Evidence for Two Distinct Affinities in the Binding of Divalent Metal Ions to Myosin*

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Binding of Mg\(^{2+}\), and Ca\(^{2+}\), and Mn\(^{2+}\) to myosin was determined by direct measurement of bound and free metals after equilibrium dialysis as well as by an indirect method involving changes in reactivity of a specific thiou group (so-called thiol-I) toward N-ethylmaleimide caused by the metal ion binding to the protein. The results indicate that with all three metal ions alone, or in mixtures, two bound with relatively high affinity per myosin molecule (equilibrium constants > 10\(^5\) M\(^{-1}\)). None was found to bind to isolated myosin heads devoid of light chain-2. Both the direct and indirect approach yielded two intrinsic equilibrium constants for all three metal ions at pH 7.6 and various conditions. At low ionic strength and 22–25°C the equilibrium constants were on average for Ca\(^{2+}\), \(K_1 = 2.8 \times 10^8\) M\(^{-1}\) and \(K_2 = 3.2 \times 10^8\) M\(^{-1}\); for Mg\(^{2+}\), \(K_1 = 1.6 \times 10^8\) M\(^{-1}\) and \(K_2 = 6.1 \times 10^8\) M\(^{-1}\); and for Mn\(^{2+}\), \(K_1 = 3.0 \times 10^8\) M\(^{-1}\) and \(K_2 = 5.1 \times 10^8\) M\(^{-1}\). Two distinct equilibrium constants of similar values were also found for binding of Mg\(^{2+}\) to myosin in the presence of 2 to 5 mM ADP. Computation analyses of equilibrium data from metal ion mixtures are compatible with the assumption that Mg\(^{2+}\) and Ca\(^{2+}\) compete independently of one another with their two intrinsic affinity constants for the two binding sites of myosin. Based on this simple model about 50% of the metal binding sites of myosin would be occupied by Ca\(^{2+}\) at around 10\(^{-5}\) \(\mu\)M free calcium and 10\(^{-2}\) \(\mu\)M free magnesium ion concentrations. At present it cannot be decided whether the two distinct affinities in myosin for metal ion binding originate from pre-existing asymmetry in the two heads of a myosin molecule or whether negative cooperativity is operative between the two binding sites.

It is now accepted that the light chain-2 subunits of myosin (the so-called 5,5'-dithiobis(2-nitrobenzoic acid) light chain or P-light chain of 19,000 daltons) contain the high affinity binding sites for divalent metal ions (1–4). The discovery that the binding of calcium ions to the homologous light chain of molluscan myosin regulates the myofibrillar ATPase activity (5) has naturally led to speculation that light chain-2 of skeletal myosin may also have some regulatory function in addition to that derived from the regulatory components, troponin and tropomyosin on the actin filament (6). Myosin-linked regulation has been described in vertebrate smooth muscle (7), and in some cases evidence for a dual system of regulation involving both myosin and actin filaments has been found (8). Several studies suggest that in vertebrate striated muscles, Ca\(^{2+}\) regulatory systems are also simultaneously operating on both filaments (2, 9, 10). Here, the binding sites for calcium ions on the myosin filament are very likely located in the light chain-2, since studies on the binding of isolated myosin heads, containing this light chain, to actin indicate that actomyosin interaction is looser in the presence of calcium ions than in their absence (11). On the other hand, Ca\(^{2+}\) regulation of the act-activated ATPase of myosin deficient in light chain-2 was found to be impaired (12, 13). But since the cause of this latter effect has been attributed to changes in the affinity of the troponin complex, and not myosin, for the calcium ions, the picture still remains unclear. A further complication is that in the muscle cell the free magnesium ion concentration may be in the range 0.1 to several millimolar whereas the calcium ion concentration ranges from values below 10\(^{-7}\) at rest to 10\(^{-5}\) to 10\(^{-4}\) M on activation. Thus, the influence of the constant high magnesium ion concentration on the binding sites for Ca\(^{2+}\) on the myosin filament needs investigation. If Mg\(^{2+}\) should also show significant binding it is then important to determine the distribution of myosin types possessing possible combinations of both ions at the physiologically relevant conditions.

Direct binding studies using 45Ca\(^{2+}\) done in the presence of variable magnesium ion concentrations indicate that about 2 mol of Ca\(^{2+}\) bind/mol of intact myosin with apparent equilibrium constants in the range of 10\(^7\) to 10\(^9\) M\(^{-1}\) (2, 14, 15). Bremel and Weber (14), using a metal ion buffer system, first noticed that at least one Ca\(^{2+}\) binds with a much higher apparent constant of the order of 10\(^9\) M\(^{-1}\), when magnesium ions are in the micromolar concentration range. However, there are also reports of lower values around 10\(^5\) to 10\(^6\) M\(^{-1}\) found in the absence of magnesium ions (16, 17). On the other hand, the binding of Mg\(^{2+}\) to myosin has been studied by only indirect means. Mg\(^{2+}\), in competition with Ca\(^{2+}\), was found to have a relatively low affinity to intact myosin of around 10\(^3\) M\(^{-1}\) (16). But, in contrast, values between 10\(^6\) and 10\(^7\) M\(^{-1}\) were observed from the quenching of the intrinsic fluorescence of heavy meromyosin (18). Earlier investigations on the inhibitory effects of magnesium ions on Ca\(^{2+}\)- and K\(^{+}\)-dependent myosin ATPases led to claims of a variety of affinities for these ions to myosin (17, 19, 20).

In an attempt to clarify the confusion surrounding the binding of Mg\(^{2+}\), we carried out systematic studies with this ion and intact myosin. The experiments were extended to involve Ca\(^{2+}\), and mixtures of both approximating to physio-
logical concentrations, since, in view of the possibility of a regulatory role for calcium ions mentioned above, some interplay between these ions may be expected. Apart from a recent short report (21), no systematic study of direct determination of ions bound to myosin, especially in the presence of both Mg$^{2+}$ and Ca$^{2+}$, has appeared.

We report here on the binding of the divalent cations Mg$^{2+}$, Ca$^{2+}$, and Mn$^{2+}$ to isolated myosin using both the direct method of atomic absorption spectroscopy and the indirect method of thiol group reactivity. The latter technique follows the change in reactivity of the essential thiol-1 group of myosin, whose modification inactivates the K$^+$-dependent ATPase but activates the Ca$^{2+}$-dependent ATPase (22) when tested at high ionic strength (23). Besides the essential thiol-1 and thiol-2 groups, myosin contains so-called nonessential thiol-3 groups which have all been shown to reside in the heavy chain and whose reactivities are allosterically affected by ligand binding (24). This indirect approach has also been used to monitor the binding of Ca$^{2+}$ to troponin-C (25), and responses of spin labels attached to thiol groups have been used to monitor the binding of Ca$^{2+}$ to troponin-C (25), and responses of spin labels attached to thiol groups have been used to follow the binding of these ions to troponin-C (13, 14) and to the isolated myosin light chain-2 (3, 26).

In the work presented here both the direct and indirect approaches yielded binding site/myosin molecule of high but distinctly different affinities for all three ions. The results suggest that, even if the binding may take place directly on light chain-2, the microenvironment of a portion of the heavy chains is affected. All three ions displayed exchangeability by direct competition for the 2 high affinity sites/myosin. Analytical computation revealed further, that the ions retained their respective intrinsic affinities unaffected when present in mixtures. In particular, it could be shown that about 50% of the binding sites may be occupied by Ca$^{2+}$ under conditions resembling those in activated muscle.

**METHODS**

Twice-distilled and deionized water was used throughout. Contamination by Mg$^{2+}$ and Ca$^{2+}$ was below 1 nM. All reagents were of the highest purity obtainable. N-ethyl-1,2-C$^{14}$ Maleimide was obtained from New England Nuclear Co., Boston, Mass.; a-chymotrypsin of bovine pancreas three times crystallized from Sigma Chemical Co.; Chelex X-100 from Bio-Rad; hydrochloric and perchloric acid supra-pure and metal standards for atomic absorption from Merck. Myosin was prepared from fast skeletal rabbit muscles and stored in the presence of 5 mM EDTA as described elsewhere (23). Several myosin preparations were also performed in the presence of 3 mM Mg$^{2+}$ and 0.5 mM diethylenetriaminepentaacetic acid instead of EDTA. Both types of preparations exhibited identical band patterns in sodium dodecyl sulfate polyacrylamide gel electrophoresis (24). On the assumption that the protein of each band stains with equal specific intensity with Coomassie brilliant blue, the averaged stoichiometry for the light chains using both types of preparations showed the ratios of light chain-1:light chain-2:light chain-3 = 1.30:1.89:0.83. To determine the degree of phosphorylation of light chain-2, polyacrylamide gel electrophoresis of a number of preparations was also performed in the presence of urea (27). In six preparations, 54 to 60% of light chain-2 was invariably in the phosphorylated state as judged from densitometric tracings of stained urea gels. This ratio did not change on storage of the proteins for up to 6 weeks. Myosin prepared in the presence of EDTA, which was used for most of the experiments presented in this paper, proved to be free of both the endogenous light chain kinase and light chain phosphatase, as incubation with Mg-ATP in the presence or absence of calcium ions left the electrophoretic pattern unchanged (28, 29). Isolated myosin heads, i.e., heavy meromyosin subfragment-1, were prepared according to Weeds and Taylor (30) and proved to be devoid of light chain 2. The K$^+$-dependent ATPase activity, measured (31) at 25°C, pH 7.6, and high ionic strength as specified earlier (23), was in the range 11.5 to 13.7 s$^{-1}$ for the myosin preparations and 5.3 to 8.7 s$^{-1}$ for the isolated heads. For calculations the following molecular weights were taken: myosin, 470,000 (32) and myosin heads, 120,000 (30).

Alkylation was performed on 0.25 to 0.40 μM protein for 5 min at 25 to 50 mM Tris-Cl or imidazole-Cl buffer, pH 7.6, in the presence of 30 to 50 mM KCl and different amounts of MalNet¹ ranging from 4 to 12.6 μM under conditions specified in the text. The reaction was stopped by addition of at least a 100-fold excess of diithiothreitol. To remove the reagents all samples were exhaustively dialyzed. Subsequently, they were analyzed for their K$^+$-dependent ATPase activities and incorporation of radioactivity in cases where [$^{14}$C]MalNet was used (33). During alkylation the concentration of metal chlorides was varied in the presence of 2 mM EDTA.

For direct metal ion binding measurements, all containers and pipettes were of suprapure HCl-washed plastic material. All buffer solutions were passed successively through Chelex X-100 and a column of parvalbumin immobilized on the polyacrylamide matrix of Bio-Gel P-60 (34) to remove as much as possible residual divalent metal ion contaminations. Equilibrium dialysis was performed with 0.6-μl samples containing 30 to 100 μM protein against 80 ml of 25 mM imidazole-Cl buffer, pH 7.6, 50 mM KCl, and 0.5 mM 2-mercaptoethanol containing various amounts of metal ions.

Before dialysis the cellophane tubing (Union Carbide Corp., Chicago, Ill.) was boiled for 2 h in water containing 0.2% sodium bicarbonate and 5 mM EDTA. After dialysis for 76 h with stirring and buffer changes, sample aliquots were drawn for protein determination. Under these experimental conditions equilibrium was usually reached after 15 to 20 h, and in one case 30 h were required; however, no difference in the content of the equilibration time on protein concentration was observed. Analyses of Mg$^{2+}$, Ca$^{2+}$, and Mn$^{2+}$ were carried out by atomic absorption (Perkin-Elmer 303 spectrometer) after deproteinization with suprapure perchloric acid. The Ca$^{2+}$ contamination in the solutions after passage over the immobilized parvalbumin column was below 10⁻⁷ M (34). However, in the composite buffer solutions containing 30 to 50 mM KCl, the sensitivity for detecting calcium ions was around 10⁻⁸ M; hence, for lower concentrations of free Ca$^{2+}$, 10⁻⁴ M EGTA was included in the dialysis buffers.

The pH of all buffer solutions was controlled at the appropriate temperature and metal ion plus chelator concentrations before and after the experiments. Protein concentrations were determined by the biuret reaction or according to Lowry et al. (35) standardized by ultramicro-Kjeldahl estimation of nitrogen (36).

For calculation of the free metal ion concentrations the following values of logarithms of association constants were taken from Ref. 37: EDTA$^{-}$/H$^{+}$, 10.3; HEDTA$^{-}$/H$^{+}$, 6.2; HEDTA$^{2-}$/H$^{+}$, 2.7; HEDTA$^{3-}$/H$^{+}$, 2.0; EGT$^{3-}$/H$^{+}$, 6.6; HEGTA$^{-}$/H$^{+}$, 8.5; HEGTA$^{2-}$/H$^{+}$, 2.7; HEGTA$^{3-}$/H$^{+}$, 2.1. ADP$^{-}$/H$^{+}$, 6.4; HADP$^{-}$/H$^{+}$, 3.8; EDTA$^{-}$/Mg$^{2+}$, 8.7; HEDTA$^{-}$/Mg$^{2+}$, 2.3; EGTA$^{-}$/Mg$^{2+}$, 5.2; HEGTA$^{-}$/Mg$^{2+}$, 3.4; ADP$^{-}$/Mg$^{2+}$, 3.3; HADP$^{-}$/Mg$^{2+}$, 1.6; Ca$^{2+}$/EGTA$^{2-}$, 10.5; HEDTA$^{-}$/Ca$^{2+}$, 3.5; EGT$^{-}$/Ca$^{2+}$, 11.0; HEGTA$^{-}$/Ca$^{2+}$, 5.3; EDTA$^{-}$/Mn$^{2+}$, 14.2; HEDTA$^{-}$/Mn$^{2+}$, 6.2; EDTA$^{-}$/K$^{+}$, 1.9; EGTA$^{-}$/K$^{+}$, 1.5; ADP$^{-}$/K$^{+}$, 0.7. Curve fitting by a nonlinear least square procedure as well as iteration calculations of the corresponding computer programs led to the equilibrium dialysis data for evaluation of binding constants. All computations were run on a computer model PDP 11/20 (Digital Equipment Corp.).

**RESULTS**

Effect of Divalent Cations on the Reactivity of the Essential Thiol-1 Group of Myosin—It has been shown that out of the four essential thiol groups in myosin, a thiol-1 always reacts first with MalNet at 25°C irrespective of ionic strength or the presence of metal ions and nucleotides (23). In the absence of nucleotides, however, a thiol-3 group, whose blockage does not affect the enzymic properties, reacts with MalNet as fast as, or even faster, than the essential thiol-1 group (33). Fig. 1 shows a time course of inactivation of the K$^+$-dependent ATPase after treatment at a given MalNet concentration at low ionic strength and 25°C in the presence of 10 mM EDTA compared to 5 mM MgCl$$_2$$. The change of slope in the semilogarithmic plot indicates that indeed more than one type of thiol groups react with MalNet under both conditions (27).

However, the rate of inactivation is much faster in the presence of magnesium ions than in their absence. The use of radioactively labeled [$^{14}$C]MalNet revealed that in both cases about the same degree of MalNet was incorporated after each

¹The abbreviations used are: MalNet, N-ethylmaleimide; EGTA, ethylene glycol bis(β-aminoethylether)-N,N,N',N'-tetraacetic acid.
Magnesium and Calcium Binding to Myosin

The inactivation of the $K^+$-dependent ATPase is the result of blockage of essential thiol groups in the heavy chains of myosin (24). On the other hand, the metal ion binding sites are located in the light chains-2 and thus the ion binding seems to alter the interaction between the light and heavy chains. A similar conclusion has been drawn from studies of Mg$^{2+}$ binding to heavy meromyosin (38), in which the quenching of intrinsic fluorescence in heavy meromyosin and the isolated light chain-2 were compared. The increase in the value of the inactivation rate with increasing added MgCl$_2$ concentration was computed as described under "Methods." Thus, an intermediate value of the inactivation slope, although the essential thiol groups show none and excess added ions, was taken as a direct measure of magnesium ion concentration produced gradual increases in inactivation rates, although the essential thiol groups show a slightly slower reaction rate toward MalNet.

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Equilibrium constants for the binding of Mg$^{2+}$ and Ca$^{2+}$ ions to myosin determined by both thiol group reactivity and equilibrium dialysis techniques at pH 7.6 as described under "Methods." The evaluation of $K_1$ and $K_2$ from the thiol group method is described in the text. $K_1$ and $K_2$ as well as the number of binding sites ($n_1$ and $n_2$) of equilibrium dialysis data were derived from linear regressions of Scatchard plots. Averages of 2 to 10 experiments each.

| Method and ion | Temperature ($^\circ$C) | Addition | $K_1$ (M$^{-1}$) | $n_1$ | $K_2$ (M$^{-1}$) | $n_2$ | $n_1 + n_2$ |
|----------------|--------------------------|----------|-----------------|------|-----------------|------|-------------|
| Thiol group reactivity | Mg | 25 | 3.3 x 10$^4$ | 3.3 x 10$^6$ | Mg | 25 | 3.2 x 10$^4$ | 2.1 x 10$^6$ | Mg | 25 | 1.4 x 10$^5$ | 8.1 x 10$^4$ | Mg | 25 | 1.4 x 10$^5$ | 6.9 x 10$^5$ |
| Equilibrium dialysis | Mg | 22 | 9.5 x 10$^6$ | 0.76 | Mg | 22 | 6.8 x 10$^7$ | 1.06 | Mg | 4 | 8.5 x 10$^7$ | 1.39 | Mg | 4 | 1.0 x 10$^9$ | 0.77 | Ca | 4 | 1.9 x 10$^5$ | 1.26 | Ca | 4 | 2.0 x 10$^6$ | 2.03 |

The values for the higher and lower affinity constants from equilibrium dialysis and the indirect alkylation technique show a good correspondence. Just one cation binds with each affinity per myosin. In the equilibrium technique the constant of the higher affinity and the number for the two binding sites varied by no more than ±20% in different experiments. Again a larger variation occurred in the constant of lower affinity.

The data in Table I are composed from experiments involving a dozen different myosin preparations some of which were prepared in the presence of MgCl$_2$ and dithiothreitol instead of EDTA. However, no significant differences could be seen between protein preparations. Beside the fact that the two equilibrium constants differ under all conditions significantly, it emerges from both techniques that for binding of Mg$^{2+}$ at low ionic strength and 25°C they are closer to one another when ADP was present. At 25°C and 4°C, the first calcium ion binds with higher affinity than Mg$^{2+}$ whereas the second one binds with a comparable affinity to that of the second magnesium ion.

In the muscle cell the concentration of Ca$^{2+}$ fluctuates according to the states of activation and rest. Since the concentration of ionized Mg$^{2+}$ is not known precisely (44) binding of Ca$^{2+}$ was also measured in the presence of different concentrations of Mg$^{2+}$. The higher the magnesium ion concentration the more the points tended to approach a straight line in the Scatchard plot whose slope indicated a lower apparent affinity constant for the Ca$^{2+}$ binding when magnesium ions were present. In the case with 1 mM MgCl$_2$, which is shown in Fig. 6, the linear regression through the first 11 points of Ca$^{2+}$ binding in the Scatchard plot yields an apparent affinity constant of 5.34 x 10$^4$ M$^{-1}$ for 1.78 sites/myosin molecule. These results agree with those reported by Morimoto and...
Magnesium and Calcium Binding to Myosin

Harrington (2) who measured Ca$^{2+}$ binding to myosin in the presence of 0.3 mM MgCl$_2$ by a method based on the partition of radioactive $^{40}$Ca$^{2+}$ between an insoluble metal ion chelator and the protein in solution. If the linear regression comprises only the first 8 points in the Scatchard plot of Fig. 6 the resulting equilibrium constant of $8.46 \times 10^4$ M$^{-1}$ is still more than three orders of magnitude lower than for the binding of the first calcium ion in the absence of MgCl$_2$. Points obtained at higher calcium ion concentrations indicate binding at sites of very low affinity in addition to the two sites of high affinity as also reported by others (2, 4, 14, 16).

The fact that the difference in $K_1$ and $K_2$ found in the absence of magnesium ions disappears in their presence in the millimolar concentration range immediately raises the question of the mode of action of these ions on the Ca$^{2+}$ binding. Curve fitting according to a nonlinear least square program was therefore carried out on the binding data when the amount of Ca$^{2+}$ bound/myosin was plotted against logarithm of free calcium ion concentration. The free magnesium ion concentration and the values of their binding constants, namely $1 \times 10^5$ and $5 \times 10^7$ M$^{-1}$ for $K_1$ and $K_2$, respectively (rounded values from Table I for Mg$^{2+}$ binding alone), were explicitly taken into account. In this calculation it was assumed that both types of ions could compete for the same sites in order to see if direct competition explains the observed apparent affinities for calcium ions. The computed values for $K_1$ and $K_2$ were $1.7 \times 10^5$ and $5.2 \times 10^4$ M$^{-1}$ which stand in good agreement with those values found for Ca$^{2+}$ in the absence of Mg$^{2+}$ (Table I). Iterative computations were also performed at each data point individually and the calculated values for $K_1$ and $K_2$ stemming from experiments involving the same magnesium ion concentration were then averaged geometrically. As Table II shows, this approach also yields values for the affinities of Ca$^{2+}$ to myosin in the presence of different amounts of MgCl$_2$ similar to those observed in the absence of Mg$^{2+}$. These results strongly indicate that the interaction of Mg$^{2+}$ with myosin does not actively affect the binding of calcium ions but simply displaced them competitively. Under this model one can calculate at any Ca$^{2+}$ concentration the relative percentage of the different species of myosin molecules bearing two cations, given the binding constants for both metal ions and the total MgCl$_2$ concentration; $5 \times 10^5$ and $5 \times 10^4$ M$^{-1}$ were used for $K_1$ and $K_2$, respectively, for Ca$^{2+}$ binding (rounded values taken from averages of Table I and II for affinity constants of calcium ions) and for $K_1$ and $K_2$ of Mg$^{2+}$ the same as above. Out of the four species, Mg$_2$MMg$_2$, Mg$_1$MCa$_2$, Ca$_2$MMg$_2$, and Ca$_1$MCa$_2$, where M stands for myosin and the subscripts 1 and 2 for the binding constants $K_1$ and $K_2$, Fig. 7 indicates that the species Ca$_2$MMg$_2$ is predominant at the point where the myosin is 50% saturated with Ca$^{2+}$.

Manganese ions, whose binding to protein is often studied because it can be conveniently followed by ESR spectroscopy, have also been investigated (4, 16, 45). Therefore, the direct binding studies reported were extended to this cation. The Scatchard plot (Fig. 8) of these data looked somewhat different from those for the other two ions, since it indicates two types of binding sites ($n = 2.35$) with the same apparent affinity ($K = 1.73 \times 10^4$ M$^{-1}$). This result is in good agreement with literature reports (4, 16), although Bagshaw (4) appears to have calculated the value of $K$ from only three data points which lie in the range between 1 and 2 bound/mysosin. The indication of only one affinity for manganese ions was obtained without addition of another valent cation, although the level of free Mg$^{2+}$ was around 1 mM according to measurement and could not be lowered further under the experimental conditions. On the other hand, there was no detectable contamination by Ca$^{2+}$ and this metal was not found bound to the protein in these experiments. Since there was no indication of a higher value for the affinity of the first manganese ion, the same types of calculations as explained above were also performed taking into account the presence of 1 mM Mg$^{2+}$. However, from the least square fitting program, which gave $K_2 = 4.1 \times 10^4$ and $K_1 = 5.4 \times 10^5$ M$^{-1}$, and the iteration procedure on each experimental point which gave on average $K_1 = 7.8 \times 10^5$ and $K_2 = 1.2 \times 10^5$ M$^{-1}$, there was indication of a significant difference between $K_1$ and $K_2$ also for this ion. Thus Mn$^{2+}$ has the lowest affinity to myosin of the three metal ions tested.

In order to corroborate the model that myosin contains two high affinity binding sites for which all three metal ions compete, the individual amounts of bound ions were measured in mixtures of Ca$^{2+}$ and Mg$^{2+}$ as well as Mn$^{2+}$ and Mg$^{2+}$. In no case is the total number of bound ions greater than 2.9/myosin indicating that the different ions do not bind specifically to

![Fig. 6](https://example.com/fig6.png)

**Fig. 6.** Scatchard plot of Ca$^{2+}$ ion binding to myosin. Equilibrium dialysis was performed as described under "Methods" at 4°C and pH 7.6 on 26 to 80 mM myosin with 1 mM MgCl$_2$ present throughout. Solid line represents the linear regression through the first 11 points.

| MgCl$_2$ (mM) | $K_1$ (M$^{-1}$) | $K_2$ (M$^{-1}$) |
|---------------|----------------|----------------|
| 0.01          | $2.1 \times 10^4$ | $1.8 \times 10^6$ |
| 0.1           | $5.4 \times 10^4$ | $1.2 \times 10^7$ |
| 1             | $3.8 \times 10^6$ | $6.7 \times 10^6$ |
distinct sites. In fact, as the amount bound for the one type of ion increased that for the second ion decreased reciprocally. In the few cases where the value of total ions bound/myosin was slightly above 2, this most probably was due to binding to nonspecific sites of low affinity as described above.

Isolated myosin heads which were devoid of light chain-2 did not exhibit changes in the reactivity of their thiol-1 group with magnesium, calcium, or manganese ions, indicating, as expected of such preparations (4, 11, 46, 47), that they do not bind metal ions. Furthermore, in the direct equilibrium dialysis, no binding was observed. Even in the presence of 10 μM free magnesium ions and 2 mM ADP where the concentration of the complex Mg·ADP would be around 40 μM, no magnesium ions were found bound to the protein. This observation confirms the deductions drawn above in the case of myosin, namely, that insignificant amounts of the metal ion are bound to the active site in the form of the complex Mg·ADP under conditions of such low free magnesium ion concentrations.

**DISCUSSION**

Divalent metal ion binding to myosin was studied by using two different techniques, equilibrium dialysis and following the changes in thiol group reactivity. The latter method has been used to follow the binding of Ca²⁺ to troponin-C (25) yielding results in agreement with those from other methods. Although it is an indirect method, it shows certain clear features in the work reported here. For example, the thiol group reactivity changes occur over two distinct concentration ranges of the metal ion. The effect also shows clear saturation characteristics and hence seems to result from formation of metal-protein complexes at two classes of binding sites. On the other hand, the results from the direct binding studies reveal that 2 metal ions bind/myosin with high but different affinities. These affinities correspond to distinct concentration ranges of the free metal ion which were found to cover just those concentration ranges where the thiol group reactivity is affected. Hence, although the experimental conditions employed in the two methods differ widely, namely 10 to 50 times higher protein concentrations and 500 to 1000 times longer equilibration times used in the direct compared to indirect method, they yielded the same binding affinities.

Since the direct equilibrium dialysis method shows that just one ion binds with each affinity, it must be concluded that the binding of each ion in succession is responsible for the thiol group reactivity changes over first the low, and then the high, free ion concentration ranges seen in the curves of Fig. 3. The rather distinct bend in the isotherms at around 50% of the saturation level indicates that both ions cause similar changes in thiol group reactivity. This effect in turn reflects similar changes in the microenvironment of the respective cysteine residues located in the myosin heavy chains (48, 49). As the metal ions are known to bind to the pair of light chains-2 (1, 2, 4), it follows that the interaction between each of these light chains and the heavy chains is altered in a similar way. These conclusions corroborate our original assumption underlying the graphical analysis that the relative change in thiol group reactivity is equal to the saturation fraction of the total of 2 metal ions binding/myosin. Further evidence for mediation of the interaction between light chain-2 and the heavy chains by cation binding has recently been reported from observations on the intrinsic fluorescence of heavy meromyosin (39). Differences in the digestion of myosin by various proteolytic enzymes between the presence and absence of divalent cations also support the view of altered interchain interactions (4, 11, 30, 47, 50).

In the present work, both approaches revealed high affinity binding of all magnesium, calcium, and manganese ions by myosin and yielded similar values for the respective equilibrium constants. Furthermore, these values are in agreement with those from other laboratories. In particular the claim that calcium ions bind more tightly than Mg²⁺ (1, 17, 21, 50, 51), and are more effective in replacing Mn²⁺ (16), is in line with our results in which calcium ions display the highest affinity of all three. In the cases of Mg²⁺ and Ca²⁺ a clear differentiation between the two high affinity sites is revealed by both methods employed in this work, whereby the first ion is bound more tightly than the second one, K¹ being on average 100 times larger than K₂. The additional presence of ADP in the millimolar concentration range did not change these findings in the case of magnesium ion binding to myosin. The second class of sites with a constant K₂ of around 10⁵ M⁻¹ for all three ions is not to be confused with the often reported low affinity sites with affinities of the order of 10⁶ M⁻¹ (2, 4, 14, 16), since with a single exception of Mg²⁺ binding in high salt at 25°C, K₂ is never lower than 10⁷ M⁻¹. Furthermore, the measurements reported here on the number of ions bound in systems containing mixtures of two different cations strongly indicate that there is a total of just 2 sites/myosin occupied by either ion at free concentrations around 10⁻⁷ M. The difference between the 2 high affinity sites does not appear to influence the competitive binding of Mg²⁺ and Ca²⁺, since good fits to the data can be calculated using the simple assumption that the ions bind independently of one another. In other words, even in the presence of millimolar concentrations of Mg²⁺ where both sites appear to have the same affinity for calcium ions (Fig. 6), one Ca²⁺ still binds with its high intrinsic affinity of around 10⁶ M⁻¹. Thus, it is just this site whose affinity for Ca²⁺ appears to be markedly affected by the presence of Mg²⁺. This may be explained by the fact that the competing magnesium ions also have a higher affinity for it than for the second site.

The shifts induced by magnesium ions in the curves of Ca²⁺ binding to myosin published by Bremel and Weber (14) are in agreement with our observations. Increasing the magnesium ion concentration from 3 μM to 1 mM raises the calcium ion concentration required for the binding of the first Ca²⁺ by a factor of about 10³, but for the second ion by a factor of only about 10 (see Fig. 3 of Ref. 14). On the other hand, the Scatchard plots of our data on Ca²⁺ binding were always linear over the range of the first ion to bind in the absence as well as in the presence of MgCl₂. They did not display the upward convex curvature found by others for Ca²⁺ binding to myosin in the absence or presence of 100 μM MgCl₂ (14, 15) which was interpreted as the result of positive cooperativity. Calculation of the statistically corrected intrinsic affinity constants by our
The role of Ca\textsuperscript{2+} binding to myosin as the trigger involved in the activation of muscle contraction has been questioned on kinetic grounds (21). The kinetic measurements indicate that the dissociation of Mg\textsuperscript{2+} is too slow to allow for a switching on mechanism. Nevertheless, calcium ion concentrations of the order of 10^{-5} M may be achieved in muscle during sustained activity, and, as the calculations presented here show, around 50% of the metal binding sites of myosin would be occupied by Ca\textsuperscript{2+} even in the presence of 1 mM free magnesium ion concentration. Since these calculations are applied to the system at equilibrium, they represent simply a solution for a state containing just one Ca\textsuperscript{2+} in the higher affinity and a Mg\textsuperscript{2+} in the lower affinity site would predominate.

Measurements made by indirect methods on isolated light chains-2 have established that they bind calcium ions with an affinity around 10^9 to 10^{10} M^{-1} and magnesium ions around 10^4 M^{-1} (1, 2). The affinity for Ca\textsuperscript{2+} is lowered by an order of magnitude when the chain becomes phosphorylated, although Mg\textsuperscript{2+} binding remains unaffected (26). A larger difference of about four orders of magnitude for Ca\textsuperscript{2+} binding has been reported for myosin to which light chain-2 in either the dephosphorylated or phosphorylated form has been reattacked (3), whereby the higher affinity was nevertheless lowered considerably by the presence of 1 mM Mg\textsuperscript{2+} (58). These observations would naturally suggest that the existence of two distinct binding affinities in myosin is due to the two states of phosphorylation of the light chain-2. The fact that just around half of these light chains is in each state in our myosin preparations would then explain the finding of two distinct affinities reported here. Consequently, questions of the cause of the metal ion binding behavior would also now apply to phosphorylation. Does the duplex myosin molecule possess heterogenous and independently functioning heads, or do the heads interact cooperatively, or do two separate myosin species exist, explaining the intermediate degree of phosphorylation? Other independent evidence points indeed to a type of intramolecular interaction. For example, the light chains-2 are identical with one another in the isolated state (59, 60), yet it appears from recombination experiments that only one is removed from each myosin molecule by treatment with 5,5'-dithiobis(2-nitrobenzoic acid) (59), indicating that they adopt an asymmetric relationship to one another. Then, out of the possibilities considered, that which envisages two populations of myosin species with different binding and phosphorylation properties seems the most unlikely. Thus myosin, with its distinguishable subunit properties arising from either intrinsic asymmetry or concerted subunit interactions, would belong to the large class of multimeric proteins in which subunit interactions are thought to play a major role in controlling their enzymic properties (61).

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