pH-Dependent Conformational Changes of Wheat Germ Calmodulin†

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ABSTRACT: Fluorescence energy transfer measurements were carried out between landmarks on wheat germ calmodulin to measure the interdomain distance. Tb^{3+} ions bound at the four Ca^{2+}-binding sites were used as energy donors, and an organic chromophore, [4-(dimethylamino)phenyl-4′-azophenyl]maleimide, attached to the single cysteine residue at position 27, was used as the acceptor. At pH's near neutrality all bound Tb^{3+} ions emit luminescence with shortened lifetimes as a result of energy transfer to the acceptor; at pH 5, however, part of the metal emission becomes unquenched. When the protein is subjected to limited digestion with trypsin in the presence of Ca^{2+}, resulting in the formation of two fragments, each corresponding to half of the molecule, the decay of Tb^{3+} emission is no longer pH sensitive. These results suggest that, like rabbit skeletal troponin C [Wang, C.-L. A., Zhan, Q., Tao, T., & Gergely, J. (1987) *J. Biol. Chem. 257*, 8372-8375], wheat germ calmodulin exists in a relatively compact conformation at neutral pH's, but becomes more elongated at pH 5.

Calmodulin (CaM)[1] is a major Ca^{2+} receptor in eukaryotic cells and modulates a wide spectrum of enzymes [for reviews, see Cheung (1980), Manalan and Klee (1984), and Cox (1988)]. Crystallographic studies (Babu et al., 1985) revealed that CaM is an elongated, dumbbell-shaped molecule with two globular domains, each containing two Ca^{2+}-binding sites: sites I and II in the N-terminal domain and sites III and IV in the C-terminal domain. The two end domains are separated by a single α-helical stretch. The entire molecule spans an end-to-end distance of 6.5 nm. This elongated structure of CaM resembles that of a homologous Ca^{2+}-binding protein, TnC, crystallized at pH 5.0 (Herzberg & James, 1985; Sundaralingam et al., 1985). According to recent interdomain distance measurements (Wang et al., 1987), however, TnC appears to exist in solution in a more compact conformation at pH ~7, although at pH 5 its length in solution is similar to that deduced from X-ray diffraction studies on the crystals. Thus, TnC may undergo a considerable conformational transition when the pH of the medium is changed from 5 to 7. More recently, Heidorn and Trewhella (1988) investigated X-ray solution scattering of CaM and found that the paired distance distribution functions of the solution and the crystal structures differed, indicating that CaM has a smaller interdomain distance in solution than in the crystal. Similar conclusions were drawn by Bayley et al. (1988) on the basis of time-resolved fluorescence anisotropy decay data of the two tyrosine residues of CaM. To address the question of whether there is a pH-dependent structural change in CaM, we have measured the distance between the two domains of CaM at different pH's by fluorescence energy transfer techniques. CaM isolated from wheat germ was chosen for this study because of its single cysteine residue at position 27 (Toda et al., 1985). Our results suggest that wheat germ CaM, similarly to skeletal TnC, also has a pH-dependent conformation, although the difference between the structures at pH 5 and pH 7 is smaller than in the case of TnC.

Materials and Methods

CaM was purified from wheat germ according to the modified procedure (Strasburg et al., 1988) of Yoshida et al. (1983). Labeling of purified wheat germ CaM was carried out by first reducing the protein with 0.1 M dithiothreitol in 6 M guanidine hydrochloride, 25 mM Hepes (pH 7.5), and 1 mM EDTA. After two dialyses against 25 mM Hepes (pH 7.5) and 0.1 M KCl, guanidine hydrochloride (6 M) and EDTA (1 mM) were added back to the solution and the protein sample was allowed to react with DAB-Mal (3 mol/mol) for 16 h, followed by exhaustive dialysis against 25 mM Pipes (pH 6.7) and 0.1 M KCl. The labeling was judged virtually complete in view of the fact that no more DAB-Mal was incorporated when the above procedure (including the reduction step) was repeated. CaM_{DAB} thus obtained exhibited an extinction coefficient of $\epsilon_{425} = 1.8 \times 10^4$ cm^{-1} M^{-1}.

Tryptic cleavage of CaM_{DAB} was carried out as described by Drabikowski et al. (1977) at a trypsin to CaM ratio of 1:50 (w/w) in 1 mM $CaCl_2$ at room temperature for 60 min. The digestion mixture was treated with 5 mM EDTA followed by dialysis against 25 mM Pipes (pH 6.7) and 0.1 M KCl to remove Ca^{2+}, and then Tb^{3+} was added for luminescence lifetime measurements.

$TbCl_3$ was purchased from Alfa Ventron (Danvers, MA). Tb^{3+} luminescence decay measurements were carried out as previously described (Wang et al., 1982a). Tb^{3+}-containing samples were excited either indirectly with a medium-intensity ultraviolet flash lamp (a Corning 7-54 cutoff filter was used to excite only the aromatic residues of the protein) or directly with a pulsed (10 Hz) nitrogen-pumped tunable dye laser (Molectron DL14). Cumarine 500 was used as the laser dye for Tb^{3+} excitation. A photomultiplier equipped with a gating circuit was used as the detector, which was gated off for 0.1

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[1] Abbreviations: CaM, calmodulin; DAB-Mal, *N*-[4-(dimethylamino)phenyl-4′-azophenyl]maleimide; DAB, the 4-dimethylaminophenylazophenyl group; CaM_{DAB}, wheat germ CaM with DAB-Mal labeled at Cys-27; TnC, the calcium-binding subunit of rabbit skeletal troponin; EDTA, ethylenediaminetetraacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Pipes, 1,4-piperazinediethanesulfonic acid; TR1C and TR2C, the N- and the C-terminal tryptic fragment of CaM, respectively.

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ms to prevent overloading with the scattered light or prompt luminescence. Decay data typically from 200 to 300 sweeps were signal-averaged in 256 channels and the digital data points fed to a PDP 11/44 microcomputer for data analysis. Decay parameters extracted from these data by the method of moments (Dyson & Isenberg, 1971) were used to calculate energy-transfer efficiencies, which in turn yielded distance information.

The efficiency of energy transfer, E, and the actual donor–acceptor separation distance, r, were calculated from the Förster equations (Stryer, 1978)

$$E = 1 - \tau_{da}/\tau_d \quad (1)$$

$$r = R_0(E^{-1} - 1)^{1/6} \quad (2)$$

where τ_{da} and τ_d are the luminescence lifetimes of the donor (Tb^{3+}) in the presence and absence of the acceptor (DAB), respectively, and R_0 is the critical distance of 50% energy transfer. τ_d and τ_{da} were determined from the luminescence decay curves of Tb_4CaM and Tb_4CaM_{DAB}, respectively. Typical errors associated with the lifetime measurements are less than 5%. R_0 is given by

$$R_0^6 = (8.78 \times 10^{-25})n^{-4}\phi\kappa^2 J \text{ (in cm}^6\text{)} \quad (3)$$

where n is the refractive index, ϕ the quantum yield, κ^2 the orientation factor, and J the donor–acceptor spectral overlap integral. In this study, n was taken to be 1.4, a typical value for protein solutions (Fairclough & Cantor, 1978); ϕ was taken to be 0.49, the same as that used for Tb^{3+} bound to TnC (Wang et al., 1982a); κ^2 was taken to be $^2/_3$, which is justified by the fact that the degenerate energy levels of Tb^{3+} ion render the energy donor virtually isotropic (Horrocks & Sudnick, 1981); and J was calculated by numerical integration from the emission spectrum of Tb_4CaM and absorption spectrum of CaM_{DAB}. Since R_0 is proportional to the sixth root of most of these terms, the error range stemming from their values is usually well less than 10%. In general, the major uncertainty in distance measurements is introduced by the orientation factor, which is around 20% (Dale & Eisinger, 1975; Stryer, 1978); with Tb^{3+} as the energy donor, however, the uncertainty is reduced to 11–12% (Latt et al., 1970). If the acceptor is also allowed to rotate freely, the range of uncertainty would be even narrower. Thus, R_0 for the Tb^{3+}–DAB couple was computed to be 3.0 ± 0.3 nm.

Results

Stoichiometric Titration of CaM with $TbCl_3$. When wheat germ CaM was titrated with $TbCl_3$, the tyrosine fluorescence increased linearly and leveled off with an end point of about 1 Tb^{3+}/CaM molecule; on the other hand, no enhancement in tyrosine-sensitized Tb^{3+} luminescence was observed until the third and fourth Tb^{3+} were added (Figure 1). This Tb^{3+} luminescence change is similar to that of mammalian CaM, whereas the changes in tyrosine fluorescence for the protein from the two sources are rather different. In the case of mammalian (e.g., bovine brain) CaM the tyrosine fluorescence exhibits a 2-fold increase as the first two Tb^{3+} ions bind to the protein, followed by a decrease as the second pair of Tb^{3+} is added (Wang et al., 1982b). The results of Tb^{3+} titration of wheat germ CaM suggest that the initially added Tb^{3+} ions bind to CaM in an ordered manner, so that the first bound Tb^{3+} ion is able to bring about conformational changes needed for the full enhancement of tyrosine fluorescence. Since wheat germ CaM has a single tyrosine residue (Tyr-139; Toda et al., 1985) within site IV, and since neither of the first two Tb^{3+} ions is sensitizable through this tyrosine, sites III and IV are

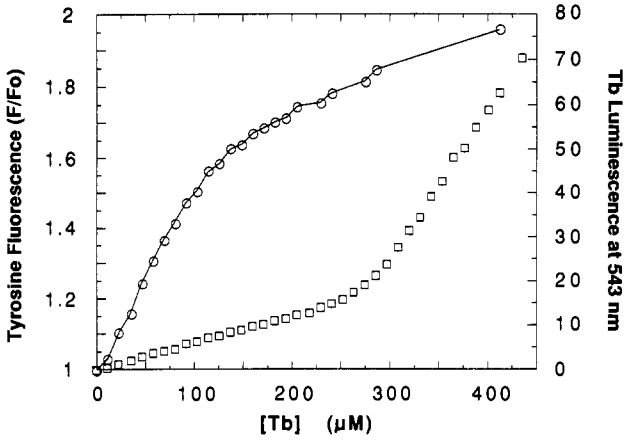

FIGURE 1: Stoichiometric titration of CaM with $TbCl_3$. CaM (0.5 mL, 136 μM) in 0.1 M KCl and 25 mM Pipes, pH 6.7, was titrated with 5.72 mM $TbCl_3$; both tyrosine fluorescence (circles, λ_{ex} = 280 nm; λ_{em} = 300 nm) and the tyrosine-sensitized Tb^{3+} luminescence (squares, λ_{ex} = 280 nm; λ_{em} = 543 nm) were monitored. Due to the high protein concentration used to establish the end point of the Tb^{3+} luminescence change, the sample became cloudy near the end of titration.

the two low-affinity sites for Tb^{3+}, in analogy to the case of mammalian CaM (Kilhoffer et al., 1980; Wang et al., 1982b). The other two Ca^{2+}-binding sites (sites I and II) would then be the high-affinity sites, of unequal affinities for Tb^{3+}. The fact that the two high-affinity sites of wheat germ CaM have different apparent affinities in the case of certain cations is also evident in an earlier paper of Yoshida et al. (1983). It remains to be seen whether this is due to intrinsic differences in the binding characters of the two sites or whether there is a negative cooperativity between them.

Luminescence Lifetime of Tb^{3+} Bound to Unlabeled CaM. To study the luminescence decay of CaM-bound Tb^{3+} ions, samples containing wheat germ CaM and varying amounts of $TbCl_3$ were excited either indirectly through the aromatic residues of the protein with a UV flash lamp or directly with a tunable dye laser. When Tb_nCaM was irradiated with UV light, a weak signal of Tb^{3+} emission, which decayed with a single lifetime of 1.3 ms, was detected only if n was greater than 2; this is consistent with the data of the steady-state fluorescence titration. Upon laser excitation, on the other hand, the emission of Tb_nCaM decayed monoexponeitially for all $n \leq 4$, the lifetime being also 1.30 (±0.03) ms. This characteristic lifetime of bound Tb^{3+} is identical with that observed for TnC and bovine brain CaM, suggesting similar environments at the binding sites of these proteins. Change of pH from 6.7 to 5.0 does not affect the lifetime of the Tb^{3+} luminescence (Table I).

Tb^{3+} Titration of DAB-Labeled CaM. When the DAB-labeled CaM was subjected to Tb^{3+} titration, both tyrosine fluorescence and the tyrosine-sensitized Tb^{3+} luminescence being monitored, the spectral changes were found very similar to those for the unmodified protein, except that the intensity of tyrosine-sensitized Tb^{3+} emission was much weaker (data not shown). The fact that both titrations have the same end points suggests that labeling of CaM at Cys-27 does not affect the binding of Tb^{3+}, whereas the decreased Tb^{3+} luminescence is a good indicator of energy transfer.

Luminescence Lifetime of Tb^{3+} Bound to DAB-Labeled CaM. Upon laser irradiation, Tb^{3+} bound to CaM_{DAB} emitted luminescence with shortened lifetimes (see below), suggesting energy transfer between Tb^{3+} and the DAB moiety. When illuminated with UV light, however, the emission intensity was too weak compared to the background to produce any useful

Table I: Summary of Fluorescence Energy Transfer Data of Tb_nCaM_{DAB} by Laser Excitation[a]

sample	n	pH	τ_1 (ms)	A_1	τ_2 (ms)	A_2
Tb_nCaM	1.0	6.7	1.33	100	–	–
	2.0		1.29	100	–	–
	3.0		1.32	100	–	–
	4.0		1.29	100	–	–
	4.0	5.0[b]	1.25	100	–	–
Tb_nCaM_{DAB}	0.7	6.7	0.93	100	–	–
	1.3		0.95	82	0.35	18
	2.0		0.96	69	0.39	31
	2.0	5.0	0.79	73	0.20	27
	2.0	7.0	1.00	61	0.61	39
	3.0	7.0	0.95	60	0.46	40
	4.0		0.95	46	0.45	54
	4.0	5.0	1.18	47	0.38	53
		7.0	1.04	72	0.26	28
Tb_nCaM_{DAB} trypsinized[c]	1.0	6.9	1.35	100	–	–
	2.0		1.36	100	–	–
	2.0	5.0	1.32	100	–	–
	3.0		1.28	100	–	–
	4.0		1.31	100	–	–
	4.0	7.0	1.35	100	–	–

[a] Decay parameters were derived from one- or two-exponential method-of-moments analyses of the decay curves. The experimental uncertainity in the lifetime determinations is 5%. Measurements were made at protein concentrations of 10–20 μM, in 25 mM Pipes and 0.1 M KCl at 25 °C. [b] The pH of the solution was adjusted by adding either 0.1 M HCl or 0.1 M KOH. [c] CaM_{DAB} was first digested with trypsin in 1 mM $CaCl_2$, followed by dialysis and the addition of $TbCl_3$.

information. Direct excitation was therefore used in the following studies.

(*a*) *Stoichiometric Titration.* CaM_{DAB} was titrated with $TbCl_3$, and the luminescence lifetime of bound Tb^{3+} was measured. When n (the ratio of added Tb^{3+} to protein concentration) equals 1, the metal emission decays monoexponentially with a lifetime of 0.93 ms (Table I). According to eq 1 and 2, this shortened lifetime corresponds to a distance of 3.5 nm between the two probes. If the initially added Tb^{3+} ion binds to the N-terminal half of the molecule where the DAB moiety is attached, as expected from its lack of tyrosine sensitization (see above), it is somewhat surprising intuitively that the observed distance is so long. On the other hand, one recognizes the fact that the DAB label itself spans about 2 nm; thus, it is not entirely unreasonable to assume that the first Tb^{3+} is located in the same half of CaM (e.g., site II) as is the DAB label, yet still maintains a rather sizable separation from it. As n increases from 1 to 4, the decay becomes multiexponential; when fitted with a biexponential equation, two lifetimes were obtained: one is about the same as the first lifetime, the other is considerably shorter (Table I), indicating that the subsequently added Tb^{3+} ions bind to sites closer (2.7 nm or less) to the DAB moiety. In no case, however, was an unquenched Tb^{3+} emission observed during the entire titration, suggesting that under these conditions CaM assumes a conformation such that all metal-binding sites are within a radius of 3.5 nm of the probe.

(*b*) *Effect of pH.* The effect of pH on the structure of CaM was examined in both the Tb_2 and Tb_4 states. With $n = 2$, changing pH from 6.7 to 5.0 had a significant effect on the decay properties of Tb^{3+} luminescence. The metal emission of Tb_2CaM_{DAB} at pH 5.0 decayed biexponentially with lifetimes of 0.20 and 0.79 ms (Figure 2). Both lifetimes were shorter than the ones observed at pH 6.7, indicating a shorter distance between DAB and the two bound Tb^{3+} ions in the more acidic medium. The change is reversible; returning of the pH to neutrality brings the decay curve nearly back to the original position. When $n = 4$, lowering the pH results in more

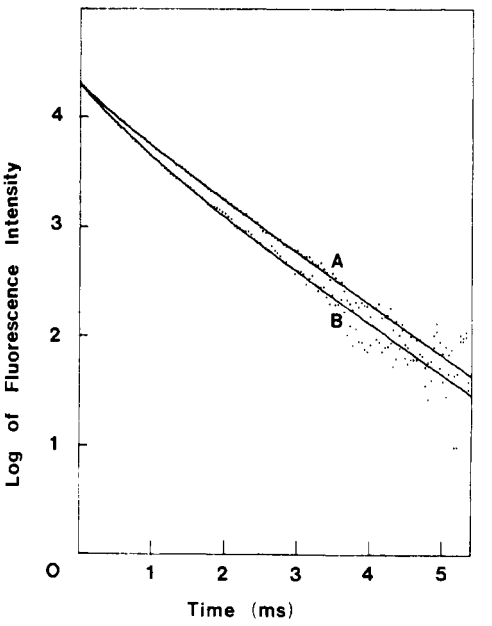

FIGURE 2: Luminescence decay curves of Tb_2CaM_{DAB} at pH 6.7 (A) and at pH 5.0 (B). Conditions were the same as described in Table I. The dots are experimental data points. The solid lines are best-fit curves calculated by using parameters derived from two-exponential method-of-moments analysis. The quality of fit was judged from the magnitude of the mean square residual defined below, where $I_{c,i}$ and

$$\chi^2/N = (1/N)\sum_{i=1}^{N}(I_{c,i} - I_{e,i})^2/I_{e,i}$$

$I_{e,i}$ are the calculated and experimental intensities, respectively, and N is the number of points. χ^2/N values of 5.0 or less were obtained for all the analyses in this work.

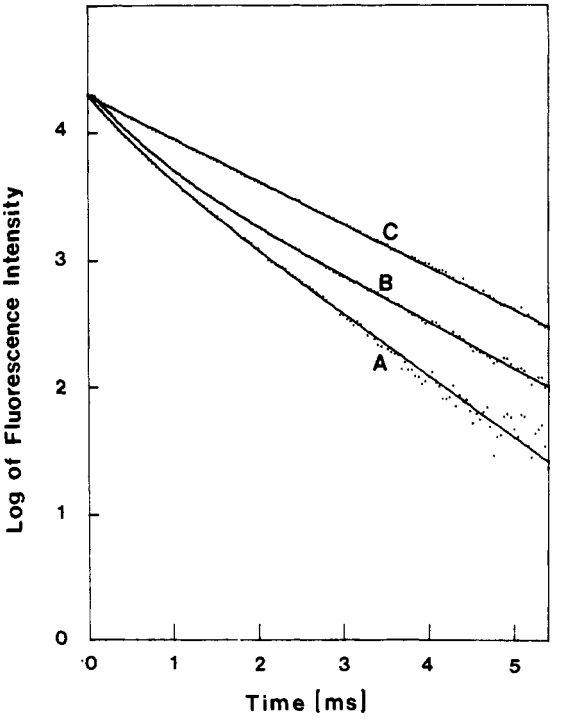

FIGURE 3: Luminescence decay curves of Tb_4CaM_{DAB} at pH 6.7 (A) and at pH 5.0 (B) and the luminescence decay curve of Tb^{3+} bound to the unlabeled CaM (C). See legend of Figure 2 and Table I for other information.

subtle changes in the decay parameters: The short-lived component remains unchanged, but the lifetime of the other component becomes even longer (Table I and Figure 3). It should be pointed out that owing to the intrinsic difficulty of resolving more than two lifetimes from a given nonexponential

decay curve, the observed lifetimes may actually represent an average of more than one species. The appearance of a decay component with a lifetime of 1.18 ms (corresponding to a donor–acceptor distance of 4.4 nm), which is accompanied by the pH change from 6.7 to 5.0, is of particular interest. Since this long-lived component was not observed when $n = 2$, it *must* be associated with the Tb^{3+} ions bound to the low-affinity sites. Thus, it seems that in the acidic medium wheat germ CaM adopts a more extended conformation so that sites III and IV in the C-terminal half are more distant than at neutral pH from the DAB label attached to Cys-27 in the N-terminal half of the molecule.

(*c*) *Effect of Trypsin Cleavage.* It has been shown that in the presence of Ca^{2+} trypsin cleaves CaM into two fragments: TR1C (residues 1–77) and TR2C (residues 78–148); each contains two Ca^{2+}-binding sites (Walsh et al., 1977). To test the hypothesis of a pH-dependent conformational change, CaM_{DAB} was treated with trypsin in 1 mM $CaCl_2$. The digestion mixture was dialyzed to remove bound Ca^{2+}, followed by addition of Tb^{3+} and lifetime measurements. It was found that the metal luminescence decayed monoexponentially with an apparently unquenched lifetime of 1.3 ms (Table I). The fact that there was no short-lived component even at low Tb^{3+}/CaM ratios suggests that part of the emission is totally quenched. According to eq 1, a distance of 1.5 nm (half of R_0) between Tb^{3+} and DAB would result in an energy-transfer efficiency of 0.985, rendering such bound Tb^{3+} essentially a "dark complex". If this were true, then the DAB moiety in the TR1C fragment must be in an environment different from that in the intact protein. More interestingly, lowering the pH from 6.8 to 5.0 at either $n = 2$ or $n = 4$ has no effect on the observed decay time. Thus, a split at the connecting sequence between the two halves of the CaM molecule apparently abolishes the pH-dependent structural transition.

Discussion

In this study we have carried out distance measurements as a function of pH between Tb^{3+} ions bound to the Ca^{2+}-binding sites and a chromophoric group (DAB) attached to Cys-27 of wheat germ CaM by fluorescence energy transfer techniques. The methodology has been successfully applied to skeletal TnC (Wang et al., 1987), in which case we found that TnC undergoes a conformational change as the pH of the medium is lowered from near neutrality to 5.0. The observations made in this work are also consistent with a similar pH-dependent structural transition for CaM. In an aqueous solution buffered at pH 6.7, CaM appears to exist in a conformational state where the two high-affinity Tb^{3+}-binding sites (sites I and II) are no farther than 4 nm from the DAB label, and the two low-affinity sites (sites III and IV) are around 3 nm from the label. As the DAB group has a maximum chain length of 2.0 nm, depending on where it is situated, CaM would have different end-to-end dimensions. In view of the hydrophobic nature of the probe, as well as the fact that Cys-27 is not readily accessible for labeling (denaturing conditions are usually required), the DAB moiety is most likely inbedded in the N-terminal domain, extending toward the C-terminal half of the molecule. An examination of the crystallographic structure of rat testes CaM (Babu et al., 1985) indicates that such an arrangement would put the tip of the DAB label beyond the midpoint of the central helix, so that the label is closer to sites III and IV than to sites I and II. Alternatively, a "bend" in the central helix would also render the DAB moiety more proximal to the C-terminal domain of the molecule. In either case the dimension of CaM would be a litter shorter than the one depicted by the crystal structure.

When the pH is lowered to 5.0, the change in the decay time of Tb^{3+} luminescence in Tb_4CaM_{DAB} is consistent with an increase in the distance between some of these bound ions and the DAB label. Since this increase in separation does not occur when $n = 2$ (in fact the interprobe distance decreases slightly), the lengthening in distance (from 2.7 to 4.4 nm) must involve Tb^{3+} ions bound at the low-affinity sites (sites III and IV) in the C-terminal domain. This would in turn imply that in acidic environments the CaM molecule becomes more stretched, in better agreement with the crystal structure obtained from the X-ray diffraction studies.

When CaM_{DAB} was digested with trypsin, the short-lived component of the Tb^{3+} emission disappeared, leaving an unquenched metal luminescence. One possible explanation for the apparent lack of energy transfer between Tb^{3+} and DAB is that the enzymic cleavage leads to an altered configuration of the DAB label, so that the label is now closer to the Tb^{3+} ions at sites I and II and totally quenches their luminescence. This explanation is consistent with the idea that in the intact protein at neutral pH the DAB label in the N-terminal domain could interact with the C-terminal half of the molecule, and the interaction is abolished when the protein is cleaved at the middle. The unquenched metal luminescence observed for the trypsinized protein must then be due to Tb^{3+} ions bound to the TR2C fragment, which is now well separated from the DAB label in the TR1C fragment. Moreover, the trypsin-digested CaM_{DAB} is no longer sensitive to pH change, indicating that the pH-dependent conformational transition does not operate once the central helix of CaM is cleaved. This phenomenon is very similar to that of skeletal TnC (Wang et al., 1987).

The indication that in a solution of neutral pH CaM (and TnC as well) exists as a relatively more compact molecule, rather than as an extended dumbbell, has been supported by recent small-angle X-ray scattering studies (Heidorn & Trewhella, 1988). These authors found that CaM undergoes a slight extension upon Ca^{2+} binding. When the pH was changed from 7.4 to 5.5 in the absence of Ca^{2+}, no significant change was observed in the overall shape of the molecule. Unfortunately, they could not study the effect of pH change in the presence of Ca^{2+} because CaM precipitated in acidic medium, maybe due to the high protein concentration used. In the present study the typical sample had a concentration of 10 μM, and there was no indication of protein aggregation upon lowering the pH. As it was pointed out in an earlier paper (Wang et al., 1987), dimer formation of the protein would only increase the energy transfer and shorten the donor's lifetime. In fact, we have observed the opposite, viz., a decreased energy transfer at lower pH. Thus, the observed change cannot be accounted for by protein aggregation.

The pH-dependent structural transition of both CaM and TnC suggests that the connecting helices of these molecules are very flexible. This idea is consistent with a number of observations. For example, the tyrosine fluorescence anisotropy of CaM has a very short (2–3 ns) decay time (Small & Anderson, 1988); recent work of site-specific mutagenesis of CaM shows that neither deletions nor alterations of residues in the central helix region of CaM significantly affect its biological activities (Persechini et al., 1988). On the other hand, it has been noted that none of the CaM fragments are capable of activating the target enzymes (Minowa et al., 1988). Thus, it is possible that the flexible stretch of CaM is essential for interacting with its target molecules. The pH-dependent conformational change observed in this study may therefore be relevant to the biological functions of CaM.

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Refolding of Denatured Ribonuclease Observed by Size Exclusion Chromatography†

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ABSTRACT: The unfolding and refolding of pancreatic ribonuclease have been observed by absorbance, fluorescence, and size exclusion chromatographic measurements in solutions of guanidinium chloride continuously maintained at pH 6.0 and 4 °C. The spectral measurements were fitted with a minimal number of kinetic phases while the chromatographic measurements were simulated from an explicit mechanism. All of the measurements are consistent with a minimal mechanism involving seven components. The folded components include the native protein and two transiently stable intermediates each having the same hydrodynamic volume. The intermediate having all native peptide isomers has an unfolding midpoint in 3.8 M denaturant while the intermediate having one nonnative peptide isomer has an unfolding midpoint in 1.3 M denaturant. The unfolded protein is distributed among four components having the same hydrodynamic volume but differing peptide isomers. At equilibrium, 10% of the denatured protein has all native isomers, 60% has one nonnative isomer, 5% has a different nonnative isomer, and 25% has both nonnative isomers. In low denaturant concentrations, the dominant component with one nonnative isomer can refold to transiently populate the compact intermediate with the same nonnative isomer.

In spite of almost 2 decades of investigation, an integrated mechanism describing the unfolding/refolding reactions of ribonuclease A in denaturants has not been agreed to (Schmid, 1986; Lin & Brandts, 1987). Since all prior kinetic measurements of unfolding and refolding have utilized spectral measurements, we were optimistic that the use of an entirely different observational probe such as hydrodynamic volume measurements might make a useful contribution to definition of the mechanism. Accordingly, a series of equilibrium and kinetic measurements of the conformational transition of ribonuclease was initiated using high-performance size exclusion

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