Spectroscopic Studies on Invertebrate Myosins and Light Chains†

Peter D. Chantler* and Andrew G. Szent-Györgyi

ABSTRACT: Myosin was isolated from 14 invertebrate muscles all of which exhibited myosin-linked regulation; 9 of these muscles solely exhibited this form of regulation. None of these myosins showed any change in Trp or Tyr fluorescence upon addition of Ca^{2+} in the presence or absence of MgATP and all myosins showed either a small or zero Trp fluorescence change upon addition of MgATP in the presence or absence of calcium. Thus a conformationally sensitive Trp, such as that present in rabbit myosin, is not a necessary requirement for the myosin ATPase. Several light chains were modified with the fluorophore *N*-iodoacetyl-*N'*-(1-sulfo-5-naphthyl)ethylenediamine and each modified light chain was added back to 0 °C-desensitized scallop myosin, and the fluorescence and fluorescence polarization were examined. No change in these parameters occurred upon addition of MgATP and/or calcium. Circular dichroism (CD) spectra of scallop myosin and desensitized scallop myosin showed no change upon addition of MgATP and/or calcium. Similarly, no change was observed in the electron spin resonance spectra of scallop myosin or myofibrils using 0 °C-desensitized preparations that had been resensitized with spin-label-modified light chains. CD studies on isolated regulatory light chains reveal a low affinity divalent-cation-dependent transition (for the scallop regulatory light chain, $pK = 3.7 \pm 0.1$ (Ca^{2+}); 3.1 ± 0.1 (Mg^{2+})) monitored at 220 nm, while an analogous change in the rabbit 5,5'-dithiobis(2-nitrobenzoic acid) light chain has a pK of 5.1 ± 0.2. These results are discussed in light of the fact that the above techniques indicate large conformational changes when applied to other calcium-binding proteins such as troponin C and parvalbumin. It is concluded that the calcium switch in regulatory myosins is of a more subtle nature.

Muscle contraction is triggered by the release of calcium ions into the sarcoplasm ($[Ca^{2+}] \geq 10^{-6}$ M) from the vesicles of the sarcoplasmic reticulum, this release being under nervous control (Weber & Murray, 1973). The calcium released is bound by control proteins which then cease to exert their inhibitory role on the actomyosin interaction. Two control systems may be defined in most species (Lehman & Szent-Györgyi, 1975). One control system is associated with the thin filament and requires troponin and tropomyosin (Ebashi, 1974). The troponin molecule possesses three subunits; troponin T, the tropomyosin binding subunit, troponin I, the inhibitory subunit capable of preventing actomyosin interaction by itself (Wilkinson et al., 1972), and troponin C, the calcium binding subunit. The other system is associated with the thick filament and is simply a regulatory myosin, the regulatory light chain–myosin heavy chain interaction facilitating calcium binding (Szent-Györgyi, 1975). Vertebrate skeletal muscles, however, only possess thin-filament control; molluscs, brachipods, echinoderms, echiuroids, and nemertine worms only possess thick-filament control (Lehman & Szent-Györgyi, 1975). These control mechanisms are further complicated by phosphorylation requirements. However, scallop myosin does not possess a phosphorylatable light chain (Frearson et al., 1976; Jakes et al., 1976) and no evidence has been found as yet that molluscan myosins require phosphorylation for calcium sensitivity.

The calcium binding subunit of troponin, troponin C, has been studied by various spectroscopic techniques (Potter et al., 1976; Murray & Kay, 1972; Van Eerd & Kawasaki, 1972; Ohnishi et al., 1975; Lehrer & Leavis, 1974) all of which suggest a major conformational change upon calcium binding. The calcium activation of the Mg^{2+}–ATPase of myosin in the presence of pure rabbit F-actin is a substantial and readily observable change exhibited by all regulatory myosins. The aim of this investigation was to try to obtain more information about this calcium switch in thick-filament-controlled species by looking at the effect of calcium ions on various optical parameters manifested by regulatory myosins and light chains. Also, considerable information about the myosin ATPase has been obtained taking advantage of the intrinsic tryptophan fluorescence change exhibited by rabbit myosin upon binding ATP[1] in the presence of Mg^{2+} (Werber et al., 1972b). The effect of MgATP on these spectroscopic parameters was also observed using regulatory myosins from invertebrates.

Materials and Methods

Myofibril Preparation. Myofibrils were prepared essentially according to Lehman & Szent-Györgyi (1975). Glycerinated muscles were washed and blended in a Sorvall Omnimixer, the buffer being 40 mM NaCl, 1.0 mM $MgCl_2$, 0.1 mM EGTA, 5 mM phosphate, pH 7.0. The suspension was spun down at 40 000*g* and the supernatant discarded. Resuspension of the pellet was followed by two further such washings, then the final pellet was suspended in the same buffer and homogenized with a Teflon plunger.

Myosin Preparation. The invertebrate myosins used in the intrinsic fluorescence studies were prepared according to Szent-Györgyi et al. (1971, 1973). For all other studies, myosins were prepared essentially by the method of Focant & Huriaux (1976); the myofibrils were spun down at 40 000*g* and resuspended in 40 mM NaCl, 0.1 mM EGTA, 5 mM phosphate, pH 7.0; NaCl and ATP were then added, on ice, to 0.6

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[1] Abbreviations used: ATP, adenosine triphosphate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid; EDTA, ethylenediaminetetraacetic acid; ESR, electron spin resonance; Mops, 4-morpholinepropanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; DTT, dithiothreitol; $NaDodSO_4$, sodium dodecyl sulfate; IAEDANS, *N*-iodoacetyl-*N'*-(1-sulfo-5-naphthyl)ethylenediamine; S-1, myosin subfragment 1; CD, circular dichroism.

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M and 5 mM, respectively, keeping the pH constant at 7.0. Actomyosin was obtained by spinning down the suspension at 40 000*g* for 10 min. $MgSO_4$ and ATP were then added to the supernatant to 20 mM and a further 5 mM respectively and a 45% ammonium sulfate cut performed keeping the pH constant using 0.5 M Na_2HPO_4. The actin precipitate was spun down at 40 000*g* for 10 min and the supernatant was made up to 65% saturation in ammonium sulfate. This pellet was spun down at 40 000*g* for 10 min and then resuspended on ice in a small volume of 0.6 M NaCl, 10 mM Tris, 1.0 mM $MgCl_2$, pH 7.4 (at room temperature). Once resuspended, the solution was dialyzed against 4 L of 10 mM phosphate, pH 7.0, and the precipitate thus formed washed twice with 40 mM NaCl, 5 mM phosphate, pH 7.0, 1.0 mM $MgCl_2$, 0.1 mM EGTA, and resuspended in the high salt buffer. These last washes remove tropomyosin which did not precipitate with the myosin. This method gives a very pure myosin preparation (see Figure 1).

Desensitization of Myosin or Myofibrils. Desensitization of myosin or myofibrils was performed at 0 °C using 10 mM EDTA as previously described (Kendrick-Jones et al., 1976).

Soluble Subfragment Preparations. Soluble subfragments of myosin were prepared by papain treatment of myofibrils in the presence of calcium and magnesium (Ca·Mg·S-1) or in the presence of EDTA (EDTA·S-1) according to the method of Drs. W. F. Stafford and E. M. Szentkiralyi (to be published). Subfragments are shown on gels in Figure 1.

Light Chain Preparation. Invertebrate light chains were prepared as described previously (Kendrick-Jones et al., 1976).

Preparation of Derivatized Light Chains. Mercenaria, Loligo, and *Spisula* regulatory light chains and the essential light chain of scallop all possess sulfhydryl groups (Kendrick-Jones et al., 1976) and all were derivatized in essentially the same manner irrespective of the reagent. The light-chain sample was dialyzed against 1.0 mM DTT, 10 mM Tris, pH 8.0, prior to dialysis against the medium of choice lacking DTT. This method was found felicitous in keeping all -SH groups in the reduced state just prior to the reaction.

The spin labels *N*-(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl)iodoacetamide and *N*-(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl)maleimide were reacted in 2–4 M excess with light chains in 0.1 M KCl, 10 mM Mops, pH 7.0, for around 8 h. Extensive dialysis against this buffer removed any unreacted labels. Calibration of the ESR spectrum with free spin labels indicated that greater than 80% of the free sulfhydryls had reacted in all cases.

Light chains in 10 mM triethanolamine, pH 8.5, were reacted with a 50 M excess of *N*-iodoacetyl-*N*-(1-sulfo-5-naphthyl)ethylenediamine (IAEDANS) at 4 °C in the dark for 2 h. Then the excess reagent was removed either by extensive dialysis or by passage through a preequilibrated (25 mM phosphate, pH 7.0) Bio-Gel A column followed by application of a phosphate gradient (25 mM–0.4 M phosphate, pH 7.0). Greater than 90% of free sulfhydryls were labeled by this technique as monitored through the Ellman reaction (Ellman, 1959).

Fluorescence. Fluorescence studies were performed with a Perkin-Elmer MPF-44 or a Perkin-Elmer MPF-3 spectrofluorimeter equipped with a thermostated cell housing maintained at a constant temperature (±0.1 °C). Excitation was at 295 nm for the intrinsic Trp (tryptophan) measurements, a wavelength where protein absorption is predominately due to Trp at pH 8.0 (Weber & Young, 1964) and which is also free from ATP absorption. Measurements were usually made

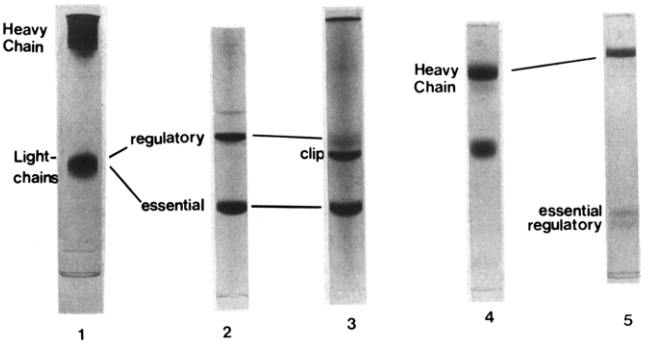

FIGURE 1: (1) 10% $NaDodSO_4$/acrylamide gel of scallop myosin prepared by the ammonium sulfate method, ~200 μg loading; (2) 10% urea/acrylamide gel of scallop myosin, ~80 μg loading; (3) 10% urea/acrylamide gel of Ca·Mg·scallop S-1, ~80 μg loading; (4) 7.5% $NaDodSO_4$/acrylamide gel of EDTA·scallop S-1, ~80 μg loading; (5) 7.5% $NaDodSO_4$/acrylamide gel of *Loligo* S-1, ~50 μg loading. Gels 2 and 3 were run at the same time. Note that the regulatory light chain has been clipped in the Ca·Mg·scallop S-1 (Stafford, W. F., & Szentkiralyi, E. M., to be published). The sample of EDTA·scallop S-1 shown in 4 has tropomyosin impurities and showed a 4.5% fluorescence enhancement; when corrected for this background, a fluorescence enhancement similar to that of the tropomyosin-free samples used in Table I is obtained. The light-chain band remaining in 4 is probably due to light-chain fragments (Stafford, W. F., & Szentkiralyi, E. M., to be published).

in 3-mL sample volumes at 25 °C (Werber et al., 1972b) containing myosin or myosin subfragments at 150 μg/mL (Seidel, 1975). For myosin solutions the buffer always contained 0.6 M NaCl, 10 mM Tris, 1.0 mM $MgCl_2$, pH 7.4 (at room temperature). The buffer for the subfragments varied as indicated in the text. Other components were added from 0.1 M stock solutions so as to cause a negligible volume change and the fluorescence was examined after each addition. Three cycles of additions were employed in all, each involving a separate protein sample. In the first cycle EGTA was added to 0.1 mM followed by ATP to 150 μM followed by Ca^{2+} to 0.2 mM; in the second cycle EGTA was added to 0.1 mM followed by Ca^{2+} to 0.2 mM followed by ATP to 150 μM; the third cycle was the same as the first except that the order of addition of EGTA and Ca^{2+} was reversed. Cells were allowed to remain in the housing for at least 2 min before a reading was taken so as to ensure thermal equilibration. The above procedure ensured that both the myosin·ADP**·P_i intermediate and the calcium switch could be observed if they gave rise to a fluorescence change. Alternatively the calcium switch was observed using tyrosine excitation (λ_{ex} = 280 nm; λ_{em} = 300–340 nm). Fluorescence polarization studies were performed using a polarization accessory supplied by Perkin-Elmer. The degree of polarization was defined as

$$P_{corr} = \frac{L_v(V_h/L_h)}{V_v + L_v(V_h/L_h)}$$

where V_h, L_h, V_v, and L_v are the intensities recorded with the excitation polarizer at 90° and the emission polarizer at 0°; the excitation polarizer at 90° and the emission polarizer at 90°; the excitation polarizer at 0° and the emission polarizer at 0°; the excitation polarizer at 0° and the emission polarizer at 90°, respectively.

Electron Spin Resonance. ESR spectra were recorded with a Varian E-109-E X-band spectrometer at 20 °C. The frequency was 9.12 GHz with a modulation width of 2 G. Subsaturating microwave power intensity was used (power setting of 10 mW).

Desensitized scallop myofibrils or desensitized scallop myosin (10–20 mg/mL) were resensitized in situ in a Varian aqueous solution sample cell using spin-labeled light chains

TABLE I: Tryptophan Fluorescence Enhancement of Various Myosins upon Addition of ATP.

type of regulation		I %[a]/I	λ_{max}^{em}[b]
	Myosin		
myosin-linked regulation	*Loligo paelei*	−1.0	335–340
	Aequipecten irradians, striated adductor	+2.0	332–333
	Mercenaria mercenaria	+1.0	335–342
	Spisula solidissima	−2.0	335–340
	Placopecten magellanicus, striated adductor	0	338–341
	Urechis caupo	−1.0	336–338
	Busycon canaliculatum	+1.0	335–338
	Crassostrea virginica, translucent adductor	0	330–340
	Placopecten magellanicus, smooth adductor	0	335–338
doubly linked regulation	*Leucophaea maderae*	+2.0	336–338
	Limulus polyphemus	+2.0	335–341
	Lethocerus cordofanus	+1.0	335–342
	Eurypelma sp.	−1.0	336–338
	Homarus americanus, crusher claw	+5.0	335–342
actin-linked regulation	*Homarus americanus*,[c] tail	+1.0	335–342
	Myosin Subfragment		
myosin linked regulation	*Loligo paelei* Ca·Mg·S-1	+1.0	330–338
	Aequipecten irradians,[d] striated adductor		
	Ca·Mg·S-1	+7.5	328–332
	EDTA·S-1	+8.4	328–330

[a] Measurements were made as described in Materials and Methods. Excitation wavelength, λ_{ex} = 295 nm. A 310-nm filter was employed. Excitation slit width was 4 nm. Emission slit width was 10 nm. Percent change is expressed as (the change in fluorescence at λ_{max} upon addition of ATP to 150 μM in the presence of 1 mM $MgCl_2$ divided by the original fluorescence at λ_{max}) × 100. Values quoted are ±1% except the subfragments which are ±2%. [b] Expressed in nm. Spectra are not corrected for instrumental variables such as lamp and photomultiplier efficiency at different wavelengths. [c] Although it has been suggested that this muscle is doubly regulated (see Lehman, 1977), work in this laboratory suggests that one retains the original classification. [d] Pure papain subfragments kindly supplied by Drs. Walter F. Stafford III and Eva M. Szentkiralyi.

at low ionic strength (40 mM NaCl, 10 mM phosphate, 2.0 mM $MgCl_2$, pH 7.0), the amount being equimolar to the myosin content. Sixty percent by weight of the myofibrils was assumed to be myosin (Szent-Györgyi et al., 1973). Sometimes the medium also contained 0.1 mM EGTA or 0.1 mM EGTA plus 0.2 mM $CaCl_2$. When a sample was tested in the presence of both Ca^{2+} and ATP an ATP-regenerating system was used. This consisted of 5 mM MgATP, 0.5 mg/mL creatine phosphokinase, and 25 mM creatine phosphate.

Circular Dichroism. CD spectra were recorded on a Cary 60 spectropolarimeter with a Model 6001 CD attachment with a programmed slit control to give a 1.5-nm band width. Concentrations were chosen so that the OD was approximately 1.0 at the wavelength of interest. In all cases the appropriate blank spectrum, i.e., the spectrum of the sample solution without the protein, was subtracted. CD spectra of the light chains were obtained in a medium containing 10 mM phosphate, pH 7.0; those of myosin were obtained in various media stated in the text. Myosin samples were clarified by a 100 000g spin for 15 min prior to obtaining the CD spectrum. Data are presented in terms of the molar ellipticity. The machine was calibrated using d-camphor-10-sulfonic acid.

Other. Protein concentrations were determined either by the Folin–Lowry procedure (Lowry et al., 1951) or by the biuret method (Gornall et al., 1949) using an OD of 0.07 cm^{-1} $(mg/mL)^{-1}$ at 550 nm.

Light chain concentrations were determined using the following extinction coefficients ($E^{0.1\%}_{1cm,280nm}$): scallop (regulatory), 0.22; scallop (essential), 0.55; *Mercenaria* (regulatory), 0.50; *Spisula* (regulatory), 0.46; *Loligo* (regulatory), 0.45; rabbit 5,5′-dithiobis(2-nitrobenzoic acid), 0.55.

Free sulfhydryl groups were determined by spectrophotometric titration using Ellman's reagent (Ellman, 1959) except that $NaDodSO_4$ was used as the denaturant.

ATPase activities were measured by following proton liberation in a pH-stat as described by Szent-Györgyi et al. (1973).

Polyacrylamide gel electrophoresis in the presence of $NaDodSO_4$ or urea/acrylamide gel electrophoresis was performed as described earlier by Kendrick-Jones et al. (1976). Calcium binding was performed using [^{45}Ca]- and [^{3}H]glucose as described earlier (Szent-Györgyi et al., 1973).

Aequipecten irradians, Placopecten magellanicus, Mercenaria mercenaria, Spisula solidissima, Loligo paelei, and *Crassostrea virginica* were obtained from the Marine Biological Laboratory, Woods Hole, Mass. Myosins from other species were derived from glycerinated preparations obtained earlier (Lehman & Szent-Györgyi, 1975).

Results

Intrinsic Fluorescence of Myosin and Myosin Subfragments in the Presence of ATP. All myosins and myosin subfragments tested gave a small or negligible tryptophan (Trp) fluorescence enhancement upon addition of ATP (Table I; the classification is according to Lehman & Szent-Györgyi, 1975). This was true irrespective of the presence or absence of calcium (0.1 mM EGTA or 0.1 mM EGTA, 0.2 mM $CaCl_2$) in the solution prior to the addition of ATP. Furthermore there was no Trp fluorescence change in any of the myosin or myosin subfragment solutions tested upon addition of Ca^{2+} to 0.2 mM to a medium containing 0.1 mM EGTA or upon addition of EGTA to 2.0 mM or 0.1 mM to a medium containing 0.1 mM $CaCl_2$ or no added calcium, respectively. All myosins possessed calcium sensitivities and activities similar to those reported earlier (Lehman & Szent-Györgyi, 1975). All subfragments were calcium insensitive in terms of their actin-activated ATPase in the presence of Ca^{2+}.

No change in tyrosine (Tyr) fluorescence occurred upon addition of calcium to scallop myosin or subfragments in the presence or absence of ATP.

Extrinsic Fluorescence of Derivatized Light Chains and Resensitized Scallop Myosin. IAEDANS derivatives of *Mercenaria, Loligo,* and *Spisula* light chains restored both calcium sensitivity and calcium binding to desensitized scallop myofibrils (Table II). They therefore seemed to act similarly to the underivatized regulatory light chains of the species. Addition of these light chains to desensitized scallop myosin also fully restored calcium sensitivity to the actin-activated Mg^{2+}-ATPase.

The fluorescence emission of IAEDANS-substituted *Mercenaria* light chain was monitored at 470 nm in 0.6 M NaCl, 10 mM Tris, 1.0 mM $MgCl_2$, 0.1 mM EGTA, pH 7.4 (at room temperature) at 25 °C, exciting at either 290 nm or 350 nm (350-nm filter). The ratio of the intensities of the emission at 470 nm exciting at 290 nm to that after excitation

TABLE II: Ca^{2+} Sensitivity and Ca^{2+} Binding of Scallop Myofibrils, Desensitized Scallop Myofibrils, and Desensitized Scallop Myofibrils Resensitized with an IAEDANS Light Chain.

type of myofibrils	Ca^{2+} sensitivity[d] (%)	Ca^{2+} bound[a] (nmol/mg of myofibril)	ratio of[c] regulatory LC to essential LC
control myofibrils	78	1.29	0.91
desensitized myofibrils	17	0.72	0.45
desensitized myofibrils + scallop EDTA-LC	90	1.30	1.02
desensitized myofibrils + *Spisula* IAEDANS-LC	82	1.28	0.91
desensitized myofibrils + *Loligo* IAEDANS-LC	86	1.29	0.94
desensitized myofibrils + *Mercenaria* IAEDANS-LC	90	N.D.[b]	1.00

[a] Determined at a free calcium concentration of 1×10^{-6} M. We thank Mr. Jim Sellers for the data in this column. [b] Not determined. [c] Obtained from urea/acrylamide gels; LC, light chain. [d] Ca^{a+} sensitivity is defined as [1 − (rate in absence of Ca^{2+})/(rate in presence of Ca^{2+})] × 100.

at 350 nm (I_{290}/I_{350}) was 0.2. These intensities were not altered by the addition of ATP or Ca^{2+} or both.

Desensitized scallop myosin does not show any fluorescence emission at 470 nm when excited at either 290 nm or 350 nm. An equimolar amount of *Mercenaria* IAEDANS-substituted light chain and desensitized scallop myosin was incubated on ice for 15 min in the above buffer. The solution was then warmed to 25 °C and the fluorescence emission determined at 470 nm exciting at either 290 nm or 350 nm. The emission intensity was invariant upon addition of ATP and/or calcium, (I_{290}/I_{350}) = 0.72. Showing that energy transfer occurred between aromatic residues on scallop myosin and the IAEDANS label of the light chains. Such increased transfer also occurred upon excitation at 295 nm indicating that one or more tryptophan residues were among the transfer donors. Similar results were obtained with *Spisula* IAEDANS-substituted light chain and *Loligo* IAEDANS-substituted light chains.

Polarization studies were performed on scallop myosin containing 1 mol of IAEDANS-substituted light chain. It was concluded that no change in the degree of polarization occurred upon addition of ATP and/or calcium within the precision of the measurement which was estimated as no better than ±10%.

Effect of Lanthanides. Actin-activated activity was not restored to *Aequipecten* myofibrils or myosin in 0.1 mM EGTA when $TbCl_3$ was added to 0.2 mM. Thus the preparation still showed an activity similar to that obtained in the absence of calcium. These preparations showed full sensitivity when Ca^{2+} was added to 0.2 mM in the absence of lanthanide. Similarly 0.2 mM $TbCl_3$, $EuCl_3$, or $GdCl_3$ did not release the inhibition of *Placopecten* myofibrils that were fully active in the presence of Ca^{2+}.

The effect of Tb^{3+} on scallop myosin was also studied spectrofluorimetrically. To scallop myosin samples in 0.6 M NaCl, 2.5 mM Tris, 1.0 mM $MgCl_2$, 33 μM EGTA, ± 0.1 mM ATP, $TbCl_3$ was added in 3 μL aliquots from a 10 mM stock. Emission at 546 nm was observed whilst exciting at 285 nm with a 390-nm filter. Perceptible emission only occurred at a 50 M excess of Tb^{3+} over myosin and this fluorescence increased and did not flatten off even at an 800 M excess of Tb^{3+} over myosin (0.4 mM Tb^{3+}) when the experiment was abandoned. Similar observations were made with 0 °C-desensitized scallop myosin.

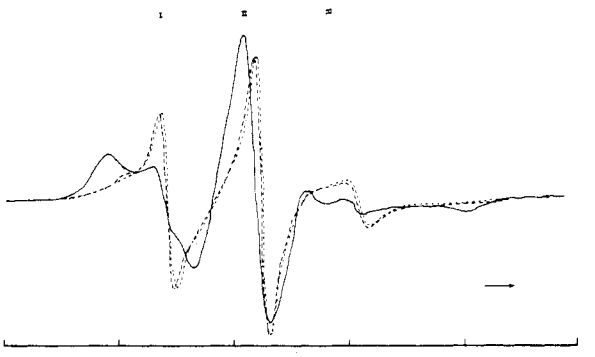

FIGURE 2: ESR spectra of maleimide spin-labeled *Loligo* regulatory light chain (- - -) in 40 mM NaCl, 10 mM phosphate, 2.0 mM $MgCl_2$, 0.1 mM EGTA, pH 7.0; desensitized scallop myosin resensitized with maleimide spin-labeled *Loligo* regulatory light chain (— —) and desensitized scallop myofibrils resensitized with maleimide spin-labeled *Loligo* regulatory light chain (—) all in the same buffer. The base line is graduated in 20-G intervals. Arrow indicates the direction of increasing field strength.

Electron Spin Resonance. Spectra of the isolated spin-labeled light chains showed only weak immobilization in all cases, i.e., the bound label showed rapid isotropic movement similar to that of the free label in solution. The maleimide spin label was always more immobilized than the iodoacetamide spin label. Maleimide spin-labeled *Loligo* regulatory light chain showed the highest degree of immobilization but this amount was still small (Figure 2). Spectra were invariant irrespective of the presence or absence of 0.1 mM free calcium.

Further immobilization occurred upon addition of spin-labeled light chains to desensitized scallop myosin and to a greater extent with desensitized scallop myofibrils. In both instances the maleimide spin-labeled *Loligo* regulatory light chain showed a greater degree of immobilization than any of the other labeled light chains but even here the spectrum did not indicate complete immobilization. These spectra were invariant in the presence or absence of 5 mM MgATP ± 0.1 mM free Ca^{2+}. Incubation of the samples for periods of longer than 30 min did not cause the spectra to alter in any way; hence, there was no evidence from the ESR spectra that a period of greater than 30 min was required for complete light-chain binding.

Circular Dichroism. (a) Scallop Myosin. The far-UV-CD spectrum from 205 to 250 nm of pure scallop myosin is seen in Figure 3. There are two negative extrema at 222 nm and 208 nm. The spectrum was found to be invariant, within the precision of measurement, when the following changes were made to the buffer: replacement of NaCl by KCl; addition of Mg^{2+} to 1.0 mM and Ca^{2+} to 0.2 mM; addition of Mg^{2+} to 1.0 mM and ATP to 50 μM; addition of Mg^{2+} to 1.0 mM, ATP to 50 μM, and Ca^{2+} to 0.2 mM. The spectrum of desensitized scallop myosin was also identical.

Near-UV-CD spectra of scallop myosin and desensitized scallop myosin were similar and showed a negative extremum at 285 nm and a positive extremum at 265 nm of magnitude around $[\theta] = 5 \times 10^4$ deg cm^2 dmol^{-1}. The curves were invariant upon addition of Ca^{2+} to 0.2 ± 1.0 mM Mg^{2+} ± 50 μM ATP within the precision of measurement.

(b) Light Chains. The far-UV-CD and the near-UV-CD spectra of invertebrate light chains have been described previously (Stafford & Szent-Györgyi, 1978) and it was observed that the far-UV-CD spectrum was invariant at calcium concentrations $\leq 10^{-4}$ M. Upon extending this range, however,

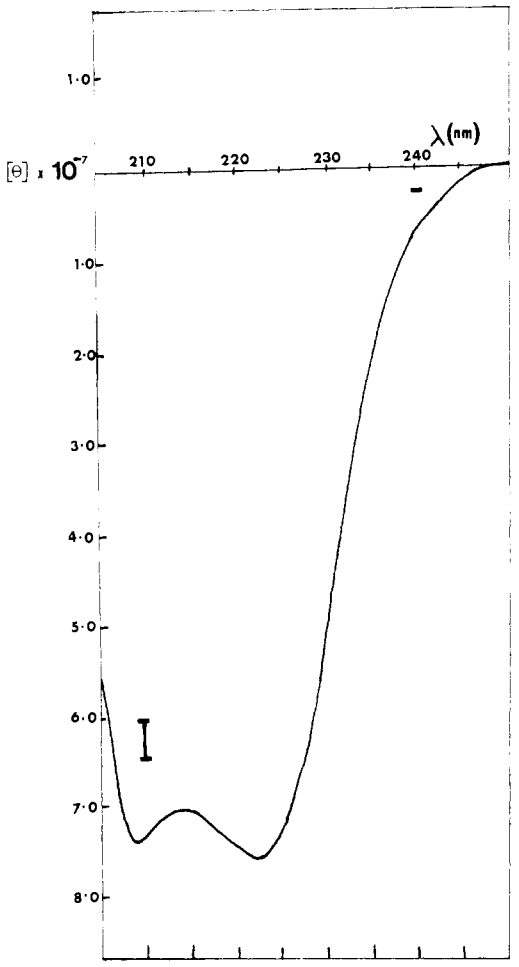

FIGURE 3: Far-UV-CD spectrum of scallop myosin in 0.6 M NaCl, 10 mM phosphate, 0.1 mM EGTA, pH 7.0. I indicates noise limits at the appropriate wavelength.

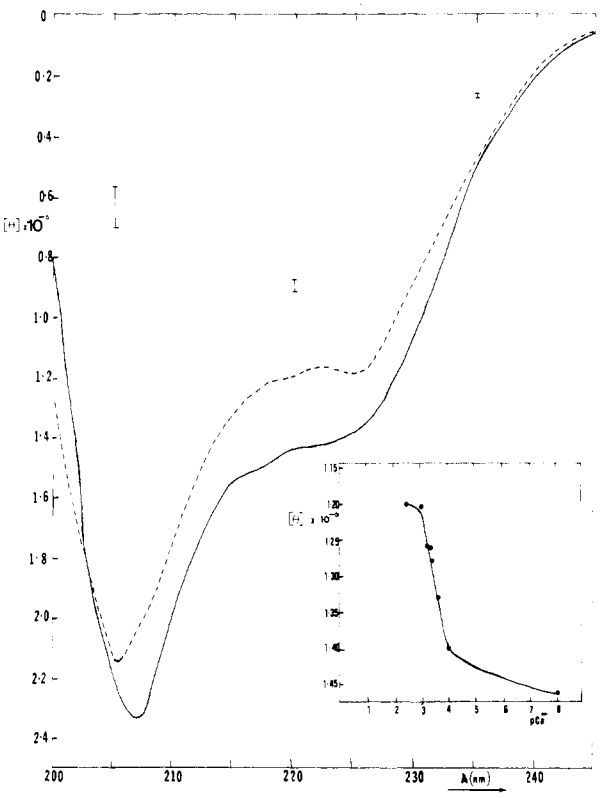

FIGURE 4: Far-UV-CD spectrum of scallop regulatory light chain in 10 mM phosphate, pH 7.0. Additions: 0.1 mM EGTA (- - -); 0.1 mM EGTA, 0.2 mM $CaCl_2$ (—). Inset: pCa^{2+} curve for charge in ellipticity at 220 nm in the scallop regulatory light chain. (The point drawn at $pCa^{2+} = 8.0$ was obtained in the presence of 0.1 mM EGTA with no added Ca^{2+}. Thus it depicts an upper limit for the calcium concentration.) I indicates noise limits at the appropriate wavelength.

it was observed (Figure 4) that a relatively large CD change occurred upon addition of calcium. This change involved the region between 205 nm and 235 nm and was maximal at 220 nm. The negative extremum also shifted from 205 nm to 207.5 nm. The change was complete upon addition of Ca^{2+} to 1.0 mM. Similarly, the same change could be elicited by the addition of Mg^{2+} ions but here 10 mM Mg^{2+} was required to complete the change. The change in ellipticity was reversible upon addition of EGTA or EDTA (the former only for the Ca^{2+}-induced change). The amount of change caused by a particular divalent cation concentration did not change with time in the machine. Addition of Mg^{2+} to a scallop EDTA light-chain solution containing 0.1 mM Ca^{2+} continued to cause an increase in the molar ellipticity at 220 nm as did addition of Ca^{2+} to a solution of light chain containing 0.1 mM $MgCl_2$. However, addition of Mg^{2+} to the scallop EDTA light-chain solution already in 1.0 mM $CaCl_2$ did not cause further change, in keeping with this phenomenon being competitive for these ions with Ca^{2+} having the greatest affinity.

pCa^{2+} curves (Figure 4, inset) and pMg^{2+} curves were constructed for several light chains including the rabbit 5,5′-dithiobis(2-nitrobenzoic acid) light chain all of which showed the same phenomenon. In the absence of Mg^{2+}, pK values for Ca^{2+} binding to the light chains, as judged by enhancement of ellipticity at 220 nm, were scallop regulatory, 3.7 (±0.1), scallop essential, 3.3 (±0.2), and rabbit 5,5′-dithiobis(2-nitrobenzoic acid), 5.1 (±0.2). pK values for Mg^{2+} binding in the absence of Ca^{2+} were scallop regulatory, 3.1 (±0.1), and scallop essential, 3.0 (±0.2). Addition of Ca^{2+} to the *Mercenaria* regulatory light chain causes a reversible change in the same wavelength range but in the opposite direction to the other light chains studied. At the end of each titration curve, the pH of the solution in the CD cell had changed by less than 0.2 pH unit.

In one experiment equimolar amounts of the scallop regulatory light chain and the scallop essential light chain were added together and titrated with Ca^{2+} monitoring the change in ellipticity at 220 nm. This change was the same as that expected for the sum of both light-chain contributions.

Discussion

Fluorescence. Werber et al. (1972) showed that the hydrolysis of ATP by rabbit myosin or myosin subfragments was accompanied by an increase in the intrinsic tryptophan fluorescence. The bulk of this fluorescence increase in the steady state was due to a conformational change from myosin (M) to M·ADP·**P_i, the predominant species during the steady-state hydrolysis of MgATP by rabbit myosin (Bagshaw et al., 1974; Taylor et al., 1970) and by relaxed scallop and *Lethocerus* myofibrils (Marston & Lehman, 1974) (where two stars indicate a species with considerable fluorescence enhancement according to the terminology of Bagshaw & Trentham). This enhancement may have been due to a Trp residue at the active site or due to a Trp residue remote from the active site but whose immediate environment was altered by a conformational change. The results presented here extend the observations of Werber et al. (1972) in that they show that the conformationally sensitive Trp is not a necessary requirement for the

myosin ATPase. Fourteen out of 15 regulatory myosins examined showed zero or a very small Trp fluorescence enhancement upon addition of ATP. Furthermore *Loligo* S-1 was shown also to have virtually zero fluorescence enhancement upon addition of ATP indicating the absence of Trp at the active site. It has also been recently reported that only a 2% fluorescence enhancement was obtained by addition of ATP to S-1 derived from frog fast muscle myosin (Ferenczi et al., 1978). Thus it is probable that Trp fluorescence enhancement during ATP hydrolysis is not in itself sufficient evidence to suggest that myosins that show such enhancement have identical mechanisms, as has been suggested (Shimizu, 1977). The fact that scallop S-1 prepared by papain digestion showed about 8% fluorescence enhancement as opposed to scallop myosin which showed only 2% enhancement (Table I) is understandable. Papain S-1 from rabbit digested in the presence of Mg^{2+} shows around 15% Trp fluorescence enhancement upon addition of ATP, whereas chymotryptic S-1 from rabbit, which has lost its 5,5′-dithiobis(2-nitrobenzoic acid) light chain, shows a 35% fluorescence increase (personal observations). This is compared with a 10% Trp fluorescence enhancement for rabbit myosin. Therefore, by simple comparison, one could expect fluorescence enhancement in the range 3–7% for scallop S-1. The fact that scallop EDTA·S-1 has the same fluorescence enhancement upon addition of ATP as Ca·Mg·S-1 means that the light chains of scallop are not involved in the mechanism of fluorescence enhancement (Figure 1). It should be noted, however, that the scallop EDTA light chain does not possess a Trp residue whereas the *Loligo* regulatory light-chain does (Kendrick-Jones et al., 1976); the lack of Trp fluorescence enhancement by Ca·Mg·*Loligo* S-1 corroborates the above conclusion.

Lobster crusher claw myosin was the only myosin to give a larger Trp fluorescence change upon addition of ATP. Here, one could predict therefore a subfragmental 1 preparation having a Trp fluorescence enhancement of the order of 8–18% upon addition of ATP. It may be noted that even the 8% enhancement of scallop S-1 is large enough for certain key features of the scallop myosin and actomyosin ATPase mechanism to be elucidated using transient-state kinetics.

The emission maxima of invertebrate myosins were usually broad (Table I) and varied between species though the wavelengths of the maxima did not alter upon addition of Ca^{2+}, ATP or both Ca^{2+} and ATP. For example, *Aequipecten* striated adductor myosin had an emission range in the region 332–333 nm, whereas lobster crusher claw myosin had an emission range in the region 335–342 nm. This probably means that the Trp residues contributing most to the fluorescence are not conserved in the same environments in different species thus leading to a change in predominance of "red" emitters and "blue" emitters between species (Elkana, 1968).

No calcium-dependent change in Trp fluorescence intensity or wavelength was observed with any of the regulatory myosins. Similarly no calcium-dependent change in Tyr fluorescence intensity, as has been observed in troponin C (Van Eerd & Kawasaki, 1972) and in the calcium-dependent protein activator of cyclic nucleotide 3′-5′-phosphodiesterase (Drabikowski et al., 1977), was observed with scallop myosin or subfragments.

No calcium-dependent change in fluorescence or polarization of fluorescence occurred with scallop myosins possessing one IAEDANS-modified regulatory light chain. This was despite the fact that the IAEDANS label is an environment-sensitive marker (Hudson & Weber, 1973; Cheung & Garland, 1978) and that the modified light chains restored both calcium sensitivity and calcium binding to desensitized scallop myofibrils (Table II) and myosin. Furthermore, the extrinsic fluorescence studies did not reveal any ATP-dependent fluorescence changes or any change in energy-transfer dependent upon ATP, Ca^{2+} or ATP plus Ca^{2+} addition. It should be noted that all fluorescence experiments were carried out in a medium containing 1.0 mM Mg^{2+} at 25 °C so that the predominant myosin species was presumably M·ADP·**P_i (Bagshaw & Trentham, 1974). In no case did the addition of actin cause a Ca^{2+} or ATP-dependent fluorescence change to be seen.

As can be seen from Figure 1, the proteins used were exceptionally pure. As the calcium sensitivities of *Aequipecten* and *Loligo* myosin were lost during the preparation of the subfragments, even when the light chains were retained, these results further substantiate the original observations of Szent-Györgyi et al. (1973) that calcium sensitivity is lost upon S-1 preparation.

The results presented here show that Tb^{3+} does not function as a calcium analogue in scallop myofibrils or scallop myosin in contrast with the fact that it may act as a calcium analogue in parvalbumins (Moews & Kretsinger, 1975) and troponin C (Miller et al., 1975). Similar results were obtained with Gd^{3+} and Eu^{3+}. It was not determined whether these lanthanides bound to the two high affinity calcium binding sites on scallop myosin. However, no fluorescence enhancement at 546 nm (exiting at 285 nm) was observed upon addition of Tb^{3+} to scallop myosin in amounts that were stoichiometric or slightly in excess of stoichiometric but this may simply reflect the fact that the Tb^{3+} ions were not located in an appropriate environment as the oscillator strengths of Tb^{3+} transitions are weak and therefore one requires close proximity to get good luminescence. As emission at 546 nm was obtained, whilst exciting at 285 nm upon addition of Tb^{3+} to scallop myosin in great molar excess, it seems unlikely that the lack of fluorescence upon addition of stoichiometric amounts of Tb^{3+} was simply due to the use of Tris buffer as has been observed in some (Donato & Martin, 1974) but not all (Kayne & Cohn, 1973) studies.

ESR Studies. Modification of *Spisula, Loligo,* and *Mercenaria* regulatory light chains with the iodacetamide and maleimide spin labels gave stoichiometric addition in all cases (1, 2, and 1 mol, respectively, per mol of light chain). ESR spectra of *Spisula* and *Mercenaria* light chains did not show much immobilization of the bound label compared with the free label. Spin-labeled *Loligo* light chain showed more immobilization than these. In all cases the maleimide spin-labeled light chains showed greater immobilization than iodoacetamide spin-labeled light chains.

The regulatory light chains of *Spisula* and *Mercenaria* contain only a single sulfhydryl group per light chain (Kendrick-Jones et al., 1976), and the ESR spectra show only a single component both before and after addition of desensitized scallop myosin or myofibrils. In contrast, *Loligo* regulatory light chain possesses two sulfhydryl groups (Kendrick-Jones et al., 1976), and its spectrum is split into two components, implying two different label environments, upon the addition of desensitized scallop myosin or myofibrils, as shown in Figure 2. The more immobilized outer peak (rotational correlation time = 10^{-8} s) is seen to a lesser extent in the resensitized scallop myosin spectrum (the light-chain tumbles with two correlation times of 15 ns and 80 ns as observed by fluorescence decay anisotropy measurements (Harvey & Cheung, as mentioned in Stafford & Szent-Györgyi, 1978)). Increased immobilization occurred upon addition of spin-labeled light chains to desensitized scallop myosin and myofibrils; that this immobilization is incomplete suggests that this is not due to the label being wedged between the light chain and the heavy

chain but rather is due to a localized conformation change in the environment of the label. This, of course, may be insignificant in terms of the gross conformation of the light chains.

All ESR spectra were invariant upon addition of 5 mM MgATP and an ATP regenerating system. Furthermore all spectra were unaffected by the presence or absence of calcium even in the presence of MgATP. This means that the environment of each spin label was not affected by the process undergone by the myosin molecule during the switching on by calcium.

The difference between the ESR spectrum of the maleimide spin-labeled regulatory light chain when added back to desensitized scallop myosin and that when added back to desensitized scallop myofibrils may not be significant. Desensitized scallop myosin is more labile alone than when present in the myofibril and it may be more difficult to add back all the light chain stoichiometrically under the conditions of the experiment. Thus the spectrum may simply be an admixture of the free light-chain spectrum and the more immobilized spectrum seen upon addition of the maleimide spin-labeled *Loligo* regulatory light chain to desensitized myofibrils.

In no instance was immobilization sufficient to permit the application of the saturation-transfer ESR technique (Thomas et al., 1976).

CD Studies. The far-UV-CD spectrum of scallop myosin (Figure 3) is similar to the spectrum of rabbit myosin (Wu & Yang, 1976), though the ellipticities at the extrema are somewhat smaller. No change is observed, within the precision of the instrument, upon addition of MgATP, which is also similar to the situation observed with rabbit myosin (Gratzer & Lowey, 1969; Cassim & Lin, 1975). Furthermore, no change is observed upon addition of Ca^{2+} to scallop myosin in the presence of MgATP. This means that no gross conformational change occurs on the myosin molecule under these conditions in contrast to the large conformational change seen in troponin C (Murray & Kay, 1972). The precision of the near-UV-CD spectra was not good enough to shed light on the controversy concerning perturbation of the myosin CD spectrum by nucleotides (Cassim & Lin, 1975; Marsh et al., 1978).

That desensitized myosin has the same conformation as intact scallop myosin as seen by CD is not very surprising: the contribution of the far-UV negative extremum of 1 mol of scallop light chain to the far-UV negative extremum of 1 mol of desensitized scallop myosin should be less than 0.1%. Thus unless a gross change in the α-helix content of the heavy chain occurred upon readdition of the light chain one would not expect to see a CD change.

The conformation of all the light chains studied is affected by the addition of Ca^{2+} or Mg^{2+} in the range 10^{-4} to 10^{-2} M (Figure 4). This effect can be shown to be competitive for these divalent cations and manifested itself most strongly by altering the ellipticity at 220 nm. The spectrum at 220 nm is most greatly influenced by the α-helix content of the protein (Greenfield & Fasman, 1969); thus in the case of both types of scallop light-chain and in the rabbit DTNB light chain it would appear that the binding of divalent cations increases the α-helix content of the light chain. By the same criterion, a decrease in α-helix content upon divalent cation binding in the millimolar range is observed in the *Mercenaria* regulatory light chain. As the pH remains virtually constant during these experiments, one is not observing the pH-dependent CD change seen earlier (Stafford & Szent-Györgyi, 1978). However, as proton binding in the range pH 7.0–1.8 does alter the same part of the CD spectrum in the same manner, it is possible that the divalent cation binding and proton binding sites are the same or overlap substantially. With the exception of the rabbit DTNB light-chain, these sites are probably nonspecific and their number and possible identity with the postulated site on the regulatory light chain involved in high affinity calcium binding in the intact myosin molecule (Jakes et al., 1976) remain to be determined.

It is unlikely that this calcium-dependent CD change represents aggregation rather than a conformational change: Alexis & Gratzer (1978) performed gel filtration studies on the rabbit DTNB light chain in the presence of millimolar concentrations of calcium and over a range of protein concentrations; they were able to conclude that the calcium-dependent effects seen by gel filtration, CD, and fluorescence were not due to self-association. Similar work has been performed in our laboratory on the scallop regulatory light chain using high-speed equilibrium ultracentrifugation in the presence and absence of millimolar calcium (Stafford & Szent-Györgyi, 1978; Dr. W. F. Stafford, personal communication) with the conclusion that calcium-dependent aggregation is extremely unlikely.

The Ca^{2+}-binding site(s) on the rabbit 5,5′-dithiobis(2-nitrobenzoic acid) light chain observed above have a pK of 5.1. This means that the change observed probably involves binding to the high affinity calcium binding site on this light chain (pK = 5.5) noted previously (Morimoto & Harrington, 1974; Werber et al., 1972a) and is the same change as that observed by Alexis & Gratzer (1978). The lower pK of 5.1 could also be due to involvement of the weaker calcium binding site (pK = 4.0) which has been observed on the rabbit 5,5′-dithiobis(2-nitrobenzoic acid) light chain by monitoring a decrease in the Trp fluorescence upon addition of Ca^{2+} ions (Werber et al., 1972a).

The fact that, in the experiment involving a mixture of scallop regulatory and essential light chains, the ellipticity at 220 nm, upon titration with calcium, was equal to the sum of the separate ellipticities suggests, but does not prove, that no calcium dependent interaction between the two sorts of light chain occurs.

Significance. Troponin C, parvalbumins, the alkali light chains, and the rabbit 5,5′-dithiobis(2-nitrobenzoic acid) light chain are evolutionarily related (Kretsinger & Barry, 1975; Weeds & McLachlan, 1974; Collins, 1976). These proteins are also related to the scallop regulatory light chain (Jakes et al., 1976). Furthermore, all regulatory light chains and the rabbit 5,5′-dithiobis(2-nitrobenzoic acid) light chain are related to each other as they all rebind to desensitized scallop myofibrils (Kendrick-Jones et al., 1976). However, secondary structure predictions indicate that the evolutionary distance between these proteins is great (Argos, 1977), especially between troponin C and the 5,5′-dithiobis(2-nitrobenzoic acid) light chain. Although troponin C is the calcium sensitizing part of the troponin-tropomyosin complex (Potter & Gergely, 1974) and the regulatory light chains are required for calcium sensitivity in regulatory myosins, troponin C and light chains have very different structures: troponin C binds Ca^{2+} by itself (Potter & Gergely, 1975) and yet the regulatory light chain does not bind calcium alone (at 1.0×10^{-6} M free calcium) (Kendrick-Jones et al., 1976); troponin C has a more globular shape as deduced from sedimentation measurements (Mani et al., 1974) than the regulatory light chains which are asymmetric with a length of 100–140 Å (Stafford & Szent-Györgyi, 1978); the regulatory light chains do not crystallize under the same conditions (personal observations) as those required for troponin C crystallization (Mercola et al., 1975). In addition, CD studies (Murray & Kay, 1972), fluorescence studies (Van

Eerd & Kawasaki, 1973; Potter et al., 1976), and ESR studies (Ohnishi et al., 1975; Potter et al., 1976) on troponin C have all revealed calcium-dependent changes that may be related to conformational alterations in the molecule. This study shows that despite the evolutionary relationship between the regulatory light chains and the other calcium binding proteins, no calcium-dependent conformational change has been detected in regulatory myosins by observation of CD spectra, intrinsic and extrinsic fluorescence, fluorescence polarization and by ESR. As various light chains from different species were used during the -SH modifications, one might also expect that a reasonable statistical sampling of light chain–heavy chain binding sites occurred. Yet, these results do not permit one to say that such a conformational change does not exist; indeed, some structural change must occur so as to enable one to witness the dramatic effect that calcium has on the actin-activated Mg^{2+}-ATPase of regulatory myosins. However, the results do indicate that the mechanism must be subtle and most probably does not involve the large conformational changes seen in troponin C and parvalbumins (Donato & Martin, 1974; Burstein et al., 1975).

At least one high affinity calcium binding site is retained in the rabbit 5,5′-dithiobis(2-nitrobenzoic acid) light chain and this has facilitated studies both on the isolated light chain (Werber et al., 1972a) and on the intact myosin (Okamoto & Yagi, 1976). As the regulatory light chains do not bind calcium ($pK = 4.0$) it may be inferred that the evolutionary distance between these molecules is also great, for there cannot simply be a deletion of the calcium binding site because the regulatory light chain binds calcium strongly ($pK = 6.0$) when in situ bound to the myosin heavy chain. Although the 5,5′-dithiobis(2-nitrobenzoic acid) light chain binds calcium, the site does not seem to be a regulatory one: the metal binding site requires concentrations of calcium about ten times higher than the range of calcium control required to fully activate muscle (Morimoto & Harrington, 1974) and an in vitro calcium requirement necessary to enhance the Mg^{2+}-ATPase of rabbit myosin when incubated with pure rabbit actin has not been demonstrated. Any remaining possibility of this site being a regulatory site has been ruled out by the finding that the off-rate constant for the displacement of Mg^{2+} from this site by Ca^{2+} is several orders of magnitude too low to fit in with the observed physiological rates (Bagshaw & Reed, 1977).

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Correlations between Subunit Distribution, Microheterogeneity, and Iron Content of Human Liver Ferritin†

Daniel J. Lavoie,‡ Kunitsugu Ishikawa,§ and Irving Listowsky*

ABSTRACT: Subunit heterogeneity of human liver ferritin was investigated by two-dimensional electrophoretic methods. The protein which ordinarily remains assembled in 10 M urea solution was dissociated into subunits in acid–urea or sodium dodecyl sulfate solutions. In agreement with earlier studies, the subunits migrated as two bands in sodium dodecyl sulfate or acid–urea gel electrophoresis systems or in two-dimensional combinations of these systems. Isoelectric focusing methods, however, resolved four major subunit bands and three to five minor bands. Each of these components migrated as either a 22 000 or a 19 000 molecular weight component in sodium dodecyl sulfate gel electrophoresis in the second dimension. The multiple subunit model, which is contrary to currently accepted representations of ferritin structure, is compatible with certain inherent properties of the protein. Thus, ferritin was fractionated on the basis of iron content to show that the relative amounts of individual subunit types were directly dependent upon the iron composition of the protein. Iron-loaded molecules were deficient in the most basic subunit types, and apoferritin was enriched in these components. Aspects of microheterogeneity of assembled ferritin molecules were correlated to subunit heterogeneity, and discrete differences in subunit populations among purified isoferritin components were demonstrated.

Ferritin, an iron-containing protein found in most mammalian tissues and widely distributed in nature, plays a prominent role in iron metabolism of eukaryotic cells (Granick, 1946; Crichton, 1973; Linder and Munro, 1973; Harrison and Hoy, 1973; Harrison et al., 1974; Harrison, 1977; Drysdale et al., 1977; Fagard and Saddi, 1977; Marcus et al., 1978). The protein subunits are arranged in the form of a hollow sphere that is uniquely suited to envelop large amounts of iron in its central cavity (Haggis, 1965; Harrison et al., 1967; Fischbach et al., 1969; Spiro and Saltman, 1969; Massover et al., 1973; Webb and Gray, 1974; Harrison et al., 1974). With improved technical developments for resolving closely related proteins, ferritins from most sources were shown to consist of a family of proteins that have been referred to as "isoferritins" (Drysdale, 1970; Urushizaki et al. 1971; Drysdale, 1974; Powell et al., 1975; Massover, 1978). It was suggested that this microheterogeneity originates from different subunit types that are present in different combinations or proportions in assembled molecules (Vulimiri et al., 1975; Adelman et al., 1975; Ishitani et al., 1975a,b; Lavoie et al., 1977; Drysdale, 1977). Ferritin undergoes a number of posttranslational structure modifications, including glycosylation (Shinjyo et al., 1975; Lavoie et al., 1977; Cynkin and Knowlton, 1977) and proteolytic processing (Niitsu et al., 1973; Ishitani et al., 1975c), and possible correlations of these factors to aspects of its microheterogeneity have been discussed in recent reviews (Marcus et al., 1978; Drysdale, 1977).

In the present report, heterogeneity of human liver ferritin is probed by two-dimensional gel electrophoretic methods. Conditions for dissociation and fractionation of the protein are defined, and a systematic relationship between subunit heterogeneity and microheterogeneity of assembled molecules is considered.

Experimental Procedures

Materials

Urea was ultrapure grade from Schwarz/Mann. Acrylamide and other electrophoretic reagents were obtained from

† From the Department of Biochemistry, Albert Einstein College of Medicine, Yeshiva University, Bronx, New York 10461. *Received May 10, 1978.* This work was supported by Grants HL-11511 and AM 17702 from the National Institutes of Health.

‡ Postdoctoral trainee on an Immunooncology training program (5T32 CA 09173).

§ Present address: Department of Medicine, Cancer Research Institute, Sapporo Medical College, Sapporo, Japan.

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