CALCIUM BINDING PROPERTIES OF CARDIAC AND SKELETAL MUSCLE MYOSINS

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1. Introduction

The homology of the calcium binding regions of the 'regulatory light chains' (DTNB light chains) of various myosins were compared [1,2], including that of cardiac tissue [3]. In the case of rabbit skeletal muscle myosin, Morimoto and Harrington [4] showed as in the studies of Werber et al. [5] that the DTNB light chains possess 2 classes of calcium binding sites, with different affinities; this same was true for native myosin [4–6]. The linear Scatchard-plots of Morimoto and Harrington [4] where myosin was analyzed in the presence of $MgCl_2$ indicate 2 high affinity binding sites with no positive cooperativity. However, in similar studies, positive cooperativity was noted in the Scatchard-plots of Bremel and Weber [7]. The studies described here were undertaken to determine if there was positive cooperativity with the binding of calcium by myosin.

2. Materials and methods

2.1. *Preparation of tissue*

Immediately after sacrificing the animals, either sheep or dog, the hearts were excised, the ventricles dissected, and immersed in acetone and dry ice, wrapped in aluminum foil and stored at −70°C until used. Atria were prepared and dissected free of ventricles as described earlier [8]. Freezing of tissue (−70°C) even for long periods of time, had no effects on the enzymatic activity of myosin [9]. For obtaining leg muscle, rabbits were killed by stunning prior to removal of tissue. Myosin was then immediately prepared from the leg muscle.

2.2. *Myosin purification*

Procedures for purifying cardiac [8–10] and skeletal muscle myosin [11] were defined earlier, except a broader $(NH_4)_2SO_4$ cut (33–45%) was used to increase the yield of myosin.

2.3. *Equilibrium dialysis*

In order to obtain a low enough free Ca^{2+} concentration for the determination of high affinity binding sites of myosin for calcium, the techniques of Potter and Gergely were used [12]. These techniques overcome the interference of Ca^{2+} contamination through the use of ethylene glycol bis (β-aminoethyl ether)-*N,N'*-tetraacetic acid (EGTA) in combination with equilibrium dialysis. Metal buffers have been used to study the metal-binding properties of skeletal muscle myosin [7]. The use of metal buffers is described in detail by Raaflaub [13]. Tris–maleate was used as the dialysate buffer since it allowed for solubilization of myosin without the addition of other cations [14]; other cations may possibly compete for the calcium binding sites of myosin [14]; Ca^{2+} present in myosin was extracted, and measured in a Perkin-Elmer spectrophotometer using 1% $CaCl_2$ 'ultrapure' as a standard. According to analyses using the atomic absorption spectrophotometer, calcium contamination was less than 10^{-6} M. The measured Mg^{2+} contamination was less than 2×10^{-7} M. The Tris-maleate buffer was passed through a Chelex-100 column as

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described by Bienfeld et al. [15], in order to remove contamination metals.

As in the procedure used by Potter and Gergely, 1.0 ml protein (myosin) (5–8 mg/ml) was dialyzed with stirring for 48 h at 4°C against 100 ml 0.2 M Tris–maleate, pH 6.5, 12.5 μCi ^{45}Ca and free Ca^{2+} (in a Ca^{2+} EGTA buffer) as indicated in the legends. All solutions used for Ca^{2+} binding were stored in either acid-washed labware or disposable plastic containers rinsed in 2.0 mM EGTA and then throughly rinsed in deionized, distilled H_2O. The dialysis containers were of a disposable plastic.

The Ca^{2+} EGTA/EGTA ratio was varied; total EGTA decreased from 400 μM at 3.3×10^{-8} M free Ca^{2+} to 1 μM at maximal free Ca^{2+} concentration. Free Ca^{2+} was calculated using the equation of Raaflaub [13]. The apparent binding constant of Ca^{2+} in a Ca-EGTA buffer, pH 6.5, using 0.2 M Tris–maleate was 3×10^5 M^{-1} [16]. For the total calcium the sum of contaminating and added Ca^{2+} were used.

After a dialysis time of 48 h, duplicate aliquots of 0.5 ml were removed from each compartment, 0.5 ml H_2O_2 was added to all vials and heated 65°C for 24 h to hydrolyze the protein. After addition of 10 ml scintillation fluor the samples were assayed in a liquid scintillation counter. The data were treated according to the method of Scatchard [17] to estimate the number of calcium-binding sites and the affinity constants of the binding sites. For determination of interaction between binding sites the equation of Edsall and Wyman was used with a least squares best fit program [18]. All binding data, including Ca^{2+} and EGTA concentrations, and Scatchard-plots were computer programmed using the B6700 Burroughs.

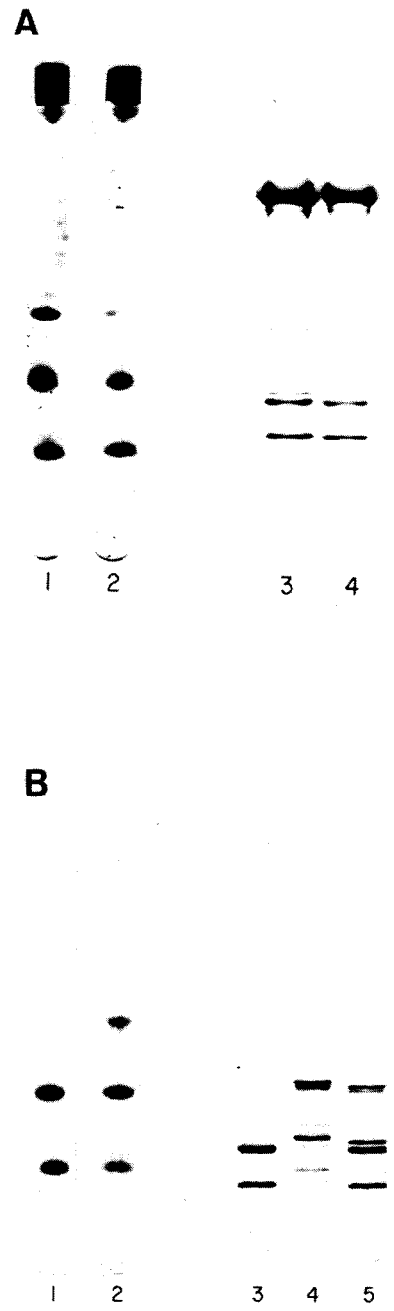

Fig.1. In A gels 1 and 3 are purified cardiac myosin before and gels 2 and 4 after water precipitation of the purified cardiac myosin. The gradient of polyacrylamide ($A_{3,4}$) in sodium dodecylsulfate (SDS) was a modification of that of O'Farrell [37], this was described in detail earlier [8]. The method of Weber and Osborn [38] was used for SDS gels electrophoresed in tubes ($A_{1,2}$). In B gels 1 and 3 are cardiac myosin light chains purified with 8 M urea from the twice H_2O-precipitated myosin. Gels 2 and 4 are the lyophilized contaminants removed with water washes, and B_5 are samples 3 and 4 combined, i.e., urea light chains and contaminants removed with water washes of the purified myosin. $B_{1,2}$ are SDS gels prepared by the Weber and Osborn method [38] whereas (B_{3-5}) are gradients of polyacrylamide.

3. Results and discussion

New criteria were used to assess the purity of myosin so as to specifically define whether contaminants were present in myosin preparations prepared as described here. What appeared to be pure myosin on 6% SDS polyacrylamide gels showed contamination on SDS slab gels of gradients of polyacrylamide (fig.1A,B). Procedures for cardiac myosin purification developed in our laboratory [8–10] were modifications of procedures for purification of skeletal muscle myosin [19–25]. These procedures are also similar to those reported by other investigators [26] for cardiac myosin preparations, including treatment of cardiac homogenates with both low and high salt concentrations, as well as water precipitations of crude myosin, i.e., in the presence of actin [26–29] and subsequent $(NH_4)_2SO_4$ fractionations [30,31] to remove possible inhibitors of myosin ATPase activity [30]. Unless the myosin after $(NH_4)_2SO_4$ fractionation (or column chromatography [32]) was twice precipitated with water, minor impurities were present (fig.1A), which did not affect myosin ATPase activity but had effects on calcium binding, and the stoichiometry of light to heavy chains. These contaminants were visible on 6% polyacrylamide gels, (fig.1A_1, A_2) and on gradients of polyacrylamide (fig.1A_3, A_4). The impurities which were removed with two water precipitations were lyophilized and analyzed; on 6% polyacrylamide (SDS) gels some of the contaminants had similar mobilities to myosin light chains (fig.1B_1, B_2) but on gradients of polyacrylamide (SDS) (fig.1B_{3-5}) they were shown to have different molecular weights as compared to myosin light chains. When studies on the stoichiometry of light to heavy chains were made by dye elution [33], using polyacrylamide gradient gels, no differences were found in the proportion of light to heavy chains between the two ventricles.

Using the methods and equilibrium constants reported here, determined according to Scatchard-plot analyses, the moles of calcium bound by rabbit skeletal muscle myosin (fig.2A) was the same as that reported in literature [34]. Many Ca^{2+} binding analyses were made but only representative plots are shown*. Left ventricular myosin from dogs (fig.2B) and sheep

*The binding of calcium, using purified tropomyosin [35] was examined, however the number of calcium binding sites, as observed by Fuchs and Briggs [36] was too low to be significant.

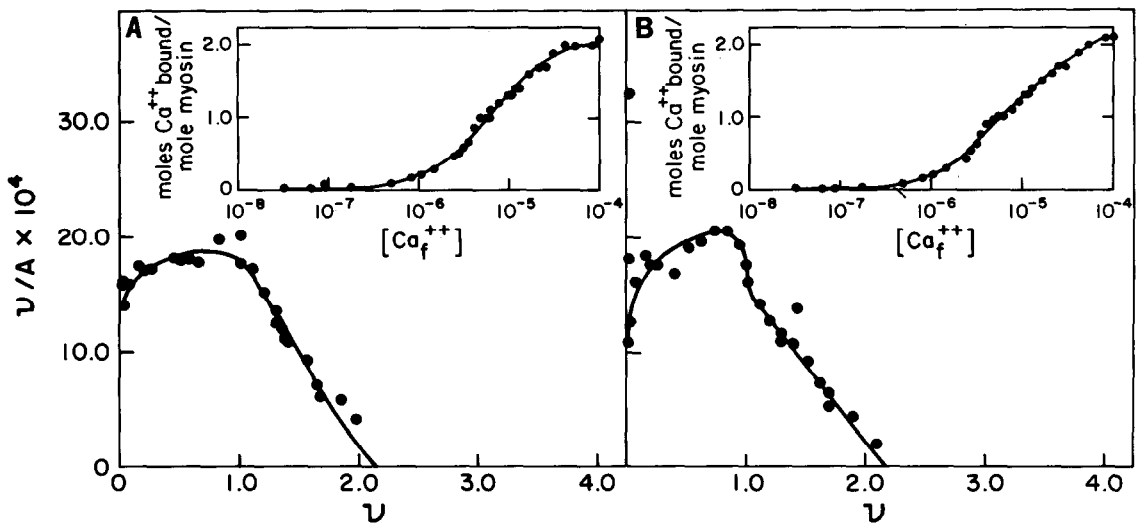

Fig.2. Representative Scatchard-plots for the binding of calcium by (A) rabbit skeletal muscle myosin. (B) dog left ventricular myosin. Values for these curves are given in table 1, columns 1 and 2. The inserts show these same calcium binding data treated according to the least-squares best-fit program as described by Edsall and Wyman [18] for determination of interaction between the calcium binding sites. Further data for these curves are given in table 1, columns 3 and 4. The molecular weight of rabbit skeletal myosin used was 500 00 [39] and for left ventricular myosin 524 00 [40].

Table 1

Tissue	Scatchard-plots		Plots for molecular interaction according to [18]			
	n	Affinity constant ($\times 10^5$)	K_1 ($\times 10^5$)	K_2	K_3	(SD)
Rabbit skeletal muscle	2.1	1.6	1.9	1.1	0.018	0.07
Dog left ventricle	2.2	1.3	2.2	1.1	0.033	0.08

bound approximately the same moles of calcium per mole of myosin as compared to rabbit skeletal muscle myosin.

To study further if the upward swing observed here in the Scatchard-plots and described previously by Bremel and Weber [7] was due to positive cooperativity in the binding of calcium by myosin, the data were treated according to the methods of Edsall and Wyman [18]. Using this equation of Edsall and Wyman for determination of interaction between binding sites, a least squares best fit program was used (fig.2, Insets). The points shown are the observed values and the curve is that for the calculated values. For all determinations $K_3 << K_1$ and K_2 and $K_1 \simeq K_2$, thus there appeared to be two binding sites with interaction between them (table 1), an observation similarly noted in the Scatchard-plots. Since the Mg^{2+} contamination was less than 2×10^{-7} M for all studies, the positive cooperativity did not appear to be due to the presence of Mg^{2+}. Similar positive cooperativity was also noted in atrial and right ventricular myosins.

Myosin may possibly have two classes of binding sites (fig.3). It appears that the number of high affinity binding sites are the same in all myosins. A thorough study has not been made of the low affinity binding sites and whether or not these low affinity binding sites may differ in the various myosins.

In conclusion, it is important to water precipitate purified cardiac myosin at least twice to remove contaminants which interfere with calcium binding and subunit stoichiometry, but not myosin ATPase activity. According to electrophoretic mobility using dodecylsulfate gels, these undefined contaminants are not troponin nor tropomyosin. According to the data obtained using a best fit program developed according to the equation of Edsall and Wyman [18], the upward swing observed in the Scatchard-plots appears to be indicative of positive cooperativity [7]. Furthermore, the positive cooperativity observed in the high affinity calcium binding sites appears to be present in the various cardiac myosins, namely left ventricular myosin, right ventricular myosin, and atrial myosin, as well as skeletal muscle myosin. This positive cooperativity may exist without any Mg^{2+} ions present.

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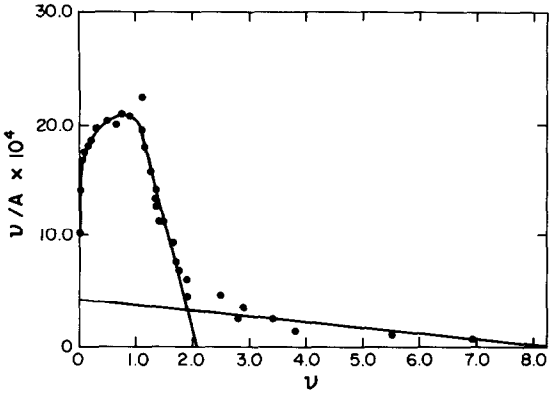

Fig.3. Scatchard-plot for Ca^{2+} binding of canine left ventricular myosin showing 2 classes of binding sites.

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References

[1] Jakes, R., Northrop, F. and Kendrick-Jones, J. (1976) FEBS Lett. 70, 229–234.
[2] Collins, J. H. (1976) Nature 259, 699–700.
[3] Leger, J. J. and Elzinga. (1977) Biochem. Biophys. Res. Commun. 74, 1390–1396.
[4] Morimoto, K. and Harrington, W. F. (1974) J. Mol. Biol. 88, 693–709.
[5] Werber, M. M., Gaffin, S. L. and Oplatka, A. (1972) J. Mechanochem. Cell Motility 1, 91–96.
[6] Beinfeld, M. C., Bryce, D. A., Kochavy, D. and Martonosi, A. (1975) J. Biol. Chem. 250, 6282–6287.
[7] Bremel, R. D. and Weber, A. (1975) Biochim. Biophys. Acta 376, 366–374.
[8] Long, L., Fabian, F., Mason, D. T. and Wikman-Coffelt, J. (1977) J. Biochem. Biophys. Res. Commun. in press.
[9] Wikman-Coffelt, J., Zelis, R., Fenner, C. and Mason, D. T. (1973) Biochem. Biophys. Res. Commun. 51, 1097–1104.
[10] Wikman-Coffelt, J., Walsh, R., Fenner, C., Kamiyama, T., Salel, A. and Mason, D. T. (1975) Biochem. Med. 13, 33–41.
[11] Fabian, F. and Muhlrad, A. (1968) Biochim. Biophys. Acta 162, 596–603.
[12] Potter, J. D. and Gergely, J. (1975) J. Biol. Chem. 250, 4628–4633.
[13] Raaflaub, J. (1956) in: Application of metal buffers and metal indicators in biochemistry (Glick, D. ed) Methods of Biochemical Analysis, Vol. 3, pp. 301–325, New York.
[14] Fenner, C., Mason, D. T., Zelis, R. and Wikman-Coffelt, J. (1973) Proc. Natl. Acad. Sci. USA 70, 3205–3209.
[15] Beinfeld, M. C., Bryce, D. A., Kochavy, D. and Martonosi, A. (1975) J. Biol. Chem. 250, 6282–6287.
[16] Ogawa, Y. (1968) J. Biochem. 64, 255–257.
[17] Scatchard, G. (1949) Ann. NY Acad. Sci. 51, 660–672.
[18] Edsall, J. T. and Wyman, J. (1958) in: Thermodynamics, electrostatics, and the biological significance of the properties of matter (Edsall, J. T. and Wyman, J. eds) Biophysical Chemistry, Vol. 1, pp. 623–626, Academic Press, New York.
[19] Rovetto, M. J., Hjalmarson, A. C., Morgan, H. E., Barrett, M. J. and Goldstein, R. A. (1972) Circ. Res. 31, 397–409.
[20] Szent-Gyorgy, A. (1947) in: Chemistry of Muscular Contraction, Academic Press, New York.
[21] Trayer, I. P. and Perry, S. V. (1966) Biochem. Zeit. 345, 87–100.
[22] Gazith, J., Himmelfarb, S. and Harrington, W. F. (1970) J. Biol. Chem. 245, 15–22.
[23] Rees, M. K. and Young, M. (1967) J. Biol. Chem. 242, 4449–4458.
[24] Tsao, T. C. (1953) Biochim. Biophys. Acta 11, 368.
[25] Wilkinson, J. M., Perry, S. V., Cole, H. A. and Trayer, I. P. (1972) Biochem. J. 127, 215–228.
[26] Swynghedauw, B., Klotz, C., Leger, J. J. and Preteseille, M. (1973) J. Mol. Cell. Cardiol. 5, 501–514.
[27] Katz, A. M., Repke, D. I. and Rubin, B. B. (1966) Circ. Res. 19, 611–621.
[28] Kleid, J. J., Rada, M., Repke, D. I. and Katz, A. M. (1972) J. Mol. Cell. Cardiol. 4, 625–632.
[29] Mueller, H., Franzen, J., Rice, R. V. and Olson, R. E. (1964) J. Biol. Chem. 239, 1447–1456.
[30] Luchi, R. J. and Kritcher, M. (1966) Circ. Res. 19, 283–294.
[31] Khaw, B. A., Beller, G. A. Haber, E. and Smith, T. W. (1976) J. Clin. Inves. 58, 439–446.
[32] Sarkar, S., Sreter, F. A. and Gergely, J. (1971) Proc. Natl. Acad. Sci. USA 68, 946–950.
[33] Fenner, C., Traut, R. R., Mason, D. T. and Wikman-Coffelt, J. (1975) Analyt. Biochem. 63, 595–602.
[34] Morimoto, K. and Harrington, W. F. (1974) J. Molec. Biol. 88, 693–709.
[35] Fenner, C., Valentine, R., Mason, D. T. and Wikman-Coffelt, J. (1975) Prep. Biochem. 5, 189–197.
[36] Fuchs, F. and Brigss, N. (1968) J. Gen. Physiol. 51, 655–675.
[37] O'Farrell, P. H. (1975) J. Biol. Chem. 250, 4007–4021.
[38] Weber, K. and Osborn, M. (1969) J. Biol. Chem. 244, 4406–4412.
[39] Gershman, L. C., Stracher, A. and Dreizen, P. (1969) J. Biol. Chem. 244, 2726–2735.
[40] Smith, A., Mason, D. T. and Wikman-Coffelt, J. (1974) FEBS Lett. 43, 104–106.