COMPARISON OF THE REACTION OF N-ETHYLMALEIMIDE WITH MYOSIN AND HEAVY MEROMYOSIN SUBFRAGMENT 1

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Summary. Myosin reacted at low ionic strength with NEM forms an actomyosin which is Ca^{++} insensitive. With HMM S-1 the reaction with NEM causes a marked loss of the actin activated ATPase activity and the Ca^{++} sensitivity is reduced but not eliminated. The presence of actin during the sulfhydryl reaction does not significantly alter this result. HMM S-1 prepared from myosin previously desensitized by NEM regains Ca^{++} sensitivity. These results indicate that the conformations of myosin and HMM S-1 are different and could reflect a difference between insoluble (filamentous) myosin and myosin, or its fragments, in solution.

Introduction. The ability of sulfhydryl reagents to remove the Ca^{++} sensitivity of natural actomyosin was recognized several years ago (1-3) and it is now generally agreed that the reactive groups are located on the myosin molecule. This effect, therefore, has generated some interest as it indicated that myosin could be involved in the regulatory process (4-6) and it became necessary to define the effect further. Initially various modifications of the sulfhydryl reaction were tested and the optimum conditions for the removal of Ca^{++} sensitivity were established (4). Next the obvious question was what caused the effect. One hypothesis which had arisen from our earlier work was that the loss of Ca^{++} sensitivity was related in some way to a conformational change on the myosin molecule elicited by the sulfhydryl reaction. During experiments designed to test this theory, it was unexpectedly found that the soluble fragments of myosin, i.e., HMM* and HMM S-1, did not respond the same as myosin when treated with NEM. These results are presented below, and

* Abbreviations: HMM, heavy meromyosin; HMM S-1, heavy meromyosin subfragment 1; NEM, N-ethylmaleimide; EGTA, 2,2-ethylenedioxybis (ethylimino diacetic acid).

could illustrate a difference in the conformations of insoluble (filamentous) myosin and the soluble fragments of myosin.

Materials and Methods. HMM was prepared using chymotrypsin by the method of Perry and Leadbeater (7). HMM S-1 was prepared from myosin by the method of Lowey, et al. (8), and from desensitized actomyosin as follows: desensitized actomyosin in 0.5 MKCl, 10 mM tris-HCl (pH 7.6) was digested with insoluble papain (8) (at an approximated weight ratio of 20 parts of actomyosin to 1 part papain) for 12 min. at 25°. The insoluble papain was removed by centrifugation at 30,000 x g for 15 minutes. The supernatant was dialyzed versus 10 volumes of 10 mM tris-HCl (pH 7.6), and then centrifuged at 30,000 x g for 10 minutes. This supernatant contained the HMM S-1, and following dialysis against 10 mM tris-HCl (pH 7.6) was used without further purification. The preparation did not contain any undigested myosin as demonstrated by electrophoresis in sodium dodecyl sulfate.

Proteins were reacted with NEM at 25° for 30 minutes, and the reaction was stopped by the addition of at least a 10-fold excess of dithiothreitol. The amount of NEM used was varied and is indicated on the figures.

The preparation of other proteins and the assay of ATPase activity was as described previously (4).

Results and Discussion. When HMM S-1 was reacted with different levels of NEM and then combined with actin, troponin and tropomyosin, the results were as shown in Figure 1. The Mg^{++} activated ATPase activity in the presence of low levels of Ca^{++} decreased rapidly and approached the level of activity obtained in the absence of Ca^{++} (i.e., plus EGTA). However, the effect of the troponin-tropomyosin complex was never completely abolished and was decreased only at very low levels of actin activation. Essentially the same pattern was obtained with HMM. These results are similar to those reported recently by Silverman, et al. (9) who found a marked reduction of actin activation on reaction of HMM with NEM at high ionic strength. However, they are different from those obtained with myosin which was reacted and assayed similarly to

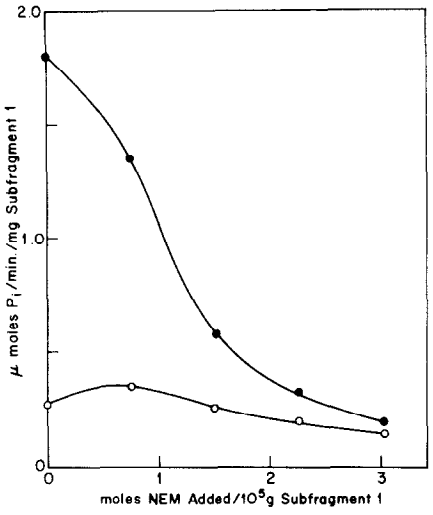

Fig. 1.

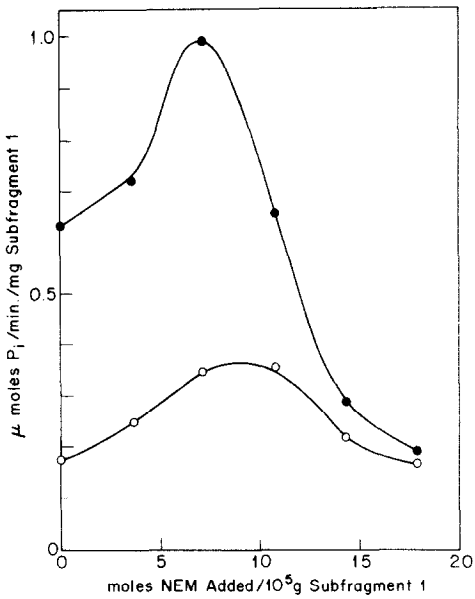

Fig. 2.

Figure 1: The reaction of NEM with HMM S-1. Conditions of the NEM reaction were: HMM S-1 (2.75 mg/ml) in 10 mM tris-HCl (pH 7.6), 25°, 30 minutes. ATPase activity was assayed in 25 mM tris-HCl (pH 7.6) 2.5 mM ATP, 2.5 mM $MgCl_2$ (●), and in tris medium plus 1 mM EGTA (○). Each assay point contained HMM S-1 (275 μg), actin (500 μg), troponin (225 μg), tropomyosin (225 μg).

Figure 2: The reaction of NEM with acto-HMM S-1. Condition of the NEM reaction were: HMM S-1 (1.17 mg/ml), actin (2.35 mg/ml) in 10 mM tris-HCl (pH 7.6) 25°, 30 minutes. Each assay point contained HMM S-1 (470 μg), actin (940 μg), troponin (400 μg), tropomyosin (400 μg). ATPase activity was assayed in the Mg^{++} medium (●), and in the Mg^{++} EGTA medium (○).

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the HMM S-1. In the case of myosin (Fig. 2) the Ca^{++} sensitivity was lost while retaining a reasonably high level of actin activation (about 40% of the original control level). The effect of NEM on myosin has been reported earlier (4) but is included here for ease of comparison.

The reaction of HMM S-1 in the presence of excess actin was then tested, and the results are shown in Figure 3. The shape is essentially the same as in Figure 1, with the possible exception that the Ca^{++} sensitivity was further reduced. Again at low levels of Ca^{++} sensitivity the actin activation was significantly lower than the control value.

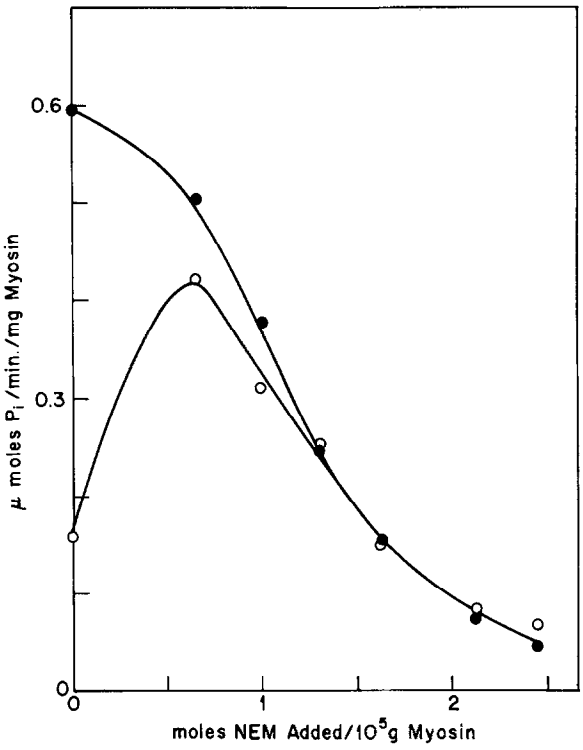

Figure 3: The reaction of NEM with myosin. Conditions of the NEM reaction were: myosin (2.56 mg/ml) in 10 mM tris-HCl (pH 7.6) 25°, 30 minutes. Each assay point contained myosin (510 μg), actin (100 μg), troponin (50 μg), tropomyosin (50 μg). ATPase activity was assayed in the Mg^{++} medium (●), and in the Mg^{++} EGTA medium (○).

The counterpart of the last curve, i.e., using actomyosin is shown in Figure 4. Here the Ca^{++} sensitivity was rapidly lost, and at the point of desensitization the activation by actin was roughly the same as control. This pattern was never observed with HMM S-1 where the actin activation was lost more readily. To explain the differences between the myosin and HMM S-1 systems, two explanations could be offered: 1) that different sites on myosin and HMM S-1 were being labelled by the NEM, or, 2) that the effects were due to different conformations of the two proteins. To distinguish between these two possibilities the following experiment was performed. Desensitized actomyosin was labelled with varying levels of NEM (the samples were those shown in Figure 4). HMM S-1 was then prepared from the labelled actomyosin samples using the procedure described earlier in Materials and Methods. The

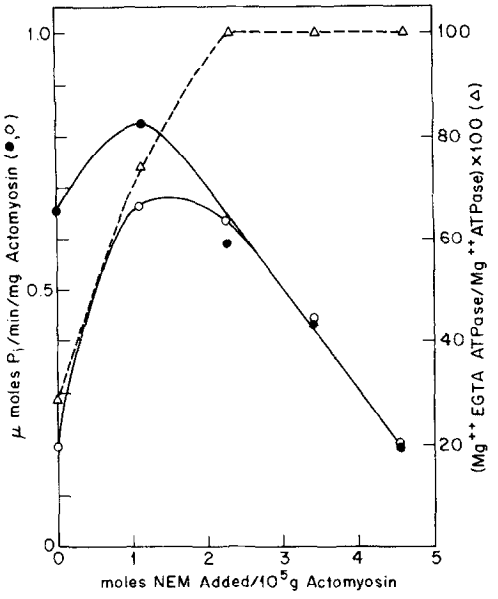

Fig. 4.

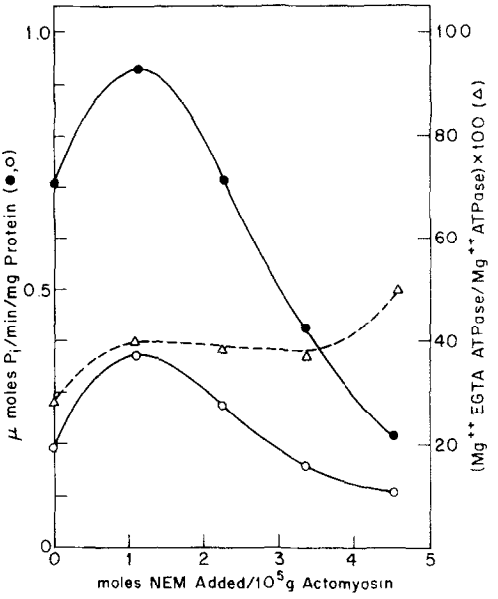

Fig. 5.

Figure 4: The reaction of NEM with desensitized actomyosin. Conditions of the NEM reaction were: actomyosin (3.65 mg/ml) in 10 mM tris-HCl (pH 7.6) 25°, 30 minutes. Each assay point contained actomyosin (365 μg), troponin (50 μg), tropomyosin (50 μg). ATPase activity was assayed in the Mg^{++} medium (●) and Mg^{++} EGTA medium (○). The ratio of these two activities (i.e., an indication of the Ca^{++} sensitivity) is also shown (△).

Figure 5: The Ca^{++} sensitivity of HMM S-1 prepared from NEM reacted desensitized actomyosin. HMM S-1 was prepared (see Materials and Methods) from the actomyosin samples shown in Figure 4. Each assay point contained HMM S-1 (400 μg), actin (400 μg), troponin (200 μg), tropomyosin (200 μg). ATPase activity was assayed in the Mg^{++} medium (●) and in the Mg^{++} EGTA medium (○). The ratio of these two activities is also shown (△).

HMM S-1 was combined with actin, troponin and tropomyosin and assayed for Ca^{++} sensitivity. The results are shown in Figure 5. The Ca^{++} sensitivity, indicated by the broken line was only slightly reduced and was definitely different from the parent NEM labelled actomyosin shown in Figure 4. It is clear that the NEM-myosin which was insensitive to the effect of troponin and tropomyosin was converted to a Ca^{++} sensitive state following proteolysis with papain.

None of the NEM labelled samples shown in Figures 4 and 5, showed an activation of the Ca^{++} activated ATPase activity at high ionic strength, which is associated with the labelling of the S_1 group. Although the K^+-EDTA moderated ATPase was inhibited at the higher NEM levels.

It was shown previously (4) that myosin separated from NEM desensitized actomyosin retained the properties of the parent actomyosin, i.e., when mixed with actin, troponin and tropomyosin yielded a Ca^{++} insensitive system. This was not the case with HMM S-1 prepared from Ca^{++} insensitive actomyosin, and since the labelling by NEM was identical in both cases, it indicates different conformations for the myosin and HMM S-1. This was also reflected in the effect of NEM on acto-HMM S-1 and actomyosin (Figs. 3 and 4), Ca^{++} sensitivity was easily removed with actomyosin but not with acto-HMM S-1. It is unlikely that the difference was due to the "single headed" nature of HMM S-1, as the double headed HMM behaved similarly. In this regard, therefore, cooperativity between the two heads of myosin appears not to be involved.

A difference in conformation between the HMM S-1 and myosin might be due to several reasons. We will consider the two that appear to us to be most likely. These are a) the difference in solubility at low ionic strength, and b) the existence of cleaved peptide bonds in the HMM S-1. It is interesting that myosin in the soluble state (i.e., at high ionic strength) does not lose Ca^{++} sensitivity when combined to form actomyosin, and although this is not conclusive, it does support the first of the two possibilities. Thus, we would like to propose that myosin in the insoluble or filamentous state is subject to different conformational constraints than soluble myosin or its soluble fragments.

These results also illustrate a general point, namely that it is dangerous to compare the effects of sulfhydryl reagents on different protein preparations and/or under different solvent conditions, since the conformation of the protein determines to some extent the subsequent effect of labelling.

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References

1. Tonomura, Y. and Yoshimura, J., Arch. Biochem. Biophys. 90, 73 (1960).
2. Maruyama, K. and Ishikawa, Y., Annot. Zool. Japan. 37, 134 (1964).
3. Kominz, D. R., Arch. Biochem. Biophys. 115, 583 (1966).
4. Daniel, J. L. and Hartshorne, D. J., Biochem. Biophys. Acta 278, 567 (1972).
5. Weber, A. and Bremel, R. D., in Contractility of Muscle Cells and Related Processes. R. J. Podolsky Ed. The Society of General Physiologists - Woods Hole, 1970. Prentice-Hall, Inc. N.J.
6. Stewart, J. M. and Levy, H. M., J. Biol. Chem. 245, 5764 (1970).
7. Leadbeater, L. and Perry, S. V., Biochem. J. 87, 233 (1963).
8. Lowey, S., Slayter, H. S., Weeds, A. G., and Baker, H., J. Mol. Biol. 42, 1 (1969).
9. Silverman, R., Eisenberg, E., and Kielley, W. W., Nature New Biology 240, 207 (1972).