Studies on the Electrophoretic Mobility of Troponin in the Presence of Ca^{2+}, Mg^{2+}, and $(Mg\text{-}ATP)^{2-}$ [1] (34606)

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The regulation of actomyosin contraction by low concentrations of Ca^{2+} ($\sim 10^{-5} M$) involves the participation of troponin, tropomyosin, Mg^{2+} and ATP[3] (1–5). The precise mechanism of action of the "Ca sensitizing factor" (troponin, tropomyosin) is unknown. Under certain experimental conditions however, the binding of 10^{-6}–10^{-5} M Ca^{2+} to the myofibrils initiated contraction (6). Fuchs and Briggs (5) have shown conclusively that troponin is the only known myofibrillar protein which bound Ca^{2+} if the concentration of this ion was 10^{-5} M. The removal of Ca^{2+} from a myofibrillar binding site and/or its replacement by Mg^{2+} was shown to occur in the relaxed state (2). Kaminer found that the "Ca sensitizing factor" required the presence of equimolar quantities of ATP and Mg^{2+} (5 mM) to produce maximal effect on actomyosin (4). In view of all these results, it appeared to us that troponin may be the only known site on the myofibrils suitable for a Ca^{2+}–Mg^{2+} exchange during the contractile event. For this reason we have investigated in a rather qualitative fashion whether or not troponin is able to bind Mg^{2+}.

If a divalent metal is bound to an ionized carboxyl group of a protein, it will change the charge of this group from $(COO)^-$ to $(COOMe)^+$. For this reason it is likely that the net charge of this protein will also change. In the subsequently described experiments we have studied the net surface charge alterations on troponin in the presence and absence of Ca^{2+}, Mg^{2+}, ATP^{4-}, and $(Mg\text{–}ATP)^{2-}$. The electrophoretic mobility was used as a qualitative or perhaps semiquantitative indicator of the surface charge on this protein.

Materials and Methods. The sodium salt of ATP was purchased from Sigma Co.; all other reagents were of reagent grade. Troponin was prepared and tested as described by Ebashi (3). Electrophoretic experiments were carried out in a microelectrophoretic apparatus supplied by Arthur Thomas Co. as described in an earlier paper (7). The total volume of liquid in the electrophoretic cell was 50 ml and always contained 0.05 M Tris buffer (pH 7.5) and 0.08 M KCl. Troponin was adsorbed for 3 hr at 25° to chromatographic quality alumina particles having an average particle size of 4–5 μ (manufactured by Merck and Co.) and used in the form of a standard suspension. Under these exeprimental conditions a protein monolayer was formed on the alumina particles (8) and the electrophoretic migration of the various troponin preparations was identical. Each experimental point is the average of 10 determinations.

Results. Figure 1 shows that the electrophoretic migration of troponin increased if EGTA, or alternatively EDTA, was added to the mixtures. These results may be attributed to the increased net negative charge on the

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[3] The following abbreviations are used in the present paper: ATP = adenosine triphosphate; EDTA = ethylenediaminetetraacetic acid; EGTA = 1,2-bis(dicarboxymethylaminoethyoxy)-ethane (ethylene glycol) bisaminoethyl ethers N,N^1-tetraacetic acid; $_s\mu$/V/cm/sec = microns per volt per centimeter per second; p ME^2 = negative logarithm of the free metal concentration.

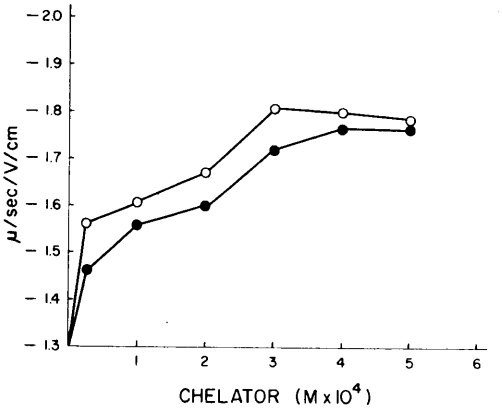

FIG. 1. Effect of chelating agents on the electrophoretic mobility of troponin: Each solution contained 1.5 ml of standard suspension (troponin adsorbed to alumina); 8×10^{-2} *M* KCl; and 5×10^{-2} *M* Tris (pH 7.5) with the following additions: (○), EGTA as specified; (●), EDTA as specified; Total final volume was 50 ml.

protein in the presence of chelators. Since the various reagents (KCl, ATP, etc.) always contain small amounts of Ca^{2+} and Mg^{2+} as contaminants ($\sim 10^{-6}$ *M*), the effect of these chelating agents may be the consequence of the removal of Ca^{2+} and Mg^{2+} from troponin. It is interesting to note that EDTA was no more effective than EGTA in increasing the electrophoretic mobility of troponin. This could be explained if troponin were to bind Ca^{2+} preferentially when both Ca^{2+} and Mg^{2+} were present only as "contaminants."

Next we studied the effect of various well-controlled concentrations of free Ca^{2+} and Mg^{2+} on the electrophoretic mobility of troponin. The effect of Ca^{2+} was studied in the presence of a 5×10^{-4} *M* EGTA–Ca buffer (9). To study the effect of Mg^{2+}, the solutions were rendered Ca^{2+}-free by the addition of 5×10^{-4} *M* EGTA. A 5×10^{-4} *M* EDTA–Mg buffer was used to ensure the proper free Mg^{2+} concentration (10). Figure 2 shows that both Ca^{2+} and Mg^{2+} in concentration higher than 10^{-8} *M* decreased the electrophoretic mobility of troponin. These results indicated that the net negative surface charge on the protein was reduced equally by the binding of Ca^{2+} and Mg^{2+}.

Figure 3 shows that the addition of 5 m*M* ATP to troponin in an essentially Ca^{2+}-free solution increased the net negative surface charge of this protein. As Fig. 1 shows the maximal electrophoretic mobility of troponin, in a medium free of divalent metals, was 1.7 μ/sec/V/cm. Since in the presence of 5 m*M* ATP the mobility of troponin was 2.5 μ/sec/V/cm, the effect of ATP cannot be explained simply on the basis of Me^{2+} binding. Indeed this result showed that $(ATP)^{4-}$ was bound to troponin. On the other hand the electrophoretic mobility of troponin decreased if Mg^{2+} was also added together with 5 m*M* ATP to this protein.

In the following experiments (Fig. 4) we studied the effect of increasing concentrations $(ATP–Mg)^{2-}$ on the electrophoretic mobility of troponin. If the Ca^{2+} concentration of these mixtures was kept at 10^{-9} *M*, the addition of 1 m*M* or more $(Mg–ATP)^{2-}$ decreased the electrophoretic mobility of troponin. On the other hand, if the Ca^{2+} concentration of the solutions was 10^{-7} *M* or higher, the addition of $(Mg–ATP)^{2-}$ up to 7 m*M* concentration did not change the electrophoretic mobility of this protein. The association constant of ATP–Mg is about 36,000 (11) and, therefore, in the presence of

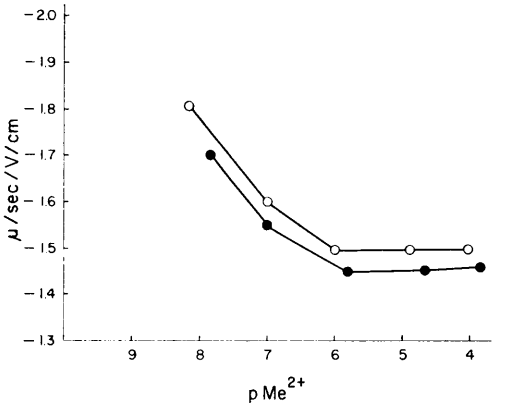

FIG. 2. Effect of Ca^{2+} on the electrophoretic mobility of troponin: Each solution contained 1.5 ml of standard suspension; 8×10^{-2} *M* KCl; and 5×10^{-2} *M* Tris (pH 7.5) to which the following were added: (○), 5×10^{-4} *M* Ca–EGTA buffer (the free Ca^{2+} concentration is specified on the abscissa); (●), 5×10^{-4} *M* EGTA and 5×10^{-4} *M* Mg–EDTA buffer (the free Mg^{2+} concentration is specified on the abscissa). Total final volume was 50 ml.

equimolar ATP and Mg^{2+}, $(Mg–ATP)^{2-}$ is the predominant ionic species. Under these experimental conditions the increase of $(Mg–ATP)^{2-}$ concentration never increased the electrophoretic mobility of troponin. Therefore, it seems to be unlikely that $(Mg–ATP)^{2-}$ was bound to this protein. On the other hand, these results would seem to indicate that the small amount of free Mg^{2+} ($\sim 10^{-6}$ *M*) in these mixtures was bound to troponin in the presence of m*M* concentration of $(Mg–ATP)^{2-}$ if the Ca^{2+} concentration was kept lower than 10^{-8} *M*.

Discussion. The muscle contains Mg^{2+} and $(Mg\text{-}ATP)^{2-}$ in m*M* concentration (12) while the Ca^{2+} concentration changes between $\sim 10^{-9}$ and $\sim 10^{-5}$ *M*. Our electrophoretic measurements (Figs. 2 and 4) showed that both Mg^{2+} and Ca^{2+} were able to alter the surface charge on troponin. Under similar experimental conditions, $(Mg–ATP)^{2-}$ had no such effect on this protein. The results depicted in Figs. 1, 2, and 4 would seem to support the view that an intermittent binding of Ca^{2+} and Mg^{2+} to troponin depending upon the available Ca^{2+} concentration is a distinct possibility.

Let us assume that the binding sites of troponin for Ca^{2+} and Mg^{2+} are not identical but still close to each other. This arrangement would account for the mutual influence these ions appear to exert on each other's

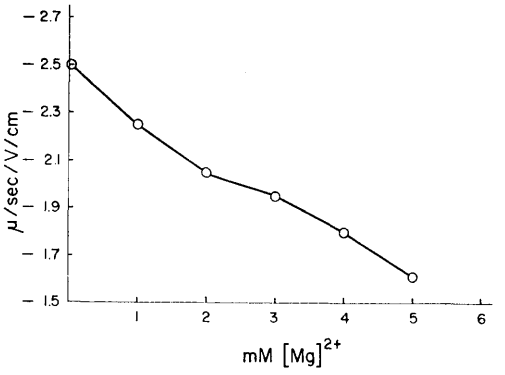

FIG. 3. Effect of Mg^{2+} on the electrophoretic mobility of troponin in the presence of ATP^{4-}: Each solution contained 1.5 ml of standard suspension; 5×10^{-4} *M* EGTA; 5×10^{-3} *M* ATP; 8×10^{-2} *M* KCl; 5×10^{-2} *M* Tris (pH 7.5); and a specified amount of Mg^{2+}. Total final volume was 50 ml.

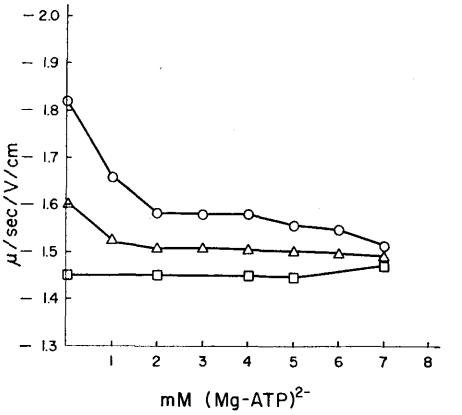

FIG. 4. Effect of $(Mg–ATP)^{2-}$ on the electrophoretic mobility of troponin: Each solution contained 1.5 ml of standard suspension; 8×10^{-2} *M* KCl; 5×10^{-2} *M* Tris (pH 7.5); and a specified amount of $(Mg–ATP)^{2-}$ to which the following additions were made: (○), 5×10^{-4} *M* EGTA; (△), 5×10^{-4} *M* EGTA–Ca buffer having 1×10^{-7} *M* free Ca^{2+}; (□) 5×10^{-7} *M* EGTA–Ca buffer having 1×10^{-6} *M* free Ca^{2+}. Total final volume was 50 ml.

binding. During contraction the intracellular Ca^{2+} concentration is raising and Ca^{2+} displaces Mg^{2+} from troponin. The result is that the Ca^{2+} binding sites of this protein are positively charged $(COO–Ca)^{+}$ and the Mg^{2+} binding sites are negative $(COO)^{-}$. During relaxation the intracellular Ca^{2+} concentration falls; consequently the troponin bound Ca^{2+} is released and Mg^{2+} may bind again to this protein. Therefore in the relaxed state the Ca^{2+} binding sites of troponin are negative $(COO)^{-}$ and the Mg^{2+} binding sites are positively charged $(COOMg)^{+}$. These local charge fluctuations on the troponin molecule close enough to the interacting surfaces of actin and myosin may indeed influence the nature of the interactions between actin and myosin and consequently regulate the contraction–relaxation process. Previous work in this laboratory has shown that alteration of surface charge on myofibrils may produce both contraction and relaxation with these muscle models (7).

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