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Association of Melittin with the Isolated Myosin Light Chains†

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ABSTRACT: Melittin is a 26-residue peptide which undergoes high-affinity calcium-dependent binding by calmodulin [Barnette, M. S., Daly, R., & Weiss, B. (1983) *Biochem. Pharmacol. 32*, 2929; Comte, M., Maulet, Y., & Cox, J. A. (1983) *Biochem. J. 209*, 269; Anderson, S. R., & Malencik, D. A. (1986) *Calcium Cell Funct. 6*, 1]. The results in this paper show that three different types of myosin light chain—the smooth muscle regulatory light chain, the smooth muscle essential light chain, and the skeletal muscle regulatory 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) light chain—also associate with melittin. The resulting complexes have dissociation constants ranging from 1.1 to 2.5 μM in the presence of 0.10 M NaCl and from ~50 to ~130 nM in solutions of 20 mM 3-(*N*-morpholino)propanesulfonic acid alone. The regulatory smooth muscle myosin light chain exhibits two equivalent melittin binding sites while each of the others displays only one. The myosin light chains evidently contain elements of structure related to the macromolecular interaction sites present in calmodulin and troponin C but not in parvalbumin. The association of melittin and other peptides with the light chains requires consideration whenever assays of the calmodulin-dependent activity of myosin light chain kinase are used to determine peptide binding by *calmodulin*. The binding measurements performed on the DTNB light chain and melittin necessitated derivation of the equation relating complex formation to the observed fluorescence anisotropy of a solution containing three fluorescent components. This analysis is generally applicable to equilibria involving the association of two fluorescent molecules emitting in the same wavelength range.

X-ray crystallography and amino acid sequencing studies revealed a family of evolutionarily related calcium-binding proteins: calmodulin, the myosin light chains, troponin C, parvalbumin, the brain-specific S-100 protein, and the intestinal calcium-binding protein [cf. Kretsinger (1980)]. Calmodulin is probably the most generalized in function of the calcium-modulated proteins. The calcium–calmodulin complex is a well-recognized activator of cyclic nucleotide phosphodiesterase (Cheung, 1967), adenylate cyclase (Cheung et al., 1975; Brostrom et al., 1975), phosphorylase kinase (Grand et al., 1981), myosin light-chain kinase [cf. Stull (1980) and Small and Sobieszek (1980)], and calcineurin [cf. Tallant and Cheung (1986)]. Calmodulin also undergoes calcium-dependent interactions with small molecules. These include a number of peptides exhibiting common structural features—notably clusters of basic amino acid residues in close conjunction with hydrophobic sequences [cf. Anderson and Malencik (1986)]. Although most of the known peptide–calmodulin associations probably do not occur in vivo, they proved to be useful models for protein binding by calmodulin. The prediction that sequences similar to those of the peptides occur in calmodulin-dependent enzymes (Malencik & Anderson, 1982) was borne out by the structures of high-affinity calmodulin-binding fragments prepared from skeletal muscle and smooth muscle myosin light-chain kinases (Blumenthal et al., 1985; Lukas et al., 1986).

Melittin, a 26-residue peptide from honey bee venom, is one of the more widely publicized calmodulin-binding peptides. Its potent inhibition of the calmodulin-dependent activity of cyclic nucleotide phosphodiesterase was first described by

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Barnette et al. (1983) and Comte et al. (1983). Structural features of melittin and several other peptides (e.g., the mastoparans) have been incorporated into a series of synthetic α-helical peptides also bound by calmodulin [cf. O'Neil et al. (1987)].

This paper describes the association of melittin with three different types of myosin light chain: the M_r 20000 regulatory light chain of smooth muscle myosin, whose reversible phosphorylation is apparently necessary for cross-bridging and contraction in smooth muscle [Sobieszek & Small, 1977; Small & Sobieszek, 1980; Suzuki et al., 1980; see review by Walsh and Hartshorne (1982)]; the M_r 17000 essential light chain, which either is a part of or is closely associated with the active site of smooth muscle myosin (Okamoto et al., 1986); and the M_r 18500 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB)[1] light chain of skeletal muscle, whose reversible phosphorylation may increase the force level in skeletal muscle at submaximal Ca^{2+} concentrations (Moore & Stull, 1984; Persechini et al., 1985).

The significance of the experiments to be described is 3-fold. First, the binding of melittin may shed light on the macromolecular interactions of the myosin light chains and provide information on their structural relationship to the other calcium-modulated proteins. Second, binding of peptides by the light chains needs to be considered whenever assays of myosin light-chain kinase (a calmodulin-dependent enzyme) are used to detect and evaluate peptide binding by *calmodulin*. Since excess concentrations of the myosin light chain are used in these assays, the fraction of peptide bound by it can be substantial. Corrections for the effect of the light chain were not included in the catalytic assay for peptide binding by calmodulin recently described by Erickson-Viitanen and DeGrado (1987). Third, the interpretation of the binding measurements performed on the DTNB light chain and melittin requires derivation of the equation relating the fraction of complex formed to the observed fluorescence anisotropy of a solution containing three fluorescent components (i.e., free melittin, free light chain, and melittin–light-chain complex). This analysis is generally applicable to equilibria in which two different fluorescent molecules associate to form a fluorescent complex.

Materials and Methods

Reagents. Tris and Mops buffers were prepared by using distilled water that had been further purified with a Milli-Q reagent water system. pH adjustments were made with HCl or KOH. Reagent-grade NaCl, magnesium acetate, and calcium acetate were used in the indicated experiments. The best available grades of Mops, Tris, 2-mercaptoethanol, dithiothreitol, EDTA, urea, and ATP were obtained from Sigma Chemical Co. Cibacron Blue–Sepharose was purchased from Pharmacia and Ultrogel AcA 54 from LKB, Inc. Melittin was supplied by Peninsula Laboratories, Inc.

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 the fluorescence anisotropy [$\bar{A} = (I_\parallel - I_\perp)/(I_\parallel + 2I_\perp)$] and total intensity ($I_T = I_\parallel + 2I_\perp$) were obtained with the SLM 4000 fluorescence polarization spectrophotometer, which is designed around the "T" format. The reproducibility of the anisotropies was ±0.001 to ±0.002. Considering that we have detected dityrosine formation during UV irradiation of calmodulin (Malencik & Anderson, 1987), we have restricted illumination of the samples to the period of measurement. No change in the intrinsic fluorescence of the myosin light chains occurred under the observation conditions. We also note that UV-induced dityrosine formation does not take place in the myosin light chains. The titrations involve the addition of microliter quantities of titrant, using Hamilton syringes, to 1.5 mL of the solution in the fluorescence cuvette. Dilution factors were minimized to 2% or less. Corrections were made for the background fluorescence of the protein and solvent blanks, which were 5% or less of the sample intensities. Mathematical analysis of the fluorescence titrations is described by Anderson (1974) and Malencik and Anderson (1984). Both fluorometers were connected to circulating water baths to maintain a constant temperature (25.0 ± 0.1 °C) in the samples.

Electrophoresis. $NaDodSO_4$ gels were run on a linear 8–20% gradient minigel system (8 cm × 10 cm) using the proper proportion of 30% acrylamide and 0.8% bis(acrylamide). The gel buffers were essentially those of the Laemmli (1970) system except that the separating gel was made 0.75 M in Tris-HCl, pH 8.8, and the running buffer (pH 8.3) was 50 mM Tris, 60 mM boric acid, and 1 mM EDTA containing 0.1% $NaDodSO_4$. The gels were run at 150 V until the bromophenol blue tracking dye reached the gel bottom (ca. 3 h).

The gels were stained in 50% methanol and 10% acetic acid containing 0.2% Coomassie Blue R-250 for 30 min. The gels were destained in 10% methanol and 10% acetic acid. Extrapolated molecular weights were determined from the following standards: phosphorylase (97K), serum albumin (68K), actin (42K), carbonic anhydrase (29K), troponin C (18K), and parvalbumin (12K).

We also employed urea–glycerol–acrylamide gel electrophoresis according to the procedure of Sobieszek and Jertschin (1986) in the characterization of phosphorylated and unphosphorylated smooth muscle myosin light chains.

Preparation of Smooth Muscle Myosin Light Chain. Smooth muscle myosin was prepared from washed turkey gizzard myofibrils, prepared as previously described (Malencik et al., 1982) using a wash buffer consisting of 50 mM Tris-HCl, 5 mM EDTA, 40 mM NaCl, 0.15% Triton X-100, and 15 mM 2-mercaptoethanol, pH 7.0. After three washes, the myosin was extracted in 4 volumes of 50 mM Tris-HCl, 2 mM EDTA, 15 mM 2-mercaptoethanol, and 5 mM ATP, pH 7.2. After centrifugation at 3000*g*, the supernatant was brought to 20 mM Ca^{2+} by the addition of 1 M calcium acetate while maintaining the pH at 7.2. The precipitated myosin was centrifuged at 3000*g* and the pellet taken up in 3 volumes of 50 mM Tris-HCl, 15 mM 2-mercaptoethanol, and 1 mM EDTA, pH 8.0 (buffer A), which was made 8 M in urea. After the solution was stirred at room temperature for 2 h, an equal volume of cold water was added and the solution then brought to 25% ethanol by addition of 95% ethanol. After centrifugation at 3000*g*, the soft, flocculent pellet was removed, and to the supernatant was added about $^1/_3$rd volume of packed DE-52 (Whatman) that had been equilibrated at pH 8.0 with 50 mM Tris-HCl. After 2 h of stirring, the DE-52 was separated on a funnel and washed with a portion of buffer A. The DE-52 was then packed into a suitable column (9 cm × 30 cm), further washed with buffer A, and eluted with 0.4 M NaCl in buffer A. The large protein peak was pooled and passed through a column (4 cm × 30 cm) of Cibacron Blue–

[1] Abbreviations: Mops, 3-(*N*-morpholino)propanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; $NaDodSO_4$, sodium dodecyl sulfate; CaM, calmodulin; MLCK, myosin light-chain kinase; LC, light chain; Mel, melittin; K_d, dissociation constant; *f*, mole fraction; $\bar{A}$, fluorescence anisotropy; *Q*, relative fluorescence yield (a proportionality factor); *F*, fluorescence intensity; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid).

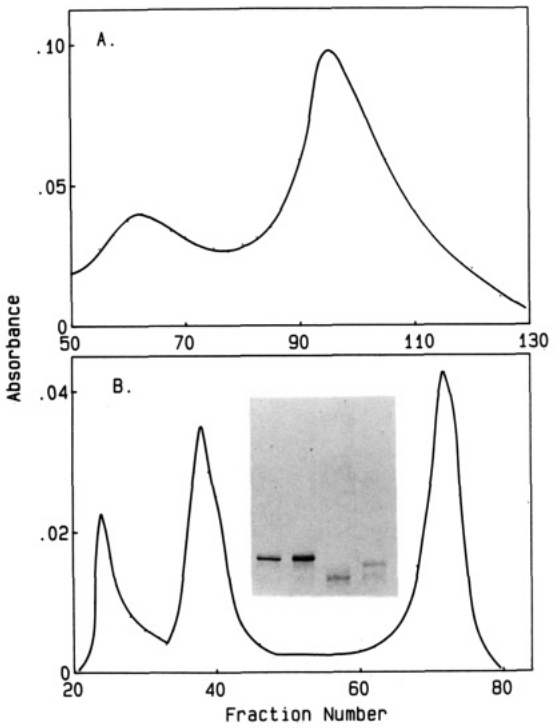

FIGURE 1: Final chromatographic steps in the purification of the turkey gizzard M_r 20000 myosin light chain. Panel A shows the results of gradient elution (0.4 → 2.0 M NaCl) of a 5 cm × 30 cm Cibacron Blue–Sepharose column. The first peak elutes at 0.6 M NaCl and contains tropomyosin and actin. The second peak elutes at 0.8 M NaCl and contains primarily the myosin light chain. Fractions contained 12.5 mL each. Panel B demonstrates the separation obtained during chromatography on an LKB Ultrogel AcA 54 sizing column (2.6 cm × 75 cm). The first peak contains an unidentified protein; the second peak, the M_r 20000 myosin light chain; and the third peak, non-protein UV-absorbing material. Fractions contained 5.5 mL each. Refer to Materials and Methods for details. The *inset* shows the results of $NaDodSO_4$ electrophoresis. Reading from left to right: (1) purified M_r 20000 myosin light chain (unphosphorylated); (2) M_r 20000 myosin light chain (phosphorylated); (3) M_r 17000 myosin light chain; (4) DTNB light chain.

Sepharose, which had previously been equilibrated with buffer A containing 0.4 M NaCl. The unbound protein fraction contains the M_r 17000 myosin light chain in a nearly pure form on the basis of $NaDodSO_4$–polyacrylamide gel electrophoresis (Figure 1B). After removal of the unbound protein, a gradient (2000 mL) running between 0.4 and 2 M NaCl (both in buffer A) was run. Two broad peaks emerged from the column: one at 0.6 M NaCl that contained mostly tropomyosin and any actin, and another at 0.8 M NaCl that was mostly 20K myosin light chain (Figure 1A). Appropriate fractions were pooled after location by $NaDodSO_4$–polyacrylamide gel electrophoresis. We note that Crouch et al. (1981) used Cibacron Blue–Sepharose chromatography in the purification of the rabbit skeletal muscle DTNB light chain. A final step utilizing an Ultrogel AcA54 sizing column (2.6 cm × 75 cm) was routinely included to completely purify the light chain (Figure 1B). At this point, the light chain is homogeneous as judged by $NaDodSO_4$–polyacrylamide gel electrophoresis (Figure 1B).

Myosin light-chain phosphorylation was performed under conditions leading to the incorporation of 1 mol of ^{32}P/mol of light chain in radioassays (Malencik et al., 1982). The reaction mixture (50 mL) contained 0.2 mg/mL M_r 20000 myosin light chain, 6 nM each of calmodulin and turkey gizzard myosin light-chain kinase, 0.2 mM calcium acetate, 2.0 mM magnesium acetate, 0.1 mM ATP, 1 mM dithiothreitol, and 50 mM Mops, pH 7.3 (25 °C). After 45 min of reaction, the solution was lyophilized, redissolved, and applied to the Ultrogel AcA 54 column. Electrophoresis on urea–glycerol–acrylamide gels according to Sobieszek and Jertschin (1986) showed that the phosphorylation reaction is complete and that the untreated light chains consist totally of the unphosphorylated species.

Miscellaneous. The DTNB light chain of rabbit skeletal muscle was prepared following the method reviewed by Wagner (1982). Porcine brain calmodulin was obtained following the procedure of Schreiber et al. (1981).

RESULTS

Melittin Binding by the M_r 20000 Regulatory Light Chain of Smooth Muscle Myosin. Teale (1960) and Weber (1961) showed that excitation at 295 nm largely excludes the fluorescence of tyrosine from the intrinsic fluorescence spectra of proteins. The fact that calmodulin and most related proteins (including the M_r 17000 and 20000 myosin light chains) contain no tryptophan allowed us to apply this principle in binding measurements on tryptophan-containing peptides—porcine glucagon, adrenocorticotropin (Malencik & Anderson, 1982), the mastoparans (Malencik & Anderson, 1983a, 1984, 1986a), and the dynorphins (Malencik & Anderson, 1984). The association of these peptides with either calmodulin or troponin C is accompanied by changes in tryptophan quantum yield and varying shifts of the emission maximum to shorter wavelengths [cf. review by Anderson and Malencik (1986)]. Determination of the fluorescence *anisotropy* $(\bar{A})^2$ is an independent method for the detection of complex formation which reflects molecular weight changes and is responsive to binding even when the fluorescence spectrum and quantum yield do not change. Application of anisotropy measurements to binding is based on the principle that in a mixture of fluorescent species, the average anisotropy equals the sum of the individual anisotropies weighed by the individual fractional contributions to the total fluorescence intensity (Weber, 1952).

The fluorescence emission maximum of a 4.0 μM melittin solution shifts from 353 nm to 335–340 nm, with a 1.6-fold increase in quantum yield, on the addition of 12 μM M_r 20000 myosin light chain. Later measurements show that 90% peptide binding occurs under the conditions (buffer = 0.10 M NaCl, 20 mM Mops, and 1 mM dithiothreitol, pH 7.5, 25 °C) used for this demonstration. The effect of the light chain on the emission spectrum of melittin is similar to that reported by Maulet and Cox (1983) for calmodulin. The anisotropy excitation spectra of melittin (Figure 2) reveal shifts in wavelength, consistent with changes in the absorption spectrum of melittin in the presence of the light chain, plus large overall increases in anisotropy. The latter values are close to those obtained for the complexes of calmodulin with mastoparan X and Polistes mastoparan (Malencik & Anderson, 1983a, 1984). They are, however, larger than those noted upon the association of melittin with calmodulin (see Figure 2; Malencik & Anderson, 1984). The results obtained in the case of calmodulin were explained by solvent accessibility of the tryptophanyl side chain, demonstrated in acrylamide quenching experiments (McDowell et al., 1985). Note that the anisotropy is critically dependent on excitation wavelength in the interval of 290–300 nm. Since it provides a favorable compromise of sensitivity and minimization of tyrosine fluorescence, we generally use

[2] $\bar{A} = (I_\parallel - I_\perp)/(I_\parallel + 2I_\perp)$ where $I_\parallel$ and $I_\perp$ are the intensities of the two linearly polarized components of the light emitted at right angles to the excitation direction. $I_\parallel$ vibrates in the direction of propagation of the exciting light, and $I_\perp$ vibrates normally to the plane corresponding to the directions of excitation and observation.

Table I: Constants for Association of Melittin with Isolated Myosin Light Chains

light chain	conditions[a]	N (sites)	K_d (μM)[h]	A_{Mel}[c]	A_{LC}	A_c	Q_c/Q_{Mel}[e]	Q_{LC}/Q_{Mel}
M_r 20 000	1	2	$\leq$0.03[b]	0.034	NA[d]	0.135	1.0	0
M_r 20 000	2	2	2.25	0.032	NA	0.143	0.67	0
M_r 20 000	3	2	4.95	0.034	NA	0.125	0.84	0
M_r 17 000	1	1	$\sim$0.13[b]	0.035	NA	0.122	1.1[g]	0
M_r 17 000	2	1	2.55	0.033	NA	0.131	1.0[g]	0
M_r 17 000	3	1	6.8	0.033	NA	0.128	1.0[g]	0
DTNB	1	1	$\leq$0.05[b]	0.033	0.082–0.092[f]	0.137	1.68	0.90
DTNB	2	1	1.1	0.034	0.090	0.133	1.68	0.90
DTNB	3	1	1.6	0.033	0.090	0.135	1.68	0.84

[a] Conditions: (1) 20 mM Mops, pH 7.5, 25.0 °C; (2) 20 mM Mops + 0.10 M NaCl; (3) 20 mM Mops, 0.10 M NaCl, 10 mM $Mg(C_2H_3O_2)_2$, and 3 mM $Ca(C_2H_3O_2)_2$. [b] Nearly total binding of melittin occurs in the micromolar concentration range. [c] A_{Mel} is the same as A_f in eq 2 and 3. [d] NA, no applicable. [e] Q_c/Q_{Mel} is same as F_c/F_0 in eq 2 and 3. [f] Concentration dependence between 0 and 3.6 μM light chain. [g] Corning glass CSO-54 filter used for the M_r 17 000 light chain. [h] Average variation in K_d is $\pm$10% or less except for the cases where nearly total binding occurs.

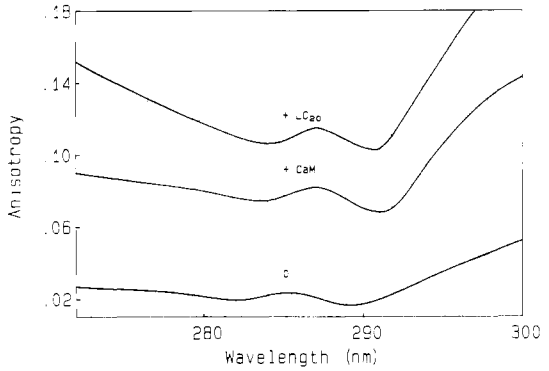

FIGURE 2: Fluorescence anisotropy excitation spectra showing the binding of melittin by the M_r 20 000 myosin light chain (LC_{20}) and calmodulin. The lower curve ("0") was obtained with a solution of 6.0 μM melittin in 20 mM Mops, pH 7.5. The middle curve corresponds to a solution containing 9.0 μM melittin, 10.0 μM calmodulin, 1 mM $Ca(C_2H_3O_2)_2$, 0.19 M NaCl, and 50 mM Mops, pH 7.5. The upper curve was recorded for a solution containing 9.0 μM melittin, 5.0 μM M_r 20 000 myosin light chain, 1 mM dithiothreitol, and 20 mM Mops, pH 7.5 (25.0 °C). The excitation bandwidth was 2 nm. The emitted light was observed through Corning glass CSO-52 cutoff filters.

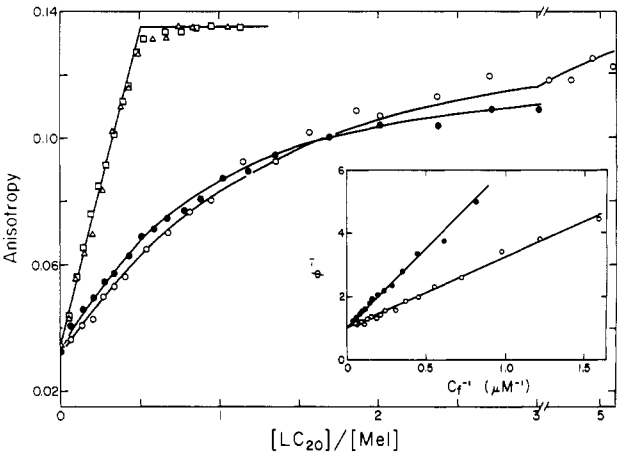

FIGURE 3: Fluorescence anisotropy titrations of melittin with the turkey gizzard M_r 20 000 regulatory light chain. Conditions: 1.5 μM melittin in 20 mM Mops [(□) using unphosphorylated light chain; (△) using phosphorylated light chain]; 2.0 μM melittin in 0.10 M NaCl and 20 mM Mops [(○) unphosphorylated light chain]; and 6.0 μM melittin in 0.10 M NaCl, 20 mM Mops, 10 mM magnesium acetate, and 3 mM calcium acetate [(●) unphosphorylated light chain]. Theoretical curves are shown for total binding (□, △), for K_d = 2.25 μM (○), and for K_d = 4.95 μM (●) with two binding sites. Other constants used are summarized in Table I. $[LC_{20}]/[Mel]$ is the ratio of the molar concentrations of the M_r 20 000 myosin light chain and melittin. The double-reciprocol plots of the fraction of melittin bound versus the concentration of unoccupied melittin binding sites ($c_f = 2[LC]_T - \phi[Mel]_T$) are shown in the inset. All solutions contained 1 mM dithiothreitol, pH 7.5, 25.0 °C. Excitation, 294 nm with 2-nm band-pass; emission filters, Corning glass CSO-52.

an excitation wavelength of 294 nm in our binding experiments.

We applied fluorescence anisotropy to the determination of the equilibrium constants and stoichiometries of melittin–myosin light-chain complexes. In the case of a simple dissociation involving two *distinguishable* fluorescent components (X and PX), PX $\rightleftarrows$ P + X, the values of $\bar{A}$ range from that of the unbound ligand X (A_f) to that of the bound ligand PX (A_c). Following the principle of additivity of anisotropies (Weber, 1952), the fraction (ϕ) of the fluorescent ligand bound is simply calculated when the relative yields of X and PX are identical under the observation conditions:

$$\phi = (\bar{A} - A_f)/(A_c - A_f) \quad (1)$$

When the yields are not identical, a related equation in which F_c/F_0 is the ratio of the fluorescence intensities of the totally bound and free ligand is applied (Evett & Isenberg, 1969; S. R. Anderson and G. Weber, unpublished results):

$$\phi = \frac{\bar{A} - A_f}{(\bar{A} - A_f) + (F_c/F_0)(A_c - \bar{A})} \quad (2)$$

An alternate form of eq 2 is used to calculate the values of $\bar{A}$ corresponding to specified values of ϕ:

$$\bar{A} = \frac{\phi[A_f - (F_c/F_0)A_c] - A_f}{\phi(1 - F_c/F_0) - 1} \quad (3)$$

Figure 3 illustrates the increases in anisotropy occurring upon the addition of varying concentrations of the non-phosphorylated M_r 20 000 regulatory myosin light chain to solutions containing fixed amounts of melittin. An excitation wavelength of 294 nm and Corning glass CSO-52 emission filters were used in the measurements. The CSO-52 cutoff filters transmit primarily at wavelengths above 357 nm, excluding both residual tyrosine fluorescence and Raman scatter. When the titrations are performed at low ionic strength (20 mM Mops), the anisotropy increases directly up to an end point of 0.5 mol of light chain/mol of melittin, indicating that the protein has two melittin-binding of melittin up to saturation, suggesting that $[Mel]_T/K_d \leq 50$ and hence $K_d \leq 30$ nM [cf. Anderson (1974)]. Parallel experiments with melittin and calmodulin confirmed the 1:1 stoichiometry reported by Maulet and Cox (1983).

The association of melittin with the M_r 20 000 myosin light chain is sensitive to increases in ionic strength. When solutions containing 2.0 μM melittin, 0.10 M NaCl, and 20 mM Mops are titrated with the light chain, both the anisotropy (Figure 3) and total intensity (not shown) change gradually. Values of A_c and F_c/F_0 (Table I) are obtained by extrapolation of the observed changes to infinite light-chain concentration.

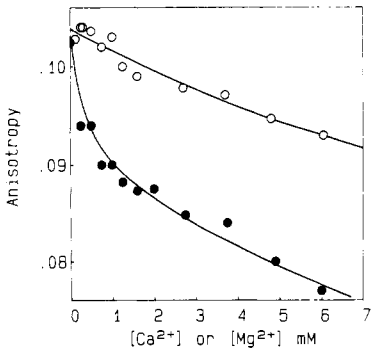

FIGURE 4: Effects of calcium and magnesium on melittin binding by the M_r 20 000 regulatory myosin light chain. Varying amounts of Ca^{2+} (●) or Mg^{2+} (○) were added to solutions containing 3.2 μM melittin, 2.35 μM light chain, 0.10 M NaCl, 20 mM Mops, and 1 mM dithiothreitol, pH 7.5, 25.0 °C. Excitation, 294 nm with 2-nm band-pass; emission filters, Corning glass CSO-54.

Equation 2 is then used to calculate ϕ, the fraction of melittin bound. When ϕ is in the range of 0.5, its *maximum* error is 7–14% (corresponding to uncertainties in anisotropy of 0.001–0.002). The concentration of unoccupied melittin-binding sites in the myosin light-chain solution (C_f) is then obtained by difference:

$$C_f = 2[LC_{20}]_T - \phi[Mel]_T$$

$[LC_{20}]_T$ is the molar concentration of the M_r 20 000 myosin light chain calculated from its molecular weight, and $[Mel]_T$ is the molar concentration of melittin. Plots of $1/\phi$ versus $1/C_f$ (inset to Figure 3) are linear, corresponding to $K_d = 2.25 \pm 0.07$ μM.[3,4] The variation in the average K_d is substantially less than the maximum error of 20–40% estimated for individual points on the titration. The smooth curve drawn through the original data points in Figure 3 was calculated by substituting the constants summarized in Table I plus specific values of ϕ into eq 3. The latter were determined from $\phi = [-b - (b^2 - 4c)^{1/2}]/2$ where $c = [Mel]_T/N[LC]_T$ and $b = -(N[LC]_T + [Mel]_T + K_d)/N[LC]_T$. N is the number of binding sites—two in this case.

The addition of millimolar concentrations of Ca^{2+} and Mg^{2+} to solutions containing 3.2 μM melittin, 2.4 μM M_r 20 000 light chain, 0.10 M NaCl, and 20 mM Mops results in partial dissociation of the complex (Figure 4). (Note that Corning glass CSO-54 filters were used in this experiment. The anisotropies differ slightly from those obtained with CSO-52 filters.) In order to determine a dissociation constant under conditions approaching those used in myosin light-chain kinase assays by Erickson-Viitanen and DeGrado (1987), we performed measurements on solutions containing 10 mM magnesium acetate, 3 mM calcium acetate, 0.10 M NaCl, and 20 mM Mops. The titration of a 6.0 μM melittin solution (Figure 3) yields linear plots of $1/\phi$ versus $1/C_f$, corresponding to a dissociation constant of 4.95 ± 0.10 μM. Although we have conducted experiments at various concentrations of melittin, we have illustrated those in which the concentration is closest to the K_d value. Weber (1965) showed that the propagation of experimental error is minimized under these conditions. The range of melittin concentrations examined is largely outside the range of self-association. To check for concentration

[3] K_d corresponds to the equilibrium concentration of unbound melittin in a solution of the light chain where half of the melittin-binding sites are occupied and to the concentration of unoccupied melittin-binding sites in a light-chain solution where half of the melittin is bound.

[4] Experiments performed in the presence and absence of 0.1 mM EDTA give the same results.

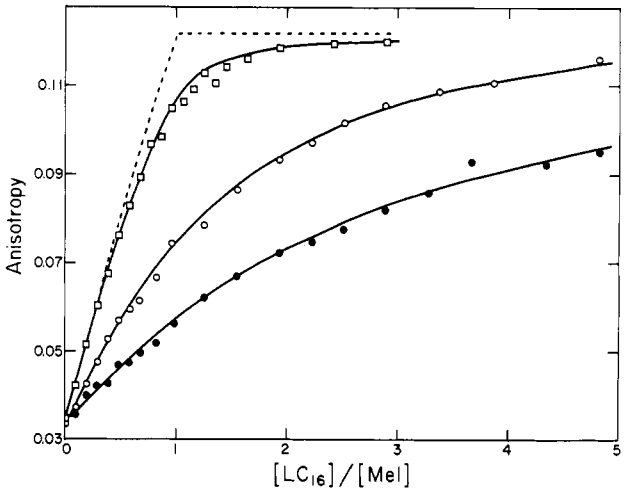

FIGURE 5: Fluorescence anisotropy titrations of melittin with the turkey gizzard M_r 17 000 essential myosin light chain. Conditions: 3.2 μM melittin in 20 mM Mops (□), in 20 mM Mops plus 0.10 M NaCl (○), and in 20 mM Mops, 0.10 M NaCl, 10 mM magnesium acetate, and 3 mM calcium acetate (●). Theoretical curves are shown for total binding (dashed line), for K_d = 130 nM (□), for K_d = 2.55 μM (○), and for K_d = 6.85 μM (●) with one binding site. Other constants used are summarized in Table I. $[LC_{16}]/[Mel]$ is the ratio of the molar concentrations of the M_r 17 000 myosin light chain and melittin. Emission filters, Corning glass CSO-54. Other conditions are given under Figure 3.

dependence, we performed a dilution experiment in which a solution containing 6.0 μM melittin and 4.0 μM myosin light chain (8 μM in binding sites) was mixed with varying proportions of a solution containing 2.5 μM myosin light chain (5 μM in binding sites). These conditions correspond to ϕ = 0.50 and a calculated anisotropy of 0.0755. The measured anisotropy values showed a small increase from 0.0758 in the presence of 6 μM melittin to 0.0799 with 0.5 μM melittin, a concentration approaching the limits of measurement. This result suggests a slight concentration dependence, with ϕ increasing to 0.54 and K_d decreasing to 4.2 μM with 0.5 μM melittin.

Experiments with the phosphorylated M_r 20 000 myosin light chain revealed only small changes in its interaction with melittin. The dissociation constant determined in the presence of 0.10 M NaCl decreased to 1.8 μM while the stoichiometry remained the same (Figure 3).

Melittin Binding by the M_r 17 000 Essential Light Chain of Smooth Muscle Myosin. Titrations of a fixed concentration of melittin (3.2 μM) with the M_r 17 000 light chain of smooth muscle myosin are shown in Figure 5. Measurements performed at low ionic strength (20 mM Mops) indicate a stoichiometry of 1 mol of melittin/mol of light chain. The smooth curve drawn through the data points was calculated for a single binding site of K_d = 130 nM, using the constants summarized in Table I. The effects of NaCl, Mg^{2+}, and Ca^{2+} are similar to those obtained with the M_r 20 000 light chain, producing a degree of dissociation which is favorable for the determination of equilibrium constants. The mathematical analysis is the same as that described in the preceding section except for the calculation of the concentration of unoccupied melittin-binding sites (C_f). In this case, $C_f = [LC_{17}]_T - \phi[Mel]_T$, where $[LC_{17}]_T$ is the molar concentration of the M_r 17 000 light chain. Plots of $1/\phi$ versus $1/C_f$ (not shown) are linear, providing K_d values of 2.55 ± 0.15 μM[4] in the presence of 0.1 M NaCl and 6.8 ± 0.6 μM in solutions containing 0.1 M NaCl, 10 mM Mg^{2+}, and 3 mM Ca^{2+} (Table I).

Melittin Binding by the DTNB Light Chain of Skeletal Muscle Myosin. Unlike the M_r 17 000 and 20 000 myosin light

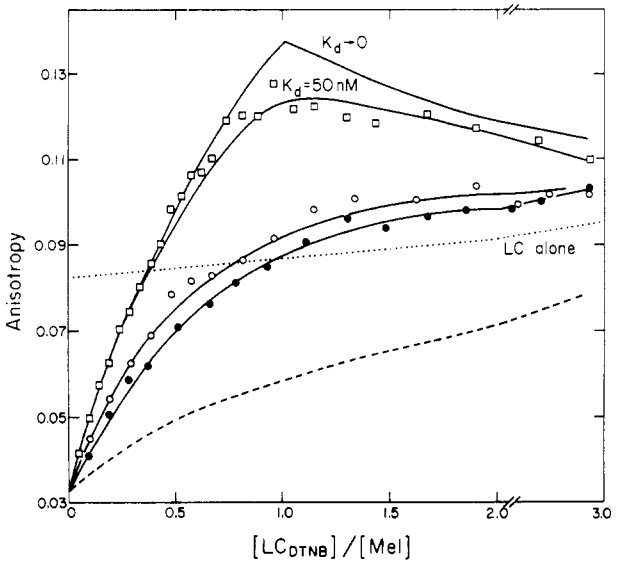

FIGURE 6: Fluorescence anisotropy titrations of melittin with the rabbit skeletal muscle DTNB light chain. Conditions: 1.8 μM melittin in 20 mM Mops (□), in 20 mM Mops plus 0.10 M NaCl (○), and in 20 mM Mops, 0.10 M NaCl, 10 mM magnesium acetate, and 3 mM calcium acetate (●). Theoretical curves are shown for total binding ($K_d \to 0$), for K_d = 50 nM (□), for K_d = 1.09 μM (○), and for K_d = 1.62 μM (●) with one binding site. Other constants are given in Table I. The dotted line shows the concentration dependence obtained on the addition of the DTNB light chain to solutions containing 20 mM Mops and zero melittin. The dashed line shows the anisotropies predicted for the addition of the light chain to melittin (20 mM Mops) in the event of no interaction. $[LC_{DTNB}]/[Mel]$ is the ratio of the molar concentrations of the DTNB light chain and melittin. Emission filters, Corning glass CSO-52. Other conditions are given under Figure 3.

chains, the DTNB light chain contains a single tryptophan residue and hence makes a significant contribution to the total fluorescence. Comparison of separate equimolar solutions of melittin and the DTNB light chain shows that they have similar intensities (1.11:1.00 for melittin *versus* protein) under our observation conditions. The anisotropy of melittin (A_{Mel}) is 0.033 while that of the light chain (A_{LC}) is 0.090, determined at varying concentrations in solutions containing 0.1 M NaCl ($\pm Mg^{2+}$, $\pm Ca^{2+}$). A slight concentration dependence, illustrated by the dotted line in Figure 6, is noted when the light chain is added to 20 mM Mops containing no added salt. The additivity principle (Weber, 1952) was used to calculate the anisotropies of *noninteracting* mixtures of melittin and the DTNB light chain. That is

$$\bar{A} = \frac{F_{Mel}A_{Mel} + F_{LC}A_{LC}}{F_{Mel} + F_{LC}} \quad (4)$$

where F_{Mel} and F_{LC} are the fluorescence intensities measured on separate solutions of melittin and the light chain. The dashed line in Figure 6 illustrates the anisotropies predicted for titrations performed in 20 mM Mops. It deviates minimally from the results derived for solutions containing 0.1 M NaCl (not shown).

Large differences between the predicted and observed anisotropies (Figure 6) bear witness to the association of the DTNB light chain with melittin. In fact, the anisotropy of the resulting complex is higher than that of the free light chain. Note that the values obtained at low ionic strength (20 mM Mops) decline after reaching a maximum at ratios near 1 mol of light chain/mol of melittin.

In order to calculate the equilibrium constants for the melittin–DTNB light-chain complex, we have extended the additivity principle to a system of three fluorescent components: the complex plus unbound melittin and unbound light chain. The anisotropy is given by the equation

$$\bar{A} = \frac{f_{LC}[LC]_T Q_{LC}A_{LC} + f_{Mel}[Mel]_T Q_{Mel}A_{Mel} + f_c[Mel]_T Q_c A_c}{f_{LC}[LC]_T Q_{LC} + f_{Mel}[Mel]_T Q_{Mel} + f_c[Mel]_T Q_c} \quad (5)$$

$[LC]_T$ and $[Mel]_T$ represent the total molar concentrations of the light chain and melittin, respectively. A_{LC}, A_{Mel}, and A_c are constants representing the anisotropies of the free light chain, of free melittin, and of the melittin–light-chain complex, respectively. Q_{LC}, Q_{Mel}, and Q_c represent the corresponding relative fluorescence yields. Note that A_c and Q_c are average values, comprising contributions from both tryptophan residues. The mole fractions (f) are defined as

$$f_{LC} = [LC]/([LC] + [LC\cdot Mel])$$

$$f_c = [LC\cdot Mel]/([Mel] + [LC\cdot Mel])$$

$$\frac{1 - f_{LC}}{f_c} = \frac{[LC\cdot Mel]/[LC]_T}{[LC\cdot Mel]/[Mel]_T} = [Mel]_T/[LC]_T$$

$$f_{Mel} = [Mel]/([Mel] + [LC\cdot Mel])$$

$$f_c + f_{Mel} = 1$$

Substitution of the mole fractions in terms of f_c, followed by rearrangement of eq 5, yields the equation relating f_c to the observed anisotropy, the total concentrations, and the constants:

$$f_c = \frac{[LC]_T Q_{LC}(A_{LC} - \bar{A}) + [Mel]_T Q_{Mel}(A_{Mel} - \bar{A})}{[Mel]_T[Q_{LC}(A_{LC} - A) + Q_{Mel}(A_{Mel} - \bar{A}) - Q_c(A_c - \bar{A})]} \quad (6)$$

When Q_{LC} is zero, this equation is the same as that given previously (eq 2).

The relative fluorescence yield of the complex, Q_c, is readily determined when melittin is tightly bound. Plots of the observed fluorescence versus $[LC]_T$ are largely linear up to the stoichiometric end point. Q_c corresponds to the fluorescence extrapolated from the linear region to a molar ratio of light chain to melittin of 1:1. The titration performed in 20 mM Mops yields Q_c = 1.86, which is slightly less than the sum of Q_{Mel} plus Q_{LC} (2.11). The value of A_c is obtained from measurements of $\bar{A}$ under conditions where $f_c \to 1$. Substitution of f_c = 1 into eq 6, followed by rearrangement, yields a close approximation of A_c from the measurements obtained at excess light-chain concentrations:

$$A_c = \bar{A} - \frac{Q_{LC}}{Q_c}\left(\frac{[LC]_T}{[Mel]_T} - 1\right)(A_{LC} - \bar{A}) \quad (7)$$

Application of eq 7 to the anisotropies measured at $[LC]_T/[Mel]_T$ ratios ranging from 1.67 to 2.86 gives a nearly constant value of A_c: 0.1325 ± 0.003.

Computation of the value of $\bar{A}$ corresponding to any specific value of f_c also follows from eq 5:

$$\bar{A} = \frac{Q_{Mel}A_{Mel}(1 - f_c) + Q_{LC}A_{LC}([LC]_T/[Mel]_T - f_c) + f_c Q_c A_c}{Q_{Mel}(1 - f_c) + Q_{LC}([LC]_T/[Mel]_T - f_c) + f_c Q_c} \quad (8)$$

Before determining equilibrium constants, we calculated theoretical curves of $\bar{A}$ versus $[LC]_T/[Mel]_T$ for total binding—i.e., $f_c = [LC]_T/[Mel]_T$ at molar ratios from zero

to one—and for high-affinity binding with K_d = 50 nM. Several values of A_c near 0.133 were tested for fit, with a final value of 0.137 used for the curves illustrated (Figure 6). The measurements obtained in 20 mM Mops correspond to essentially complete binding up to ratios of 0.7 mol of light chain/mol of melittin. Dissociation detected at higher ratios places K_d in the range of about 50 nM or less.

Experiments performed in 0.10 M NaCl indicate a degree of dissociation for which equilibrium constants can be accurately determined. However, since comparatively high concentrations of light chain are required for $f_c \rightarrow 1$, we adopted the values of A_c and Q_c determined in 20 mM Mops as first approximations. The values of f_c calculated from eq 6 and the concentrations of unbound light chain, $c_f = [LC]_T - f_c[Mel]_T$, were then examined on double-reciprocal plots such as those used in the preceding sections (see Figure 3, inset). Linearity of these plots over a wide concentration range and demonstration that $f_c = 1$ when $c_f^{-1} = 0$ require application of the correct values of A_c and Q_c, as well as of the other constants. Only small adjustments in A_c, which have a slight effect on the calculated values of K_d, were made in computation of the theoretical curves (Figure 6) corresponding to $K_d = 1.1 \pm 0.1$ μM (in 0.10 M NaCl)[4] and to $K_d = 1.62 \pm 0.14$ μM (in 0.10 M NaCl, 10 mM Mg^{2+}, and 3 mM Ca^{2+}). All the constants involved are summarized in Table I. The propagation of the error in anisotropy is somewhat greater for the three-component system. We calculate *maximum* errors in f_c of 10–20%, corresponding to maximum error in individual K_d values of 30–60%. However, the variation in the final determination of K_d is on the order of ±10%.

Discussion

The moderate binding of melittin by the myosin light chains, with K_d values of 1.1–2.5 μM determined in 0.10 M NaCl solution, suggests that they retain peptide-binding sites related to those occurring in calmodulin and troponin C. The failure of parvalbumin, which apparently has no protein recognition sites (Blum et al., 1977), and even high concentrations of serum albumin to associate with melittin (Malencik & Anderson, 1985) demonstrates a degree of specificity in the melittin–light-chain interactions. On the other hand, the stability of the melittin–calmodulin complex, with $K_d \leq 1$ nM (as discussed later), far surpasses that of any melittin–light-chain complex. Previous observations on the dynorphins, strongly basic "random coil" peptides, and mastoparan X, an α-helical tetradecapeptide, corroborate this distinction. The dissociation constants obtained with mastoparan X were ~0.9 nM for calmodulin (Malencik & Anderson, 1983a), ~0.2 μM for skeletal muscle troponin C (Malencik & Anderson, 1984),[5] 21 μM for the M_r 20000 myosin light chain, and ∞ (no binding detected) for parvalbumin (Anderson & Malencik, 1986). With dynorphin$_{1-17}$, the values were 0.65 μM for calmodulin, 2.4 μM for skeletal muscle troponin C, and ∞ for both the M_r 20000 myosin light chain and parvalbumin.

X-ray crystallographic studies of calmodulin, performed at 3.0-Å resolution, show that the molecule consists of two globular lobes connected by an exposed α-helix containing amino acid residues 65–92 (Babu et al., 1985). Although the locations of any peptide recognition site(s) in calmodulin are unknown, the central helix is under scrutiny as a possible target for peptides and pharmacological agents. The calcium-dependent binding of mastoparan X by proteolytic fragments of calmodulin containing amino acid residues 72–148 and 1–106 (Malencik & Anderson, 1984) supports this possibility. A similar helix occurs in the three-dimensional structure of troponin C (Herzberg & James, 1985) but not in parvalbumin (Kretsinger & Nockolds, 1973). The myosin light chains have not been crystallized. However, the amino acid sequence of the M_r 20000 myosin light chain [cf. Pearson et al. (1984) and Maita et al. (1981)] shows substantial amounts of possible helix, including a continuous segment comprising residues 99–145. Note that the effects of calcium ion on the organization and/or accessibility of the melittin-binding sites in calmodulin and the myosin light chains differ. Calcium strongly stabilizes the melittin–calmodulin complex (Maulet & Cox, 1983) but destabilizes the melittin–light-chain complexes. Zimmer and Hofmann (1984) reported association of the cardiac myosin regulatory light chain with nonpeptide calmodulin antagonists: trifluoperazine, W-7, and R24571.

The interaction of three different types of myosin light chain with melittin is apparently related to some common function, possibly association with the myosin heavy chain. Nonetheless, variations among the light chains exist. The combination of the DTNB light chain with melittin is stronger and less sensitive to the destabilizing effects of Ca^{2+} and Mg^{2+} than that of either the M_r 17000 or the M_r 20000 myosin light chain. The latter light chain is distinguished by the presence of two melittin-binding sites while each of the others displays only one. The excellent fit of a model containing two fluorescent components (free and bound melittin) to the data shows that the two peptide-binding sites of the M_r 20000 light chain are occupied at about equal frequencies. The measurements are not expected to distinguish between the complexes containing one and two molecules of melittin since the anisotropies would be close to the limiting value in either case. Various spectroscopic measurements on calmodulin point to a single binding site whose affinity for the peptides studied surpasses that of any additional sites [cf. Anderson and Malencik (1986)]. However, evidence that there is a secondary site in the case of β-endorphin was provided by covalent cross-linking experiments—performed at high concentrations of both peptide and protein (Giedroc et al., 1983).

The association of melittin with the myosin light chains occurs in a favorable concentration range, permitting the accurate determination of binding constants and investigation of the effects of ionic strength variation etc. Similar experiments with calmodulin have been difficult, however, since the association is essentially complete at the lowest melittin concentrations accessible to fluorescent measurement. Radiotracer methods would permit the use of lower concentrations, in the nanomolar range. However, loss of both proteins and peptides by adsorption to glass etc. becomes a major source of error. Added carriers, on the other hand, may interact with one of the ligands.

Competition experiments permit comparison of dissociation constants even when the individual values are too small for direct evaluation. We developed a competitive displacement method, based on the calmodulin-dependent binding of 9-anthroylcholine by smooth muscle myosin light-chain kinase, for evaluation of calmodulin binding by intermediate-affinity proteins and peptides (Malencik et al., 1982; Malencik & Anderson, 1983b). This analysis was extended forthwith to high-affinity peptides: the mastoparans (Malencik & Anderson, 1983a) and melittin [cf. review by Anderson and Malencik (1986)]. An alternate approach in this direction was introduced by Comte et al. (1983), who used the fractional stimulation of cyclic nucleotide phosphodiesterase as a measure of the equilibrium concentration of unbound calmodulin in

[5] Troponin C also binds melittin (Malencik & Anderson, 1985).

assay mixtures containing melittin. However, the conditions used resulted in ~95% binding of melittin and, consequently, uncertainty in the concentration of unbound melittin. The reported dissociation constant was 3 nM.

Erickson-Viitanen and DeGrado (1987) recommended a competitive assay using chicken gizzard myosin light-chain kinase. Solution conditions include 0.5 nM kinase, varied concentrations of calmodulin and peptide, 8 μM M_r 20 000 regulatory myosin light chain, 0.1 mM ATP, 10 mM $MgCl_2$, 3 mM $CaCl_2$, 0.1 M KCl, and 20 mM Tris (pH 7.3). Half-maximum stimulation of catalytic activity in this assay requires 6 nM calmodulin in the absence of added peptides and 18 nM calmodulin when 20 nM melittin is present. When the concentration of calmodulin bound by the enzyme is ignored, the concentration of the calmodulin–melittin complex was calculated by difference (18 nM − 6 nM) to be 12 nM. The concentration of unbound calmodulin, [CaM], was taken as 6 nM and that of melittin as 8 nM (20 nM − 12 nM). The resulting value of K_d was 4 nM.

However, we have shown that the M_r 20 000 myosin light chain associates appreciably with melittin under the above conditions. Accordingly, the calculation of the dissociation constant (K_d) for the melittin–calmodulin complex from the results of the assay needs to consider the binding of melittin by the light chain. Whenever $[LC]_T \gg [Mel]_T$

$$K_d = \frac{([Mel]_T - [CaM]_T + [CaM])[CaM]}{(1 + N[LC]_T/K_{LC})([CaM]_T - [CaM])}$$

$[CaM]_T$ is the total concentration of calmodulin, [CaM] is the concentration of free calmodulin deduced from the activity measurements, and N is the total number of melittin-binding sites on the myosin light chain. Substitution of K_{LC} = 4.95 μM suggests a correction factor of 4.2, with the true dissociation constant of the melittin–calmodulin complex [derived from the measurements of Erickson-Viitanen and DeGrado (1987)] being 1 nM. Applicability of this correction depends on the purity of the myosin light-chain preparation used, including whether the M_r 17 000 light chain was also present. (Myosin light-chain kinase assays often use mixed light chains.) The corrected dissociation constant of the melittin–calmodulin complex is still larger than the values reported by us. Competition experiments monitored through measurements of 9-anthroylcholine binding by turkey gizzard myosin light-chain kinase, of the fluorescence anisotropy of dansylcalmodulin [cf. Anderson and Malencik (1986)], and of the hemolytic activity of melittin (Malencik & Anderson, 1985) consistently gave values near 0.1 nM or less. A relatively high concentration of Ca^{2+} (3 mM) is an unusual feature of the assay of Viitanen and DeGrado (1987). Myosin light-chain kinase assays generally are performed in solutions containing 0.1–0.2 mM Ca^{2+}. The K_m for calmodulin and the enzyme reported by these authors (6 nM) is also larger than the value (1 nM) obtained under different conditions by Conti and Adelstein (1981), Malencik et al. (1982), and Malencik and Anderson (1986).

Continuing interest in assays for high-affinity peptide binding by calmodulin is certain to follow future preparations of calmodulin-binding fragments from enzymes and other proteins. In characterizing the binding of peptide analogues of the heat-stable protein inhibitor of cAMP-dependent protein kinase by calmodulin (Malencik et al., 1986), we performed myosin light-chain kinase assays in which the myosin light chain was substituted with the synthetic tridecapeptide substrate described by Kemp et al. (1983). However, we prefer the competitive displacement method using 9-anthroylcholine since continuous titrations spanning a range of concentrations can be performed on a single sample. True dissociation constants are readily obtained by application of a small correction factor determined by the known dissociation constants of the 9-anthroylcholine–MLCK and 9-anthroylcholine–MLCK–CaM complexes [Malencik et al., 1982; Malencik & Anderson, 1983a,b; see review by Anderson and Malencik (1986)].

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^{15}N and ^{1}H NMR Studies of *Rhodospirillum rubrum* Cytochrome c_2[†]

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ABSTRACT: ^{15}N-Enriched cytochrome c_2 was purified from *Rhodospirillum rubrum* that had been grown on $^{15}NH_4Cl$, and the diamagnetic iron(II) form of the cytochrome was studied by ^{15}N and ^{1}H NMR spectroscopy. ^{15}N resonances of the four pyrrole nitrogens, the ligand histidine nitrogens, the highly conserved tryptophan indole nitrogen, and some proline nitrogens are assigned. The resonances of the single nonligand histidine are observed only at low pH because of severe broadening produced by proton tautomerization. The resonances of exchangeable protons bonded to the nitrogens of the ligand histidine, the tryptophan, and some amide groups are also assigned. The exchange rates of the nitrogen-bound protons vary greatly: most have half-lives of less than minutes, the indolic NH of Trp-62 exchanges with a half-time of weeks, and the ligand histidine NH proton exchanges with a half-time of months. The latter observation is indicative of extreme exclusion of solvent from the area surrounding the ligand histidine and lends credence to theories implicating the degree of hydrophobicity in this region as an important factor in adjusting the midpoint potential. The dependence of the ^{15}N and ^{1}H NMR spectra of ferrocytochrome c_2 on pH indicates neither the Trp-62 nor the ligand His side chains become deprotonated to any appreciable extent below pH 9.5. The His-18 NH remains hydrogen bonded, presumably to the Pro-19 carboxyl group, throughout the pH titrations. Because neither deprotonated nor non-hydrogen-bonded forms of His-18 are observed in spectra of the ferrocytochrome, the participation of such forms in producing a heterogeneous population having different g tensor values seems unlikely. A single ionization, occurring with a pK of 6.8, causes the resonances of the pyrrole nitrogens and several groups near the heme to shift. This ionization, attributed to the protonation/deprotonation of His-42 or a His-42–heme propionate hydrogen-bonded pair, appears to affect a wide range of groups near the heme, perhaps by altering the packing of the Ω loops which cover that region of the protein.

The cytochromes c_2 are a class of electron-transport proteins found in the purple non-sulfur bacteria. The importance of their function in photosynthetic electron transport and their structural homology to the mitochondrial cytochrome c have made them the subject of a great deal of study in recent years. Many structural and functional parameters have been measured, and equally many attempts have been made to relate structure to function.

A property of obvious importance to the cytochromes is the midpoint oxidation–reduction (redox) potential of the heme iron. The character of the heme groups of the cytochromes is determined both by the *ligands* provided by the protein and by the *environment* created by the folded peptide chains. Several theories concerning the control and diversity of the midpoint potentials of the cytochromes have been proposed.

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