On the Location of the Divalent Metal Binding Sites and the Light Chain Subunits of Vertebrate Myosin†

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ABSTRACT: The divalent metal ion binding sites of skeletal myosin were investigated by electron paramagnetic resonance (EPR) spectroscopy using the paramagnetic Mn(II) ion as a probe. Myosin possesses two high affinity sites ($K < 1$ μM) for Mn(II), which are located on the 5,5′-dithiobis(2-nitrobenzoate) (DTNB) light chains. Mn(II) bound to the isolated DTNB light chain gives rise to an EPR spectrum similar to that of Mn(II) bound to myosin and this indicates that the metal binding site comprises ligands from the DTNB light chain alone. Myosin preparations in which the DTNB light chain content is reduced by treatment with 5,5′-dithiobis(2-nitrobenzoate) show a corresponding reduction in the stoichiometry of Mn(II) binding, but the stoichiometry is recovered on reassociation of the DTNB light chain. Chymotryptic digestion of myosin filaments in the presence of ethylenediaminetetraacetic acid yields subfragment 1, but digestion in the presence of divalent metal ions produces heavy meromyosin. Myosin with a depleted DTNB light chain content gives rise to subfragment 1 on proteolysis, even in the presence of divalent metal ions. It is proposed that saturation of the DTNB light chain site with divalent ions protects this subunit against proteolysis, which, in turn, inhibits the cleavage of the subfragment 1–subfragment 2 link. Either the DTNB light chain is located near the region of the link and sterically blocks chymotryptic attack, or it is bound to the subfragment 1 moiety and affects the conformation of the link region. When the product heavy meromyosin was examined by sodium dodecyl sulfate gel electrophoresis, an apparent anomaly arose in that there was no trace of the 19 000-dalton band corresponding to the DTNB light chain. This was resolved by following the time course of chymotryptic digestion of the myosin heavy chain, the DTNB light chain, and the divalent metal binding site. The 19 000-dalton DTNB light chain is rapidly degraded to a 17 000-dalton fragment which comigrates with the alkali 2 light chain. The divalent metal site remains intact, despite this degradation, and the 17 000 fragment continues to protect the subfragment 1–subfragment 2 link. In the absence of divalent metal ions, the 17 000-dalton fragment is further degraded and attack of the subfragment 1 link ensues. Mn(II) bound to cardiac myosin gives an EPR spectrum basically similar to that of skeletal myosin, suggesting that their 19 000-dalton light chains are analogous with respect to their divalent metal binding sites, despite their chemical differences. The potential of EPR spectroscopy for characterizing the metal binding sites of myosin from different sources and of intact muscle fibers is discussed.

Contraction of vertebrate skeletal muscle is initiated through the release of Ca(II), which binds to the troponin–tropomyosin complex associated with the actin filament, thereby relieving the inhibition of the actin-activated myosin ATPase activity (see Weber and Murray, 1973). The existence of Ca(II) binding sites on the myosin thick filaments has raised the possibility that other events are controlled by Ca(II) release (Morimoto and Harrington, 1974; Bremel and Weber, 1975). However, as yet there is no clear evidence that vertebrate skeletal muscle possesses a myosin-linked regulatory system of the type found in many invertebrate muscles (Lehman and Szent-Györgyi, 1975) since, in the absence of the troponin–tropomyosin components, Ca(II) has no major effect on the in vitro actomyosin ATPase rate. As a basis for discussion of the significance of the Ca(II) binding sites of myosin, it is pertinent to review the structural features of this molecule.

Myosin (480 000 daltons) is a hexamer comprising two heavy chains (200 000 daltons) and four light chains (approximately 20 000 daltons). Each heavy chain folds to make up a roughly globular region and a long filamentous region which intercoils with its partner heavy chain to form a rod. At physiological ionic strength, the rods aggregate to provide the backbone of the myosin thick filament. Two light chains are associated with each globular region of the myosin molecule. This structure emerged from studies in which the myosin molecule was subjected to limited proteolytic digestion (Lowey et al., 1969). A susceptible region is present in the myosin rod which, on proteolysis, yields light meromyosin (140 000 daltons) and a water-soluble fragment, heavy meromyosin (340 000 daltons). Heavy meromyosin contains the two globular moieties (subfragment 1, 2 × 110 000 daltons) connected by a short rod region (subfragment 2, 60 000 daltons) and may be further digested to release these fragments. Each subfragment 1 bears one ATPase site and one to two light chains. Since these products can be obtained by digestion with a number of proteolytic enzymes of differing specificity, it is thought that the peptide chain may be uncoiled in the susceptible regions and, hence, these may represent the positions of flexible joints in the myosin filament (Huxley, 1969).

In the case of vertebrate fast skeletal muscle, three types of light chains have been identified (21 000, 19 000, and 17 000 daltons), giving rise to the possibility of myosin isoenzymes. The 19 000-dalton component can be partially but selectively removed from skeletal myosin, without loss of ATPase activity, by a combined treatment with DTNB[1] and EDTA and is

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[1] Abbreviations used: A1-LC, alkali 1 light chain; A2-LC, alkali 2 light chain; DTNB, 5,5′-dithiobis(2-nitrobenzoate); HMM-HC, heavy meromyosin heavy chain; $PhCH_2SO_2F$, phenylmethanesulfonyl fluoride; S1-HC, subfragment-1 heavy chain; EPR, electron paramagnetic resonance, EDTA ethylenediaminetetraacetic acid.

termed the DTNB light chain (Gazith et al., 1970; Weeds and Lowey, 1971). Myosin contains two DTNB light chains and binds 2 mol of Ca(II) with high affinity. There is growing evidence that the high affinity Ca(II) sites of myosin are associated with the DTNB light chains (Werber et al., 1972, 1973; Morimoto and Harrington, 1974; Margossian et al., 1975). Werber et al. (1972) speculated that these subunits are located near each subfragment 1–subfragment 2 joint because the DTNB light chain is degraded during the papain digestion of myosin to yield subfragment 1, but it is retained in heavy meromyosin prepared by tryptic digestion. However, Margossian et al. (1975) showed that subfragment 1 could be prepared with a nearly stoichiometric DTNB light chain content, provided divalent metal ions were present during the papain digestion. In the present study the relationships between divalent metal ion binding, the DTNB light chain, and the susceptibility of the subfragment 1–subfragment 2 joint to proteolysis are reexamined taking advantage of two recent experimental findings.

During an investigation of metal binding to subfragment 1, Bagshaw and Reed (1976) found a high affinity site (termed L site) for the paramagnetic Mn(II) ion which was different from the active site. The stoichiometry of the L site depended on the conditions employed for the papain digestion of myosin and was found to correlate with the DTNB light chain content. Beinfeld et al. (1975) demonstrated that Ca(II) was more effective than Mg(II) in displacing Mn(II) from the high affinity site. Hence Mn(II) appears to be an analogue of Ca(II) in binding to the DTNB light chain site (Bagshaw and Reed, 1976). EPR spectroscopy being a reasonably rapid and sensitive technique (~1 min per scan and ~1 μM free Mn$(H_2O)_6^{2+}$) offers a convenient method to study this site. In this particular case EPR spectroscopy presents a further advantage in that the X-band signal of Mn(II) bound to the myosin L site is not extensively broadened, as is frequently observed for macromolecular complexes of Mn(II) (Cohn and Townsend, 1954; Reed and Ray, 1971). Thus under appropriate conditions EPR spectroscopy can be used to monitor, continuously and specifically, the occupancy and structure of this site with Mn(II).

Recently Yagi and Otani (1974) have established conditions for the proteolysis of myosin, by chymotrypsin, which do not lead to extensive cleavage of the heavy chain as is observed with trypsin (Bálint et al., 1975) or papain (Stone and Perry, 1973). While these latter enzymes yield apparently homogeneous heavy meromyosin and subfragment 1 preparations, the polypeptide chain is nicked, so that under the dissociating conditions of sodium dodecyl sulfate gel electrophoresis, a multi-banded heavy chain pattern is observed which complicates the identification of the digestion products. An interesting property of chymotryptic attack was revealed when myosin filaments were digested. Subfragment 1 was produced when the digestion was carried out in the presence of EDTA, but heavy meromyosin was the product in the presence of divalent cations (Weeds and Taylor, 1975). Other studies have demonstrated an effect of divalent metal ions on the digestion of myosin by trypsin, but the site of metal binding was not identified (Biró et al., 1972; Bálint et al., 1975). A plausible mechanism for these findings is that saturation of the divalent metal site protects the DTNB light chain from digestion and, in turn, this subunit blocks proteolytic attack of the subfragment 1–subfragment 2 joint. This hypothesis is tested since it may clarify the mode of interaction between divalent metal ions and the myosin thick filament.

The light chain subunits of myosin extracted from other muscles differ from those of fast skeletal muscle. Typically a component of about 19 000 daltons is present, although it may not be susceptible to dissociation by DTNB treatment. While differences in the 19 000-dalton components are apparent from their precise electrophoretic mobilities on sodium dodecyl sulfate gels and their peptide maps (Lowey and Risby, 1971; Weeds and Pope, 1971), there are also analogies between them in their ability to be phosphorylated by a specific kinase (Frearson and Perry, 1975) and their ability to hybridize with desensitized invertebrate myosins and restore the Ca(II) sensitivity of the myosin-linked regulatory system (Kendrick-Jones et al., 1976). It is of interest to investigate the EPR spectrum of Mn(II) bound to myosin isolated from other sources. This may provide a rapid and sensitive probe for examining the divalent metal sites of light chains analogous to the DTNB light chain of fast skeletal muscle. A preliminary study with rabbit cardiac myosin is reported.

Materials and Methods

Proteins. Skeletal myosin was prepared from the back and leg muscles of the rabbit by extraction of a muscle mince with 0.3 M KCl–0.15 M potassium phosphate and 1 mM ATP and purified by repeated dilution–precipitation steps essentially as described by Szent-Györgyi (1947). Cardiac myosin was prepared similarly from rabbit hearts.

DTNB–EDTA treatment of myosin was carried out as described by Weeds and Lowey (1971). After the 10-min reaction time, the myosin solution was either diluted tenfold to lower the ionic strength and precipitate the myosin with a reduced DTNB light chain content (DTNB–EDTA myosin), or it was diluted tenfold with 0.5 M NaCl and treated with 2 mM dithiothreitol to allow the DTNB light chain to reassociate (reconstituted myosin). As a control, myosin was treated with DTNB in the presence of 10 mM Ca(II) and was isolated by a dilution–precipitation step before treatment with dithiothreitol (DTNB–Ca(II) myosin).

After these treatments, all the myosin samples were dialyzed against 0.5 M NaCl–1 mM dithiothreitol–50 mM Tris adjusted to pH 8.4 with HCl at 4 °C for 5 h to unblock the thiol groups and then dialyzed against 20 mM Tris-HCl at pH 8.4. The myosin precipitates were collected by centrifugation and then washed three times with 20 mM Tris-HCl to remove unbound light chains and the residual DTNB. Finally the myosin precipitates were dissolved in 0.5 M NaCl to a concentration of about 15 mg/ml and dialyzed against 0.5 M NaCl–50 mM Tris-HCl at pH 8.4. Myosin concentrations were determined by the method of Lowry et al. (1951) which had been previously standardized by micro-Kjeldahl analysis.

The supernatant from the DTNB–EDTA treatment, which contained the DTNB light chains, was treated with 4 mM dithiothreitol and was concentrated in a Minicon B15 macrosolute ultrafilter. It was washed free of 5-thio-2-nitrobenzoate in this apparatus with 1 mM dithiothreitol–50 mM Tris-HCl at pH 8.4.

Chymotryptic Digestion. Myosin filaments were formed by dialysis against 0.12 M NaCl–20 mM sodium phosphate at pH 7.0 and were digested with α-chymotrypsin (type 1-s from bovine pancreas, Sigma Chemical Co.) as described by Weeds and Taylor (1975). Digestion was terminated by addition of $PhCH_2SO_2F$; nevertheless, some residual proteolytic activity was noted which caused further degradation over a period of several days. The soluble products of digestion were recovered by dialysis of the digest against 5 mM sodium phosphate (pH 7.0) and removal of the insoluble protein by centrifugation.

Sodium Dodecyl Sulfate Gel Electrophoresis. Samples were prepared for sodium dodecyl sulfate gel electrophoresis by mixing a solution of the protein (1–4 mg/ml) with an equal volume of a solution containing 2% sodium dodecyl sulfate, 2% β-mercaptoethanol, 20% glycerol, 20 mM sodium phosphate at pH 7.0, and bromophenol blue tracking dye. The mixture was immediately heated to 100 °C for 2 min to circumvent residual proteolytic activity. Discontinuous zonal electrophoresis was carried out on 7.5 or 12.5% acrylamide gels in Tris-glycine buffer and in the presence of sodium dodecyl sulfate, essentially as described by Potter (1974). The gels were stained with 0.1% fast green and scanned in a Gilford 240 spectrophotometer with a linear transport attachment. The molecular weights of the myosin components were calculated from their mobilities using phosphorylase *a*, actin, and hemoglobin as standards (Weber and Osborn, 1969). The values reported here serve as a means of identifying components rather than providing an accurate measure of their molecular weights.

EPR Spectroscopy. EPR spectra were recorded at 9.0 GHz (X-band) using a Varian E-3 spectrometer. Samples (approximately 50 μl) were contained in high purity quartz capillary tubing. The six-line EPR spectrum characteristic of $Mn(H_2O)_6^{2+}$ in aqueous solution generally becomes extensively broadened on complex formation and allows binding to be monitored in a titration simply by following the disappearance of the free Mn(II) signal (Cohn and Townsend, 1954). However, Mn(II) bound to the high affinity site (L site) of myosin contributes significantly to the observed EPR signal (Yazawa and Morita, 1974; Bagshaw and Reed, 1976). At 20 °C the amplitude of the signal of Mn(II) bound to the L site is approximately 20% of that of an equivalent concentration of free $Mn(H_2O)_6^{2+}$ and this percentage is increased at 0 °C because only the free $Mn(H_2O)_6^{2+}$ signal is reduced by the temperature change. While the L-site spectrum is not extensively broadened, it differs from that of $Mn(H_2O)_6^{2+}$ in that the amplitudes of the inner peaks are reduced relative to the outer peaks (see, for example, Figure 8). The relative contribution from $Mn(H_2O)_6^{2+}$ and Mn(II) bound at the L site therefore may be evaluated using eq 1

$$P_{1f} = \frac{R_{obsd} - R_L}{R_f - R_L} \quad (1)$$

where P_{1f} is the fraction of the observed amplitude of the 1st (low-field) peak arising from the free $Mn(H_2O)_6^{2+}$, R_{obsd} is the observed ratio of the amplitudes of the 2nd to 1st peak, R_L is the ratio for the L-site alone, and R_f is the ratio for free $Mn(H_2O)_6^{2+}$. Typically $R_L = 0.6$ and $R_f = 1.1$, but these ratios were checked for each experiment since the precise values showed some dependence (~5% variation) on the spectrometer adjustments. R_L was obtained from the observed spectrum of a Mn(II)–myosin mixture which had been equilibrated with a known low concentration of free $Mn(H_2O)_6^{2+}$ by dialysis.

Titrations were carried out by adding a known concentration of $MnCl_2$ (30–120 μM) to solutions of myosin (15–20 mg/ml) and recording the EPR spectra. The amount of myosin added (~100 μl) was checked by weight to avoid the pipetting error incurred with such viscous solutions. The spectra were analyzed according to eq 1. Mn(II) binding could then be evaluated from the disappearance of the free $Mn(H_2O)_6^{2+}$ or the appearance of the L-site spectrum. The former was standardized against $MnCl_2$ of known concentration and provided a measure of the total Mn(II) bound, i.e., both to the high affinity L sites and the low affinity N sites (Bagshaw and Reed, 1976).

TABLE I: Relative DTNB Light Chain Content and L-Site Stoichiometry of Native and DTNB-Treated Myosins.

Myosin Sample[a]	Native[b]	DTNB–EDTA	DTNB–Ca(II)	Reconstituted
DTNB light chain	1.00[c]	0.59	1.00	0.92
L site	1.00[d]	0.61	0.95	0.89

[a]See Materials and Methods section for the terminology of myosin samples. [b]The data are normalized with respect to native myosin. [c]Corresponds to 2 mol of DTNB light chain per myosin molecule (Weeds and Lowey, 1971). [d]Corresponds to 1.8 mol of Mn(II) bound with high affinity (Figure 1).

Results

Mn(II) Binding to the DTNB Light Chain. In a previous study it was noted that the stoichiometry of Mn(II) binding to the high affinity site of subfragment 1 depended on the conditions used for its preparation. Inclusion of a divalent metal ion (Mg(II), Ca(II), or Mn(II)) during the papain digestion of myosin increased the fraction of subfragment 1 molecules bearing an intact DTNB light chain and high affinity Mn(II) binding site, compared with subfragment 1 preparations obtained by digestion in the presence of EDTA (Bagshaw and Reed, 1976). While no major difference was detected in the heavy chain content of these preparations by sodium dodecyl sulfate gel electrophoresis (see also Margossian et al., 1975), the possibility remained that the Mn(II) binding site was located on the heavy chain and its correlation with the DTNB light chain was coincidental. It is therefore important to demonstrate this relation with myosin preparations which have not undergone proteolysis and, if possible, with the isolated DTNB light chain.

Selective removal of the DTNB light chain from myosin was achieved by DTNB–EDTA treatment as described in the Materials and Methods section. The myosin samples were subject to sodium dodecyl sulfate gel electrophoresis and their light chain contents were determined by densitometry (Table I). Mn(II) binding to these samples, as determined by EPR spectroscopy, is illustrated in the Scatchard plots of Figure 1. Native myosin binds 1.8 mol of Mn(II) with high affinity ($K_L < 1$ μM). The equilibrium constant is an upper limit owing to the competition from contaminating Ca(II) and Mg(II). Atomic absorption measurements indicate the metal binding site is initially near saturated with Ca(II). DTNB–EDTA treatment reduces the stoichiometry of Mn(II) binding to 1.1 mol, but a stoichiometry of 1.6 mol is restored if the sample is treated with dithiothreitol prior to precipitation of the myosin (reconstituted myosin). Mn(II) binding measured by the appearance of the L-site spectrum (open symbols) parallels the Scatchard plots for Mn(II) binding to the high affinity sites (solid symbols). For a system with one class of sites, the superposition of the solid and open symbols is expected since this factor depends only on the accuracy of measuring the peak heights (<5% error) and the estimation of the total Mn(II) concentration. The deviation of the solid and open symbols at high levels of saturation ($\bar{\nu}_{Mn}$) suggests that Mn(II) bound to the weak N sites contributes less to the observed spectrum, although the error in these data is large since free $Mn(H_2O)_6^{2+}$ dominates the EPR signal. A systematic error in estimating the contributions of the free Mn(II) and of Mn(II) bound to the L site to the observed spectrum, that would arise from errors in the values of R_f and R_L used in eq 1, leads to a nonlinear Scatchard plot. In practice this error would not be revealed

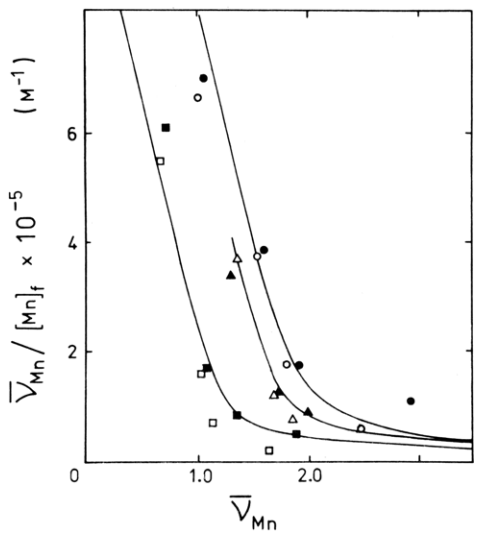

FIGURE 1: Scatchard plot of Mn(II) binding to myosin samples. Data are given for native myosin (●, ○), DTNB–EDTA myosin (■, □), and reconstituted myosin (▲, △) in 0.5 M NaCl, 50 mM Tris-HCl at pH 8.0 and 20 °C. Mn(II) binding was determined by EPR spectroscopy using eq 1 to separate the contributions from $Mn(H_2O)_6^{2+}$ and Mn(II) bound to myosin. Data obtained from the disappearance of the free $Mn(H_2O)_6^{2+}$ signal (●, ■, ▲) were standardized against solutions of known free Mn(II) concentration and $\bar{\nu}_{Mn}$ is expressed as mol of Mn(II) bound per 480 000 g of myosin. Data obtained from the appearance of the characteristic bound spectrum (○, □, △) were first plotted in arbitrary units (mm of peak height/g of myosin) and they were found to parallel the high affinity binding data shown by the solid symbols. Adjusting the scale of one set of data to superimpose the corresponding solid symbols gave a reasonable fit for the remaining sets. This normalization confirmed that the amplitude of the first peak for Mn(II) bound to the L site is 20% that for free $Mn(H_2O)_6^{2+}$ at 20 °C (Bagshaw and Reed, 1976), The solid lines are computed Scatchard plots for a system with two classes of noninteracting sites with dissociation constants $K_L = 1\ \mu M$ and $K_N = 1$ mM. A stoichiometry of 20 was used for the weak N sites. Stoichiometries for the high affinity L sites are from left to right 1.1, 1.6, and 1.8 mol of Mn(II) per mol of myosin.

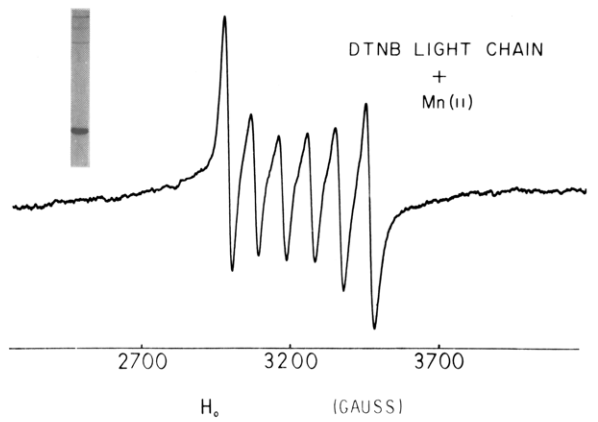

FIGURE 2: EPR spectrum of Mn(II) bound to the isolated DTNB light chain. The supernatant from the DTNB–EDTA treatment of myosin was concentrated in a Minicon B15 ultrafilter in the presence of 4 mM dithiothreitol and sufficient Mn(II) was added to just complex with the EDTA. The concentrated protein was washed with 1 mM dithiothreitol–50 mM Tris-HCl until colorless and then examined in the EPR spectrometer. Sufficient Mn(II) remained to be readily detected and, although its actual concentration was not determined, the observed spectrum shows the free Mn(II) makes little contribution, i.e., $[Mn(H_2O)_6^{2+}] < 3\ \mu M$. Mn(II) bound to EDTA makes no contribution under these conditions. The protein concentration was 6 mg/ml and comprised 93% DTNB light chain as judged by 12.5% sodium dodecyl sulfate gel electrophoresis (see insert).

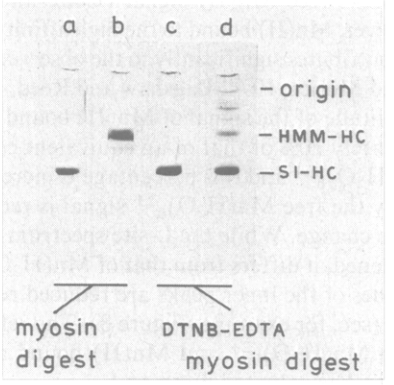

FIGURE 3: Sodium dodecyl sulfate gel electrophoresis of the products of digestion of myosin and DTNB–EDTA myosin. Native myosin (a, b) and DTNB–EDTA myosin (c, d) were digested with chymotrypsin in the presence of EDTA (a, c) or Ca(II) (b, d) as described in the text. The water-soluble products were subject to electrophoresis on 7.5% sodium dodecyl sulfate gels to resolve the heavy chain content. The light chains are eluted under these conditions but could be resolved on 12.5% gels (see text). The subfragment 1 (S1-HC) and heavy meromyosin (HMM-HC) heavy chains were identified on the basis of their mobility with respect to phosphorylase *a* and myosin heavy chain markers.

owing to the nonlinearity resulting from Mn(II) binding to the weak N sites. Analysis of the data obtained here, using high myosin concentrations ($\gg K_L$), shows that the calculated equilibrium constant (K_L) is very sensitive to such an error, but the estimated stoichiometry of the high affinity site is practically unaffected (±3%) when extreme values for R_f of 1.0–1.15 and R_L of 0.55–0.65 were substituted in eq 1. Thus the parameter of interest, the L-site stoichiometry, can be obtained with reasonable accuracy by this method.

Table I shows the correlation between the relative DTNB light chain content and the L-site stoichiometry obtained from the experiments described above and also includes data for DTNB–Ca(II) myosin. DTNB treatment is only effective in dissociating the DTNB light chain and reducing the L-site stoichiometry when carried out in the presence of EDTA. The recovery of Mn(II) binding when the DTNB light chain is allowed to reassociate indicates that a possible irreversible modification of the heavy chain during the DTNB treatment is not responsible for the loss of Mn(II) binding in the DTNB–EDTA sample.

Freshly isolated DTNB light chain binds Mn(II) to give the characteristic L-site spectrum (Figure 2) and unambiguously shows the location of this binding site. The isolated DTNB light chain readily loses its affinity for Mn(II) with age and as yet a complete titration with this preparation has not been attempted. The similarity of the EPR spectra for Mn(II) bound to the isolated light chain and the intact myosin (see Figure 8) shows that the L site does not require the heavy chain for its structural integrity, although the heavy chain appears to stabilize the DTNB light chain in vitro. Since myosin contains two DTNB light chains (Weeds and Lowey, 1971), the titrations above show that, in the absence of nucleotide, this site is the only high affinity Mn(II) binding site present in the intact myosin molecule.

Products of Chymotryptic Digestion. The native and DTNB-treated myosin samples examined in the previous section were digested with chymotrypsin in the presence of 1 mM EDTA or 5 mM Ca(II) at low ionic strength, as described by Weeds and Taylor (1975). After removal of the insoluble protein by centrifugation, the supernatant protein concentration was measured and a known quantity (~30 μg) was subjected to sodium dodecyl sulfate gel electrophoresis. Figure 3 shows the digestion products of native myosin and

TABLE II: Soluble Products of Chymotryptic Digestion in the Presence of EDTA or Ca(II).

Myosin Substrate[a] (67 μM)	Ca(II)[b]	Product Yields (μM) HMM-HC	S1-HC	$1 - ([S1]_{Ca}/[S1]_{EDTA})$[c]
Native	−	0.5	16	
	+	2.9	0.38	0.98
DTNB-EDTA	−	0.48	21	
	+	1.6	7.7	0.64
DTNB-Ca(II)	−	0.37	12	
	+	2.4	1.1	0.91
Reconstituted	−	0.35	14	
	+	2.4	3.1	0.78

[a]See Materials and Methods section for terminology. [b]−Ca(II) corresponds to digestion in the presence of 1 mM EDTA. [c]This expression provides an approximate measure of the fraction of subfragment 1-subfragment 2 links which are protected by Ca(II) against chymotryptic attack.

DTNB-EDTA myosin after electrophoresis on 7.5% gels. Digestion in the presence of EDTA yields predominantly (>98%) a heavy chain (80 000 daltons) characteristic of subfragment 1, regardless of the DTNB light content of the parent myosin. Digestion of native myosin in the presence of Ca(II) releases considerably less soluble protein, and this comprises mainly (88%) a heavy chain (140 000 daltons) corresponding to heavy meromyosin. However, digestion of myosin samples with a reduced DTNB light chain content, in the presence of Ca(II), yields a substantial amount of subfragment 1. Table II summarizes these results which are based on the amount of protein released into the supernatant during digestion and the ratio of subfragment 1 to heavy meromyosin heavy chains produced, as estimated by gel densitometry. Under the dissociating conditions of sodium dodecyl sulfate gel electrophoresis, double and single-headed heavy meromyosin (Margossian and Lowey, 1973) would not be distinguished since both would give rise to HMM-HC. Patterns of chymotryptic digestion similar to those illustrated in Figure 3 were obtained when Mn(II) was substituted for Ca(II).

The data are in agreement with the hypothesis that divalent metal ions bound to the DTNB light chain cause protection of the subfragment 1-subfragment 2 link and leave heavy meromyosin as the major digestion product. Further studies are required to evaluate the kinetics of digestion but the product ratios of Table II indicate that the unprotected subfragment 1-subfragment 2 joint (i.e., in the absence of DTNB light chain and/or in the presence of EDTA) is cleaved five to ten times faster than the heavy meromyosin-light meromyosin joint of the myosin filament. When protected by the metal-DTNB light chain complex, the subfragment 1-subfragment 2 joint is cleaved five to ten times slower than the heavy meromyosin joint.

Myosin samples with a depleted DTNB light chain content, on digestion in the presence of Ca(II), rapidly release subfragment 1 from the unprotected links but slowly yield subfragment 1 from the protected links. An estimate (or at least a lower limit) for the fraction of myosin heavy chains in which the subfragment 1-subfragment 2 link is protected is given by the expression $1 - ([S1]_{Ca}/[S1]_{EDTA})$, where $[S1]_{Ca}$ is the subfragment 1 yield obtained by digestion in the presence of Ca(II), and $[S1]_{EDTA}$ is yield obtained in the presence of EDTA, at an appropriate time point. The value obtained for this expression after 10 min digestion (Table II) shows a reasonable agreement with expected degree of protection based on the DTNB light chain contents listed in Table I.

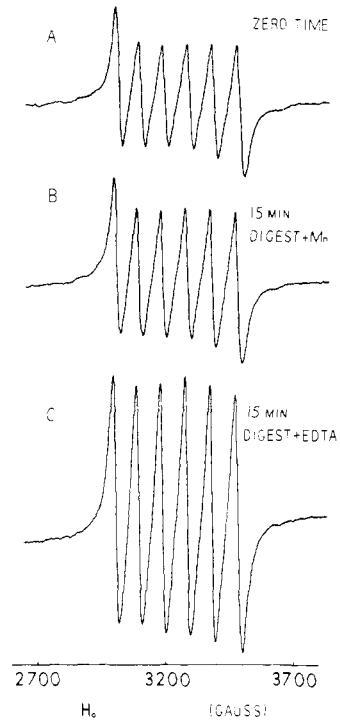

FIGURE 4: EPR spectra to determine the time course of L-site digestion. (A) The spectrum for a solution containing 125 μM $MnCl_2$, 23 mg/ml myosin in 0.12 M NaCl, 10 mM sodium phosphate at pH 7.0 and 20 °C. (B) Chymotrypsin (0.05 mg/ml) was added to the above solution and the spectrum was recorded after 15-min digestion time. (C) Chymotrypsin (0.05 mg/ml) was added to a solution containing 125 μM EDTA, 23 mg/ml myosin in 0.12 M NaCl, 10 mM sodium phosphate (pH 7.0). After 15-min digestion 0.4 mM $PhCH_2SO_2F$ was added, followed by 2 × 125 μM $MnCl_2$, and the spectrum was recorded. The first aliquot of $MnCl_2$ complexed with the EDTA and gave no observable spectrum. The spectrometer settings were the same throughout. A reduction in Mn(II) bound to the L site is apparent from the decrease in the difference between the 1st and 2nd peaks and a release of free $Mn(H_2O)_6^{2+}$ is indicated by an overall increase in the peak amplitudes.

However, when the samples of the digestion products were examined on 12.5% gels (cf. Figure 3), to resolve the light chains, a paradox arose. The heavy meromyosin contained no trace of the 19 000-dalton DTNB light chain, although the 17 000-dalton alkali 2 light chain appeared more dense than the 21 000-dalton alkali 1 light chain band. The protection hypothesis may stand if one of the following explanations applies. Either the 19 000-dalton DTNB light chain of heavy meromyosin is degraded by the residual proteolytic activity, after stopping the myosin digestion with $PhCH_2SO_2F$, or the 19 000-dalton DTNB light chain is cleaved during the 10-min digestion, but this degradation is not sufficient to destroy the divalent metal binding site and the fragmented DTNB light chain continues to protect the subfragment 1-subfragment 2 link. In order to test these explanations, a method for continuously monitoring the presence of the divalent metal ion site is required. The EPR spectrum of Mn(II) bound to the L site provides a unique approach.

The Time Course of DTNB Light Chain and L-Site Digestion. $MnCl_2$ (125 μM) was added to a suspension of 23 mg/ml myosin filaments (i.e., 96 μM in L sites) and the EPR spectrum was recorded. Figure 4A shows there is a considerable contribution from Mn(II) bound to the L site. At time zero 0.05 mg/ml chymotrypsin was added and digestion was al-

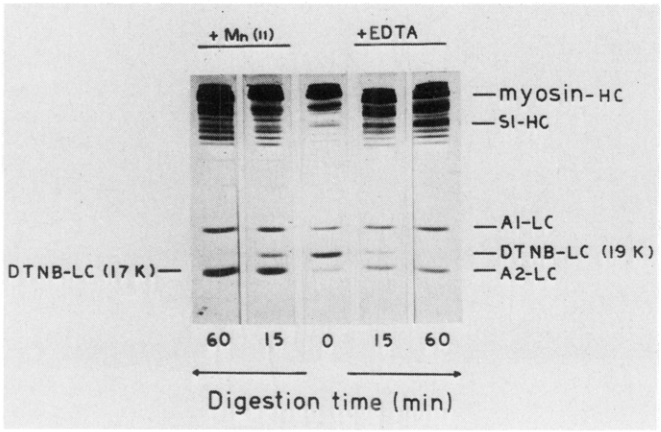

FIGURE 5: 12.5% sodium dodecyl sulfate gel electrophoretograms showing the time course of chymotryptic digestion of myosin. This experiment was run parallel with the L-site determination and details of the solutions are given in the legend of Figures 4B and 4C for digestion in the presence of Mn(II) and EDTA, respectively. Aliquots were removed from the digestion mixture and immediately treated with $PhCH_2SO_2F$ and prepared for sodium dodecyl sulfate gel electrophoresis at the times indicated. The additional bands in the heavy chain region appear to arise from the proteolysis of insoluble components (e.g., myosin rods) since they are not present in the supernatant after centrifugation (cf. Figure 3).

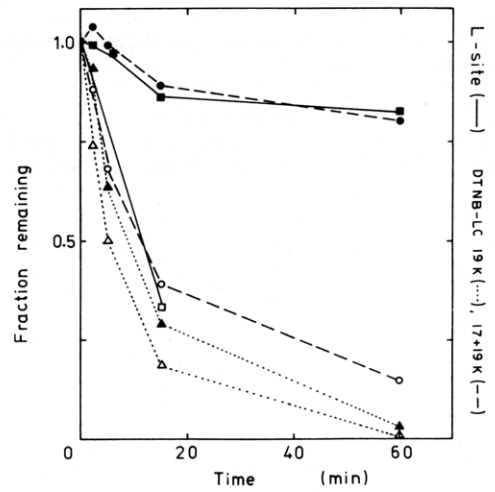

FIGURE 6: Time course of DTNB light chain and L-site degradation. The digestion of the DTNB light chain in the presence of Mn(II) (▲, ●) or EDTA (△, ○) was determined by densitometry of gels such as those illustrated in Figure 5. The intact DTNB light chain content (▲, △) was determined from the area of the 19 000 peak, after normalizing against the alkali 1 light chain content to compensate for errors in loading. The sum of the 19 000-dalton DTNB light chain content and its 17 000-dalton fragment (●, ○) were evaluated from peak areas after allowing for a constant contribution to the 17 000-dalton band from the alkali 2 light chain. The relative L-site contents of the Mn(II) (■) and EDTA (□) digests were determined from EPR spectra such as those shown in Figure 4, using eq 1 to separate the contributions from free $Mn(H_2O)_6^{2+}$ and Mn(II) bound to the L site.

lowed to proceed. The EPR spectrum was scanned at intervals and concomitantly aliquots were taken from the incubation mixture and treated with $PhCH_2SO_2F$. These samples were prepared for analysis by sodium dodecyl sulfate gel electrophoresis as described in the Materials and Methods section. The time course of the digestion of the myosin light and heavy chains was evaluated from sodium dodecyl sulfate gels such as those illustrated in Figure 5. In the presence of near stoichiometric [Mn(II)], the 19 000-dalton DTNB light chain is rapidly degraded and is barely detectable after 60 min of incubation (Figures 5 and 6). However, the decrease in the 19 000-dalton band is accompanied by an increase in the 17 000-dalton band. The intensity of the 21 000-dalton band associated with the alkali 1 light chain (A1-LC) remained constant and, indeed, was used to calibrate the exact amount of protein loaded on each gel. The time course of the loss of L site was evaluated from the observed EPR spectrum using eq 1. Figure 4B shows it is remarkably resistant to chymotryptic attack and more than 80% remained after 60 min of digestion (Figure 6). While the time course of the L-site digestion clearly does not follow that of the 19 000-dalton light chain alone, a good correlation is observed if the L site is compared with the sum of the 19 000-dalton material and the additional material produced at 17 000 daltons (Figure 6). It is concluded that, in the presence of divalent metal ions, chymotrypsin cleaves the DTNB light chain to give a derivative which, on sodium dodecyl sulfate gel electrophoresis, superimposes the 17 000-dalton alkali 2 light chain (A2-LC). This limited digestion does not affect the L site and the 17 000-dalton fragment is protected from further degradation by divalent metal ions.

When digestion is carried out in the presence of 125 μM EDTA, the 19 000-dalton DTNB light chain is degraded to the 17 000-dalton derivative but this is further digested to give smaller peptides (Figures 5 and 6). The L site cannot be continuously monitored under such conditions, but at a known time interval a sample may be removed, the reaction stopped with $PhCH_2SO_2F$, and a two times stoichiometric amount of $MnCl_2$ (250 μM) added. The first equivalent of Mn(II) binds to the EDTA and gives no observable EPR spectrum. Figure 4C shows after 15-min digestion the resultant spectrum is dominated by free $Mn(H_2O)_6^{2+}$, compared with the spectrum 4B obtained after a 15-min digestion in the presence of Mn(II). Figure 6 shows that the residual L site (34% the initial value), after 15-min digestion in the presence of EDTA, corresponds to the sum of the residual 19 000- and 17 000-dalton components.

A control was carried out in which $PhCH_2SO_2F$ was added to the myosin filaments prior to the chymotrypsin. After 60 min no loss of L site was detected, but 10% of the 19 000-dalton light chain was converted to its 17 000-dalton fragment. EPR measurements were also carried out to check the equivalence of the stock $MnCl_2$ and EDTA solutions and to demonstrate that EDTA could compete effectively with the L site for Mn(II) at these concentrations.

The production of subfragment 1 was monitored in the above experient by the appearance of a band at 80 000 daltons. Using isolated subfragment 1 of known concentration as a standard, the fraction of myosin heavy chains converted to subfragment 1 was estimated by densitometry. The time course of subfragment 1 formation and DTNB light chain digestion (19 000 + 17 000 dalton components) are compared in Figure 7. Increasing the EDTA concentration to 1 mM results in a more rapid loss of DTNB light chain and a more rapid formation of subfragment 1, which suggests that in the presence of 125 μM EDTA contaminating metal ions (presumably Mg(II) and Ca(II)) can still offer some protection. More importantly Figure 7 shows that subfragment 1 formation does not exceed DTNB light chain destruction under any one condition. Since μM concentrations of Mn(II) were sufficient to protect the subfragment 1–subfragment 2 link, these data confirm the postulate that the effect of Mn(II) is mediated through its binding to the high affinity L-site rather than the weak N-sites.

Mn(II) Binding to Cardiac Myosin. Cardiac myosin contains two kinds of light chains with sodium dodecyl sulfate gel

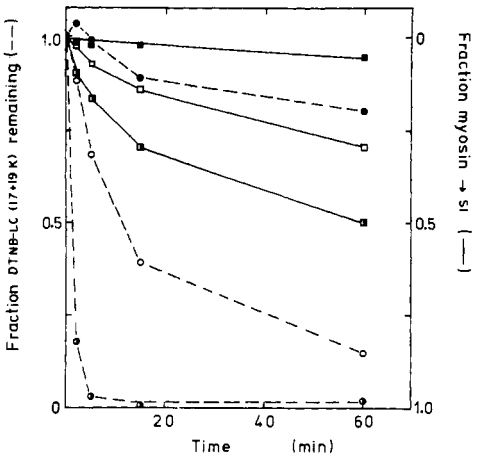

FIGURE 7: Time course of the DTNB light chain digestion and subfragment 1 formation. The digestions of the 19 000-dalton DTNB light chain plus its 17 000-dalton fragment (●, ○, ◑) were monitored by densitometry of gels such as those in Figure 5. The fraction of myosin heavy chains cleaved to give subfragment 1 (■, □, □) was determined from the appearance of a band at 80 000 daltons. Digestion was initiated by the addition of 0.05 mg/ml chymotrypsin to 23 mg/ml myosin in 0.12 M NaCl, 10 mM sodium phosphate at pH 7.0 and 20 °C in the presence of 125 μM $MnCl_2$ (●, ■), 125 μM EDTA (○, □), or 1 mM EDTA (◑, □).

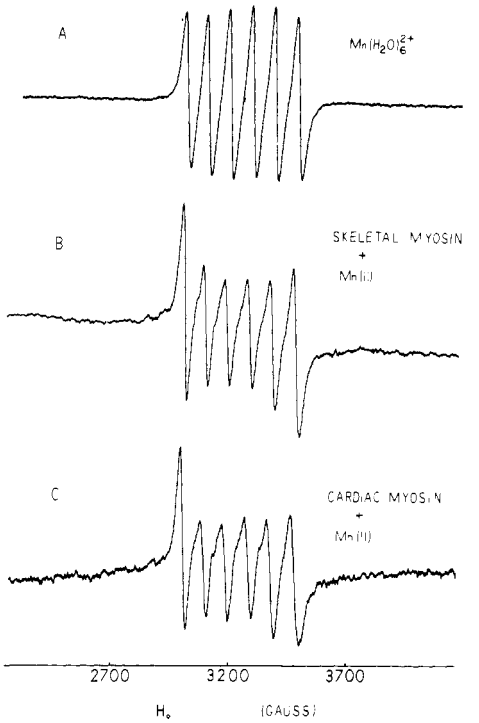

FIGURE 8: EPR spectra of $Mn(H_2O)_6^{2+}$, Mn(II) bound to skeletal myosin and Mn(II) bound to cardiac myosin. Compositions of the solutions were (A) 65 μM $MnCl_2$, 0.5 M NaCl, 50 mM Tris-HCl at pH 8.4 and 0 °C; (B) 91 μM $MnCl_2$, 44 mg/ml skeletal myosin (188 μM in L sites), 0.5 M KCl, 50 mM Tris-HCl at pH 8.4 and 2 °C; (C) 69 μM $MnCl_2$, 24 mg/ml cardiac myosin (presumably 100 μM in L-type sites), 0.5 M NaCl, 50 mM Tris-HCl at pH 8.4 and 0 °C. The spectrometer setting were the same (20 G modulation), except the receiver gain which was (A) 2.5×10^5, (B) 6.2×10^5, and (C) 8×10^5. Thus on binding Mn(II) to skeletal or cardiac myosin the amplitude of the first peak of the EPR signal is quenched to 35–40% the value for $Mn(H_2O)_6^{2+}$ at 0 °C.

electrophoretic mobilities corresponding to molecular weights of about 27 000 and 19 000. The 19 000 component is specifically phosphorylated by myosin light chain kinase, like the 19 000 DTNB light chain of skeletal myosin (Frearson and Perry, 1975), but it is not dissociated by DTNB–EDTA treatment and has a different amino acid composition (Weeds and Pope, 1971). Figure 8 compares the EPR spectra observed on adding Mn(II) to an excess of skeletal and cardiac myosins. Clearly cardiac myosin contains a high affinity site for Mn(II) which has an analogous structure to the skeletal myosin L site. The ratio of the 6th to 1st peak heights of the cardiac myosin L-site is noticably lower than that of skeletal myosin. However, further studies are required to resolve whether this is due to an intrinsic difference in the metal binding site of cardiac myosin. A modifying effect caused by the state of phosphorylation of the light chain remains a possibility.

Discussion

Divalent metal ion binding to myosin is complex, but at least two classes of sites have now been specifically identified. Under physiological conditions, the metal ion involved in the ATPase reaction, namely Mg(II), associates as its ATP complex and the active site shows no detectable affinity for divalent metal ions in the absence of nucleotide (Bagshaw and Trentham, 1974; Bagshaw and Reed, 1976). Divalent cations do, however, bind to skeletal myosin in the absence of nucleotide and have led to the identification of a Ca(II) binding site associated with the DTNB light chain (Werber et al., 1972; Morimoto and Harrington, 1974). The properties of the Mn(II) binding site reported in this study are similar to the properties of the Ca(II) binding site discussed by Morimoto and Harrington (1974) and suggest that these ions bind to a common L site. In the absence of nucleotide, it appears that a maximum of 2 mol of divalent metal ions (i.e., the sum of Mn(II), Ca(II), and Mg(II)) per mol of myosin are bound with high affinity, as estimated by atomic absorption spectroscopy, which therefore may be totally accounted for by the DTNB light chain site (J. M. Sykes and C. R. Bagshaw, unpublished observations). Competition experiments indicate that the relative affinities for the L site are Mn(II) > Ca(II) > Mg(II) (Beinfeld et al., 1975). Determinations of equilibrium constants in the presence of metal ion buffers are more reliable and yield values of 0.01 to 0.1 μM for Ca(II) binding, the affinity of Mg(II) being about 100-fold weaker (Sugden and Nihei, 1969; Bremel and Weber, 1975; Bálint et al., 1975). The true value of K_L for Mn(II) is therefore probably <0.1 μM. The competition between Ca(II) and Mg(II) binding raises an important question concerning the physiological role of the DTNB light chain. From equilibrium studies Bremel and Weber (1975) concluded this site might become partially occupied by Ca(II) on activation of the muscle. Even so, since Mg(II) itself is bound tightly (Kiely and Martonosi, 1969), its rate of displacement by Ca(II) may preclude the DTNB light chain as a candidate for controlling early events of contraction.

The finding that the EPR spectrum for Mn(II) bound to the isolated light chain is the same as that bound to myosin confirms the Morimoto and Harrington (1974) suggestion that the DTNB light chain can exist in the same conformation when free in solution. However, the isolated light chain shows a marked tendency to lose its metal binding capacity and also fails to recombine completely with DTNB–EDTA myosin on aging. In some instances it was noted that recombination of the DTNB light chain did not always result in full reconstitution of the L site, suggesting that this subunit can recombine in a nonnative state. These preparations also showed incomplete protection of the subfragment 1–subfragment 2 link on digestion in the presence of divalent metal ions. The latter technique provides an extremely sensitive assay for reconsti-

tution (as opposed to mere recombination) since subfragment 1 is released from the unprotected links against the almost zero background level from native myosin (Table II).

The line shape of the EPR spectrum of Mn(II) bound to the L site reflects its structural characteristics, but interpretation is difficult and requires measurements at different frequencies and temperatures. Generally the spectra of slowly tumbling Mn(II)-protein complexes are of low intensity due to their solid-state characteristics arising from the incomplete averaging of the anisotropic magnetic environment (Reed and Ray, 1971). The narrow line width of the EPR spectrum observed for Mn(II) bound to the L site, together with its low solvent proton relaxation rate enhancement (Bagshaw and Reed, 1976), suggests that the L site comprises a rather symmetrical environment in which the amino acid residues shield the metal ion from the solvent water. Amino acid residues of the myosin heavy chain do not appear to be coordinated to the metal ion of the DTNB light chain site. If this were the case, solvent water molecules might then be expected to gain free access to the Mn(II) ion bound to the isolated DTNB light chain and give rise to a broadened EPR spectrum (Reed et al., 1971).

Even without a detailed understanding, the characteristic EPR spectrum of the L site is a useful structural probe for the divalent metal ion site. It presents a routine technique to classify the metal binding sites of myosin molecules isolated from different muscles. In this respect the preliminary results with cardiac myosin are encouraging since Frearson and Perry (1975) consider that, despite their chemical differences, the 19 000 components of skeletal and cardiac myosin are analogous on the basis of their ability to be phosphorylated. Further the 19 000 components of various myosins appear analogous in their ability to restore Ca(II) sensitivity of the ATPase on hybridization with desensitized scallop myosin. However, the incorporated DTNB light chain of the hybrid appears to play an indirect role since Ca(II) binding is not totally recovered. Presumably the Ca(II) switch operates through the remaining regulatory light chain (Kendrick-Jones et al., 1976). Neither Mg(II) nor Mn(II) effectively competes with Ca(II) for the high affinity Ca(II) site of native scallop myosin (Szent-Györgyi et al., 1973; C. R. Bagshaw and A. G. Szent-Györgyi, unpublished observations). The physiological significance of this specificity is clear since, in muscle, a Ca(II) switch must operate in the presence of near mM concentrations of Mg(II). However, as a consequence, the use of Mn(II) as a spectroscopic probe may prove to be limited to less specific metal binding sites, such as that of the DTNB light chain for which the competition offered by Mg(II) leaves its competency as a Ca(II) switch in question (Bremel and Weber, 1975).

Despite this limitation EPR spectroscopy promises to be a useful tool to test the predicted homologies in metal binding proteins (Kretsinger, 1976). From the knowledge of the primary sequence of the DTNB light chain and its homology with parvalbumin and troponin C, Collins (1976) predicted that the metal binding site involves residues 37-48. The finding that Mn(II) binding is unaffected by digestion to a 17 000 dalton-fragment is consistent with, but not a stringent test of, Collins' prediction. It is possible that the resultant 2000-dalton fragment remains bound to the myosin by noncovalent forces. Mn(II) does not appear to bind to the alkali light chains with any significant affinity, as deduced from studies with subfragment 1 prepared by papain digestion (Bagshaw and Reed, 1976) and the close agreement between the stoichiometry of Mn(II) binding and the content of the DTNB light chain alone, reported here. It has been suggested that the lack of metal binding sites in the alkali light chains has arisen through mutation of the ancestral protein in the Ca(II) binding loop (Weeds and McLachlan, 1974).

EPR spectroscopy also finds use in experiments where it is necessary to monitor the metal binding site continuously, exemplified here by the study of the time course of chymotryptic digestion. These results clearly demonstrate a relationship between the metal binding site, the DTNB light chain, and the suceptibility of the subfragment 1-subfragment 2 link to digestion and can explain the observations of Weeds and Taylor (1975). These authors also reported that digestion at high ionic strength yields heavy meromyosin regardless of the divalent metal ion content. This result has been confirmed and it was also noted that the overall yield of soluble protein was higher. It appears that monovalent metal ions increase the heavy meromyosin to subfragment 1 ratio simply by solubilizing the myosin filament and allowing a more rapid attack of the heavy meromyosin joint compared with the subfragment 1 joint. The partial degradation of the DTNB light chain may depend on the particular sample of chymotrypsin used since the inability of $PhCH_2SO_2F$ to inhibit the proteolytic activity completely suggests the presence of contaminating proteases. The DTNB light chain seems to have suffered less degradation in the heavy meromyosin reported by Weeds and Taylor (1975, Figure 1). The experiments reported here were carried out with one batch of chymotrypsin, but similar results have been obtained using another sample from the same supplier.

Two mechanisms of protection of the subfragment 1-subfragment 2 joint can be envisaged. Either the DTNB light chain is located near the subfragment 1-subfragment 2 joint and sterically blocks chymotryptic attack, or the DTNB light chain is located on the subfragment 1 moiety but affects the conformation of the link. Since papain can yield subfragment 1 with an intact DTNB light chain, its site of attack is presumably different from that of chymotrypsin. However, the resultant subfragment 1 preparations are difficult to compare on sodium dodecyl sulfate gels since the heavy chain is nicked elsewhere by papain. No difference was detected by ultracentrifugation (Weeds and Taylor, 1975).

While divalent metal ions have an effect on the subfragment 1-subfragment 2 link, mediated by the DTNB light chain, it remains to be established if this can account for cross-bridge movement observed in muscle (Huxley, 1972; Haselgrove, 1975). Morimoto and Harrington (1974) have reported an effect of the addition of Ca(II) on the sedimentation coefficient of myosin filaments, but it has not been determined if the cross-bridges can respond specifically to Ca(II) and do so under physiological metal ion concentrations. EPR spectroscopy can be extended to examine these more organized systems. Indeed on diffusing Mn(II) into glycerinated psoas muscle the characteristic EPR spectrum of the DTNB light chain was obtained with a signal-to-noise ratio equal to that recorded with isolated myosin (C. R. Bagshaw and G. H. Reed, unpublished observations). A contribution from Mn(II) bound to troponin was not apparent, although this is less likely to be detected, since solution studies indicated its EPR spectrum is somewhat broader (less intense) than that of the DTNB light chain (J. Loscalzo, personal communication). However, the scope of this approach in following events during contraction might be limited because, on addition of ATP, Mn(II) will also act as a Mg(II) analogue and may perturb the ATPase reaction (Bagshaw, 1975).

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