Table IV: Comparison of Constants Obtained from Single Half-Turnover and Steady-State Kinetics

constant	single half-turnover[a]	steady state[b]
k_1 (s^{-1})	9	87
k_{-1} (s^{-1})	5.2	10.8
k_2/K_{So} (mM^{-1} s^{-1})	66	190
k_{-2}/K_{Si} (mM^{-1} s^{-1})	31	23
E_a for k_{-1} (kcal/mol)	28.2[c]	41
E_a for k_{-2}/K_{si} (kcal/mol)	17.4[d]	18

[a] Values in this study at 10 °C. [b] Values from Lowe and Walmsley (1986) at 10 °C. [c] Value from condition C in Figure 4. [d] Value from condition D in Figure 4.

energies for the individual constants contributing to these composite constants. Through the use of this assumption, it was then possible to calculate the values of all the individual constants in Scheme I.

Our values and those of Lowe and Walmsley at 10 °C are presented in Table IV. With the exception of the values for k_1, which differ by a factor of 10, the values for the other rate constants agree within a factor of 3. Moreover, our values and theirs of the activation energies for k_{-1} and k_{-2}/K_{Si} are similar. When one considers that results from the steady-state measurements are for the intact erythrocyte, with its asymmetric distribution of membrane lipids (op den Kamp, 1979), whereas our results are for the purified transporter reconstituted in unsealed membranes of erythrocyte lipids, this agreement is very good. It shows that the purified, reconstituted transporter functions very similarly to the transporter in the erythrocyte.

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Turkey Gizzard Caldesmon: Molecular Weight Determination and Calmodulin Binding Studies†

Dean A. Malencik, Juan Ausio, Christine E. Byles, Brett Modrell, and Sonia R. Anderson*

Department of Biochemistry and Biophysics, Oregon State University, Corvallis, Oregon 97331-6503

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ABSTRACT: Sedimentation equilibrium and sedimentation velocity measurements demonstrate that turkey gizzard caldesmon is an elongated molecule of molecular mass 75 ± 2 kDa. The frictional ratio (2.14) is consistent with a prolate ellipsoid of axial ratio 24, corresponding to an apparent length and width of 516 and 21.5 Å, respectively. As was previously determined for chicken gizzard caldesmon [Graceffa, P., Wang, C.-L. A., & Stafford, W. F. (1988) *J. Biol. Chem. 263*, 14196–14202], this molecular weight is appreciably smaller than the value (~135 000) estimated from the results of $NaDodSO_4$ gel electrophoresis experiments. However, a significant difference between the true molecular weights of turkey and chicken gizzard caldesmons—75 000 versus 93 000—also points to probable molecular weight variations within the subclass. Binding measurements, based on perturbation of the intrinsic tryptophan fluorescence of caldesmon in the presence of calmodulin, show that the interaction between the two proteins is strongly ionic strength and temperature dependent. Dissociation constants of 0.075 and 0.38 μM were determined in solutions containing 0.1 and 0.2 M KCl, respectively, at 24.3 °C. Fluorescence emission spectra and fluorescence anisotropy excitation spectra indicate that the tryptophanyl residues of caldesmon are located in solvent-accessible regions of the molecule, where they exhibit a high degree of mobility even when calmodulin is bound.

The caldesmons, major calmodulin and actin binding proteins in smooth muscle and nonmuscle cells, comprise two classes of immunoreactive polypeptides having apparent molecular weights of 120 000–150 000 and 71 000–77 000 (Bretscher & Lynch, 1985). Caldesmon was originally identified in chicken gizzard as the 120–150-kDa species and shortly thereafter found to inhibit the actin-activated adenosinetriphosphatase activity of phosphorylated myosin (Sobue et al., 1981, 1982).

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* Address correspondence to this author.

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Inhibition occurs only when actin-bound tropomyosin is present (Marston et al., 1984; Smith & Marston, 1985; Lash et al., 1985; Sobue et al., 1985; Horiuchi et al., 1986) and is reversed by the addition of excess Ca^{2+}–calmodulin (Sobue et al., 1982; Ngai & Walsh, 1984). Vascular smooth muscle caldesmon inhibits force production in "desensitized" skeletal muscle fibers, confirming the assumption that the inhibition of adenosinetriphosphatase activity is related to inhibition of contraction (Taggart & Marston, 1988). The mechanism of caldesmon regulation is apparently similar to that established for skeletal muscle troponin and smooth muscle myosin phosphorylation in that all three systems modulate the rate of ADP and P_i release from actomyosin (Marston, 1988). The emerging function of caldesmon indicates that elements of both thin- and thick-filament Ca^{2+} control operate in smooth muscle. Recall that the latter involves reversible phosphorylation of the 20K myosin light chain—catalyzed by the calmodulin-dependent enzyme myosin light chain kinase [Sobieszek & Small, 1977; cf. reviews by Small and Sobieszek (1980), Hartshorne and Siemankowski (1981), and Perry et al. (1984)].

The two molecular weight forms of caldesmon are derived either from a single gene by alternative splicing events or from separate but closely related genes. Peptide mapping studies of chicken liver caldesmon (75 kDa) and chicken gizzard caldesmon (125 kDa) showed that the primary structures of the corresponding terminal regions of the two proteins may be identical. However, sequences occurring in the central core of the 125-kDa protein are absent in the 72-kDa protein (Ball & Kovala, 1988). Limited chymotryptic digestion of chicken gizzard caldesmon results in three major polypeptides, including a 40-kDa fragment plus an 18-kDa degradation product which retain the ability to bind F-actin and Ca^{2+}–calmodulin (Szpacenko & Dabrowska, 1986). Both the 40-kDa chymotryptic fragment and a functionally related 25-kDa fragment obtained through specific cleavage at cysteine residues have been placed at the carboxyl terminus of caldesmon (Riseman et al., 1989).

The initial molecular weight determinations of the caldesmons were based on the results of sodium dodecyl sulfate ($NaDodSO_4$)[1] gel electrophoresis and/or sedimentation velocity experiments. That the true molecular weight of chicken gizzard caldesmon is actually less than originally estimated became evident in sedimentation equilibrium measurements, which provided a molecular mass of 93 ± 4 kDa (Graceffa et al., 1988). In the meantime, we had undertaken sedimentation equilibrium studies of turkey gizzard caldesmon which also indicated a molecular weight smaller than the apparent value.

This two-part paper summarizes (1) the results of sedimentation equilibrium and sedimentation velocity experiments conducted with turkey gizzard caldesmon and (2) observations on the intrinsic tryptophan fluorescence of caldesmon, including determination of equilibrium constants for the calmodulin–caldesmon interaction.

Materials and Methods

Reagents and Preparations. Caldesmon from fresh turkey gizzards, donated by the Oregon Turkey Growers, was prepared according to the procedures described by Bretscher (1984) for chicken gizzard caldesmon. The purified protein was characterized by $NaDodSO_4$ gel electrophoresis in 7.5% polyacrylamide according to Laemmli (1970) and by amino acid analysis (Moore & Stein, 1963; Knecht & Chang, 1986). The electrophoresis experiments (gel photo included for reviewer's use) revealed a single component of apparent molecular weight ~135 000, determined relative to standards (debranching enzyme, 160K; phosphorylase *b*, 97K; bovine serum albumin, 68K; actin, 42K; carbonic anhydrase, 29K; troponin C, 18K; parvalbumin, 12K). The results of the amino acid analyses (Table I) are generally similar to those obtained for chicken caldesmon, as summarized by Graceffa et al. (1988). By assuming an average residue weight of 115, we calculated from these analyses that the total protein in a solution of absorbancy 0.52 at 280 nm is 1.0 ± 0.1 mg/mL.[2] The ratio of tyrosine to tryptophan was found to be 0.53 ± 0.03 from the results of absorption measurements performed on solutions containing 0.1 M NaOH [cf. procedure of Bencze and Schmid (1957)].

Table I: Amino Acid Analysis of Turkey Gizzard Caldesmon[a]

amino acid	mol % of amino acid	amino acid	mol % of amino acid
Asx	7.1	Met	1.1
Glx	25.2	Ile	1.6
Ser	5.1	Leu	5.0
Thr	4.2	Phe	1.2
Gly	4.7	Cys[b]	0.24
Ala	11.0	Lys	14.3
Pro	3.5	His	0.42
Val	4.1	Tyr	0.54
Arg	9.7	Trp[c]	1.0

[a] Average of five determinations. The variations were generally less than 10%. [b] Determined as (carboxymethyl)cysteine. [c] Determined according to the procedure of Bencze and Schmid (1957).

Bovine brain calmodulin, prepared according to Schreiber et al. (1981), was subjected to a final purification step by affinity chromatography on a fluphenazine–Sepharose matrix (Charbonneau & Cormier, 1979). The calmodulin concentrations are based on $E^{1\%}_{280nm} = 2.0$ and a molecular weight of 16680 (Watterson et al., 1980). Rabbit skeletal muscle myosin light chain kinase was prepared and assayed according to the procedure of Blumenthal and Stull (1980). Melittin was purified by reverse-phase high-performance liquid chromatography of a commercial preparation supplied by Sigma Chemical Co.

Tris and Mops buffers were prepared by using distilled water that had been further purified with a Milli-Q reagent water system. Reagent-grade or best available grade chemicals were employed throughout the experiments.

Fluorescence Measurements. Fluorescence spectra were recorded with an SLM-Aminco 500 SPF fluorescence spectrophotometer and corrected for the wavelength dependence of the grating transmission and detector response. Measurements of fluorescence anisotropy were obtained with the SLM 4000 fluorescence polarization spectrophotometer, which is designed around the "T" format. Considering that we have detected dityrosine formation during UV irradiation of calmodulin (Malencik & Anderson, 1987), illumination of the samples was restricted to the period of measurement. Both instruments were connected to circulating constant-temperature baths. The experimental procedures and data analyses were described previously by us in binding experiments with both smooth muscle myosin light chain kinase (Malencik et

[1] Abbreviations: Mops, 3-(*N*-morpholino)propanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; $NaDodSO_4$, sodium dodecyl sulfate; CaM, calmodulin; Cldsm, caldesmon; K_d, dissociation constant; *f*, mole fraction; *F*, fluorescence intensity.

[2] That is, the solution was found to contain 9.6 ± 0.09 μmol of total residues/mL.

al., 1982) and various tryptophan-containing peptides [Malencik & Anderson, 1982; see reviews by Anderson and Malencik (1986, 1989)]. The concentrations of caldesmon finally employed in these measurements are similar in magnitude to the dissociation constants, thus minimizing the propagation of error (Weber, 1965). They are 20–100-fold lower than the concentrations at which a slight self-association occurs.

Analytical Ultracentrifuge Analysis. Sedimentation velocity analysis was carried out using the Beckman Model E analytical ultracentrifuge equipped with a UV-scanner optical system. The scans were recorded at 280 nm and analyzed according to the procedure of van Holde and Weischet (1978). The initial concentration of the sample examined in these experiments corresponded to A_{280} = 0.4 (~0.7 mg/mL).[3] The temperature of the rotor was equilibrated to 20 °C prior to the start of the run and subsequently regulated by the RTIC unit. The rotor speed was fixed at 44000 rpm.

Sedimentation equilibrium analysis was carried out in aluminum-filled epon synthetic boundary double-sector cells with sapphire windows. The initial concentration of the sample in fringes was determined at the end of each run using the same cell (Chevrenka, 1969). The speed for the sedimentation equilibrium runs was routinely 20000 rpm and the temperature maintained at 20 °C. Interference and schlieren optics were used simultaneously. The interference patterns were analyzed as described by Yphantis (1964). Refractometric units (in fringes) were converted to concentration units by using the relation 4.1 fringes = 1 mg/mL (Babul & Stellwagen, 1969). The schlieren patterns were analyzed according to Lamm (1929) in order to obtain the apparent z-average molecular weight:

$$M_z^{app} = \frac{2RT}{\omega^2(\partial\rho/\partial c_2)_\mu} \frac{\mathrm{d}\ \ln\ [(1/r)(\mathrm{d}c_2/\mathrm{d}r)]}{\mathrm{d}r^2} \qquad (1)$$

The distribution of M_z^{app} as a function of the radial position was obtained from the local slopes at each radial position, using the approach described by Yphantis (1964). The partial specific volume was estimated from the amino acid composition (Cohn & Edsall, 1943) and found to be 0.703 g/cm^3.

The buffer used in all the analytical ultracentrifuge experiments contained 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.1 mM dithiothreitol, 0.1 mM phenylmethanesulfonyl fluoride, and 50 mM NaCl. In the sedimentation velocity experiments, the salt concentration was changed as indicated in the text. At the conclusion of each experiment, the caldesmon samples were subjected to NaDodSO$_4$ gel electrophoresis to confirm their integrity.

Results

Sedimentation Studies. Figure 1 shows the distribution of the different apparent molecular weight averages (n, w, and z) as a function of concentration. Both the number- and the weight-average molecular weights (M_n^{app} and M_w^{app}) coincide fairly well within the range of concentrations analyzed. Yet, careful inspection of these plots reveals a minimal decrease in M_w^{app} with decreasing concentration. This behavior suggests a slight nonideality of the sample, superimposed upon which there is a noticeable aggregation tendency. Nevertheless, the coincidence of M_n^{app} and M_w^{app} in the region close to the meniscus clearly indicates sample homogeneity in that region.

[3] The caldesmon concentrations are based on the results of the amino acid analyses, an average residue weight of 115, and a molecular weight of 75000.

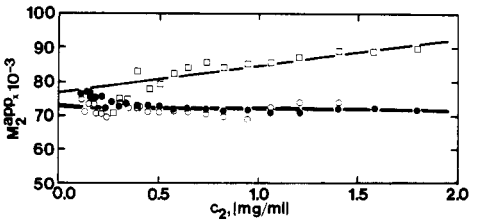

FIGURE 1: Equilibrium ultracentrifugation of caldesmon. The observed apparent molecular weight averages as a function of the protein concentration are presented: (●) M_n^{app}; (○) M_w^{app}; (□) M_z^{app}. Rotor speed = 20000 rpm and T = 19 °C. The measurements correspond to the interference patterns obtained after 96 h. Buffer: 50 mM NaCl, 10 mM Tris, 1 mM EDTA, 0.1 mM dithiothreitol, and 0.1 mM phenylmethanesulfonyl fluoride (pH 7.5).

This fact facilitates determination of the molecular weight (M_2) for nonaggregated caldesmon. Furthermore, application of the following equations eliminates nonideality effects from M_2 (Roark & Yphantis, 1969):

$$2(M_n^{app})^{-1} - (M_w^{app})^{-1} = M_2^{-1} \qquad (2)$$

The value of M_2 turns out to be 75K ± 2K after extrapolation of all the molecular weight averages, including M_z^{app}, to zero concentration. Notice that, as a result of the method employed in the analysis of the sedimentation equilibrium data (see Materials and Methods), the values for M_z^{app} and for M_n^{app} are determined independently. Whereas the weight and number averages are obtained from interference patterns, the z average is derived from the schlieren images. Additionally, the M_z^{app} values thus obtained do not depend on knowledge of the initial starting concentration of the sample.

The self-association properties of caldesmon can be analyzed from the concentration dependence of the apparent z-average molecular weight (M_z^{app}) shown in Figure 1. Through the use of the equation

$$(M_z^{app})^{-1} = M_2^{-1} + 4A_2C_2 + \ldots \qquad (3)$$

we obtained the virial coefficient $A_2 = -1.68 \times 10^{-4}$ mol/(mL·g^{-2}). The negative value for A_2 is consistent with the tendency of caldesmon to self-associate. Figure 2 shows the results of a sedimentation velocity analysis performed according to van Holde and Weischet (1978). In this representation, the number of lines converging toward a common s value is proportional to the fraction of the sample represented. Thus, approximately 25% of the caldesmon sediments with an $s_{20,w}$ greater than 2.95 S, the value corresponding to the nonaggregated monomer. In order to analyze the ionic strength dependence of the association reaction, sedimentation velocity analysis was carried out at different salt concentrations. The results in Figure 3 show that aggregation is not appreciably ionic strength dependent.

The data obtained by sedimentation equilibrium together with the sedimentation velocity results allow us to obtain some information about the conformational parameters of caldesmon. The frictional parameters of the molecule can be established from the relationships (Eisenberg, 1976):

$$f = \frac{M_2}{s_{20,w}\mathcal{N}}\left(\frac{\partial\rho}{\partial c_2}\right)_\mu \qquad (4)$$

$$\left(\frac{\partial\rho}{\partial c_2}\right)_\mu = (1 + \xi_1) - (\bar{v}_2 + \xi_1\bar{v}_1) \equiv 1 - \phi_2' \qquad (5)$$

where f = the fractional coefficient, M_2 = the molecular weight of the protein, $\mathcal{N}$ = Avogadro's number, ρ = the density of the solution, c_2 = the protein concentration, ξ_1 = the preferential hydration parameter, $\bar{v}_1$ = the partial specific volume

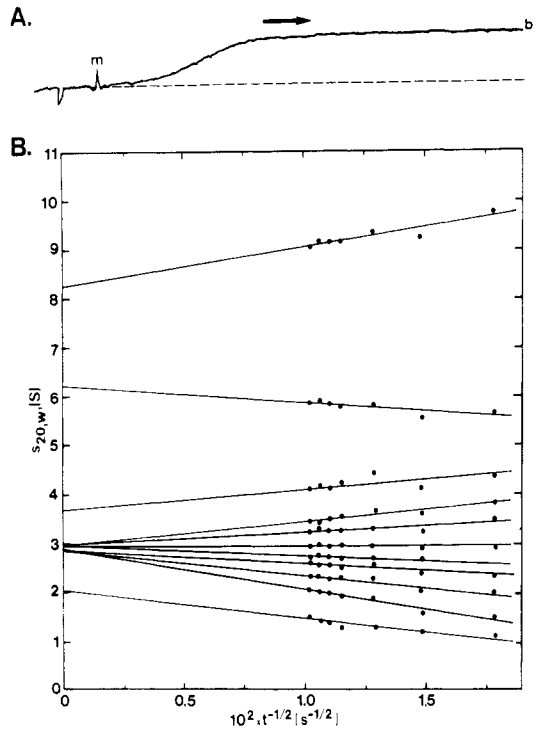

FIGURE 2: (A) Ultraviolet scanner trace of caldesmon recorded at 280 nm. Initial protein concentration was 0.7 mg/mL. Rotor speed = 44 000 rpm. T = 20 °C. m = position of the meniscus, b = cell bottom; the arrow indicates the direction of sedimentation. The composition of the buffer is described under Figure 1. (B) Boundary analysis of the sedimentation velocity experiment according to the method of van Holde and Weischet (1978).

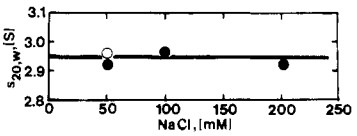

FIGURE 3: Ionic strength dependence of the sedimentation coefficient of caldesmon. The experimental conditions are as in Figure 2. (●) Caldesmon obtained by conventional chromatography; (○) caldesmon obtained by HPLC.

of water, $\bar{v}_2$ = the partial specific volume of the protein, ϕ_2' = the apparent partial specific volume. The frictional coefficient (f^0) of a hypothetical equivalent sphere of radius R_0 is given by

$$f^0 = 6\pi\eta R_0 \tag{6}$$

where

$$R_0 = \left[\frac{3M_2}{4\pi\mathcal{N}}(\bar{v}_2 + \xi_1\bar{v}_1)\right]^{1/3} \tag{7}$$

The Stokes radius is defined by

$$R_s = (f/f^0)R_0 \tag{8}$$

Assuming a compromise value for the hydration of the protein of ξ_1 = 0.3 g of H_2O/g of protein, we obtained an f/f^0 = 2.14. This indicates an extremely asymmetric molecule, corresponding to a prolate ellipsoid of a/b = 24 and a Stokes radius of 66 Å. Such a shape can be ascribed to an ellipsoid of length $L = 2a$ and cross-sectional diameter $d = 2b$ (Tanford, 1961) with a volume

$$v_h = \frac{M_2}{\mathcal{N}}(\bar{v}_2 + \xi_1\bar{v}_1) = (4/3)\pi ab^2 \tag{9}$$

We thus obtain L = 516 Å and d = 21.5 Å, with a corresponding radius of gyration $R_G = L/(12)^{1/2}$ = 149 Å. A rigid rod of equivalent length and volume corresponds to d = 17.6 Å. The relationships between the results of our hydrodynamic studies and those previously reported for other sources of caldesmon are considered under Discussion.

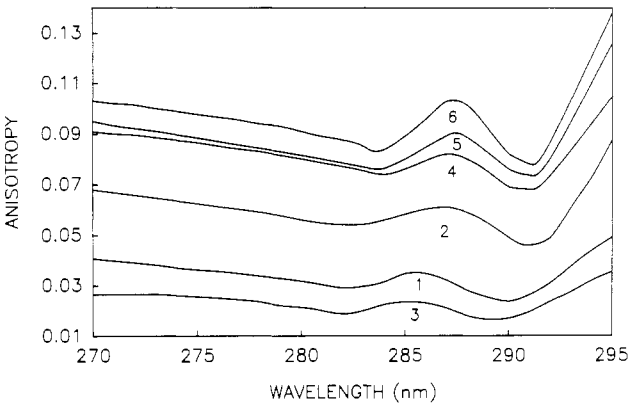

FIGURE 4: Fluorescence anisotropy excitation spectra. (1) 1.0 μM caldesmon. (2) 1.0 μM caldesmon + 10 μM calmodulin. (3) 9.0 μM melittin. (4) 9.0 μM melittin + 10 μM calmodulin. (5) 1.0 μM skeletal muscle myosin light chain kinase. (6) 1.0 μM myosin light chain kinase + 1.4 μM calmodulin. Conditions: 0.10 M KCl, 50 mM Mops, 0.8 mM dithiothreitol, and 1 mM $CaCl_2$, pH 7.3 (25 °C). The excitation band width was 2 nm. Emitted light was observed through Corning Glass CSO-52 cut-off filters.

Fluorescence Binding Measurements. (A) Spectra. Teale (1960) and Weber (1961) found that excitation at 290–295 nm largely excludes the fluorescence of tyrosine from the intrinsic emission spectra of proteins. Since calmodulin contains tyrosine but no tryptophan, we have applied this principle in demonstrating interactions between calmodulin and various tryptophan-containing peptides and proteins [cf. reviews by Anderson and Malencik (1986, 1989)]. Fluorescence spectra, obtained with an excitation wavelength of 294 nm, show that the association of turkey gizzard caldesmon with calcium–calmodulin is accompanied by a shift in the emission maximum (350 to 337 nm) similar to that recently reported for chicken gizzard caldesmon (Shirinsky et al., 1988). Spectral broadening occurs in both cases, suggesting that the six or so tryptophan residues of caldesmon are unequally affected by the association.

Fluorescence anisotropy excitation spectra reflect the relative rigidities of tryptophanyl residues, providing information not obtainable from emission spectra (Weber, 1960b, 1966; Anderson & Weber, 1966). Comparison of the anisotropy excitation spectrum of caldesmon to the spectra determined for melittin [a 26-residue peptide with high affinity for calmodulin first reported by Barnette et al. (1983) and Maulet and Cox (1983)] and for skeletal muscle myosin light chain kinase (a 90-kDa calmodulin-dependent enzyme) indicates that the tryptophanyl[4] residues of caldesmon generally have a high degree of rotational mobility (Figure 4). In fact, the anisotropies are only slightly larger than those observed with melittin. The relatively uniform decrease in anisotropy at all excitation wavelengths rules out energy transfer among tryptophan residues as a major source of depolarization in caldesmon. Cases of energy transfer are recognizable by an especially abrupt rise in anisotropy at the long-wavelength edge of the absorption band, with values approaching those obtained in the absence of transfer (Weber, 1960a,b; Anderson & Weber, 1969).

[4] The emission cut-off filters (Corning Glass CSO-52) employed in these measurements largely exclude the fluorescence of tyrosyl residues.

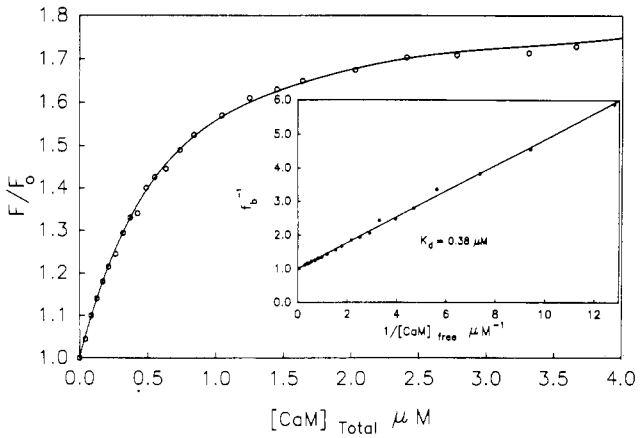

FIGURE 5: Fluorescence titration of turkey gizzard caldesmon with bovine brain calmodulin. The relative fluoroescence (F/F_0) was measured at 320 nm. The smooth curve through the data points was calculated for the binding of 1 mol of CaM/mol of caldesmon with K_d = 0.38 μM and F_∞/F_0 = 1.82. The double-reciprocal plot of the fraction of caldesmon bound versus the concentration of free calmodulin is shown in the inset. Conditions: 0.30 μM caldesmon in 0.196 M KCl, 50 mM Mops, 0.8 mM dithiothreitol, and 1 mM $CaCl_2$, pH 7.2 (24.3 °C). Excitation: 290 nm with 2.5-nm band-pass. Emission band-pass = 5 nm.

The addition of calmodulin has distinctive effects on the anisotropy excitation spectra of the three proteins. In the case of either melittin or caldesmon, the spectrum is significantly displaced toward longer wavelengths. The overall increases in anisotropy found with caldesmon and myosin light chain kinase signify either actual increases in the rigidities of the tryptophan residues or alterations in quantum yield which enhance the relative contributions of the more rigid residues. In the instance of melittin, the large changes in anisotropy can be due to decreased local mobility of the single tryptophan residue and/or to the slower rate of Brownian motion characteristic of the melittin–calmodulin complex. (Brownian motion of molecules as large as caldesmon or myosin light chain kinase is expected to make little *direct* contribution to the changes in intrinsic fluorescence anisotropy.) Note that caldesmon–calmodulin exhibits the highest degree of localized flexibility of the three complexes.

(*B*) *Equilibrium Constants.* The intrinsic tryptophan fluorescence of caldesmon was monitored in experiments to determine both the stoichiometry of the caldesmon–calmodulin complex and the values of the dissociation constant (K_d) under varying experimental conditions. For a simple equilibrium (PX $\rightleftarrows$ P + X) in which P (calmodulin) is nonfluorescent, the fraction (f_b) of ligand X (caldesmon) bound is determined from the fluorescence enhancement:

$$f_b = (F/F_0 - 1)/(F_\infty/F_0 - 1)$$

F_0 is the intensity of caldesmon measured in the absence of calmodulin. The value of F_∞, the fluorescence of totally bound caldesmon, is determined through extrapolation of the changes in fluorescence to infinite calmodulin concentration. Double-reciprocal plots of f_b^{-1} vs $[CaM]_{free}^{-1}$ (which is calculated according to $[CaM]_{free} = [CaM]_{total} - f_b[Cldsm]_{total}$) are linear, allowing calculation of K_d.

Figure 5 illustrates the relative increase in fluorescence intensity monitored at 320 nm when varying concentrations of calmodulin are added to a solution containing 0.30 μM caldesmon,[3] 0.196 M KCl, 1.0 mM $CaCl_2$, 0.8 mM dithiothreitol, and 50 mM Mops, pH 7.5 (24.3 °C). An excitation wavelength of 290 nm was employed in order to maintain reasonable sensitivity at low concentrations of caldesmon. The theoretical curve drawn through the data points corresponds to the binding of 1 mol of caldesmon/mol of calmodulin with K_d = 0.38 μM and F_∞/F_0 = 1.82. The linearity of the double-reciprocal plot (inset) over values of F_0 from 0.17 to 0.90 and extrapolation of f_b to 1.0 vindicate the assumptions underlying the calculations. Titrations monitored at 330 and 340 nm yield the same value of K_d, but with smaller fluorescence enhancements. Experiments performed at varying ionic strengths and temperatures are also consistent with the simple model. That the caldesmon–calmodulin interaction is strongly ionic strength dependent is evident in the results summarized in Table II. The van't Hoff plot (not shown) based on the dissociation constants determined at three different temperatures (0.1 M KCl) is linear, yielding $\Delta H°_{diss}$ = 7.0 kcal/mol. The corresponding values for $\Delta G°$(25 °C) and $\Delta S°$ are 9.7 kcal/mol and 0.009 kcal mol^{-1} $°C^{-1}$.

Table II: Effects of Ionic Strength and Temperature Variation on the Dissociation Constant of the Caldesmon–Calmodulin Complex[a]

temp (°C)	[KCl] (M)	[Cldsm] (μM)	K_d (μM)[b]
13.0	0.1	0.14	0.026
24.3	0.1	0.14	0.075
37.8	0.1	0.14	0.35
24.3	0.196	0.30	0.38
38.5	0.192	0.70	1.3

[a] Conditions: 50 mM Mops, 1 mM $CaCl_2$, 0.8 mM dithiothreitol, indicated concentrations of KCl, and indicated temperature, pH 7.5. [b] Reproducibility was ±10% or better.

In view of the self-association tendency of caldesmon (Cross et al., 1987), we checked the calmodulin binding equilibria for protein concentration dependence. Samples containing 7.8 μM caldesmon plus concentrations of calmodulin calculated to produce 50% saturation—i.e., 3.98 μM when [KCl] = 0.1 M and 4.28 μM when [KCl] = 0.2 M (refer to Table II)—were added stepwise to solutions containing 0.075 and 0.38 μM calmodulin, respectively. The fluorescence intensities, determined at 320 nm after each addition, showed direct proportionality to caldesmon concentration over the range 0.05–1.2 μM. Small deviations from linearity (i.e., 3.5% at 2.8 μM in the sample containing 0.1 M KCl) were detected at higher concentrations. Since proportionality necessitates constancy of f_b and hence of K_d (Anderson & Weber, 1965), the assumption that the self-association of caldesmon makes little contribution to calmodulin binding equilibria determined in dilute solutions is vindicated.

Comparison of our observations to those previously reported for other calmodulin binding proteins, including chicken gizzard caldesmon, is considered under Discussion.

Discussion

Perturbation of the intrinsic tryptophan fluorescence of turkey gizzard caldesmon in the presence of calmodulin allowed us to verify the one-to-one stoichiometry of complex formation and to determine dissociation constants. These experiments revealed an interaction which is both temperature dependent and ionic strength dependent. The calmodulin binding domain of caldesmon may contain strongly basic amino acid sequences similar to those observed in calmodulin binding peptides [cf. reviews by Anderson and Malencik (1986, 1989)], and in fragments derived from skeletal muscle (Blumenthal et al., 1985) and smooth muscle (Lukas et al., 1986) myosin light chain kinases. Nevertheless, the interaction of caldesmon with calmodulin is comparatively weak: The results in Table II suggest a dissociation constant of ~1 μM the ionic strength and temperature are in the physiological range. However, the presence of proteins such as tropomyosin and

F-actin may stabilize the association in vivo. The isolated complexes of skeletal and smooth muscle myosin light chain kinases with calmodulin correspond to dissociation constants of 15 nM (Olwin et al., 1984) and 2.8 nM (Malencik & Anderson, 1986), respectively.

The temperature dependence of the equilibrium constants demonstrates that enthalpy and entropy changes both contribute to the stabilization of the caldesmon–calmodulin complex, with the former predominating. Similar contributions of enthalpy to the free energy of binding have been determined for the complexes of calmodulin with skeletal muscle myosin light chain kinase (Blumenthal & Stull, 1982), porcine glucagon (Malencik & Anderson, 1983), smooth muscle myosin light chain kinase (Mamar-Bachi & Cox, 1987), and seminal plasmin, for which $\Delta S^\circ = 0$ (Milos et al., 1988). (The latter authors noted that the avid binding of melittin by calmodulin is unusual in that both the enthalpy and entropy terms are positive.)

Comparison of the equilibrium constants determined for turkey gizzard caldesmon to those previously reported for chicken gizzard caldesmon reveals substantial differences. For example, we find that $K_d = 0.075$ μM in solutions containing 0.1 M KCl, pH 7.5 (24.3 °C), while Shirinsky et al. (1988) report that $K_d = 0.55$ μM in solutions containing 0.1 M KCl, pH 7.4 (25 °C). This apparent discrepancy is readily resolved. First, the calculated concentration of chicken gizzard caldesmon (2.2 μM) was based on the incorrect molecular weight (140 000). Consideration of the true molecular weight, 93K (Graceffa et al., 1988), shows that the concentration was actually 3.3 μM. Second, the fluorescence titration published by Shirinsky et al. (1988) is incompatible with $K_d = 0.55$ μM. For example, saturation is attained at 8.0 μM calmodulin, suggesting that $K_d \leq 0.1$ μM. Estimations of K_d from the results obtained at lower degrees of saturation provide values ranging from 0.2 μM to less than 0.1 μM. Inability to extract a precise K_d from the data reflects the excess protein concentration used (Weber, 1965), a factor which may be compounded by the self-association of caldesmon. Recall that our experiments were performed at a protein concentration of 0.14 μM. Mills et al. (1988) have reported a dissociation constant of 0.25 μM for chicken gizzard caldesmon and a fluorescent conjugate of spinach calmodulin in solutions containing 0.09 M KCl (temperature unspecified).

Fluorescence anisotropy excitation spectra show that the six or so tryptophanyl residues of turkey gizzard caldesmon are apparently located in solvent-accessible regions of the molecule, where they exhibit a high degree of mobility, even in the complex with calmodulin. Comparison of our spectra with those obtained for other proteins including skeletal muscle myosin light chain kinase (Figure 4), smooth muscle myosin light chain kinase (Malencik et al., 1982), calcineurin (unpublished results), and beef heart and muscle lactate dehydrogenase (Anderson & Weber, 1966) makes manifest the unique characteristics of caldesmon. In this particular regard, caldesmon resembles small peptides such as melittin and even some unfolded proteins [cf. Weber (1960) and Anderson and Weber (1966)].

The difference between the molecular weights reported for chicken gizzard [93K + 4K according to Graceffa et al. (1988)] and turkey gizzard (75K + 2K) caldesmons is well above the error of sedimentation equilibrium determinations. Calculation of frictional ratios from the molecular weights and sedimentation coefficients show that the two caldesmons differ primarily in length. Assuming a hydration of 0.3 g of H_2O/g of protein and a partial specific volume of 0.703 g/cm^3, turkey gizzard caldesmon can be represented by a prolate ellipsoid which is 516 Å long and 21.5 Å in diameter. The hydrodynamic ellipsoid deduced for chicken gizzard caldesmon measures 730 Å by 20.8 Å (Graceffa et al., 1988). The distinctive results found in independent studies of chicken and turkey gizzard caldesmons point to the probable existence of molecular weight differences within the subclass, a phenomenon which is consistent with the alternative gene splicing model. Since the two major subclasses indeed differ in the size of the central core (Ball & Kovala, 1988), the "71–77-kDa" caldesmons must also have true molecular weights smaller than the values estimated from $NaDodSO_4$ gel electrophoresis. The elongated structure of caldesmon, possibly together with an asymmetric charge distribution, may explain the nonideal behavior observed in $NaDodSO_4$ gel electrophoresis experiments.

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