Volume 234, number 1, 218–223 FEB 06027 July 1988

Frictional resistance to motions of bimane-labelled spinach calmodulin in response to ligand binding

Shixing Yuan and Alfred Haug

Department of Microbiology and Pesticide Research Center, Michigan State University, East Lansing, MI 48824, USA

Received 4 April 1988; revised version received 12 May 1988

The single cysteinyl residue 26 of spinach calmodulin was labelled with the thiol-specific bimane fluorescence probe. Following application of stoichiometric quantities of Ca^{2+} or aluminum ions to the protein, temperature-dependent fluorescence changes (anisotropy, lifetime) could be monitored via the label. From these data the Y function could be constructed which, as a function of temperature, seems to consist of two linear regions which intersect at the critical temperature, T_c. From the Y function the thermal coefficient, $b(T)$, of the frictional resistance to fluorophore rotation could be determined. $b(T)$ was dependent on the type and stoichiometry of the ligand(s) bound to calmodulin. Changes of the thermal coefficient apparently resulted in part from ligand-triggered structural pertubations transmitted over a considerable distance to calmodulin region I, the site of the fluorophore.

Calmodulin; Protein flexibility; Fluorescence anisotropy; Fluctuation

1. INTRODUCTION

Calmodulin (CaM) is a small (M_r 15700), acidic protein, which mediates Ca^{2+}-dependent regulatory processes in cells. An intriguing feature of calmodulin is its capability to interact with and stimulate numerous specific enzymes [1]. The biochemically active form appears to be $(Ca^{2+})_4$-calmodulin which has a pronounced tendency to bind to a basic amphiphilic α-helix in a variety of target peptides [2]. The protein's intrinsic flexibility probably contributes to its versatility [3]. Thanks to its flexibility and associated rapid local motion, calmodulin can apparently control and thus correlate molecular events [4,5], e.g., those triggered by specific ligands bound to specific locations. This motion and associated conformational changes are thought to be crucial for its biochemical activity [5].

To analyze the conformational changes, a small fluorophore attached to the protein may be used. For example, by measuring the temperature dependence of fluorescence polarization and lifetime of the probe, the Y function can be established [6]. This function describes the frictional resistance associated with conformational changes to the probe's rotational motion.

Conformational changes at a defined locus on calmodulin may be the result of transfer of structural perturbations initially generated by ligands bound to protein regions distant from or close to the defined locus [7]. Examination of these kinds of ligand-triggered conformational changes via a fluorophore requires studies of the relative motion of calmodulin's constituent regions.

To monitor ligand-triggered conformational changes through a fluorophore, we therefore selected spinach calmodulin which harbors a single cysteinyl residue at site 26, i.e., region I of the polypeptide chain [7,8]. Our results indicate that through a strategically anchored fluorophore, conformational changes could be determined in response to ligand-triggered structural perturbations.

Correspondence address: S. Yuan, Department of Microbiology and Pesticide Research Center, Michigan State University, East Lansing, MI 48824, USA

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/88/$3.50

2. MATERIALS AND METHODS

The fluorescence probe monobromotrimethylammoniobimane was purchased from Calbiochem Company (San Diego, CA) under the commercial name of thiolyte MQ. Phenyl-Sepharose CL-4B was obtained from Pharmacia Company (Uppsala, Sweden). The other chemicals were of the highest quality available.

2.1. *Purification of spinach calmodulin*

Spinach calmodulin was purified from fresh spinach leaves according to a method described [7]; a slight modification was made by replacing phenothiazine Affigel with phenyl-Sepharose CL-4B. The purity of the protein was judged by the characteristic ultraviolet absorption spectrum and the absence of tryptophan fluorescence. Moreover, following electrophoresis on SDS gels, only one band was observed [7].

2.2. *Labelling of spinach calmodulin*

Freshly isolated calmodulin was labelled with a fluorescence probe, thiolyte MQ. This probe is highly reactive with reduced thiol groups. The probe concentration was evaluated by measuring its absorbance (ϵ_{378} = 5700 $M \cdot cm^{-1}$) [9]. The protein concentration was determined by the Bradford assay [10]. The molar ratio of [label]/[calmodulin] was found to be unity [7].

2.3. *Fluorescence studies of labelled calmodulin*

Fluorescence experiments were performed on an SLM spectrofluorimeter, model 4800 (Urbana, IL), which was interfaced with a Hewlett-Packard desk top computer, HP-85, and a plotter to aid in data acquisition and analysis. The steady-state fluorescence anisotropy, r, was measured by using the T-format method. Two Corning filters, CS-72, were placed in the emission pathways. The excitation wavelength was at 385 nm. Fluorescence lifetimes were determined according to the phase shift and demodulation methods [11].

2.4. *Analysis of the data*

The Y function is dependent on the thermal coefficient of frictional resistance, $b(T)$, to rotation of the fluorophore [6]. If a fraction, f_1, of the protein molecules is in substate 1, while the other fraction, f_2, occupies substate 2, the corresponding thermal coefficients are b_1 and b_2, respectively. The ratio, $f_2/f_1 = K$, is the equilibrium constant, and

$$b(T) = b_2 f_1 + b_2 f_2 \quad (1)$$

The Y function is defined as

$$Y = \ln(\eta_o/\eta) = b(T) \cdot (T - T_o)$$
$$= \ln\{[r(0)/r] - 1\} - \ln(RT\tau/V\eta_o) \quad (2)$$

where η and η_o are viscosities at temperature T and a reference temperature, T_o (293.1 K), respectively. r is the anisotropy measured at temperature, T, $r(0)$ is the apparent limiting anisotropy [11]. For the bimane-labelled protein, we obtained a value of $r(0)$ = 0.2142 from a Perrin plot [7]. V is the molecular volume of the fluorophore probe, τ is the fluorescence lifetime measured, and R is the gas constant. Since the Y function is the logarithm of a ratio of viscosities, a low value of the Y function is associated with restricted fluorophore rotation because of a high viscosity.

The standard enthalpy at the critical temperature T_c can be derived from

$$\Delta H = [(dY/dT)_{T_c} - 0.5(b_1 + b_2) \cdot 4RT_c^2/t_c(b_2 - b_1)] \quad (3)$$

T_c is the critical temperature, where $K = 1$, and $t_c = T_c - T_o$. The critical temperature was obtained from a linear regression analysis of the data points. The slope at this temperature was determined after fitting the experimental data to theoretical curves using a non-linear regression scheme (Marquardt's algorithm) on an IBM-XT computer. The correlation coefficient for these kinds of statistical analyses was better than 0.98.

3. RESULTS

Fluorescence anisotropy and lifetime studies were performed on bimane-labelled spinach calmodulin in Mops buffer over the temperature range 10–40°C. From these data corresponding Y function values (fig.1) were calculated using eqn 2.

Upon binding of the first two Ca^{2+} to spinach calmodulin, the Y function values increased compared with corresponding values of apocalmodulin, at all temperatures tested (fig.1). Following binding of the first two Ca^{2+} to calmodulin's high affinity binding sites III and IV [1], a perturbational signal was locally elicited, producing conformational changes which, in turn, can be monitored at the bimane-labelled region I. Relative to apocalmodulin, the probe's rotational motion became apparently less restricted in $(Ca^{2+})_2$-calmodulin. In the presence of saturating Ca^{2+} concentrations, where Ca^{2+} also occupies region I, the Y function values were higher at temperatures below 298 K, while those above that temperature were lower compared with corresponding values derived from apocalmodulin (fig.1). Below 298 K and in the presence of saturating Ca^{2+} concentrations, the probe experienced less rotational constraint compared with that above this critical temperature. This temperature dependence is probably a manifestation of conformational changes occurring at the critical temperature of 298 K. At a molar ratio of [Al]/[calmodulin] = 3:1, addition of aluminum produced a change which, in turn, restricted the probe's rotation more than that observed in apocalmodulin above 293 K. Below this temperature the rotational characteristics were reversed (fig.1).

The thermal resistance coefficients, b_1 and b_2, are listed in table 1. In the low temperature range, the values of the slopes, b_1, were larger than those

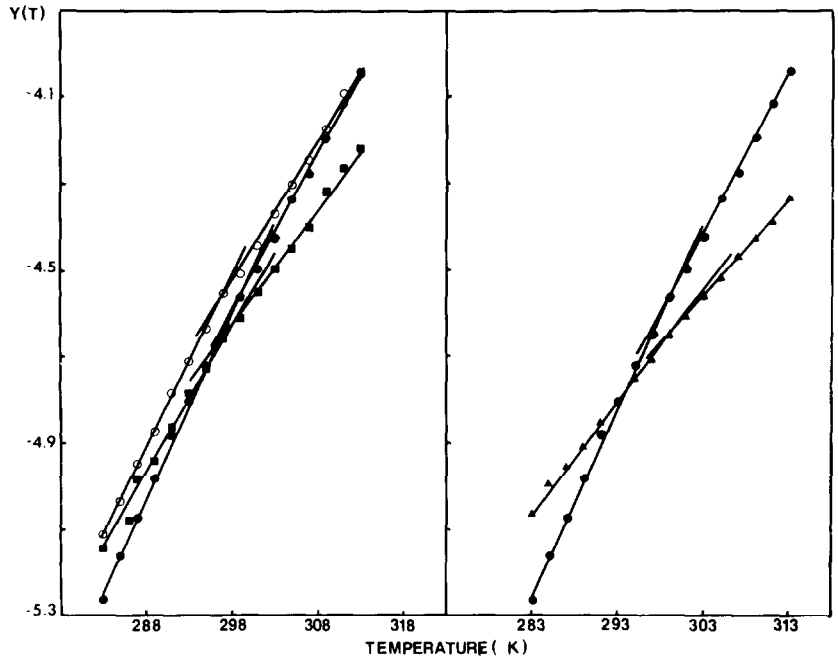

Fig.1. $Y(T)$ vs temperature for bimane-labelled spinach calmodulin. Fluorescence studies were performed on an SLM spectrofluorimeter, model 4800. The labelled calmodulin was dissolved in Mops buffer (10 mM, pH 6.5, 0.1 M KCl) to reach a concentration of 7 μM. (●) Apocalmodulin; (○) $2Ca^{2+}$; (■) calmodulin + $5Ca^{2+}$; and (▲) calmodulin + $5Ca^{2+}$ + 3Al.

in the higher temperature range, b_2. All b_1 and b_2 values decreased as the Ca^{2+} concentration of the solution increased.

Concerning changes of the thermal resistance coefficient (table 1), it appeared that the effective resistance to rotation experienced by the fluorophore comprises two temperature-dependent contributions, viz., the protein conformations and the solvent viscosity. It is known that Ca^{2+}- and Al-binding to calmodulin lead to pronounced conformational changes in the protein [1,7,12,13]. Ligand-triggered changes are also reflected in the enthalpy values (table 1) observed at the critical temperature, where the protein was being transformed from substate 1 into substate 2. For apocalmodulin this enthalpy change was 8.0 kcal·mol^{-1}·K^{-1}, while for $(Ca^{2+})_2$-calmodulin the enthalpy value decreased to 2.9 kcal·mol^{-1}·K^{-1}. The probe residing in region I seemingly became more sensitive to temperature changes following binding of two Ca^{2+} at regions III and IV. Structural perturbations at these latter sites produced conformational changes which could be monitored at the defined site of the fluorophore.

Following further addition of Ca^{2+} until saturation, one of the Ca^{2+} bound to Ca^{2+} binding region I. Consequently new bonds were established which rendered the probe's rotational motion less prone to temperature changes, as indicated by ΔH = 5.3 kcal·mol^{-1}·K^{-1}. When aluminum (3:1) was present, the probe's motion appeared to be less disposed to temperature changes as indicated by ΔH = 8.2 kcal·mol^{-1}·K^{-1}. These Al-related changes of motion may be a consequence of con-

Table 1

Physical parameters[a] for spinach calmodulin in the presence of ligands

Calmodulin	b_1	b_2	b_1/b_2	T_c	ΔH	ΔS
Apocalmodulin	4.7	3.4	1.4	299	8.0	26.8
CaM + $2Ca^{2+}$	4.2	3.0	1.4	297	2.9	9.8
CaM + $5Ca^{2+}$	3.7	2.5	1.5	298	5.3	17.8
CaM + $5Ca^{2+}$ + 3Al	2.7	2.2	1.2	299	8.2	27.4

[a] b_1 and b_2 are listed as percent decrease per degree, T_c is the critical temperature in K with an error of ± 1 K, ΔH in kcal per mol, ΔS in kcal per mol per degree

formational changes monitored at the probe's monitoring site.

4. DISCUSSION

Regarding calmodulin's multifunctional roles [1], the inherent flexibility of the polypeptide chain, in the presence and absence of Ca^{2+}, is apparently of great import [3]. This flexibility is in part determined by rotational barriers generated by bonds such as those of peptide linkages and hydrogen bonds in helix structures. Like that of other globular proteins in a thermal bath, calmodulin's flexible structure rapidly fluctuates around its equilibrium configuration in solution. Associated local motions are generally thought to be crucial for biochemical activities [4,5]. Molecular dynamics analysis of proteins indicated that there exist a multitude of conformational substates which are accessible by rapid structural fluctuations of the protein [14]. When triggered locally by the first two Ca^{2+}, bound to the carboxyl-terminus lobe of the calmodulin dumb-bell [15], conformational changes can be monitored through the strategically anchored fluorophore. These may originate from changes of segmental motion and/or global protein motion which, in turn, had been produced by ligand-triggered perturbations of local protein structure. Therefore, these types of perturbations are becoming manifest in changes of physical properties of fluorophore rotation.

We have also shown that spinach calmodulin, both in its apoform and in the presence of metals, undergoes a substate change at a temperature of 25°C. This temperature value is significantly below the major thermal unfolding of the protein, viz., at 55°C for apocalmodulin and at >90°C for calmodulin in the presence of Ca^{2+} [16]. In biochemically active calmodulin, i.e., $(Ca^{2+})_4$-calmodulin generated in the presence of saturating Ca^{2+} (table 1), the enthalpies lie below those of calmodulin which is biochemically inactive (apocalmodulin) or whose regulatory capacity has been greatly diminished by aluminum ions [13]. Furthermore, in $(Ca^{2+})_4$-calmodulin the lower enthalpy seems to reflect the sensitive dynamic aspects of calmodulin, essential for the transfer of information within the macromolecule in response to regulatory stimuli. Again in biochemically active calmodulin, the Ca^{2+}-protein interaction characteristics probably remained intact [17] in both structures associated with the substances below and above the critical temperature. The temperature-dependent changes observed in apocalmodulin versus $(Ca^{2+})_4$-calmodulin (fig.1) may be related in part to their respective degree of hydration. This is illustrated, for example, in studies indicating that water molecules are being removed at each coordination site of Ca^{2+} [18]. Furthermore, in biochemically active calmodulin a Ca^{2+}-induced exposure of hydrophobic surface was observed [19]. Similarly, concomitant with the breakage of hydrogen bonds [13], changes in solvation structure are to be expected upon application of aluminum ions to $(Ca^{2+})_4$-calmodulin.

In summary, thanks to its finely tuned, Ca^{2+}-dependent flexibility, calmodulin is capable of fulfilling regulatory functions.

Acknowledgements: The work was supported in part by grant 1-R01-ES04468-01 from the National Institutes of Health. The authors thank Dr Ken Poff, Plant Research Laboratory, for use of the SLM spectrophotometer.

REFERENCES

[1] Klee, C.B. and Vanaman, T.C. (1982) Adv. Protein Chem. 35, 213–321.
[2] O'Neil, K.T., Wolfe, H.R., Erickson-Vittanen, S. and DeGrado, W.F. (1987) Science 236, 1454–1456.
[3] Lambooy, P.K., Steiner, R.F. and Sternberg, H. (1982) Arch. Biochem. Biophys. 217, 517–528.
[4] Karplus, M. and McCammon, J.A. (1981) CRC Crit. Rev. Biochem. 9, 293–349.
[5] Careri, G. (1984) Order and Disorder in Matter, The Benjamin/Cummings Publ., Menlo Park, CA.
[6] Scarlata, S., Rholam, M. and Weber, G. (1984) Biochemistry 23, 6789–6792.
[7] Yuan, S. and Haug, A. (1988) Eur. J. Biochem., in press.
[8] Vanaman, T.C. (1983) Methods Enzymol. 102, 296–310.
[9] Kosower, N.S., Kosower, E.M., Newton, G.L. and Raney, H.M. (1979) Proc. Natl. Acad. Sci. USA 76, 3382–3386.
[10] Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
[11] Lakowicz, J.R. (1984) Principles of Fluorescence Spectroscopy, Plenum Press, New York.
[12] Weis, C. and Haug, A. (1987) Arch. Biochem. Biophys. 254, 304–312.
[13] Siegel, N. and Haug, A. (1983) Biochim. Biophys. Acta 744, 36–45.
[14] Elber, R. and Karplus, M. (1987) Science 235, 318–321.
[15] Babu, Y.S., Sack, J.S., Greenhough, T.J., Bugg, C.E., Means, A.R. and Cook, W.J. (1985) Nature 315, 37–40.

[16] Martin, S.R. and Bayley, P.M. (1986) Biochem. J. 238, 485–490.
[17] Permyakov, E.A., Shnyrov, V.L., Kalinichenko, L.P. and Orlov, N.Y. (1985) Biochim. Biophys. Acta 830, 288–295.
[18] Mulqueen, P., Tingey, J.M. and Horrocks, W.DeW. (1985) Biochemistry 24, 6639–6645.
[19] LaPorte, D.C., Wierman, B.M. and Storm, D.R. (1980) Biochemistry 19, 3814–3819.