PRELIMINARY NOTE

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The importance of sulfhydryl groups for the calcium-sensitive response of natural actomyosin

It has been known for many years that treatment of natural actomyosin with sulfhydryl reagents, *e.g.* *p*-chloromercuribenzoate (PCMB), can prevent the response corresponding to relaxation[1,2] which is effected by the removal of calcium. Because natural actomyosin consists of several components (including myosin, actin, tropomyosin, troponin A and troponin B) the location of the critical sulfhydryl group(s) was not obvious. Troponin binds calcium[3,4] and was therefore considered to be a likely location for this sulfhydryl group(s). The effect of sulfhydryl reagents on the functioning of troponin has led to conflicting conclusions. YASUI *et al.*[5] found that PCMB reduced the activity of troponin, whereas STAPRANS *et al.*[6] found its activity to be unaffected by *N*-ethylmaleimide. EBASHI *et al.*[7] confirmed both reports. It was suggested[6,7] that the apparent discrepancy between these results was due to the possibility that the PCMB attached to troponin could migrate to one of the other components of the natural actomyosin complex and reduce relaxation, whereas *N*-ethylmaleimide being linked to troponin by a more stable bond would remain fixed. The object of the present study is to clarify the situation with regard to the importance of troponin's sulfhydryl groups and to investigate whether or not a transfer of PCMB from troponin to another protein occurs.

In Fig. 1 it is shown that the complex of troponin and tropomyosin (1:1 weight ratio) is not affected by reaction with an excess of the following reagents: iodoacetamide, iodoacetic acid, and *N*-ethylmaleimide. Using ^{14}C-labeled reagents it was found that under the conditions given in Fig. 1 the extent of incorporation for each of the three reagents was approx. 4.4 moles/10^5 g of the troponin–tropomyosin complex. In each case the inhibition of the Mg^{2+}-activated ATPase activity of the desensitized actomyosin in the absence of calcium was similar to that obtained with control troponin and tropomyosin. Similar results were obtained when either troponin or tropomyosin were reacted with the above reagents and then combined with its untreated partner. The reactions were also carried out under different conditions, *e.g.* at pH's between 7 and 9, with and without 5 M guanidine hydrochloride, at zero and 1 M KCl concentrations and at 0° and room temperature. Using the above reagents there was no indication that the sulfhydryl groups of either troponin or tropomyosin were essential for the calcium-dependent response of actomyosin. However when troponin was labeled with PCMB and combined with tropomyosin, the inhibition of ATPase activity in the absence of calcium was reduced. Shown in Fig. 1 are the results obtained at a titer of 2 moles of PCMB per 10^5 g troponin. When approx. 2 nmoles of PCMB had been added (equivalent to 200 μg of the troponin–tropomyosin mixture) no inhibition was observed.

Abbreviations: PCMB, *p*-chloromercuribenzoate; EGTA, ethylene glycol bis-(β-aminoethyl ether)-*N*,*N*^{1}-tetraacetic acid.

To test for the possibility that certain sulfhydryl groups on troponin were reactive to PCMB but not to *N*-ethylmaleimide the following experiment was performed: troponin (2 mg/ml) in 5 M guanidine hydrochloride, 50 mM Tris–HCl (pH 7.6) was reacted with *N*-ethylmaleimide (40 mM) overnight at 4°. The solution was dialyzed against 10 mM Tris–HCl (pH 7.6) and then reacted with ^{14}C-labeled PCMB (2 mM) for 30 min. After dialysis to remove excess PCMB the extent of labeling was determined and found to be approx. 0.15 mole of PCMB per 10^5 g of *N*-ethylmaleimide-treated troponin. (Control troponin (not reacted with *N*-ethylmaleimide) gave approx. 6 moles PCMB incorporated per 10^5 g.)

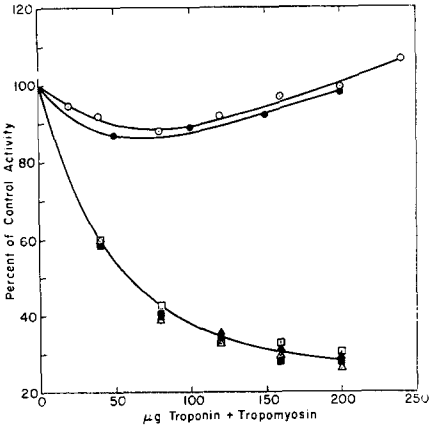

Fig. 1. Effect of sulfhydryl reagents on the inhibitory action of troponin and tropomyosin. Assay conditions: 2.5 mM $MgCl_2$, 2.5 mM ATP, 1 mM ethylene glycol bis-(β-aminoethyl ether)-*N,N'*-tetraacetic acid (EGTA), 25 mM Tris–HCl (pH 7.6), 0.28 mg/ml desensitized actomyosin, final volume 2 ml, temperature 25°. Troponin and tropomyosin combined in an equal weight ratio. □, control troponin and tropomyosin; ○, PCMB-labeled troponin (2 moles PCMB per 10^5 g protein) and control tropomyosin; ●, PCMB-labeled tropomyosin (2 moles PCMB per 10^5 g protein) and control troponin. The complex of troponin and tropomyosin labeled with iodoacetic acid (△), *N*-ethylmaleimide (▲), iodoacetamide (■). For the latter three points the conditions under which the complex was reacted were: 12.5 mM sulfhydryl reagent, 50 mM Tris–HCl (pH 7.6), protein concentration 1 mg/ml, reaction time 24 h at 4°, followed by dialysis against 10 mM Tris–HCl (pH 7.6) to remove excess reagent.

The presence of "selectively reactive" sulfhydryl groups on troponin was therefore unlikely. This was confirmed by assaying the effect of PCMB-labeled tropomyosin (2 moles PCMB per 10^5 g protein) mixed with control troponin. Again the calcium-dependent inhibition of the actomyosin–ATPase activity was lost (Fig. 1). A logical explanation for the above data is that PCMB was transferred from troponin or tropomyosin to a critical site on myosin or actin. NAKATA AND YAGI[8] have demonstrated recently that the transfer of PCMB between actin and heavy meromyosin can occur.

When PCMB was added to a mixture containing desensitized actomyosin (0.56 mg), troponin (0.1 mg) and tropomyosin (0.1 mg), the inhibition of ATPase activity, in the absence of calcium, was lost when approx. 25 % of the total sulfhydryl groups were titrated. This corresponded to the reaction of 12 nmoles sulfhydryl groups out of a total of approx. 50 nmoles. At this level of PCMB the Mg^{2+}-activated ATPase activity was the same in the absence and presence of calcium, and was slightly higher (7 %) than the control (zero PCMB) activity. Increasing the PCMB concentration

caused a reduction of ATPase activity. Similar results were obtained when Subfragment 1 and actin (2.7:1 weight ratio, respectively) were used instead of desensitized actomyosin. Recently WATANABE[9] has also shown that PCMB alters the superprecipitation of myosin B.

The amount of PCMB required to block the relaxation of actomyosin was less when labeled troponin or tropomyosin was used than when PCMB was added to a mixture of all of the proteins. From the results illustrated in Fig. 1, assuming that all of the PCMB was transferred from either troponin or tropomyosin to another site, no more than approx. 2 nmoles of PCMB were required. This would imply that the migration of PCMB from labeled troponin or tropomyosin was directed towards a specific site, possibly due to the proximity of the PCMB donor and acceptor.

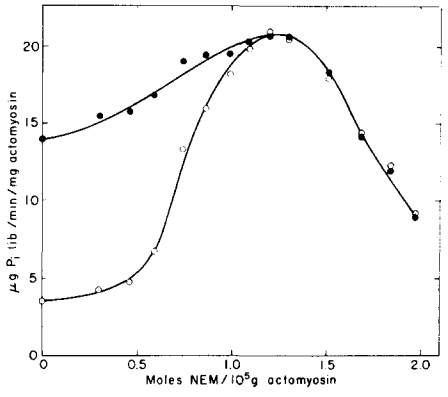

Fig. 2. Effect of *N*-ethylmaleimide (NEM) on the calcium-sensitive response of actomyosin. Desensitized actomyosin (2.6 mg/ml) was reacted in 25 mM Tris–HCl (pH 7.6) with varying amounts of *N*-ethylmaleimide ranging from 8 to 80 μM, at 25° for 30 min. The reaction was stopped by the addition of 20 mM dithiothreitol (0.2 ml). The extent of reaction was determined using ^{14}C-labeled *N*-ethylmaleimide. The reacted actomyosin was assayed in the presence of troponin (50 μg) and tropomyosin (50 μg) in two assay media: ●, 2.5 mM $MgCl_2$, 2.5 mM ATP, 25 mM Tris–HCl (pH 7.6); ○, 2.5 mM $MgCl_2$, 2.5 mM ATP, 1 mM EGTA, 25 mM Tris–HCl (pH 7.6).

From the above experiments the location of the critical sulfhydryl group has been restricted to either actin or myosin. This is confirmed in Fig. 2 where the effect of labeling desensitized actomyosin with *N*-ethylmaleimide is shown. After reaction with *N*-ethylmaleimide a constant amount of troponin and tropomyosin was added, and the Mg^{2+}-activated ATPase activity was determined in the absence and presence of calcium. At approx. 1.2 moles of *N*-ethylmaleimide per 10^5 g actomyosin the inhibition in the absence of calcium was eliminated, *i.e.* the Mg^{2+}- and the Mg^{2+}–EGTA-activated ATPases were the same.

It is necessary now to establish whether the *N*-ethylmaleimide effect was due to the reaction of the sulfhydryl groups of actin or myosin. F-actin was reacted with *N*-ethylmaleimide under conditions similar to those described by MARTONOSI[10] (these were: 3–4 mg/ml F-actin, 0.5–2 mM *N*-ethylmaleimide, 10 mM Tris–HCl (pH 7.6), final volume of 1 ml, reacted for 30 min at 25°, followed by the addition of 20 mM dithiothreitol (0.2 ml) to stop the reaction). The reacted actin was then mixed with either Subfragment 1 or myosin (1:2.7 and 1:4 weight ratios, respectively) and assayed for calcium sensitivity with troponin and tropomyosin. The *N*-ethylmaleimide-

treated actin behaved identically to control actin (see also ref. 11), and this therefore suggests that the critical sulfhydryl group(s) is located on myosin. Recently A. WEBER AND D. BREMEL (personal communication) have also found that myosin contains sulfhydryl groups which are essential for the calcium-sensitive response of actomyosin. When myosin alone was reacted with *N*-ethylmaleimide and then combined with F-actin the calcium sensitivity was gradually reduced but its loss was not sharply defined as was the case with *N*-ethylmaleimide *plus* actomyosin. The influence of actin on the availability of the sulfhydryl groups of myosin is being investigated further.

The results presented above suggest that the sulfhydryl groups of troponin are not essential for its functioning. The effect of PCMB can be explained by assuming a migration of the PCMB from troponin to a specific site on myosin. At the moment there are not sufficient experimental data to identify this site as an inhibitory site distinct from the ATP hydrolysis site (see ref. 12). WEBER[13] has proposed that the relaxation of myofibrils is dependent upon the extent to which the hydrolysis site of myosin is saturated with ATP and she suggested that the concept of a separate inhibitory site was not consistent with her data. In view of this we must also consider the possibility that the effect of the sulfhydryl reagents was due to an alteration of only the hydrolysis site.

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Chemistry Department,
Carnegie-Mellon University,
Pittsburgh, Pa. 15213 *(U.S.A.)*

D. J. HARTSHORNE
J. L. DANIEL

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