Properties of the Non-Specific Calcium-Binding Sites of Rabbit Skeletal-Muscle Myosin

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The non-specific Ca^{2+} -binding sites of skeletal-muscle myosin are located on the light chains; with the dissociation of light chains there is a corresponding decrease in the number of Ca^{2+} -binding sites on light-chain-deficient myosin. The released light chains have a decreased binding affinity. Myosin heavy chains indirectly influence the Ca^{2+} -binding properties of light chains by increasing the affinity of light chains for bivalent cations; this influence varies with pH. Because of light-chain dissociation at low Ca^{2+} and/or Mg^{2+} concentrations, anomalies may exist when analyses of non-specific Ca^{2+} -binding properties of myosin are assessed by dialysis equilibrium.

There appears to be a combined interaction of the heavy and light chains of myosin with bivalent cations (Okamoto & Yagi, 1976; Kuwayama & Yagi, 1977, 1979; Higuchi *et al.*, 1978) resulting in a firmer complex (Szent-Gyorgyi, 1975; Kasman & Kakol, 1977; Higuchi *et al.*, 1978). Werber (1978) assessed the fluorescence quenching of tryptophan present in myosin induced by metals and was able to show that Ca^{2+} produced a 6.7% quenching of heavy meromyosin at pH 7.4, whereas the Ca^{2+} -induced quenching at pH 6.4 was negligible. On the other hand, pH did not affect the intrinsic fluorescence of the purified Ca^{2+} -binding light chain, LC_2 , in the presence of Ca^{2+} . His data indicate an interaction between the heavy and light chains of myosin with respect to the binding of metal ions and that there may be alterations in this interaction with changes in pH. Thus, the studies described in the present paper were undertaken to assess the Ca^{2+} -binding characteristics of myosin at a neutral pH and at a pH of 6.5.

Experimental

Rabbit skeletal-muscle myosin was purified as described previously (Fabian *et al.*, 1977). Protein concentration was determined by the method of Lowry *et al.* (1951).

For Ca^{2+} -binding a Ca^{2+} /EGTA buffer was used (Fabian *et al.*, 1977). Below $10\ \mu\text{M}$ free Ca^{2+} , Ca^{2+} was fixed and EGTA varied from $0.4\ \text{mM}$ at $10\ \text{nM}$ free Ca^{2+} to $0.04\ \text{mM}$ at $10\ \mu\text{M}$ free Ca^{2+} . At higher free Ca^{2+} concentrations EGTA was fixed and appropriate amounts of Ca^{2+} were added to adjust

the free Ca^{2+} concentrations (Bremel & Weber, 1975; Fabian *et al.*, 1977). Free Ca^{2+} was calculated at different pH values by using the computer program of Potter & Gergely (1975), a modification of that of Perrin & Sayce (1967), where the cumulative logarithm of the six dissociation constants of ionized EGTA and Ca^{2+} -EGTA complexes was taken into consideration (Perrin & Sayce, 1967; Bulos & Sacktor, 1979; Holroyde *et al.*, 1979). The amount of contaminating Ca^{2+} in the protein was assessed as described in previous reports using atomic absorption on the supernatant after an EGTA extraction (Fabian *et al.*, 1977). All buffers, as well as other appropriate solutions, were passed through a column of Chelex-100 to remove contaminating Ca^{2+} in the chemicals. Each flask contained $12.5\ \mu\text{Ci}$ of $^{45}\text{Ca}^{2+}$ (New England Nuclear). In experiments with Mg^{2+} , all dialysis solutions contained either 0.01 , 0.1 or $1\ \text{mM}$ - Mg^{2+} . A calculated amount of Ca^{2+} was added to achieve the desired free Ca^{2+} concentration at a defined pH and ionic strength; free Ca^{2+} in the presence of Mg^{2+} was recalculated on the basis of the cumulative logarithm of eight association and dissociation constants, including four ionized EGTA forms, two Ca^{2+} -EGTA complexes, and two Mg^{2+} -EGTA complexes as described by Bulos & Sacktor (1979). The chelator, EGTA, was prepared as the free acid and buffered to pH 6.5 and 7.0 with KOH. For preparing a stock solution of Ca^{2+} , CaCO_3 (AR grade) was heated to 120°C for 4 h, cooled in a desiccator under vacuum and the desired amount was dissolved in 1 litre of $0.1\ \text{M}$ -HCl and neutralized with KOH. Myosin was precipitated first in $0.1\ \text{M}$ - $\text{KH}_2\text{PO}_4/\text{KOH}$, pH 7.0,

containing 0.5 M-KCl, 1 mM-EDTA and 0.5 mM-dithiothreitol and dialysed in the same buffer; the second dialysis mixture contained the same additions, except no EDTA was added, and the third dialysis mixture contained the same additions, except EGTA (0.1 mM) was substituted for the EDTA (Holroyde *et al.*, 1979). For the dialysis equilibrium 1 ml of protein (5 mg/ml) was dialysed against 100 ml of buffer. The dialysis buffer consisted of 0.2 M-Tris/maleate, pH 6.5, and 0.1 M-KCl. Where analyses were made at pH 7.0, 0.2 M-Tris/maleate, pH 7.0, containing 0.1 M-KCl was used. After a dialysis for 24 h proteins from both inside and outside the bag were treated as described previously (Fabian *et al.*, 1977). Duplicate samples (0.1 ml) of the protein solution inside the dialysis bag were taken for determination of protein concentration and determined by the Bio-Rad assay [Bio-Rad Laboratories Technical Bulletin 1051 (1977)].

For light-chain dissociation, myosin was incubated for 5 min at 35–37°C at 0.1 mg/ml in the following solution: 0.65 M-KCl, 5 mM-EDTA, 1 mM-dithiothreitol, 5 mM-ATP (sodium salt) and 0.1 M-Tris/HCl, pH 7.0. A neutral pH was used for retaining maximal enzymic activity (Higuchi *et al.*, 1978). At the end of the incubation period myosin was immediately precipitated by bringing it to 45% $(\text{NH}_4)_2\text{SO}_4$ saturation, stirred for 5 min and immediately centrifuged. This treatment was repeated a second time. Conditions were chosen for maximal dissociation of light chain LC_2 with no corresponding loss in ATPase activity (Wikman-Coffelt & Srivastava, 1979b). The light chains remaining in the supernatant were precipitated by bringing the solution to 80% $(\text{NH}_4)_2\text{SO}_4$ saturation. After centrifugation, light-chain-deficient myosin as well as the dissociated light chains were solubilized in 0.05 M-Tris/HCl, pH 7.5, buffer containing 0.5 M-KCl and 1 mM-dithiothreitol.

Polyacrylamide gradient slab gels containing sodium dodecyl sulphate were prepared as described previously (Long *et al.*, 1977). For determination of the protein concentration present in Coomassie Blue-stained polyacrylamide gels, the dye was removed with 25% pyridine as described previously, and the protein concentration was determined (Fenner *et al.*, 1975).

Results and Discussion

The downward concavity of Scatchard plots, indicative of decreased binding in the low Ca^{2+} concentration ranges and/or positive co-operativity was obtained for rabbit skeletal-muscle myosin (Bremel & Weber, 1975; Fabian *et al.*, 1977) and cardiac myosin (Fabian *et al.*, 1977; Higuchi *et al.*, 1978). Similar results were obtained for rabbit skeletal-muscle myosin at pH 6.5 by using a 0.2 M-

Tris/maleate buffer containing 0.1 M-KCl (Fig. 1a); when $10 \mu\text{M-Mg}^{2+}$ was added to the equilibrium-dialysis system, there was diminished binding of Ca^{2+} in the low Ca^{2+} concentration ranges and the peak of the Scatchard plot shifted to the right (Fig. 1b). On the other hand, the peak of the Scatchard plot shifted to the left when analyses were made at pH 7.0 and where Mg^{2+} was present at less than $0.1 \mu\text{M}$ (Fig. 1c). A similar shift was obtained previously for frog skeletal-muscle myosin when a pH 7.0 phosphate buffer was used, compared with the pH 6.5 Tris/maleate buffer (Wikman-Coffelt & Srivastava, 1979a). The myosin Ca^{2+} -binding sites analysed in the present paper are the non-specific binding sites that have been described by Bagshaw & Kendrick-Jones (1979), since they were displaced by 1 mM-Mg^{2+} (Fig. 2). Since the myosin light-chains are not pH-sensitive (Chantler & Szent-Gyorgyi, 1978; Stafford & Szent-Gyorgyi, 1978), it is likely

Fig. 1. Scatchard plots of rabbit skeletal-muscle myosin (a) At pH 6.5 and $\text{Mg}^{2+} < 10^{-7} \text{ M}$ ($n = 2.0$; $K = 1.8 \times 10^5 \text{ M}^{-1}$); (b) at pH 6.5 and $\text{Mg}^{2+} = 10 \mu\text{M}$ ($n = 2.0$; $K = 1.4 \times 10^5 \text{ M}^{-1}$); (c) at pH 7.0 and $\text{Mg}^{2+} < 10^{-7} \text{ M}$ ($n = 2.0$; $K = 1.3 \times 10^5 \text{ M}^{-1}$).

Fig. 2. Ca^{2+} binding of native myosin in the presence of various Mg^{2+} concentrations. Bound Ca^{2+} (mol) are plotted against free Ca^{2+} values for native rabbit skeletal-muscle myosin with increasing Mg^{2+} concentrations and at pH 6.5.

that the difference observed in the present paper in Ca^{2+} binding at pH 6.5 relative to pH 7.0 may be due to contributions from the heavy chains, which are susceptible to conformational changes with alterations in pH (Ishigami & Morita, 1977). Alterations in heavy chains with pH may either influence the Ca^{2+} -binding properties of the light chains indirectly or may affect the heavy-chain–light-chain oligomeric complex.

Once the Ca^{2+} -binding light chain was released from the myosin oligomer the number of Ca^{2+} -binding sites on the heavy chains was proportionately lost (Fig. 3a), and consistent with the observations of Kuwayama & Yagi (1977, 1979) and Okamoto & Yagi (1976), as well as previous reports from our laboratory (Higuchi *et al.*, 1978), the dissociated light chains retained the Ca^{2+} -binding site, but with a decreased binding affinity (Fig. 3b). Light-chain-deficient myosin is shown in Fig. 4 (gel 2) and the released light chains in Fig. 4 (gel 3). Conditions for dissociation of light chains were chosen so that there was no loss in myosin ATPase activity with the 70–75% release of light chain LC_2 (Wikman-Coffelt & Srivastava, 1979b).

We analysed whether the downward concavity of Scatchard plots was due to partial dissociation of the light chains at low Ca^{2+} concentrations by assessing the degree of light-chain dissociation in a Ca^{2+} /EGTA buffer at a pCa^{2+} of 8 relative to the same analyses at a pCa^{2+} of 5. As shown in Fig. 5

Fig. 3. Scatchard plots showing Ca^{2+} binding of (a) light-chain-deficient myosin and (b) of light chains

Light-chain-deficient myosin after two consecutive dissociations as described in Experimental section. Myosin treated in this manner was shown previously to have 100% ($\text{K}^+ + \text{Ca}^{2+}$)-activated ATPase activities (Wikman-Coffelt & Srivastava, 1979b). The degree of light-chain release was quantified by gel electrophoresis (Fenner *et al.*, 1975) and it was shown that 74% of the light chains were dissociated from myosin heavy chains in the sample used in (a). ($n = 0.4$; $K = 1.8 \times 10^5 \text{ M}^{-1}$). (b) Shows the released light chains after dissociation as described in (a). The molecular weight of light chain LC_2 was taken as 18000 and 40% of the protein present in the dissociated light chains was shown by gel electrophoresis to be comprised of light chain LC_2 ($n = 1.0$; $K = 2.8 \times 10^4 \text{ M}^{-1}$).

(gel 2) and described in the legend, 15% of the Ca^{2+} -binding light chain was dissociated from the heavy chain at low Ca^{2+} concentrations with a pCa^{2+} of 8. However, when the pCa^{2+} concentration was 5 there was no light-chain dissociation (Fig. 5, gel 3). Thus, the low Ca^{2+} concentrations are conducive to light-chain LC_2 dissociation. Chelating agents have been shown previously (Kasman & Kakol, 1977) to promote light-chain LC_2 dissociation, and bivalent cations have been shown to produce a firmer complex of light chain LC_2 with the heavy chain (Szent-Gyorgyi, 1975).

The non-specific Ca^{2+} -binding sites described in the present paper are located on the Ca^{2+} -binding light chain, LC_2 . These light-chain binding sites have a higher affinity when associated with the heavy chains than when in the dissociated state (Szent-Gyorgyi *et al.*, 1973; Higuchi *et al.*, 1978; Bagshaw & Kendrick-Jones, 1979). Thus in the low Ca^{2+} concentration ranges where the light chains may be par-

Fig. 4. Native myosin (1) and partial dissociation of light chains after treatment of myosin in the presence of EDTA (2) and the released light chains (3)

Myosin was treated as described in Experimental section for release of light chains. After treatment myosin retained 100% ($\text{K}^+ + \text{Ca}^{2+}$)-stimulated ATPase activities, but there was a 15% decrease in actin-activated myosin ATPase activity. Conditions for assessing the various ATPase activities were described previously (Wikman-Coffelt & Srivastava, 1979b). Standard errors were less than 5%. In these conditions there was a 5–10% release of light chains LC_1 and LC_3 , but a 70–75% dissociation of light chain LC_2 .

Fig. 5. Native myosin (1) and partial dissociation of light chain LC_2 after treatment at low Ca^{2+} concentrations (1 nM) (2) and after treatment at $10 \mu M$ - Ca^{2+} (3)

Myosin (0.1 mg/ml) was incubated at $25^\circ C$ for 10 min in 0.6 M-KCl, Ca^{2+} /EGTA buffer of pCa^{2+} 8 and pCa^{2+} 5, and 0.2 M-Tris/maleate, pH 6.5. The solution was prepared as described in Experimental section to minimize bivalent cation contamination. At the end of the incubation period myosin and light-chain-deficient myosin were precipitated with $(NH_4)_2SO_4$ to separate them from the dissociated light chains as described in the Experimental section, and analysed on polyacrylamide gradient gels for assessment of light-chain dissociation. (1), Normal myosin; (2), myosin after treatment as described above with a pCa^{2+} value of 8; (3), myosin after treatment at a pCa^{2+} of 5. The percentage of the Ca^{2+} -binding light chain present was determined as described in Experimental section. For three analyses with standard errors of less than 5% the data were: (2), 15% LC_2 release; (3), no release of light chain LC_2 . In both cases there was no dissociation of light chains LC_1 and LC_3 .

higher Ca^{2+} binding in the low Ca^{2+} concentration ranges with increases in pH; alterations in pH could also affect the conformation of myosin and thus alter its Ca^{2+} -binding properties. As described by Bagshaw & Kendrick-Jones (1979) and Bagshaw & Reed (1977) the binding of Ca^{2+} and/or Mg^{2+} by myosin may be essential to the structure of myosin as shown in the present paper. Such binding may not be related to regulation in contraction in vertebrate striated muscle.

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tially dissociated from the heavy chains a lower degree of Ca^{2+} binding would occur. This dissociation may vary with pH and thus account for the