NATIVE TROPOMYOSIN: EFFECT ON THE INTERACTION OF ACTIN WITH HEAVY MEROMYOSIN AND SUBFRAGMENT-1

Evan Eisenberg and W. Wayne Kielley
Section on Cellular Physiology, Laboratory of Biochemistry, National Heart and Lung Institute, National Institutes of Health, Bethesda, Md. 20014

Received May 18, 1970

SUMMARY. In the absence of Ca^{++}, native tropomyosin markedly inhibits the acto-subfragment-1 ATPase as well as the acto-heavy meromyosin ATPase despite the fact that subfragment-1, in contrast to heavy meromyosin, has only a single site for ATP. Double reciprocal plots of acto-heavy meromyosin ATPase vs. actin concentration both in the presence and absence of native tropomyosin, show that the native tropomyosin has little effect on the acto-HMM-ATPase but acts mainly by affecting the binding of actin to the HMM-ATP complex.

It is now generally accepted that contraction of muscle is triggered *in vivo* by release of Ca^{++} from the sarcoplasmic reticulum (1,2). This Ca^{++} binds to native tropomyosin along the actin filament, which in turn allows the actin and myosin filaments to interact in such a way that contraction occurs (2,3,4). In the absence of Ca^{++}, on the other hand, the actin-myosin interaction which causes contraction is inhibited by the native tropomyosin and the muscle relaxes (1,2). Analogously *in vitro*, in the absence of Ca^{++}, native tropomyosin inhibits both superprecipitation of actomyosin and the acto-myosin ATPase activity, whereas in the absence of native tropomyosin, Ca^{++} has no effect (2,5).

None of these studies made it clear whether the native tropomyosin is reducing the binding of actin to myosin-ATP, the turnover rate of the acto-myosin-ATP complex or perhaps both. This question is very difficult to approach with actomyosin because it is in the precipitated state at low ionic strength, but with heavy meromyosin (HMM) linear double reciprocal plots of ATPase vs. actin concentration can be obtained and therefore the binding of the actin to the HMM-ATP complex (K_a) and the turnover rate of the acto-HMM-ATP complex (Vmax) can be determined independently (6). In a recent model of muscle contraction, one of the authors proposed that *in vivo* in the absence of Ca^{++}, native tropomyosin blocks the binding of actin to myosin-ATP

rather than the turnover rate of the actomyosin-ATP complex (7). Using the reciprocal plot technique with acto-HMM, however, Szentkiralyi and Oplatka reported preliminary results which suggest that tropomyosin affects Vmax rather than Ka(8).

In the present study we have investigated this question using HMM and have also investigated whether the effect of native tropomyosin can be demonstrated with subfragment-1 (S-1), a further tryptic digestion product of HMM which, in contrast to the two ATP binding sites on HMM, has only a single site for ATP (9,10). We find that the native tropomyosin affects both S-1 and HMM suggesting that the two ATP sites on HMM are not required for the effect of native tropomyosin. We further find that the major effect of native tropomyosin is on the binding of actin to HMM-ATP, with little if any effect on the turnover rate of the acto-HMM-ATP complex.

MATERIALS AND METHODS

Myosin was prepared by the method of Kielley and Harrington (11). HMM and S-1 were also prepared as described previously using G-200 Sephadex gel filtration to purify the S-1 (7,12). Actin was prepared using a Sephadex G-200 column as described by Adelstein et al. (13). The resulting G-actin was then polymerized to F-actin, pelleted twice by centrifugation at 30,000 RPM and finally taken into solution as described previously (12). Native tropomyosin was prepared as described by Hartshorne and Mueller (14) with the exception that 5mM 2-mercaptoethanol rather than 2mM dithiothietol was used in all solutions. ATPase was measured at 25°C, pH=7.0 using a pH-stat as described previously (12). Protein concentrations were also measured as described previously (7). The extinction coefficient used for native tropomyosin was 380 cm^2/g at 278mμ (14).

RESULTS AND DISCUSSION

Table I shows the effect of Ca^{++} and ethyleneglycol-bis(aminoethylether) tetraacetic acid (EGTA) on the ATPase activity of acto-HMM and acto-S-1 both with and without native tropomyosin present. In the absence of native

TABLE I

Effect of Native Tropomyosin on Acto-HMM and Acto-S-1 ATPase

Protein	ATPase (μmole/mg-min) Excess EGTA	Excess Ca^{++}
Acto-HMM	1.48	1.36
Acto-HMM+Native tropomyosin	0.09	2.26
Acto-S-1	0.85	0.82
Acto-S-1+Native tropomyosin	0.16	1.00

For the experiment with HMM all samples contained 1.6mM ATP, 0.8mM $MgCl_2$, 18mM KCl, and 0.4 mg/ml actin. There was 0.24 mg/ml HMM in the sample with excess EGTA and native tropomyosin, and 0.12 mg/ml HMM in the other samples. Native tropomyosin when added equaled 0.5 mg/ml. Samples with excess EGTA contained 5.0mM EGTA and samples with excess Ca^{++} contained 5.0mM EGTA and 5.2mM $CaCl_2$. For the experiments with S-1 all samples contained 2mM ATP, 1mM $MgCl_2$, 20mM KCl 0.8 mg/ml actin, and 0.20 mg/ml S-1. Native tropomyosin when added equaled 1.0 mg/ml. Samples with excess EGTA contained 1mM EGTA and samples with excess Ca^{++} contained 0.5mM $CaCl_2$.

tropomyosin the acto-HMM ATPase was essentially the same whether excess Ca^{++} or EGTA was present. This was also true for the acto-S-1 ATPase. In the presence of native tropomyosin with excess Ca^{++} also present, the native tropomyosin increased both the acto-HMM and acto-S-1 ATPases. A similar activating effect of native tropomyosin in the presence of Ca^{++} was reported by Hartshorne and Mueller for the actomyosin ATPase (15). However it was in the absence of Ca^{++}, that the major effect of the native tropomyosin occurred for, as can be seen, with EGTA present the native tropomyosin caused marked inhibition of both the acto-HMM and acto-S-1 ATPases. Thus it is clear that native tropomyosin not only affects the acto-HMM ATPase, as has previously been reported by Kominz (16) but also affects the acto-S-1 ATPase despite the fact that S-1 has only a single site for ATP.

We next investigated the effect of native tropomyosin on the double reciprocal plot of acto-HMM ATPase vs. actin concentration. Clearly this experiment cannot be performed by adding a constant amount of native tropo-

myosin since the fraction of actin saturated with native tropomyosin would then decrease as the actin concentration increased. Similarly if a constant ratio of native tropomyosin to actin were employed, but the actin were not completely saturated with native tropomyosin, the fraction of actin complexed with native tropomyosin might tend to decrease at low actin concentrations due to partial dissociation of the actin-native tropomyosin complex. We therefore employed a ratio of native tropomyosin to actin where, as is shown in Table I, even at the lowest actin concentration used in the reciprocal plot, the acto-HMM ATPase was more than 90% inhibited by EGTA i.e. the actin was essentially completely saturated with native tropomyosin. We then employed an EGTA-Ca^{++} buffer to hold the free Ca^{++} concentration constant so that, in effect, the fraction of actin complexed with Ca^{++}-free native tropomyosin was constant throughout the range of actin concentration employed in the reciprocal plot.

Other conditions also had to be carefully chosen for this experiment. With Mg^{++} in excess over ATP, the native tropomyosin was affected by Ca^{++} in a range where the EGTA was a poor Ca^{++}-buffer. Furthermore in the presence of excess Mg^{++} an unusual effect of native tropomyosin was noted in which the ATPase rate became markedly activated as the ATP was hydrolyzed -- an effect which seemed to be increased by high ratios of HMM to actin. Both of these difficulties were avoided by working at a two to one ratio of ATP to Mg^{++}. We also noted that in the absence of excess Mg^{++}, the action of native tropomyosin almost completely disappeared at a KCl concentration a little below 15mM, an effect which may be related to the dissociation of native tropomyosin from actin at very low ionic strength (17). Since determinations of Vmax and Ka from the reciprocal plots is most accurate at very low ionic strength, we worked at the lowest KCl concentration where the maximal effect of the native tropomyosin was still observed.

Taking all of these factors into consideration, we chose the conditions given in Figure 1 to perform our experiment. Figure 1 shows double reciprocal

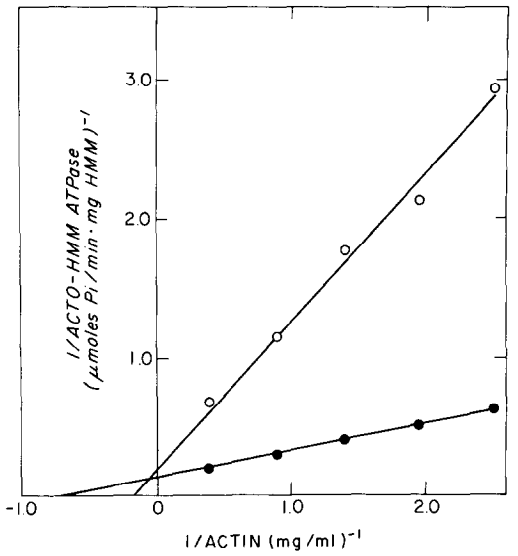

FIGURE 1

Double reciprocal plots of acto-HMM-ATPase vs. actin concentration with and without native tropomyosin. All samples contained 1.6mM ATP, 0.8mM $MgCl_2$, 19mM KCl, 5mM EGTA, 2mM $CaCl_2$. (O) Native tropomyosin added at a concentration equal to 1.25 times the actin concentration, HMM concentration varied from 0.14-0.27 mg/ml. (●) No native tropomyosin added. HMM concentration varied from 0.06-0.12 mg/ml. As described previously for this type of plot (6), in all cases the ATPase of the HMM in the absence of actin was subtracted from the measured ATPase rate to give the acto-HMM ATPase rate, the reciprocal of which is plotted on the ordinate.

---

plots of acto-HMM ATPase vs. actin concentration both in the presence and absence of native tropomyosin at a free Ca^{++} concentration where the native tropomyosin causes partial inhibition of the acto-HMM-ATPase. As can be seen both plots are linear and the native tropomyosin appears to cause a marked change in the abscissa intercept of the reciprocal plot (Ka), but only a small change in the ordinate intercept (Vmax). Repeat experiments yielded essentially identical data. Even the small change observed in Vmax may be erroneously high, since the point on the reciprocal plot at the highest native tropomysoin concentration is, if anything, above its true value i.e. the ATPase rate observed under this condition is probably too low. This is because at the highest native tropomyosin concentration employed,

trace amounts of myokinase and AMP-deaminase in the native tropomyosin produced enough IMP and NH_3 from ADP to cause a slow rise in pH to occur in the reaction mixture after all of the ATP was hydrolyzed, and therefore even during the initial course of the reaction enough NH_3 may have been produced to reduce the rate of H^+ production as measured with the pH-stat by a few percent. We thus conclude that under our conditions, although a small effect on the turnover rate of the acto-HMM-ATP complex cannot be ruled out, the major effect of native tropomyosin appears to be a reduction in the binding of actin to the HMM-ATP complex. Using a similar approach Hartshorne has recently obtained a comparable result (D. Hartshorne, personal communication).

This result may correlate with recent experiments on skinned muscle fibers which suggest that decreasing the Ca^{++} concentration has little effect on the unloaded velocity of contraction but does markedly reduce force development (18). It is also consistent with *in vivo* studies which suggest that the actin and myosin filaments are dissociated during relaxation of skeletal muscle, since relaxed as opposed to activated muscle shows very little resistance to stretch (19).

ACKNOWLEDGEMENTS

We would like to thank Mr. Louis Dobkin for his excellent technical assistance.

REFERENCES

1. Weber, A., Herz, R., and Reiss, I., Proc. Royal Soc. Series B., 160, 489 (1964).
2. Ebashi, S. and Endo, M., Prog. in Biophys. and Molecular Biol. 18, 123 (1968).
3. Endo, M., Nonomura, Y., Masaki, T., Ohtsuki, I., and Ebashi, S., J. Biochem. 60, 605 (1966).
4. Fuchs, F. and Briggs, F.N., J. Gen. Physiol. 51, 655 (1968).
5. Ebashi, S. and Ebashi, F., J. Biochem. 55, 604 (1964).
6. Eisenberg, E. and Moos, C., Biochemistry 7, 1486 (1968).
7. Eisenberg, E., Zobel, C.R., and Moos, C., Biochemistry 7, 3186 (1968).
8. Szentkiralyi, E.M. and Oplatka, A., J. Mol. Biol. 43, 551 (1969).
9. Eisenberg, E., Barouch, W.W., and Moos, C., Federation Proc. 28, 536 (1969).
10. Morita, F. and Shimizu, T., Biochim. Biophys. Acta 180, 545 (1969).
11. Kielley, W.W. and Harrington, W.F., Biochim. Biophys. Acta 41, 401 (1960)
12. Eisenberg, E. and Moos, C., J. Biol. Chem. 242, 2945 (1967).
13. Adelstein, R., Godfrey, J.E. and Kielley, W.W., Biochem. Biophys. Res. Comm. 12, 34 (1963).
14. Hartshorne, D.J. and Mueller, H., Biochim. Biophys. Acta 175, 301 (1969).

15. Hartshorne, D.J. and Mueller, H., Biochem. Biophys. Res. Comm. 31, 647 (1968).
16. Kominz, D.R., Arch. Biochem. and Biophys. 115, 583 (1966).
17. Martonosi, A., J. Biol. Chem. 237, 2795 (1962).
18. Teicholz, L.E. and Podolsky, R.J., Biophys. J. 10, 218a (1970).
19. Hill, D.R., J. Physiol. 199, 637 (1968).