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Metal binding to myosin and to myosin DTNB-light chain

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Summary. The effects of various divalent cations, Ca^{2+}, Mg^{2+} and Mn^{2+} on the intrinsic fluorescence of heavy meromyosin (HMM) and myosin 5,5′-dithio-bis-(2-nitrobenzoate) DTNB-light chain of rabbit striated muscle, are compared. At pH 6.4, the fluorescence change induced by the metal ions is present only in the isolated light chain and disappears in HMM, thus indicating an interaction between the heavy and light chains with respect to the binding of the metal ions. Whereas Mg^{2+} binds more strongly than Ca^{2+} to myosin, this order is reversed in the case of the DTNB-light chain.

In vertebrate striated muscle, it is believed that Ca^{2+}-regulation is controlled by thin filaments components, troponin and tropomyosin[2]. Myosin-linked regulation has been described in molluscan muscle[3] and in vertebrate smooth muscle[4], and in some cases a dual system of regulation, involving both myosin and the thin filaments components, has been found[5]. Some evidence for the existence of myosin-linked regulation in vertebrate striated muscle has also previously been presented[6-9]. This hypothesis is supported by the finding that striated muscle myosin and its fragment, heavy meromyosin (HMM), bind Mg^{2+} [10,11] and Ca^{2+} [12,11] with dissociation constants in the micromolar range. Because tight binding sites of Ca^{2+} are also present on the light chains of myosin which can be released by 5,5′-dithio-bis-(2-nitrobenzoate) (DTNB-light chains), it has been suggested[13] that they might constitute part of the regulatory system of striated muscle. Evidence in favor of this assumption came from studies[14] in which it was shown that the DTNB-light chain from rabbit striated muscle could replace a scallop myosin light chain, which is responsible for Ca^{2+} regulation in this molluscan muscle, and restore the Ca^{2+} sensitivity of the system.

It has previously been speculated[13] that the metal-binding DTNB-light chain might be located at the hinge between subfragment-1 and subfragment-2 of myosin. This hypothesis is supported by the fact that both with trypsin[15,16] and chymotrypsin[17] either subfragment-1 or heavy meromyosin is produced, depending on whether digestion is carried out in the presence of EDTA or divalent cations. Recent work[18,19] has established that saturation of the DTNB-light chain site on myosin with divalent ions protects this subunit against proteolysis, and this in turn inhibits the cleavage of the heavy chain at the subfragment-1–subfragment-2 hinge. The present work describes the effect of Ca, Mn and Mg divalent ions on the intrinsic fluorescence of rabbit myosin DTNB-light chain and of HMM. The binding constants of these metal ions to myosin, HMM and the DTNB-light chain are compared in an attempt to evaluate the role of the light chain in metal-binding and regulation in the thick filaments.

Table 1. Effect of metal ions or metal substrate complexes on the intrinsic fluorescence of HMM[a]

Metal ion or metal-substrate complex	$-\Delta I/I$[b] pH 7.4[c] (%)	pH 6.4[d] (%)
Mg^{2+}	6.2	0.5
Ca^{2+}	6.7	0.3
Mn^{2+}	5.7	-
Mg-ATP	−17.0	−17.0
Mn-ATP	−14.3	−17.0

[a]HMM concentration 134 μg/ml; [b]decrease of tryptophan fluorescence at maximum of emission spectrum, $\lambda = 350$ nm (uncorrected spectrum); [c]in 50 mM Hepes (N-hydroxyethylpiperazine-N′-ethane sulfonic acid) buffer, final pH 7.41; [d]in 50 mM cacodylate buffer, final pH 6.35.

Materials and methods. Myosin was extracted from the back muscles of white rabbits. Myosin and HMM were prepared as previously described[20]. DTNB-light chains were purified by preparative gel electrophoresis[21] from a crude light chain extract obtained by urea treatment of myosin[22]. All reagents were of analytical grade. Double distilled water was used throughout.

Fluorescence measurements were performed at 25 °C as previously described[13]. The excitation wavelength used was 295 nm, so as to ensure that only tryptophan residues were excited[20]. The difference spectra induced by metal ions were similar to those previously published, with no shift in wavelength of emission in the case of the DTNB-light chain[13] and with a small blue shift in the case of HMM[11]. The fluorescence effects were expressed as $\Delta I/I$ values (in %), the ratio of the changes in fluorescence intensity at the peak of the spectrum, induced by the metal ions, ΔI, to the intensity measured in their absence, I. The correction for dilution effects (< 5%) was estimated by the addition of aliquots of a stock solution of metal ion to a tryptophan solution. The observed fluorescence intensities were compared to that of a tryptophan solution used as an external standard to correct for source intensity fluctuations. The concentration of HMM was 134 μg/ml ($A_{295} < 0.03$) and that of the DTNB-light chain-120 μg/ml ($A_{295} < 0.02$).

Results and discussion. The effect of Mg^{2+}, Ca^{2+} and Mn^{2+} on the intrinsic fluorescence of HMM is shown in table 1. At pH 7.4, all 3 metal ions induce a quenching of about 6% of the fluorescence, whereas the effect is negligible at pH 6.4. However, as also shown in table 1, there is no decrease in the magnitude of the fluorescence enhancement due to ATP, by lowering the pH from 7.4 to 6.4 with either Mg^{2+} or Mn^{2+}. This is evidence that the pH lowering did

not cause denaturation and indeed the magnitude of the effect of ATP on the fluorescence of HMM is almost independent of pH over a large pH range - from pH 6 to 9.5 (unpublished observations). Moreover, the intrinsic fluorescence of HMM itself is independent of the type of buffer and pH in this range. The effect of the same metal ions on the fluorescence of the DTNB-light chain is shown in table 2. Here again, the effects of Mg^{2+} and Ca^{2+} are very similar at pH 7.4, whereas a slightly higher value is obtained in the case of Mn^{2+}. On the other hand, in this case, contrary to the situation with HMM, all 3 metal ions retain all - or almost - the full effect at pH 6.4. The fluorescence change accompanying the binding of the metal ions to HMM most probably originates from the quenching of the fluorescence of the tryptophan residues of the DTNB-light chain and not those of the heavy chain, since the latter's fluorescence by itself is not affected by the presence of metal ions[6]. However, although at pH 7.4, the tryptophans of the DTNB-light chain are quenched also when they are part of HMM, causing a small blue shift of the residual fluorescence[11], at pH 6.4 this effect has disappeared. It might therefore be that, at pH 6.4 at least, the heavy chain is playing a role in the binding of the metal ions. This occurs either directly, by having the metal ion bridge between the heavy and the DTNB-light chains, or through the DTNB-light chain.

In principle, it could also be that, at pH 6.4, the fluorescence quenching of the DTNB-light chain is counterbalanced by an enhancement in the fluorescence of the heavy chain of HMM. In any case, the interaction is reflected in a change in the efficiency of fluorescence quenching caused by metal ions. The fact that metal ions protect the heavy chain from proteolytic cleavage at the subfragment 1-subfragment 2 hinges[15-17] can be regarded as evidence in favor of the 'bridging hypothesis'. It would be tempting to speculate that lowering the pH of HMM solutions from pH 7.4 to 6.4 causes a local conformational change, which changes the distance between the metal ion and the tryptophan residues of the DTNB-light chain. In order to probe for such a possibility, it was attempted to bind Tb^{3+} ions to the DTNB-light chain and to HMM. Unfortunately, no such binding could be demonstrated, and instead, in some cases, turbidity developed. This is consistent with the recent finding by others[23] that the total myosin light chains failed to bind terbium ions.

The binding constants of the various metal ions to myosin, HMM and the DTNB-light chain are compared in table 3. It is very interesting to note that whereas in the case of myosin, Mg^{2+} binds 12fold more strongly than Ca^{2+}, this order is reversed in the case of the DTNB-light chain. The latter actually possesses sites for Ca^{2+} with the same binding constant as those of myosin, whereas the sites for Mg^{2+} are 200fold weaker. This is, of course, the result of more than 2000fold decrease in the Mg^{2+} binding constant when going from myosin or HMM to the DTNB-light chain. It thus appears that although the Ca^{2+} and Mg^{2+} sites on the DTNB-light chain are identical[13], in myosin Ca^{2+} can be bound without the help of the heavy chain, whereas the latter is required for the strong binding of Mg^{2+}. In addition, the binding of Mg^{2+} to HMM[8] - and probably to myosin as well - is cooperative.

In conclusion, the fact that the dissociation constants of Ca^{2+} and Mg^{2+} from myosin are influenced in such a different manner by the proximity of the heavy chain suggests that Mg^{2+} binds by bridging between the heavy and DTNB-light chains, whereas Ca^{2+} binds only to the latter. Mn^{2+} occupies an intermediary position between the 2 other ions, i.e., it might be partially bridging. It is therefore not easily displaced by either Ca^{2+} or Mg^{2+}, thus giving rise in competition experiments to apparent inhibition constants, which are much higher than the true dissociation constants of the high affinity sites[24]. The combined interaction of the heavy and light chains with the divalent cations probably ensures a very sensitive mechanism of regulation of the metal ion switch in the thick filaments.

Table 2. Effect of various metal ions on the intrinsic fluorescence of the DTNB-light chain of myosin[a]

Metal ion	$-\Delta I/I$[b] pH 7.4[c] (%)	pH 6.4[d] (%)
Mg^{2+}	18.7	16.2
Ca^{2+}	17.0	17.4
Mn^{2+}	22.5	17.5

[a]Light chain concentration 190 µg/ml; [b]decrease in tryptophan fluorescence at maximum of emission spectrum, $\lambda = 350$ nm (uncorrected spectrum); [c]in 50 mM Hepes buffer, final pH 7.41; [d]in 50 mM cacodylate buffer, final pH 6.33.

Table 3. Comparison of binding constants of metal ions to myosin, HMM and DTNB-light chain at pH 7.4

	$10^{-4} \times K\ M^{-1}$ Mg^{2+}	Ca^{2+}	Mn^{2+}
Myosin	>200[a]	0.1; 17[b]	0.1; 100[c]
HMM	115; 1660[d]	-	-
DTNB-light chain	0.09[e]	1.0; 17.9[e]	2.0[f]

[a]Kiely and Martonosi[10], 2 high affinity sites; [b]Morimoto and Harrington[12], 2 equivalent strong binding sites; [c]Beinfeld et al.[24], 2 equivalent strong binding sites; [d]Werber et al.[11], corrected for statistical factors; [e]Werber et al.[13]; [f]unpublished results.

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