Interaction of Skeletal Myosin Light Chains with Calcium Ions†

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ABSTRACT: Light chains have been purified from rabbit skeletal muscle myosin. The 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) light chains have been prepared in the fully phosphorylated and dephosphorylated states, by the use of endogenous light chain kinase and phosphatase. The addition of calcium or magnesium ions leads to a considerable change in intrinsic fluorescence, the fluorescence of a covalently bound label, circular dichroism and high-resolution proton magnetic resonance spectra. The circular dichroism change is consistent with an increase in α-helix content of some 5%. Binding of calcium was followed by fluorescence and circular dichroism titrations. Magnesium ions are bound competitively, but much more weakly, and saturation is not complete even at 2 mM free magnesium concentration. Phosphorylation brings about a considerable diminution of affinity for calcium ions. A further consequence of calcium binding is a large change in Stokes radius. The loss of an N-terminal peptide of molecular weight 2000 from the DTNB light chains does not diminish the ability of the molecule to bind calcium, but the effect on the Stokes radius is reduced by about one-half. The DTNB, as well as both the A1 and A2 alkali light chains, are highly asymmetric, as judged by their apparent Stokes radii. The DTNB light chains have an estimated axial ratio, based on a prolate ellipsoid model of the order of 10:1 in the absence of divalent cations, corresponding to a length of about 150 Å. In the presence of calcium ions this falls to about 100 Å. The spectroscopic properties of the light chains, however, indicate that large proportions of the residues are contained in globular domains. This suggests a structure containing globular and elongated or flexible parts.

The work of Szent-Györgyi and his colleagues has established that in molluscan and many other invertebrate myosins the regulatory element, which is responsible for the capture of the calcium ions that activate the contraction cycle, is a light chain, situated in the myosin heads (Kendrick-Jones et al., 1976). In vertebrate striated muscle, on the other hand, the primary site of regulation is the complex of proteins associated with the thin filaments, to one of which, troponin C, the calcium ions are bound. In some invertebrate phyla the two modes of regulation coexist (Lehman & Szent-Györgyi, 1975). There are indications, however, that in vertebrate striated muscle a mechanism involving only a change in the thin filaments, contingent on the binding of calcium by troponin C, is insufficient to account completely for the phenomena associated with calcium activation. In particular, there is evidence of an effect of calcium ions on the structure of the thick filament (Morimoto & Harrington, 1974; Haselgrove, 1975), and on its interaction with unregulated actin (Lehman, 1977). The corresponding binding site is evidently on one of the myosin light chains—the DTNB[1] light chain—which has indeed been found to bind calcium, both in situ (Beinfeld et al., 1975; Bagshaw, 1977) and in the isolated state (Werber et al., 1972; Kuwayama & Yagi, 1977). This light chain possesses extensive sequence homologies with troponin C, as well as with the other class of skeletal myosin light chains, the alkali light chains (Weeds & McLachlan, 1974; Collins, 1976), although the latter have not been reported to be capable of binding calcium ions. The DTNB light chains can moreover be made to replace a regulatory light chain in molluscan myosin, with restoration of calcium sensitivity (Kendrick-Jones et al., 1976).

The binding of calcium to the DTNB light chains in situ has a conformational effect on the myosin, which reveals itself in a greatly increased resistance to proteolysis of the subfragment 1–subfragment 2 junction (Bagshaw, 1977; Weeds & Pope, 1977). A further property of the light chain is that it serves as a substrate for a highly specific myofibrillar calcium-dependent kinase (Perrie et al., 1973), which catalyzes the phosphorylation of a single serine residue near the N-terminal end and close in the sequence to the presumed calcium binding site (Collins, 1976).

We have examined the effects on the conformation of the DTNB light chains from rabbit skeletal muscle of the attachment of calcium and magnesium ions. We show that the binding is accompanied by a substantial change in secondary structure and overall shape of the molecule, that these effects are facilitated when the phosphoryl group is removed, and that they are partially eliminated in the protein truncated by scission near the N terminus.

Materials and Methods

Preparative Procedures. Rabbit skeletal muscle myosin was prepared by the method of Perry (1955). Total light chains were prepared by dissociation of the myosin at 10–20 mg/mL in 5 M guanidine hydrochloride at 20 °C for 1 h followed by precipitation of the heavy chains with ethanol (Holt & Lowey, 1975). The phosphorylation state of the DTNB light chain was controlled by prior enzymic phosphorylation or dephosphorylation of the myosin. Fractionation of the light chains was accomplished by column chromatography on DE52 ion-exchange cellulose (Holt & Lowey, 1975). The column (30 × 1.1 cm) was equilibrated with 0.08 M phosphate buffer, pH 6.0, containing 0.1 mM dithiothreitol. The light chains were eluted with a linear gradient to a final potassium phosphate concentration of 0.25 M.

Crude myosin light chain kinase was prepared by the method of Pires et al. (1974), and the phosphatase as described by Morgan et al. (1976), omitting, however, the final purification step. It was found that complete dephosphorylation ensued after 1 h at 25 °C in a buffer containing 12 mM mag-

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[1] Abbreviation used: DTNB, 5,5′-dithiobis(2-nitrobenzoic acid).

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nesium chloride, 5 mM EGTA, 50 mM Tris-HCl, 0.5 mM dithiothreitol, pH 7.6. The procedures of Pires et al. (1974) were followed in generating fully phosphorylated and dephosphorylated myosin samples. Phosphorylation and dephosphorylation could be readily monitored by gel electrophoresis in the presence of urea (see below).

The DTNB light chain fragment of 17 000 molecular weight, which comigrates with the alkali light chain, A2, on polyacrylamide gels in the presence of sodium dodecyl sulfate (Bagshaw, 1977; Weeds & Pope, 1977), was prepared by chymotryptic digestion of myosin in the presence of calcium ions for 1 h under the conditions of Bagshaw (1977), followed by column chromatography, under the conditions described above. The fragment elutes between the DTNB and the A_2 light chains.

The DTNB light chains were labeled with the fluorescent dansyl chromophore by exposure to the thiol-specific reagent dansylaziridine (Scouten et al., 1974), purchased from Pierce Chemical Co. The reagent was dissolved in acetone at 10 mg/mL, and 50 μL of this solution was added to 1 mL of protein at 2 mg/mL, in 0.1 M sodium chloride, 50 mM Tris, pH 8.0. The mixture was gently agitated at 4 °C for 5 h, and excess reagent removed by a brief spin in a bench-top centrifuge, followed by dialysis. Spectrophotometric analysis, taking the molar residue absorptivity of the dansyl chromophore at 340 nm as 3400 (Hartley & Massey, 1956), revealed the incorporation of two dansyl groups per molecule of DTNB light chain as expected.

Gel Electrophoresis. Gel electrophoresis in the presence of sodium dodecyl sulfate was carried out in the system of Weber & Osborn (1969). To resolve phosphorylated and unphosphorylated chains, the urea system of Perrie & Perry (1970) was used, namely, 8 M urea, 0.12 M glycine, 20 mM Tris, pH 8.6, in a 10% flat-bed acrylamide gel. After staining in 0.05% Coomassie Brilliant Blue G250 in 2-propanol:acetic acid:water (25:12.5:62.5 v/v) and destaining in 2-propanol:acetic acid:water (12.5:12.5:75 v/v), the gels were evaluated by densitometry in a Joyce-Loebl instrument. Areas under peaks were measured by planimetry.

Gel Filtration and Sedimentation. Sedimentation velocities were determined with a Spinco Model E ultracentrifuge, at 60 000 rpm, using schlieren optics and protein concentrations in the range 1–10 mg/mL. For gel filtration a 90 × 1.1 cm column of Sephadex G-100 (superfine) was used and eluted with 0.1 M sodium chloride, 20 mM borate, 0.05 mM dithiothreitol, 0.1 mM EGTA, pH 8.0. When required, 2 mM calcium or magnesium chloride was incorporated. The flow rate was maintained at 3 mL/h, and the eluate was monitored at 280 nm, or when low protein concentrations were used, 210 nm. It was found that excellent data for sets of proteins could be economically obtained on mixtures. The fractions were individually analyzed by gel electrophoresis, as described, and quantitative densitometry of stained protein zones in each fraction (13 per gel slab) allowed an elution curve to be constructed for each component. As many as 6 proteins, incompletely resolved on the column, could thus be applied simultaneously. This allowed very precise comparisons of, for example, alkali and DTNB light chains, the latter phosphorylated and dephosphorylated, and the chymotryptic fragment, in a single column experiment. The interstitial volume of the column and the total available volume were determined from the elution volumes respectively of Blue Dextran and Bromophenol Blue.

Fluorimetry. Fluorometric titrations were performed in a Hitachi MPF-3L spectrofluorimeter, the cell housing thermostated at 14 °C. Absorbances at the excitation wavelength of 295 nm were kept below 0.08, and the emission intensity was measured at 345 nm. The buffer was 0.1 M sodium chloride, 0.1 M Hepes, 0.01 M EGTA, 100 μM dithiothreitol, pH 7.2, containing calculated concentrations of calcium (or magnesium) ions. These were added in the form of a concentrated stock solution of calcium (or magnesium) chloride; correction was made for dilution, the maximum correction being no more than about 2%. Titration of the chelating agent with calcium ions led in this buffer system to a drop in pH of some tenths of a unit. This was monitored, and taken into account in computing the free calcium or magnesium ions, by way of standard formulations (Perrin & Dempsey, 1974; Portzehl et al., 1964). If necessary, and routinely at the end of a titration, the pH was carefully readjusted. In the conditions of our experiments the fluorescence was insensitive to pH over a wide range on either side of neutrality. Reversibility was checked by addition of excess EGTA. To guard against any long-term drifts, a solution of the protein in the above buffer, without added calcium, in another cell was used as reference. Fluorescence of the dansyl chromophore in the modified protein was excited at 344 nm and measured at 483 nm, using the same titration routine.

Circular Dichroism. Measurements were made in a Cary 61 instrument with a thermostated cell housing. Path lengths were in general 1 cm for the near-ultraviolet (aromatic) region of the spectrum, and 2 mm below 250 nm. For titrations, the same solvent system was used as in fluorimetry. Because of the presence of dithiothreitol, the lower working limit of wavelength was 222 nm, and titrations with calcium and magnesium were performed at this point. Essentially the same procedure was used as in the fluorimetry.

Proton Magnetic Resonance. Spectra were measured in a 270-MHz Bruker pulsed Fourier transform spectrometer, made available to us by Dr E. M. Bradbury. Protein concentrations were in the range 4–12 mg/mL and the solvent was 0.1 M sodium chloride, 10 mM phosphate, 0.2 mM EGTA, 0.2 mM dithiothreitol, apparent pD 7.6, with excess calcium chloride when required. The D_2O was redistilled before use. Generally 10^4 to 10^5 free induction decays were accumulated, with a pulse length of 12 μs. To restrict H_2O contamination, the protein samples were dialyzed with several changes into the D_2O-containing buffer; adjustments of pD were made with DCl and NaOD.

Results

The chromatographic basis for the preparation of pure light chain components is illustrated in Figure 1. To obtain satisfactory yields of purified phosphorylated and dephosphorylated DTNB light chains, the myosin was subjected to the action of endogenous kinase or phosphatase in the manner described by Pires et al. (1974). The DE52 column affords partial resolution of the phosphorylated and dephosphorylated DTNB light chains and the A1 alkali light chain (Figure 1). After prior phosphatase treatment of the myosin, the trailing part of the peak associated with the dephosphorylated DTNB light chain yields a pure component. Conversely, chromatography of the light chains from fully phosphorylated myosin allowed the isolation of both phosphorylated DTNB and alkali light chains in a high state of purity. Figure 2 shows that the interconversion of the phosphorylation states can be monitored by urea gel electrophoresis. The results, which are in accord with the observations of Perry and co-workers (Perrie et al., 1973; Pires et al., 1974; Morgan et al., 1976), also confirm that no modification of the alkali light chains occurs. We also find (Figure 2) that the urea gels are able to resolve the truncated DTNB chain, generated by chymotryptic cleavage in situ, from the A2 light chains, from which it is indistinguishable in sodium

TABLE I: Calcium and Magnesium Dependent Physical Properties of Skeletal Myosin Light Chains.

Light chain	K_{assoc}(appt),[b] Ca^{2+} (M^{-1})	K_{assoc}(appt),[b] Mg^{2+} (M^{-1})	Quenching of intrinsic fluorescence by calcium % intensity change)	Molar residue ellipticity at 222 nm (deg cm^2 $dmol^{-1}$)	Partition coefficient, K_d[c]	App Stokes radius (Å)[d]	Length of prolate ellipsoid of revolution (Å)[e]	
DTNB, dephosphorylated	2.5 (± 0.5) × 10^5	1.2 (± 0.1) × 10^3	14 ± 1	−8 900	0.235	30.5	150	$-Ca^{2+}$
				−10 000	0.288	26	105	$+Ca^{2+}$
DTNB, phosphorylated	4.9 (± 1.2) × 10^4	1.2 (± 0.1) × 10^3	17.5 ± 2	−7 800	0.245	29.5	140	$-Ca^{2+}$
				−9 300	0.292	26	105	$+Ca^{2+}$
DTNB fragment[a]	1.4 (± 0.3) × 10^5	1.0 (± 0.5) × 10^2	13 ± 1	nd	0.267	27.5	125	$-Ca^{2+}$
				nd	0.293	25.5	105	$+Ca^{2+}$
Alkali 1				−8 900	0.212	32.5	170	
Alkali 2				−9 900	0.300	25	105	

[a] Chymotryptic fragment, missing N-terminal segment, dephosphorylated (see text). [b] Refers to strong binding sites; obtained from free divalent cation concentration at midpoint of transition profiles. [c] The linear calibration for globular proteins is given by: $erf^{-1}(1 - K_d) = 0.418 \log M - 1.15$. [d] From elution volume on gel filtration column. The volume reproducibility corresponds to a reproducibility in apparent Stokes radius of 0.5 Å. [e] Calculated from Stokes radius, assuming hydration of 0.4 g of water per g of protein and a prolate equivalent hydrodynamic ellipsoid of revolution.

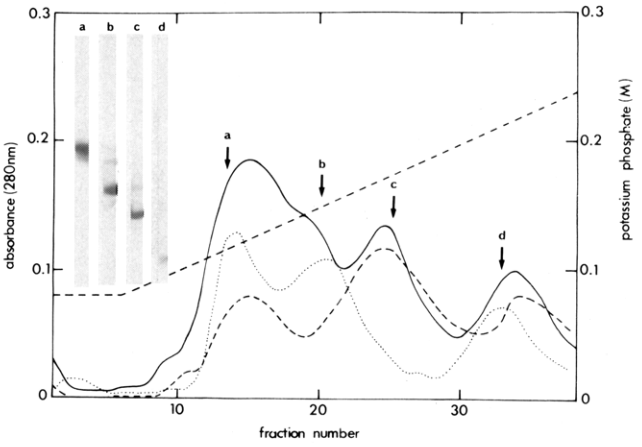

FIGURE 1: Column chromatography of skeletal myosin light chains on DEAE-cellulose. Right-hand ordinate axis refers to the phosphate gradient (broken line). The elution curves represent light chains of untreated myosin, containing a mixture of phosphorylated and unphosphorylated DTNB light chains (—), enzymically dephosphorylated myosin (···), and fully phosphorylated myosin (- - -). The inset shows polyacrylamide gels after electrophoresis in the presence of 8 M urea of column fractions from the elution profile of untreated myosin light chains; the positions of the fractions are as indicated.

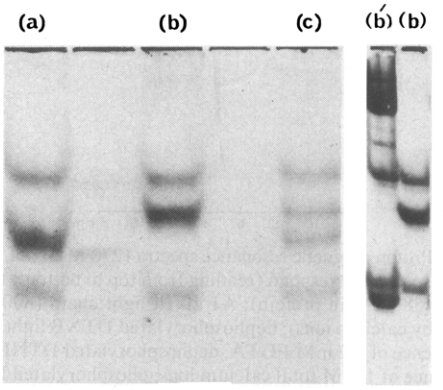

FIGURE 2: Polyacrylamide gel in 8 M urea of myosin light chains (a) after phosphorylation with endogenous light chain kinase, (b) after dephosphorylation with endogenous phosphatase, and (c) without prior enzyme treatment; (b′) shows chymotryptic conversion of the DTNB light chains into a fragment of molecular weight 17 000, migrating just ahead of the smaller alkali light chain (A2).

dodecyl sulfate gels (Bagshaw, 1977; Weeds & Pope, 1977).

As judged by circular dichroism, the phosphorylated and dephosphorylated DTNB light chains differ appreciably from each other (Table I). When calcium ions are added to a solution of either, the spectroscopic properties undergo considerable changes. In the first place there is a diminution in the quantum yield of the intrinsic fluorescence, as previously noted by Werber et al. (1972), amounting to some 15% at saturation. That this is a reflection of a conformational change, rather than merely a perturbation of the indole chromophore on binding of calcium in its immediate vicinity, is shown by the accompanying change in circular dichroism. The increased negative ellipticity in the peptide absorption region corresponds on saturation with calcium to an increase in the total α-helix content of some 5%. At the same time changes occur in the system of Cotton effects arising from the aromatic residues. Such Cotton effects are a consequence of a defined, rigid structure around the chromophores, the environments of some of which are evidently considerably altered (Figure 5, inset). Some inferences about the conformations of the light chains and the effects of calcium follow from an examination of the high-resolution proton magnetic resonance spectra. The spectra of all light chains differ from simulated spectra based on a mixture of amino acids; thus in the native state a sizeable proportion of the side chains are not in a solvent environment. A particularly striking feature of the spectra of both alkali light chain species (A1 and A2) is the presence of a set of resonances in the high-field region (Figure 3), which arises from ring-current shifts. These result from the close proximity of aliphatic groups to the planes of aromatic system, and may be regarded as diagnostic of close-packed, and rigid, i.e., globular, structure (see, e.g., Sternlicht & Wilson, 1967). Calcium ions have no effect whatever on these spectra. In the case of the DTNB light

FIGURE 3: Proton magnetic resonance spectra (270 MHz) of myosin light chains. The spectra correspond (reading from top to bottom) to: A2 alkali light chain (580 μM in protein); A1 alkali light chain (600 μM) (both unchanged by calcium ions); dephosphorylated DTNB light chain, (275 μM) in presence of 0.2 mM EGTA; dephosphorylated DTNB light chain in the presence of 1 mM total calcium ions; phosphorylated DTNB light chain (300 μM) in the presence of 0.2 mM EGTA; phosphorylated DTNB light chain in the presence of 0.6 mM calcium ions; dephosphorylated DTNB light chains in the presence of 0.7 mM cobalt(II). The small signal near 0 ppm in the spectra of the dephosphorylated DTNB chains is an organic contaminant. Arrows mark the most prominent ring-current-shifted aliphatic proton resonances.

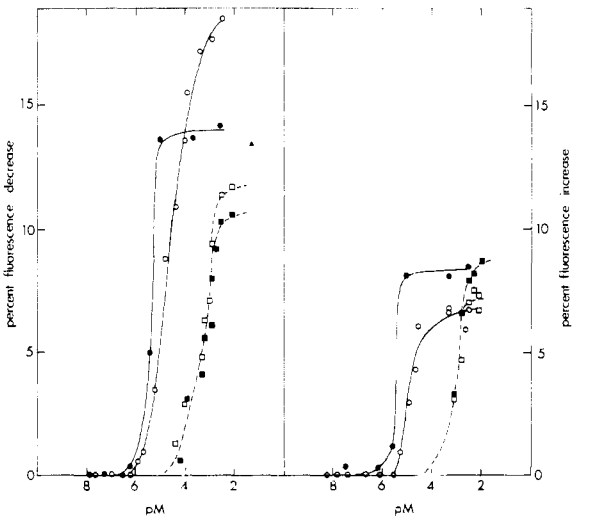

FIGURE 4: Fluoresence titrations of DTNB light chains with calcium and magnesium ions. The left-hand panel refers to intrinsic fluorescence, the right-hand panel to the fluorescence of attached dansyl chromophores (see text). Full lines and circles refer to calcium, broken lines and squares to magnesium, both with dephosphorylated (●, ■) and phosphorylated (○, □) proteins. The effect of calcium (6 mM) in the presence of a saturating level of magnesium ions is also shown (▲).

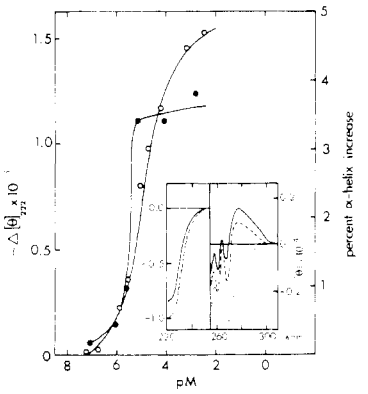

FIGURE 5: Circular dichroism titrations with calcium ions of DTNB light chains in the phosphorylated (○) and dephosphorylated (●) states. The wavelength is 222 nm. The right-hand axis refers to the change in apparent α-helix content corresponding to the ellipticity changes. Inset: circular dichroism spectra of DTNB light chains in the peptide and aromatic Cotton effect regions,in the absence (—) and presence (- - -) of excess calcium ions. The dotted curves shows A2 alkali light chain for comparison. The ellipticity scale for the peptide (backbone) circular dichroism (left-hand ordinate of inset) is referred to molar residue concentration, that for the aromatic Cotton effect system (right-hand ordinate) to concentration in molarity of total protein. The data for all light chains agree satisfactorily with those of Wu & Yang (1976).

chains, on the other hand, calcium ions elicit a number of changes in the spectrum, which are most obvious in the high-field region, in which signals of aliphatic methyl and methylene protons primarily lie (Figure 3). The spectrum of the DTNB light chain shows little superficial resemblance to that of troponin C (Seamon et al., 1977; Levine et al., 1977); neither does the addition of calcium ions bring about the large changes in line widths and chemical shifts reported for this protein. Phosphorylation of the DTNB light chains gives rise to minor changes in the spectrum, including a diminution in the presumed β-methylene signal of serine at 3.95 ppm. The calcium-induced changes affect at least ten aliphatic proton resonances; it is difficult to make unambiguous assignments, but from literature values of chemical shifts (see, e.g., McDonald & Phillips, 1969) some of them can be tentatively identified as stemming from the β- and γ-methylene protons of lysine and proline, β-methylene protons of serine, glutamic acid, and/or methionine, and the methyl group of threonine. When calcium is replaced by a paramagnetic ion, cobalt(II), a very similar pattern of changes is observed, with no obvious additional broadening effects. This suggests that there is little direct perturbation of signals in consequence of interaction of the metal ion with side chains at the binding site, and that the observed effects result from an overall conformational change.

The changes in fluorescent intensity (Figure 4) and in ellipticity (Figure 5) of the dephosphorylated DTNB light chain on addition of calcium ions are sufficient to allow determination of a binding profile. The curves can be fitted very satisfactorily with a single binding constant of 2.5×10^5 M^{-1}. On phosphorylation, the spectroscopic transition shifts to higher free calcium concentration, to an extent corresponding to a diminution in binding constant of somewhat less than an order of magnitude. Moreover the profile is not symmetrical and gives evidence of a second weaker binding process, as also in-

ferred by Werber et al. (1972) for DTNB light chains of unknown phosphorylation state. Similar binding curves for the phosphorylated and dephosphorylated light chains are obtained by fluorimetric titration of the modified protein, containing two dansyl groups attached to the thiols (Figure 4). The binding constant is unaffected by the substituent groups. At the same time the dansyl fluorescence undergoes a blue shift on introduction of calcium ions, indicating the passage of the chromophores into a less polar environment. This shift is about 5 nm in the phosphorylated and 3 nm in the dephosphorylated state. These results, taken together with the small but reproducible differences between the magnitudes of the calcium-induced changes in intrinsic fluorescence and circular dichroism of the unmodified protein in the phosphorylated and dephosphorylated forms, indicate that they are not conformationally identical even when saturated with calcium.

The changes in fluorescence and circular dichroism of the DTNB chains could be effected also by magnesium ions, though at much higher concentration (Figure 4). In this case there was practically no effect of phosphorylation on the affinity. Moreover, even at high magnesium concentrations, near the physiological range, for example, 2 mM, saturation of the change in optical properties is not attained, and in these conditions calcium ions still produce the expected residual effect at relatively low concentration (Figure 4). In order to determine whether the conformational changes described above are also associated with a change in gross shape or asymmetry, a sedimentation velocity study was attempted. Calcium-induced changes could be observed, suggesting a diminution in frictional coefficient on binding, but extrapolation as a function of protein concentration revealed a tendency for the protein to associate, at least toward the upper end of the concentration range of these experiments, and there was, moreover, evidence of time-dependent aggregation effects in these conditions. We therefore resorted to gel filtration, which allows the use of low protein concentrations, as a measure of apparent Stokes radius (Warshaw & Ackers, 1971). The column of Sephadex G-100 was calibrated with standard proteins, and the molecular weights were plotted against $\mathrm{erf}^{-1}(1 - K_d)$, following Ackers (1970), where K_d, the partition coefficient for the protein between the buffer and the inside of the Sepharose beads, is obtained from the elution volume.

The gel filtration medium was chosen to give linearity with sufficient discrimination in the relatively low-molecular weight range, with which we are here concerned. The calibration standards lie on a straight line, and all three light chains are found to have very much lower partition coefficients than would correspond to globular proteins of the same molecular weights (Table I); they are therefore either highly asymmetric, or open and unfolded structures. Moreover addition of calcium ions causes a very considerable contraction in Stokes radius of the DTNB light chains. If the calibration is recast in terms of the Stokes radius, R_s, derived from literature data for the sedimentation coefficients, s, and partial specific volumes, $\bar{v}$, of the proteins, so that

$$R_s = M)1 - \bar{v}\rho)/6\pi\eta_0 Ns$$

(where M is the molecular weight, ρ the density, and η_0 the viscosity of the solvent, and N the Avogadro number), one may use it to obtain the apparent Stokes radii of the light chains. The calibration is again linear over the range of standards used, with $\mathrm{erf}^{-1}(1 - K_d) = 0.0195R_s + 0.248$. Nozaki et al. (1976) have shown that this procedure must be approached with caution, because highly asymmetric molecules are apparently able to penetrate the gel matrix by an orientation selection mechanism, and are therefore retarded to a greater degree than

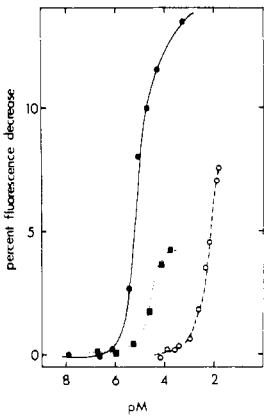

FIGURE 6: Fluorescence titrations of chymotryptically truncated dephosphorylated DTNB light chains with calcium (●) and magnesium (○), and calcium ions in the presence of 20 mM magnesium ions (■).

would be predicted on the basis of Stokes radius. It is not likely that this effect will be important for the relatively modest asymmetries with which we are dealing, but we cannot exclude the possibility that the estimates of Stokes radius are low, and that the asymmetries therefore represent a *lower* limit. At all events, the binding of calcium causes the apparent Stokes radius of the dephosphorylated DTNB light chains to fall from 31 to 26 Å, and that of the phosphorylated molecule from 30 to 26 Å. Qualitatively similar effects are produced by magnesium, but the change is somewhat smaller, with a Stokes radius in the presence of 2 mM magnesium ions of about 28 Å.

From the Stokes radii we may obtain the frictional coefficient, $f = 6\pi\eta_0 R_s$, and hence the frictional ratio, (f/f_0), corresponding to a sphere of identical molecular weight and partial specific volume, i.e., $f_0 = 6\pi\eta_0(3M\bar{v}/4\pi N)^{1/3}$. If, in the usual way, (f/f_0) is decomposed into contributions of hydration and asymmetry, one may deduce the hydration contribution for an assumption, say, of 0.4 g of water per g of protein effective hydration (see, e.g., Kuntz & Kauzmann, 1974). For a prolate ellipsoid model, according to Perrin, we then obtain an axial ratio for DTNB light chains (dephosphorylated) in the absence of divalent cations of 9, and in the presence of calcium of 5. From the volume of the prolate ellipsoid of revolution we obtain for the length (major axis) of the molecule 150 and 100 Å, respectively (with 130 Å in the presence of 2 mM magnesium ions and no calcium). Table I summarizes the corresponding data for all light chains. It is important to note that the elution volumes are reproducible and entirely independent of protein concentration down to 10 μg/mL, i.e., over a range of more than an order of magnitude. This renders it improbable that the calcium dependent effects which we observe are related to self-association rather than to a conformation change.

We have examined finally the chymotryptically truncated DNTB light chain, which is generated by digestion of myosin in the presence of calcium ions (Bagshaw, 1977; Weeds & Pope, 1977). In situ this retains its calcium binding capacity. As isolated by column chromatography, it is also sensitive to calcium and magnesium by spectroscopic criteria (Figure 6). The binding constant for calcium is similar to that of the corresponding intact dephosphorylated light chain, but the binding of magnesium appears to be much weaker. The elution volume from the gel filtration column reveals that the Stokes radius in the presence of calcium ions differs little from that of the intact light chain. The change induced by calcium is, however, greatly reduced (Table I). Thus although the calcium binding site is evidently left undisturbed by removal of the terminal

peptide, the conformational consequences are diminished.

Discussion

The results described above indicate in the first place that the isolated DTNB light chains are functional, in the sense that their specific divalent cation binding site is intact. Comparison of the binding constant with that of the light chain in situ is difficult, because there is as yet poor agreement between data obtained for myosin by various methods (Morimoto & Harrington, 1974; Beinfeld et al., 1975; Bremel & Weber, 1975; Bagshaw, 1977). Whether the local activity of magnesium ions can be equated with the total magnesium concentration in the myofibril is not clear, but so far as our data relate to the situation in the intact tissue, effective competition by calcium cannot be excluded, at any rate in the unphosphorylated state.

We stress, however, that there are as yet insufficient grounds to assert that binding of calcium, or indeed magnesium ions to light chains must have physiological significance. Viewed in terms of a simple competition, the slow rate of replacement of magnesium by calcium on the DTNB light chain binding site (Bagshaw & Reed, 1977) argues against a light-chain dependent control mechanism operating on the time scale of a single twitch. The extent of the change in the conformation of the light chain in situ that can be brought about by calcium ions in the presence of physiological concentrations (in the mM range) of magnesium must also remain uncertain until it has been resolved whether the affinity of the binding site is higher when the light chain is incorporated in myosin.

That phosphorylation has a physiological function in striated muscle is suggested by the observation of phosphorylation in the course of a single tetanus in frog muscle (Bárány & Bárány, 1977). In vertebrate smooth muscle a thick-filament-based control system has already been shown to operate, and the implication of phosphorylation in the regulation process in this system has been convincingly argued (Sobieszek & Small, 1977). Our data raise the possibility that it may also play a part in calcium regulation in mammalian striated muscle and support the suggestion (Lehman, 1977) that a thick-filament control mechanism operates in such systems. If indeed the DTNB light chains do play a regulatory role, one might conjecture that they control the calcium ion-dependent change in cross-bridges orientation, inferred from physical and structural studies (Morimoto & Harrington, 1974; Haselgrove, 1975). The change may be small, involving perhaps a rotary motion of the myosin heads, since these appear throughout to remain in contact with the filament surface (Sutoh & Harrington, 1977). Such a model for the action of calcium is rendered more attractive by the evident relationship of the DTNB light chain to the conformational character of the proteolytically labile subfragment 1–subfragment 2 junction of the myosin (Bagshaw, 1977; Weeds & Pope, 1977). It has also been reported (Margossian & Lowey, 1977) that calcium promotes the self-association of subfragment 1, and that this interaction depends on the presence of the DTNB light chains. This suggests the possibility of a calcium-induced interaction between the heads of each myosin molecule; as was shown earlier, by cross-linking experiments in which intermolecular interactions are rigorously excluded (d'Albis & Gratzer, 1976), the heads in intact myosin are at least partly in contact.

The calcium binding site of the DTNB light chain can be tentatively identified from a comparison of its sequence with that of troponin C (Collins, 1976), and evidently lies near the N terminus of the chain. It has been reported that the C-terminal end is located in the myosin head (Kendrick-Jones & Jakes, 1976). If it is permissible, on the basis of the results described above, to infer that the light chain structure consists of a globular domain and one or more elongated or flexible elements, it seems probable that any physiological activity would depend on a retraction or deformation of the latter, in response to the binding of calcium in the globular part. It would be premature to devise models for the mechanisms by which the DTNB light chain protects peptide bonds against proteolytic attack, or controls changes in the myosin head orientation. We note only that the length of the chain, at least as interpreted on the primitive (and in a complex system rather arbitrary) prolate ellipsoid model, is about 130 Å in the presence of magnesium ions in the physiological concentration range, and therefore sufficient to run at least a large part of the length of the myosin head and into the hinge region. The shape of the head is a matter of debate, but its principal axis must be expected to be between about 100 and 200 Å in length (Lowey, 1971; Mendelson et al., 1973, etc.). It should be remarked that the regulatory EDTA light chain of scallop muscle appears also to be asymmetric, on the basis of sedimentation studies, with a suggested axial ratio of 10 (Szent-Györgyi, 1975).

As regards the N-terminal peptide, its loss on exposure of myosin to chymotrypsin may be unrelated to events at the subfragment 1–subfragment 2 junction. The fragment is evidently not needed to sustain the (presumably) globular domain containing the binding site. It is not clear whether such a fragment of less than 20 residues could exert a sufficiently large direct effect on the Stokes radius, or whether the failure of calcium to produce the maximal shape change in the truncated light chain is due to a loss of conformational information with the peptide.

It is unexpected that the alkali light chains, A1 and A2, also display considerable asymmetry. Again, however, the spectroscopic data, most of all the presence of a series of ring-current shifted aliphatic resonances in the proton magnetic resonance spectra, make it clear that both species contain globular regions. If it is valid to describe the alkali light chains also in terms of a structure comprising a globular head and an elongated tail, then this tail would again be able to span a large part of the length of the myosin head. It appears that the very considerable sequency homology between the alkali and DTNB light chains (Collins, 1976) may be matched by a corresponding similarity in their structures, despite the absence of a functional binding site for divalent cations in the alkali light chains.

Note Added in Proof

A paper has now appeared, by W. F. Stafford & A. G. Szent-Györgyi ((1978) *Biochemistry 17*, 607–614), in which regulatory light chains from a variety of sources have been examined by gel filtration and hydrodynamic methods, all in the presence of calcium ions. The results for DTNB light chains, interpreted on a prolate ellipsoid model, agree very well with the data reported here. In addition, evidence from fluorescence anisotropy decay kinetics favors such an asymmetric, rather than an open, coil-like structure.

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