THE SIGNIFICANCE OF THE SLOW DISSOCIATION OF DIVALENT METAL IONS FROM MYOSIN 'REGULATORY' LIGHT CHAINS

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Received 13 July 1977

1. Introduction

It is well established that Ca^{2+} binding to troponin C, located on the actin filament, is involved in the activation of contraction of vertebrate skeletal muscle by relieving the inhibitory effect of the troponin–tropomyosin system [1]. More recently the discovery of a Ca^{2+} binding site on the DTNB light chain of the myosin filament has led to the proposal of this being an additional site engaged in the control of contraction [2,3]. These ideas received impetus from the finding that the vertebrate DTNB light chain can substitute for the regulatory light chain of scallop myosin in conferring Ca^{2+} sensitivity to the ATPase of scallop myofibrils, although the DTNB light chain does not appear to participate directly [4]. Vertebrate skeletal myosin itself does not exhibit a Ca^{2+} sensitive, actin-activated ATPase in vitro in the absence of troponin and tropomyosin [5,6]. Nevertheless it remains plausible that Ca^{2+} binding to the DTNB light chain initiates the movement of the myosin crossbridges towards the actin filaments [2,3,7] and such an effect might not be revealed by the actomyosin ATPase in the steady-state, particularly in preparations which lack the structural integrity of the myofibril. The finding that the DTNB light chain is located near and/or has an effect on the subfragment 1– subfragment 2 hinge region appears consistent with the function of controlling crossbridge mobility [8,9]. However, one obstacle in the proposal of any kind of Ca^{2+} sensitive switch mechanism is the rather low specificity of the divalent metal ion site of the DTNB light chain ($K_{Ca} \sim 10^{-8}$ M and $K_{Mg} \sim 10^{-6}$ M). At physiological concentrations of Mg^{2+} ($\sim$ 1 mM) the effective binding constant for Ca^{2+} is therefore only about 10^{-5} M [3,5] which is the same order of magnitude as the free $[Ca^{2+}]$ during contraction. These equilibrium measurements indicate Ca^{2+} could partially displace Mg^{2+} from the DTNB light chain, so leaving the competency of this site as a switch in debate. A further criterion which must be met if the DTNB light chain is to be implicated in activation, is that this displacement should occur fast enough to account for the observed rate of tension generation. This aspect is examined here and in view of the slow displacement reaction, alternative functions of the divalent metal ion site are discussed.

Abbreviations: DTNB light chain, 19 000 dalton subunit dissociated by treatment with 5,5′-dithiobis-(2-nitrobenzoate); EGTA, ethyleneglycolbis-(β-aminoethylether)-*N,N*′-tetraacetic acid; EPR, electron paramagnetic resonance

2. Materials and methods

Myosin and subfragment 1 containing the DTNB light chain were prepared from rabbit skeletal muscle

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as described previously [10]. Glycerinated rabbit psoas muscle fibre bundles were prepared as described by Marston [11]. The instrumentation used for X-band EPR spectroscopy [10] and fluorescence stopped-flow spectrophotometry [12] has been reported. Metal ions bound to myosin were determined, after quenching with 4% perchloric acid, using a Perkin-Elmer atomic absorption spectrophotometer (model 403).

3. Results

The two DTNB light chains totally account for the high affinity binding (i.e., $K<10^{-5}$ M) of divalent metal ions to vertebrate skeletal myosin prepared by standard procedures. This is indicated by the competition between Ca^{2+}, Mg^{2+} and Mn^{2+} binding (table 1). The latter, although not physiologically relevant, allows the application of EPR spectroscopy which has demonstrated that the Mn^{2+} binding sites are located solely on the DTNB light chains [8]. The apparent binding constant for Mn^{2+} of 10^{-6} M [8,10] was estimated in the presence of about 5 μM contaminating Ca^{2+} and therefore K_{Mn} is calculated to be about 2×10^{-9} M, knowing the value of K_{Ca}. The myosin ATPase sites only bind divalent metal ions in the presence of nucleotide and do not interfere with these measurements [10].

The data of table 1 allow conditions to be established in which the DTNB light chain site is initially occupied by Ca^{2+} or Mg^{2+}. The dissociation rates of these metal ions were measured by stopped-flow spectrophotometry [12] utilizing the protein fluorescence change noted by Werber et al. [2]. Subfragment 1 containing the DTNB light chain [10] was used since the isolated light chain is liable to denaturation [8]. The rate constants for dissociation of Ca^{2+} and Mg^{2+} from the DTNB light chain of subfragment 1 are 0.46 s^{-1} and 0.057 s^{-1}, respectively (fig.1). For the simplest mechanism this slow rate of Mg^{2+} dissociation will limit its displacement by Ca^{2+}, and this is indicated to be so by the effect of Ca^{2+} addition on the Mg^{2+}-subfragment 1 complex at a known interval before mixing with an excess of EDTA. The fluorescence enhancement induced by EDTA addition is now biphasic, and the amplitude of the slow phase corresponds to the expected concentration of the Mg^{2+}-subfragment 1 complex at the time of EDTA addition.

The binding of Ca^{2+} or Mg^{2+} to metal-free subfragment 1 causes a quenching of fluorescence with a rate

Table 1
Equilibrium binding of divalent metal ions to myosin

No.	Free concentrations (μM)			Moles bound/mole myosin			
	Mg^{2+}	Ca^{2+}	Mn^{2+}	Mg^{2+}	Ca^{2+}	Mn^{2+}	Total
1	0.01	0.0001	0	0.1	0.26[a]	0	0.36
2	10	0.001	0	2.0	0.88[b]	0	2.88
3	11	9	0	0.42	2.24	0	2.66
4	904	15	0	[c]	1.02	0	[c]
5	19	10	11	0.2	0.54	1.94	2.68
6	100	6	1	1.08	1.22	0.76	3.06

[a,b]Possibly upper limits owing to incomplete equilibration of CaEDTA and CaEGTA, respectively
[c]Not determined

Rabbit skeletal myosin (10 mg/ml) was dialysed against divalent metal ions at the concentrations shown, in 0.5 M NaCl, 50 mM Tris/HCl, at pH 8.4 and 4°C, and the metal ion content was analysed by atomic absorption spectroscopy. The total metal bound exceeds 2 mol/mol myosin owing to binding to weak sites [3,5,8]. In numbers 2, 3 and 5 the high affinity sites are predominantly occupied by Mg^{2+}, Ca^{2+} and Mn^{2+}, respectively, and demonstrate that the relative affinities are $Mn^{2+} > Ca^{2+} > Mg^{2+}$. Number 4 corresponds approximately to the concentrations in contracting muscle and indicates partial occupancy by Ca^{2+} (cf. ref. [5])

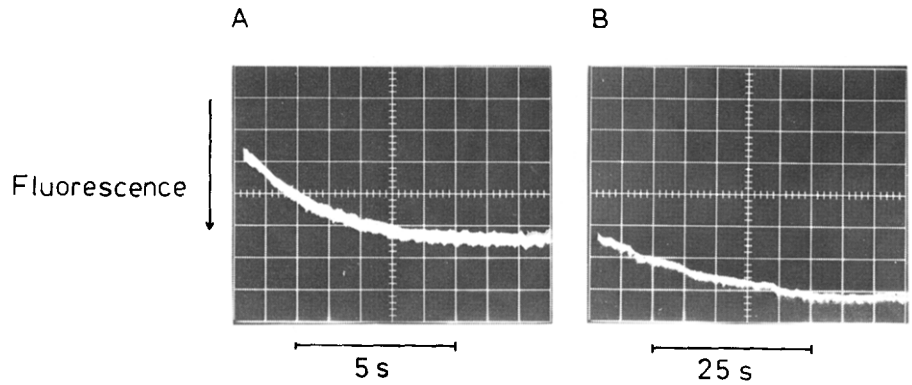

Fig.1. The rates of dissociation of Ca^{2+} and Mg^{2+} from subfragment 1. The protein fluorescence enhancement (5%) was monitored in a stopped-flow apparatus (excitation 290 nm, emission 350 nm) [12]. The subfragment 1 contained 0.6 mol DTNB light chain [10]. One syringe contained 1 mM EDTA and the other 8 μM subfragment 1 plus in (A) 10 μM Ca^{2+}, and in (B) 10 μM Mg^{2+} and 1 mM EGTA (reaction chamber concentrations). The buffer was 0.1 M KCl, 50 mM triethanolamine/HCl, at pH 7.1 and 22°C. Logarithmic plots of the traces yield values for the dissociation rate constant for Ca^{2+} of 0.46 s^{-1} (A) and for Mg^{2+} of 0.057 s^{-1} (B).

constant of 50 s^{-1}, which is independent of the metal ion concentration in the range 0.5–5 mM. Binding is therefore at least a two-step process in which the fluorescence change reflects a protein isomerization step. The apparent second order rate constants for the formation of the initial binary complex between the protein and metal were not determined in these studies but the value for Mg^{2+} is likely to be about three orders of magnitude lower than that for Ca^{2+}, owing to the rate-limiting displacement of the water molecules from the coordination sphere of the metal ion [13]. This factor, in part, would bring the overall ratio of the association and dissociation rate constants in line with the observed affinities.

The effective rate of Ca^{2+} binding to the DTNB light chain, controlled by Mg^{2+} dissociation, is several orders of magnitude too slow to participate in the activation of muscle contraction, which is complete within 100 ms [14]. In an attempt to justify the assumption that these rates are applicable in vivo, the properties of the metal ion site of the DTNB light chain of a glycerinated psoas muscle were examined by EPR spectroscopy. The power of this technique lies in the unusually intense spectrum (i.e., narrow line widths) of Mn^{2+} bound to the DTNB light chain compared with other components such as actin and troponin [8,15]. The spectra of Mn^{2+} bound to subfragment 1, myosin and glycerinated psoas muscle are virtually identical (fig.2) suggesting that no changes in the coordinating ligands of the DTNB light chain have occurred during the isolation of the proteins used for the rate and equillibrium studies. Addition of MgATP, containing sufficient MnATP to minimize changes in free $[Mn^{2+}]$, caused contraction of the psoas muscle, but the characteristic Mn^{2+}–DTNB light chain EPR spectrum remained. Hence the DTNB light chain site also exhibits a high affinity and low specificity for divalent metal ions within a muscle at least after glycerination, and therefore the constituent rate constants are likely to be similar to those measured in solution.

The slow tumbling of Mn^{2+}–protein complexes results in powder-type spectra and in addition the individual lines are often broadened owing to rapid electron spin relaxation [16]. The narrow line widths observed in the Mn^{2+}-DTNB light chain spectrum (presumably arising from the least orientation dependent, fine structure transition; $-\frac{1}{2} \rightarrow +\frac{1}{2}$) indicate a relatively slow electron spin relaxation rate which may be explained, in part, by the shielding of the Mn^{2+} from bombardment by solvent water molecules. Complementary studies on the nuclear relaxation rate of the solvent water protons suggest that the low enhancement induced by the bound Mn^{2+} occurs because there are no rapidly exchangeable water molecules in the first coordination sphere [10]. Possibly all the coordinating ligands are donated by amino acid residues of the DTNB light chain, which would be in harmony with the stopped-flow studies in requiring a change in protein

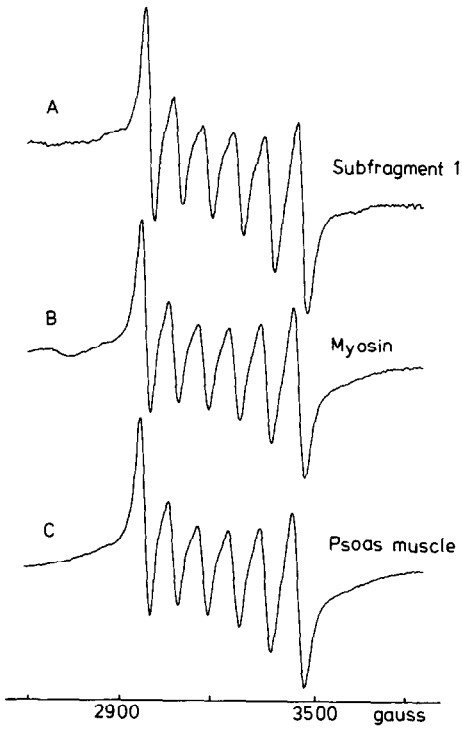

Fig.2. EPR spectra of Mn^{2+} bound to subfragment 1, myosin and glycerinated psoas muscle. These signals arise predominantly from Mn^{2+} bound to the DTNB light chain (cf. ref. [8], fig.2). (A) 315 μM subfragment 1 containing 0.6 mol DTNB light chain [10] plus 80 μM Mn^{2+}. (B) 160 μM (sites) rabbit skeletal myosin. (C) 4 × 0.2 cm glycerinated psoas muscle. Samples B and C were equilibrated with 4 μM free Mn^{2+}, at pH 7.4 and 4°C. Spectrometer settings were the same except the receiver gain which was 2.5×10^5 in A and 1.25×10^5 in B and C. The relative amplitudes of spectra B and C indicate a DTNB light chain concentration of 190 μM within the muscle after correction for the surrounding buffer. This compares with 230 μM myosin nucleotide binding sites as determined by Marston [11] for a similar preparation.

structure (i.e., a first order process) to accomodate the metal ion.

4. Discussion

These results indicate that the divalent metal ion site of the DTNB light chain is not competent to exert control during the early events of contraction. It remains to be established if this site plays a passive structural role or controls later events of contraction. Ca^{2+} binding and release from the DTNB light chain would not compete with the troponin system during activation or relaxation owing to its slow kinetics. This situation suggests a possible role of the light chain site in acting as a kinetic buffer to counteract any overshoot in Ca^{2+} release during a prolonged tetanus, thereby minimizing the lag-time in the subsequent relaxation phase (cf. the ideas of Pechère et al. [17] for the more positive role of parvalbumin in relaxing muscle). However, analogue computer simulation shows that, for reasonable estimates of the kinetic and concentration parameters involved, the effect of 190 μM (fig.2) DTNB light chain on the time course of Ca^{2+} accumulation is barely significant. Nevertheless, while the modulation of the free Ca^{2+} concentration is unlikely to be its function per se, the DTNB light chain might contribute to the slow changes in the free Ca^{2+} concentration observed in frog muscle fibres during prolonged stimulation [14].

While the reported studies are restricted to the DTNB light chain of rabbit skeletal muscle, EPR spectroscopy has revealed analogous non-specific sites on the regulatory light chains* from a variety of species, including the mollusc *Spisula solidissima*, which in addition exhibits myosin-linked regulation in the classical sense (C.R. Bagshaw and J. Kendrick-Jones, unpublished observations). This finding suggests that the vertebrate DTNB light chain site is related more to the molluscan Mg^{2+} binding site which is implicated to have a structural role [4], rather than the Ca^{2+} site which is involved in regulation. It should be emphasized that the present studies do not rule out the existence of some kind of myosin-linked regulation in vertebrate skeletal muscle, whose site of interaction is lost during the extraction of the myosin [6,7], but rather they indicate that the so-called Ca^{2+} site of the DTNB light chain is not involved, at least on the time scale of activation.

Acknowledgements

We are grateful to Drs A. Weber and G. K. Radda for their support and comments. We are also indebted to

* The term regulatory light chain is used broadly [4] to distinguish this type from a second class related to the alkali extractable light chains

Drs J. F. Eccleston, P. W. Kuchel, D. R. Trentham and Mr J. M. Sykes for their help. Part of this work was done during the tenure of a Research Fellowship of the Muscular Dystrophy Associations of America to C.R.B. Financial support was provided by the Science Research Council, England and the National Institutes of Health USA (Grant HL 15692).

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