1976) and Klee (1977). TnC was prepared from rabbit skeletal muscle as previously described (Potter & Gergely, 1974). Purity of proteins was examined by amino acid analysis and by gel electrophoresis

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[1] Abbreviations: TnC, troponin C; Pipes, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); DPA, 2,6-pyridinedicarboxylic acid (dipicolinic acid); CN, coordination number; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

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in the presence of urea or sodium dodecyl sulfate. Protein concentrations were routinely checked by steady-state fluorescence titration with $TbCl_3$, monitoring both the tyrosine emission and the enhanced metal luminescence [excited at 280 nm; see Wang et al. (1982a)]. $TbCl_3$ and $DyCl_3$ were purchased from Alpha-Ventron (Danvers, MA) in the hexahydrated form (99.9% purity). All kinetic measurements were made at 25 °C by rapidly mixing equal volumes of protein and metal/chelator solutions containing 25 mM Pipes, pH 6.8, and 0.1 M KCl in a Dionex (Model 13000) stopped-flow spectrophotometer equipped with a flurescence detector, dead time ~2 ms. Typically, the final protein concentration was 5–10 μM. The excitation wavelength was set at 280 nm. The digitized fluorescence signal was fed to a PDP-11/03 computer and fitted with single- or double-exponential equations by a nonlinear regression method; whenever a double-exponential equation did not yield a better fit (judged from the sums of χ^2), the best fit with a single exponential is reported. Suitable cutoff filters (Corning 7-60 and 3-72) were used for tyrosine fluorescence and Tb^{3+} luminescence measurements to eliminate undesired emission and light scattering.

Results

Binding of Ca^{2+} to Apocalmodulin. The rates of binding and dissociation of Ca^{2+} were monitored by changes in the intrinsic (tyrosine) fluorescence of the protein. Addition of two Ca^{2+} ions per apocalmodulin induces an increase in the steady-state tyrosine fluorescence (F/F_0 = 2.5 at 300 nm). The rate of the increase varies with the Ca^{2+} concentration and follows first-order kinetics when the Ca^{2+} level is in excess of the protein concentration. Practically, the range of measurable rates is limited by two factors: With protein concentrations less than 1 μM, the resulting fluorescence changes are too small to detect; with higher protein concentrations (and therefore higher Ca^{2+} levels), the rates are too fast to detect (the instrument limit is about 350 s^{-1}). Thus, it is difficult to obtain an exact value for the second-order rate constant from these pseudo-first-order rates; rather, a lower limit is estimated to be 2×10^7 M^{-1} s^{-1}.

Addition of more than two Ca^{2+} ions per calmodulin produces only a small spectral change beyond that observed for the binding of the first two ions and could not, therefore, yield information on the rate constants for the binding to the lower affinity sites. Removal of Ca^{2+} from calmodulin with EDTA (in either the 2Ca or the 4Ca state), monitored by the decrease in tyrosine fluorescence, yields a rate constant of 14 s^{-1}. If one assumes that this corresponds to the off rate of Ca^{2+} from the high-affinity sites, a binding constant of $K = 7.1 \times 10^6$ M^{-1} is obtained for these sites, assuming that the on rate is diffusion limited (i.e., $k_{on} \sim 10^8$ s^{-1} M^{-1}).

Binding of Tb^{3+} to Apocalmodulin. Tb^{3+} binding to calmodulin was monitored either by changes in tyrosine fluorescence or by the appearance of Tb^{3+} luminescence when more than two Tb^{3+} ions bind to the protein. Addition of two Tb^{3+} ions per apocalmodulin induces a tyrosine fluorescence increase which is completed within the instrument mixing time, suggesting a diffusion-limited binding of Tb^{3+} to sites I and II of the protein (F/F_0 = 1.9 for the steady-state fluorescence intensity at 300 nm). Further Tb^{3+} binding, presumably to sites III and IV, is detected by the large increase in the metal ion luminescence. The rate of appearance of Tb^{3+} luminescence increases with the $TbCl_3$ concentration, the second-order rate constant being 2.75×10^6 M^{-1} s^{-1}.

The removal of Tb^{3+} from sites III and IV with EDTA results in a single-exponential loss of Tb^{3+} luminescence with a value of k_{off} = 18.5 s^{-1} (Figure 1). The removal of Tb^{3+} from sites I and II by EDTA monitored by the loss of tyrosine fluorescence is also single exponential with a value of k_{off} = 5.5 s^{-1} (Figure 2). However, if Tb^{3+} is removed from these sites by mixing with excess dipicolinic acid (DPA), which forms a strongly fluorescent complex with Tb^{3+} (Barela & Sherry, 1976), two apparent rate constants are obtained, 0.59 and 5.6 s^{-1} (Figure 3). This result raises the possibility that the off rates from the two high-affinity Tb^{3+}-binding sites in calmodulin are different and that the process with a rate constant of 5.5 s^{-1} observed for the tyrosine fluorescence change is only associated with one of the two sites. Assuming diffusion-controlled binding, $k_{on} \sim 10^8$ s^{-1} M^{-1}, and using the off rates obtained with DPA, one obtains 1.7×10^8 M^{-1} and 1.8×10^7 M^{-1} as binding constants for Tb^{3+} to the two high-affinity sites and 1.5×10^5 M^{-1} to the low-affinity sites.

Binding of Two Tb^{3+} Ions to 2Ca–Calmodulin. If Ca^{2+} binds preferentially to sites I and II as does Tb^{3+}, then upon adding two Tb^{3+} ions for each 2Ca-calmodulin the added Tb^{3+} would be expected initially to bind to the unoccupied sites (sites III and IV) with an increase in the metal ion luminescence, assuming that the diffusion-controlled on rate of Tb^{3+} to these empty sites remains unchanged. However, since some of the bound Ca^{2+} will eventually be displaced from sites I and II and bind to sites III and IV owing to the higher affinity of

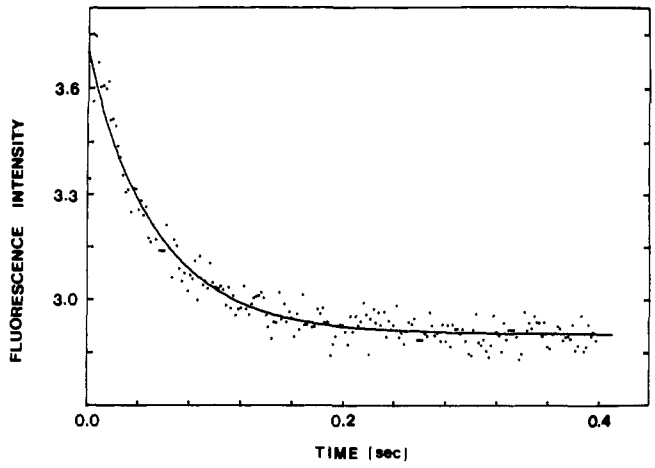

FIGURE 1: Typical stopped-flow trace of Tb^{3+} release from sites III and IV of calmodulin. 15 μM 4Tb–calmodulin in Pipes buffer (25 mM Pipes and 0.1 M KCl, pH 6.8) was mixed with 1 mM EDTA in the same buffer, and Tb^{3+} luminescence was monitored. The solid line is the fitted curve. Fluorescence intensity is in arbitrary units.

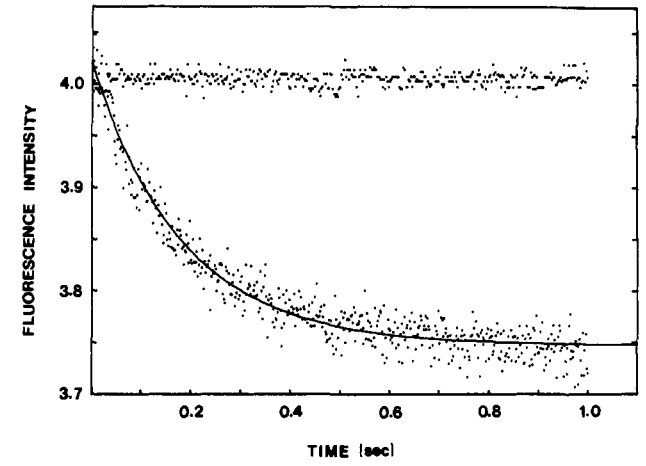

FIGURE 2: Typical stopped-flow trace of Tb^{3+} release from sites I and II of calmodulin. 13 μM 2Tb–calmodulin in Pipes buffer was mixed with 2 mM EDTA in the same buffer, and tyrosine fluorescence was monitored. The solid line is the fitted curve. The upper trace results from the mixing of 2Tb–calmodulin with buffer only.

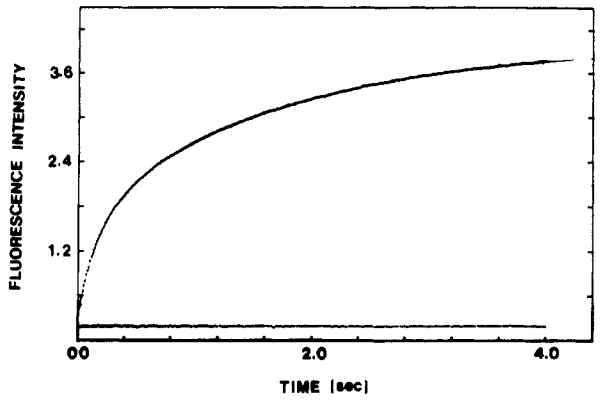

FIGURE 3: Typical stopped-flow trace of Tb^{3+} release from sites I and II of calmodulin by DPA. 13 μM 2Tb–calmodulin in Pipes buffer was mixed with 80 μM DPA in the same buffer, and Tb^{3+} luminescence was monitored. The solid line is the fitted curve based on a biexponential equation (see Materials and Methods). The lower trace represents the mixing of 2Tb–calmodulin with buffer only.

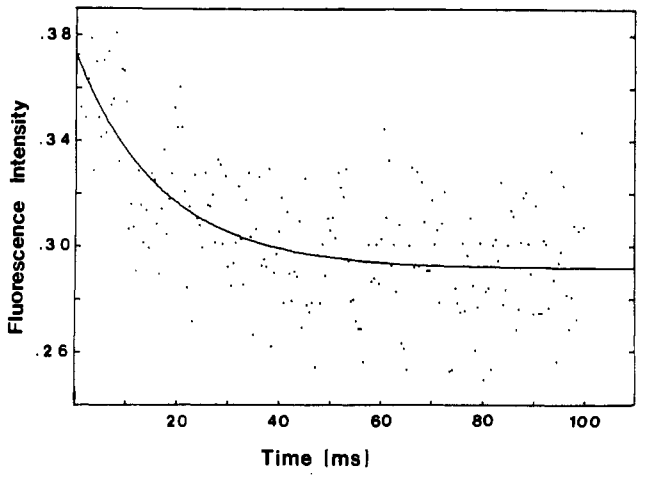

FIGURE 4: Stopped-flow trace of Tb^{3+} binding to 2Ca–calmodulin. 10 μM 2Ca–calmodulin was mixed with 20 μM $TbCl_3$, and tyrosine fluorescence was monitored. Because of the small change in the fluorescence signal and the small time constant (0.1 ms) used, the data points were quite scattered. However, the change is very reproducible. The trace shown here represents an average of six identical experiments. The solid line is the fitted curve. The fluorescence intensity is in arbitrary units.

Tb^{3+} for the first two sites, the metal luminescence would then decrease again as Tb^{3+} binds at sites I and II. The rate of the decrease would be limited by the Ca^{2+} off rate from sites I and II. If, on the other hand, one assumes that Tb^{3+} and Ca^{2+} exhibit different binding site preferences, viz., Ca^{2+} preferentially occupies sites III and IV, then the immediate Tb^{3+} binding to sites I and II would not be impeded by the presence of the two bound Ca^{2+} ions.

To investigate this question, we carried out rapid mixing of 10 μM 2Ca–calmodulin with 20 μM $TbCl_3$: No transient increase in Tb^{3+} luminescence was observed, supporting the view that sites III and IV are the high-affinity sites for Ca^{2+}. There was an accompanying small, but reproducible, decrease in the tyrosine fluorescence with a rate constant of 59 s^{-1} (Figure 4). The fact that this rate is approximately 4 times the Ca^{2+} off rate (Table I) again suggests that Tb^{3+} and Ca^{2+} do not compete for the same sites but, instead, form a hybrid complex, 2Tb–2Ca–calmodulin, in which the Tb^{3+} ions occupy sites I and II and Ca^{2+} occupies sites III and IV. The rate of 59 s^{-1} is likely to represent the rate of a conformational change induced in the protein by Tb^{3+} binding to 2Ca–calmodulin.

Table I: Summary of Kinetic Parameters of Ca^{2+} and Tb^{3+} Binding to Calmodulin and Troponin C[a]

syringe A	syringe B	monitoring and spectral change	k (s^{-1})
2Ca–calmodulin	EDTA	tyrosine fluorescence decrease	14 ± 1
	$2Tb^{3+}$	tyrosine fluorescence decrease	59 ± 6
		Tb^{3+} luminescence, no change	
	$4Tb^{3+}$	tyrosine fluorescence decrease	14.0 ± 1.6
		Tb^{3+} luminescence increase	15.0 ± 1.2
4Ca–calmodulin	EDTA	tyrosine fluorescence decrease	13.6 ± 0.8
	$4Tb^{3+}$	tyrosine fluorescence decrease	13 ± 2
		Tb^{3+} luminescence increase	14 ± 1
2Tb–calmodulin	EDTA	tyrosine fluorescence decrease	5.5 ± 0.6
	DPA	Tb^{3+} luminescence increase (biphasic)	5.6 ± 0.9 0.59 ± 0.8
4Tb–calmodulin	EDTA	Tb^{3+} luminescence decrease	18.5 ± 0.7
	2 mM Ca^{2+}	tyrosine fluorescence increase (biphasic)	17.2 ± 2.2 1.38 ± 0.06
2Dy–calmodulin	$2Tb^{3+}$	Tb^{3+} luminescence, rapid increase[b] followed by slower decrease	0.36 ± 0.05
2Ca–TnC	$2Tb^{3+}$	tyrosine fluorescence decrease	0.6 ± 0.1
		Tb^{3+} luminescence increase	0.7 ± 0.1

[a] Conditions: a solution containing 10–20 μM protein (in syringe A) was mixed rapidly with an equal volume of a solution containing metal ion or chelator (in syringe B); both solutions were in 25 mM Pipes (pH 6.8) and 0.1 M KCl, equilibrated at 25 °C. Rate constants shown are means ± SD (n = 4–6). [b] The spectral change of the rapid phase finished within mixing time.

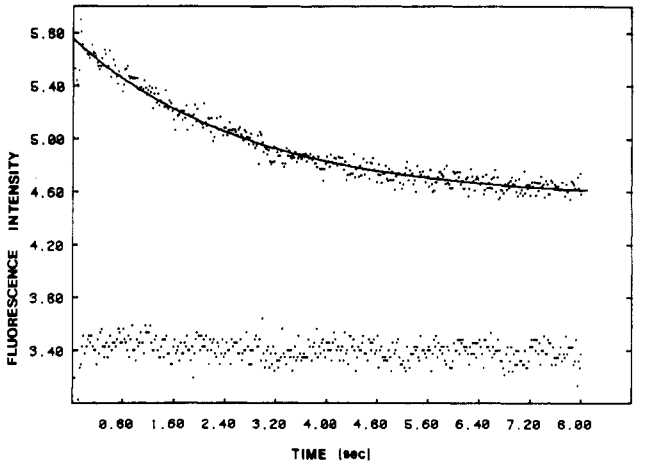

FIGURE 5: Averaged (n = 5) stopped-flow trace of Tb^{3+} binding to 2Dy–calmodulin. 9 μM 2Dy–calmodulin was mixed with 18 μM $TbCl_3$, and Tb^{3+} luminescence was monitored. The solid line is the fitted curve. The lower trace results from the mixing of 2Dy–calmodulin with buffer only.

Binding of Two Tb^{3+} Ions to 2Dy–Calmodulin. The fact that the chemical properties of Dy^{3+} are essentially identical with those of Tb^{3+} led us to assume that Dy^{3+}, like Tb^{3+}, will bind to calmodulin first at sites I and II. Dy^{3+} is not luminescent and will therefore not interfere with the Tb^{3+} luminescence changes. Addition of Tb^{3+} results in a rapid rise

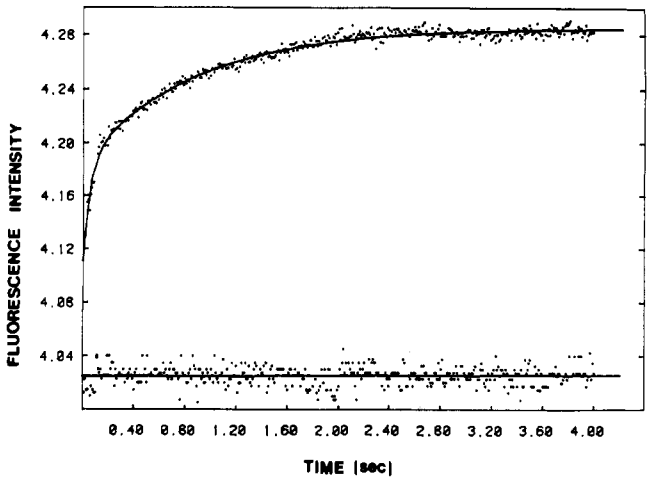

FIGURE 6: Averaged ($n = 6$) stopped-flow trace of Tb^{3+} release from calmodulin by Ca^{2+} displacement. 13 μM 4Tb–calmodulin was mixed with 2 mM $CaCl_2$ while tyrosine fluorescence was monitored. The solid line is the fitted curve based on a biexponential equation (see Materials and Methods). The lower trace results from the mixing of 4Tb–calmodulin with the buffer only.

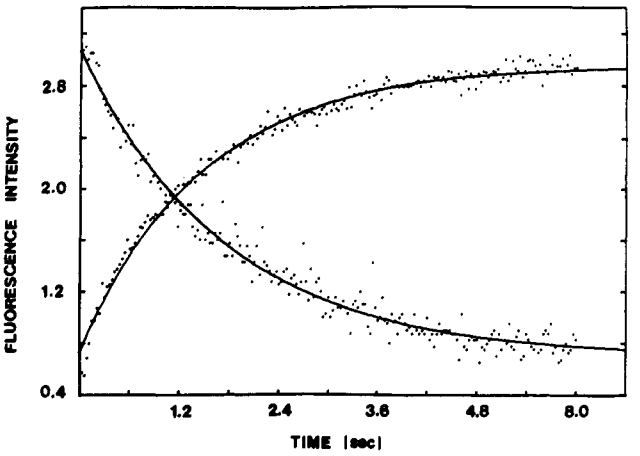

FIGURE 7: Averaged ($n = 6$) stopped-flow traces of Ca^{2+} release from TnC by Tb^{3+} displacement. 10 μM 2Ca–TnC was mixed with 20 μM $TbCl_3$, and both tyrosine fluorescence (the decreasing signal) and Tb^{3+} luminescence (the increasing signal) were monitored. Solid lines are fitted curves. The fluorescence intensity is in arbitrary units.

in Tb^{3+} luminescence, owing to initial binding to sites III and IV, followed by a slower decrease as Tb^{3+} replaces Dy^{3+} at sites I and II (Figure 5). This is the expected result if both ions bind sites I and II with higher affinity than sites III and IV (see above). The rate of decrease in Tb^{3+} luminescence ($k = 0.36\ s^{-1}$), which corresponds to the off rate of Dy^{3+}, is comparable to the Tb^{3+} off rate determined by using DPA (Table I).

Binding of Four Tb^{3+} Ions to 2Ca- and 4Ca–Calmodulin. The addition of 40 μM Tb^{3+} to 10 μM 2Ca- or 4Ca–calmodulin produces changes in Tb^{3+} luminescence and tyrosine fluorescence with a value of 13–14 s^{-1} for the rate constant. These changes indicate the replacement of Ca^{2+} at sites III and IV with Tb^{3+} and agree with the Ca^{2+} off rate from 2Ca–calmodulin determined with the use of EDTA (Table I).

Binding of Ca^{2+} to 4Tb–Calmodulin. Rapid mixing of 13.5 μM 4Tb–calmodulin with excess (2 mM) $CaCl_2$ results in an increase of tyrosine fluorescence. The spectral change can be best fitted with a biexponential curve, the two rate constants being 17.2 and 1.38 s^{-1} (Figure 6). The same experiment, but monitored by the decrease of Tb^{3+} luminescence, yields only a single rate constant of 21 s^{-1}. Since the Tb^{3+} luminescence decrease reflects Tb^{3+} replacement by Ca^{2+} at sites III and IV, the biphasic change of tyrosine fluorescence was interpreted as follows: the faster phase (17.2 s^{-1}) corresponds to Ca^{2+}–Tb^{3+} exchange at sites III and IV, while the slow phase (1.38 s^{-1}) reflects exchange at sites I and II. The latter process, in particular, is considerably slower than the removal of Tb^{3+} from the same sites with EDTA measured by tyrosine fluorescence (5.5 s^{-1}, see Table I), suggesting that the presence of Ca^{2+} at sites III and IV may influence the dissociation rate constant of Tb^{3+} at sites I and II.

Binding of Tb^{3+} to 2Ca–TnC. It is well-known for TnC that both Ca^{2+} and Tb^{3+} bind preferentially to sites III and IV, resulting in a tyrosine fluorescence increase in the case of Ca^{2+} binding and a Tb^{3+} luminescence enhancement in the case of Tb^{3+} binding (Wang et al., 1981). On rapid mixing of 2Ca–TnC with Tb^{3+}, there is a simultaneous decrease in tyrosine fluorescence and an increase in Tb^{3+} luminescence with values of $k = 0.6$–$0.7\ s^{-1}$ for both spectral changes (Figure 7). The low values for the rate constants (0.6–0.7 s^{-1}) are consistent with the interpretation that the increase in Tb^{3+} luminescence follows the dissociation of Ca^{2+} from sites III and IV. This behavior clearly contrasts with that of calmodulin, for which under similar conditions there is no increase in Tb^{3+} luminescence.

Discussion

Terbium binds readily to Ca^{2+}-binding sites in proteins because its ionic radius and coordination properties are similar to those of Ca^{2+} (Prados et al., 1974). In several intracellular Ca^{2+}-binding proteins that bind more than one Ca^{2+}, Tb^{3+} has been shown to bind to all of the Ca^{2+}-binding sites—in the same order of preference exhibited by Ca^{2+}. In TnC, for example, both ions bind first to the higher affinity sites III and IV followed by binding to sites I and II (Wang et al., 1981). Under the experimental conditions in our earlier paper (Wang et al., 1982a), calmodulin exhibited two classes of binding sites for both Ca^{2+} and Tb^{3+}. In that work, steady-state Tb^{3+} titrations of calmodulin suggested that the ion binds preferentially to sites I and II in the N-terminal half of the protein and subsequently binds to sites III and IV. Results of tyrosine fluorescence studies and competitive Ca^{2+}–Tb^{2+} titrations appeared consistent with the view that Ca^{2+} behaved similarly.

This view has been challenged by recent studies employing ^1H and ^{113}Cd NMR that provide compelling evidence that Ca^{2+} binds first to sites III and IV rather than to sites I and II. Close similarities were revealed between the ^{113}Cd NMR spectra of 2Cd–calmodulin and of proteolytic fragments of the protein containing only sites III and IV in the presence of the ion, indicating that the ion binds first to these sites (Andersson et al., 1983). A ^1H NMR comparison of Ca- and Cd–calmodulin and its fragments yielded nearly identical spectra, confirming that Ca^{2+} also initially occupies sites III and IV (Forsén et al., 1983). Our earlier results are reconcilable with these findings only if Tb^{3+} and Ca^{2+} exhibit different preferences for the binding sites on the protein. The kinetic studies reported here support this view.

Addition of either ion to apocalmodulin results in rapid binding ($k \sim 10^6$–$10^8\ M^{-1}\ s^{-1}$). If Tb^{3+} binds preferentially to sites I and II and Ca^{2+} to sites III and IV, then the addition of two Tb^{3+} ions to 2Ca–calmodulin or two Ca^{2+} ions to 2Tb–calmodulin might also be expected to give rapid on rates since, in each case, the hybrid complex 2Tb–2Ca–calmodulin would be formed without displacing any of the initially bound ions. If, on the other hand, both ions preferred the same pair of sites, then one could expect the added ion to bind initially

to the lower affinity unoccupied sites and subsequently to displace some of the initially bound ion from the preferred sites, the final distribution of the ions depending on their relative affinities for each class of sites. In this second case, the rate of the initial binding to the unoccupied sites would be rapid, but the redistribution would be limited by the relatively slow off rates of the initially bound ions from the preferred sites (cf. Table I).

The rapid mixing of Tb^{3+} with 2Ca–calmodulin produces a small enhancement in the tyrosine fluorescence that yields a rate constant of 59 s^{-1}, about 4 times faster than the rate of Ca^{2+} displacement from the protein. Perhaps more significant in this experiment is the absence of any transient Tb^{3+} luminescence that would have indicated its initial binding to sites III and IV (assuming the two Ca^{2+} ions were initially bound to sites I and II). This experiment provides strong evidence that the two ions prefer different sites in calmodulin. Addition of Tb^{3+} to 4Ca–calmodulin results in a decrease in tyrosine fluorescence and a similar increase in Tb^{3+} luminescence ($k \sim 15$ s^{-1}); both spectral changes indicate exchange at sites III and IV. Similar results have recently been reported by Tudor & White (1983).

It should be pointed out that although Tb^{3+} could indeed bind to calmodulin nonspecifically at high Tb^{3+}/protein ratios, as evidenced by protein precipitation upon addition of more than six Tb^{3+} ions, all the experiments described in this paper were carried out at Tb^{3+}/calmodulin ≤ 4.

In our earlier study on the kinetics of metal binding to TnC (Wang et al., 1983), we showed that the Ca^{2+} off rate from sites III and IV was reduced when Tb^{3+} occupied sites I and II. This indicates interaction between the two classes of sites in TnC such that the binding characteristics of half of the molecule are modulated by the binding of metal ions to the other half. A similar observation was made in calmodulin. When Tb^{3+} was displaced from calmodulin by mixing 4Tb–calmodulin with Ca^{2+}, the slower phase of the tyrosine fluorescence increase ($k = 1.38$ s^{-1}, Table I) was 4 times slower than the decrease of tyrosine fluorescence observed when Tb^{3+} is removed from the protein with EDTA ($k = 5.5$ s^{-1}). Assuming that the tyrosine change in each case results from the dissociation of Tb^{3+} from sites I and II, this would suggest that in the first experiment Ca^{2+} bound to sites III and IV increases the Tb^{3+} affinity for sites I and II.

The two control experiments (Figure 4 and 5) show the results obtained under conditions where the ions compete for the same sites. In one, Dy^{3+} is employed as a Tb^{3+} analogue bound to sites I and II in calmodulin. Rapid mixing of 2Dy–calmodulin with Tb^{3+} results in a transient rise in Tb^{3+} luminescence as the ion initially binds to sites III and IV followed by a slower decrease owing to the displacement of part of the Dy^{3+} from sites I and II. In the other experiment, addition of Tb^{3+} to 2Ca–TnC results in initial binding to the free TnC sites I and II followed by Ca^{2+} displacement from sites III and IV as monitored by the appearance of Tb^{3+} luminescence ($k \sim 0.6$–0.7 s^{-1}).

In view of the extensive sequence homology that exists between calmodulin and TnC, it is instructive to compare the two proteins with respect to their Ca^{2+}- and Tb^{3+}-binding characteristics and the spectral changes accompanying the binding.

(i) Both proteins bind Ca^{2+} preferentially at sites III and IV which can be monitored by an increase in intrinsic fluorescence attributable to Tyr-109 in TnC and Tyr-99 and Tyr-138 in calmodulin. Binding of the second pair of Ca^{2+} ions to the lower affinity sites I and II produces no further change in fluorescence in either protein.

(ii) The first pair of Tb^{3+} ions added to TnC binds to sites III and IV as indicated by the appearance of Tyr-109-sensitized Tb^{3+} luminescence that exhibits a stoichiometry of 2 (Wang et al., 1981). The second pair binds to sites I and II with no further change in the ion luminescence. The fluorescence of Tyr-109 does not exhibit the enhancement upon Tb^{3+} binding that occurs when Ca^{2+} binds to sites III and IV. This may result from quenching of the tyrosine by hydroxyl ions that are coordinated to the bound Tb^{3+} (see below).

(iii) The first pair of Tb^{3+} ions added to calmodulin binds to sites I and II. The binding produces an increase in the fluorescence of Tyr-99 and/or Tyr-138; however, the absence of any metal ion luminescence suggests that the two tyrosine residues are not close to the bound ions. Thus, the tyrosine enhancement is likely to be a long-range effect arising from a conformational change in the molecule. This is supported by the observation of Tb^{3+}-induced changes in the circular dichroism of calmodulin (Wang et al., 1982a). The second pair of Tb^{3+} ions binds to sites III and IV, producing a large enhancement in the ion luminescence with Tyr-99 and/or Tyr-138 acting as energy donors. The tyrosine fluorescence from these residues decreases as Tb^{3+} occupies sites III and IV, possibly also involving quenching by hydroxyl ions as stated above.

Consideration of the above results gives rise to a number of questions. For example, the binding of Tb^{3+} to sites III and IV of both TnC and calmodulin quenches the tyrosine fluorescence although, in both cases, Ca^{2+} binding to the same sites enhances it. Since these tyrosines act as donor fluorophores, it is tempting to attribute the quenching to energy transfer to the vicinally bound Tb^{3+}. However, the extremely low extinction coefficient for Tb^{3+} in the wavelength range of tyrosine emission precludes significant quenching by energy transfer. Furthermore, in earlier studies (Wang et al., 1981, 1982b), the same quenching was observed when lanthanide ions having no spectral overlap with tyrosine were substituted for Tb^{3+}. Although the differences between Ca^{2+}-induced and lanthanide-induced tyrosine changes might result from different conformations of the protein induced by the ions, another mechanism may involve quenching by hydroxyl groups that are brought into proximity with the tyrosines as part of the coordination sphere of the bound lanthanide. Ca^{2+}, with its lower coordination number, would be expected to contain fewer coordinated –OH moieties (see below).

The ability of lanthanides to replace Ca^{2+} in a variety of proteins stems from the similarities in the chemical properties of the ions. Both bind to sites that provide oxygen as the ligands rather than nitrogen. Both also exhibit variable coordination numbers with a high degree of flexibility of bond distances (Williams, 1976). In the case of Ca^{2+} bound to proteins, the ligands exhibit an octahedral geometry, CN = 6, and the ionic radius is 0.1 nm. Tb^{3+}, on the other hand, favors higher coordination numbers of 8 or 9, and the ionic radius is 0.104 and 0.110 nm, respectively (Shannon, 1976). Typically, the nearly equal ionic radii of Ca^{2+} and Tb^{3+} coupled with the higher charge to volume ratio of the latter account for its ready replacement of Ca^{2+}.

In many known Ca^{2+}-binding proteins, Tb^{3+} (and other trivalent lanthanide ions) can replace bound Ca^{2+} at a given site, the lanthanides having a higher affinity. It is therefore somewhat surprising to find that these two ions exhibit opposite binding preferences for the two classes of metal-binding sites in calmodulin. Yet it is not unusual in comparing the affinities of a pair of ions for a series of small chelators to find different,

and even reversed, ratios. For instance, both Ca^{2+} and Mg^{2+} bind EDTA with about the same binding constants, $\sim 10^{10}$ M^{-1}, while for EGTA, the affinity of Ca^{2+} is 10^4 times higher than that of Mg^{2+}. For glycine, on the other hand, the preference is reversed: Mg^{2+} binds more strongly than Ca^{2+} (Williams, 1970). Similar situations may exist in the metal–protein complexes. Since the binding strength of a metal ion to a protein depends on the number of ligands and their geometry, the structure of the protein may undergo distortion to different extents to achieve the optimal conformations for different metal ions. In the case of calmodulin or other multisite proteins, the problem can be more complicated, because the occupancy of one or more sites may also affect the ease with which another site assumes the optimal geometry. High-resolution X-ray crystal structures of calmodulin containing various metals are likely to provide the necessary information for interpreting the binding properties of the various sites for different metals.

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Registry No. Ca, 7440-70-2; Tb, 7440-27-9; Dy, 7429-91-6.

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