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THE EFFECT OF Ca^{2+} ON THE SULFHYDRYL REACTIVITY OF TROPONIN: EVIDENCE FOR A Ca^{2+}-INDUCED CONFORMATIONAL CHARGE

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SUMMARY

Troponin prepared from rabbit skeletal muscle in the presence of dithiothreitol (SH-troponin) was found to have a sulfhydryl content of about 4 moles/$1 \cdot 10^5$ g in the presence of a Ca^{2+}-chelating agent. The addition of physiological concentrations of Ca^{2+} reduced the reactive sulfhydryl content to 2.0–2.5 moles/$1 \cdot 10^5$ g. These sulfhydryl groups are evidently not direct participants in the inhibition of actomyosin superprecipitation, since treatment with *N*-ethylmaleimide had no effect on the Ca^{2+}-sensitizing activity of SH-troponin.

Troponin prepared in the absence of dithiothreitol (S–S-troponin) showed a significant reduction in Ca^{2+}-sensitizing activity, relative to SH-troponin. The sulfhydryl groups of S–S-troponin, approx. 2 moles/$1 \cdot 10^5$ g were not appreciably affected by Ca^{2+}.

It is postulated that the Ca^{2+}-sensitive sulfhydryl groups exist at a site which is essential for the regulatory function of troponin and which undergoes a conformational change upon the binding of Ca^{2+}.

INTRODUCTION

Muscle contraction is activated by the binding of Ca^{2+} to the myofibrils[1]. The requirement for Ca^{2+} is conferred by two myofibrillar proteins, tropomyosin and troponin[2]. Recent studies[3,4] have provided convincing evidence that it is the troponin which contains the physiologically relevant Ca^{2+} receptor sites. The molecular mechanism whereby the Ca^{2+}–troponin complex promotes the interaction between actin and myosin, the contractile proteins of the myofibril, remains obscure.

In an earlier report[5] from this laboratory data were presented showing that the intact sulfhydryl groups of troponin were essential for the Ca^{2+}-sensitive inhibition of actomyosin superprecipitation produced by the tropomyosin–troponin complex. This conclusion has been contested by other investigators[4,6,7]. In the course of re-examining this question it was observed that if a Ca^{2+}-chelating agent was present in the reaction mixture the sulfhydryl content of troponin was consistently elevated. In this report data are presented showing that physiological concentrations of Ca^{2+}

Abbreviations: EGTA, ethylene glycol bis-(β-aminoethylether)-*N*,*N*′-tetraacetic acid; DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid).

cause a decrease in the sulfhydryl reactivity of troponin which can be attributed to a conformational change in the protein induced by Ca^{2+} binding.

METHODS

Troponin was prepared from rabbit skeletal muscle according to the method of YASUI *et al.*[5], with 1 mM dithiothreitol being present in all preparative solution unless otherwise indicated. As in the previous publication[5] troponin prepared in the presence of dithiothreitol is referred to as SH-troponin whereas that prepared in the absence of dithiothreitol is referred to as S–S-troponin.

The methods for the preparation of tropomyosin and desensitized actomyosin were as described previously[5]. The ability of the tropomyosin–troponin complex to inhibit the superprecipitation of desensitized actomyosin was assayed by the light scattering technique,[8] as described by YASUI *et al.*[5].

Sulfhydryl determinations were carried out by the method of ELLMAN[9] and protein concentration was estimated by the method of LOWRY *et al.*[10]. To separate SH-troponin from dithiothreitol aliquots of the stock troponin solution were applied to columns of Bio-Gel P-10 (0.9 cm × 20 cm) equilibrated with 50 mM KCl, 10 mM phosphate (pH 7.5) and eluted with the same solution. The final assay mixture contained 0.20 or 0.40 mM ethylene glycol bis-(β-aminoethylether)-*N*,*N*′-tetraacetic acid (EGTA), and divalent cation as required, in a total volume of 3 ml. 30 min after the addition of 5,5′-dithiobis-(2-nitro-benzoic acid) (DTNB) the absorbance at 412 mμ was measured with a Coleman–Hitachi 124 spectrophotometer equipped with a Coleman 165 recorder. Calculations were based on a molar absorbance coefficient of $1.36 \cdot 10^4$ (ref. 9), verified with a standard cysteine solution.

To determine whether there was any loss of sulfhydryl groups during the time which elapsed between the separation of the protein from the dithiothreitol and the reaction with DTNB a few determinations were also made using the modification of BUTTERWORTH *et al*,[11] in which DTNB is added to the protein solution in the presence of dithiothreitol. The protein was then isolated by gel filtration with Bio-Gel P-10 and the thionitrophenylate ion liberated by the addition of excess dithiothreitol[11]. The results were the same with both methods.

RESULTS

The SH-troponin preparations employed in this study had approx. 4 moles sulfhydryl per $1 \cdot 10^5$ g protein, as determined in the presence of EGTA (mean, 3.75 ± 0.15; $n = 23$). The addition of $CaCl_2$ at a concentration approximately equal to the EGTA concentration caused a marked inhibition of the reaction of DTNB with troponin (Fig. 1). In most cases the reactive sulfhydryl content was reduced to 2.0–2.5 moles/$1 \cdot 10^5$ g. As shown in Table I this effect was dependent on the native conformation of the protein. In the presence of 8 M urea the total sulfhydryl content was approximately doubled (with respect to the "Ca^{2+}-free" state) and was not affected by Ca^{2+}. As a further check it was shown that neither EGTA nor Ca^{2+} had any effect on the reaction of DTNB with cysteine. Thus the results shown are ascribed to a specific effect of Ca^{2+} on protein conformation rather than to interference with the analytical reaction.

TABLE I

EFFECT OF Ca^{2+} ON THE SULFHYDRYL CONTENT OF NORMAL AND DENATURED TROPONIN

Reaction conditions are described in METHODS. Protein concentration, 0.60 mg/ml.

	Moles sulfhydryl/1 · 10⁵ g	
	No urea	*8 M urea*
SH-Troponin		
+ 0.40 mM EGTA	3.92	7.60
+ 0.40 mM EGTA, 0.40 mM $CaCl_2$	2.50	7.66
S–S-Troponin		
+ 0.40 mM EGTA	1.84	5.51
+ 0.40 mM EGTA, 0.40 mM $CaCl_2$	1.41	5.09

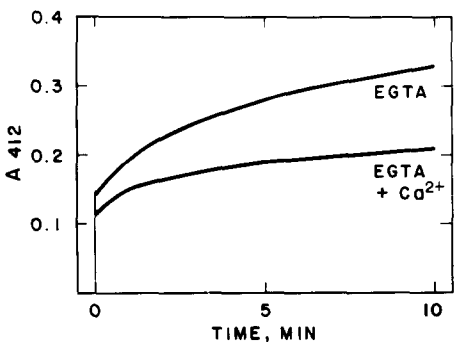

Fig. 1. The time-course of the reaction of DTNB with SH-troponin in the presence of EGTA and EGTA + Ca^{2+}. Reaction conditions are described in METHODS. The EGTA and $CaCl_2$ concentrations were 0.40 mM; protein concentration, 0.60 mg/ml.

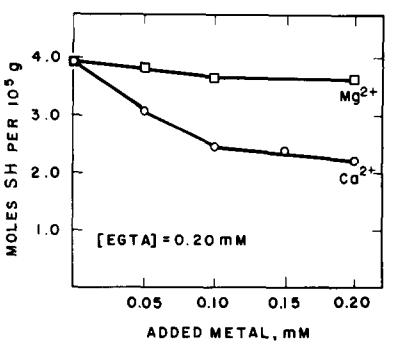

Fig. 2. The effect of added $CaCl_2$ or $MgCl_2$ on the reactive sulfhydryl content of SH-troponin in the presence of 0.20 mM EGTA. Reaction conditions are described in METHODS. Protein concentration, 0.43 mg/ml.

In the experiment illustrated in Fig. 2 varying concentrations of Ca^{2+} or Mg^{2+} (up to 0.20 mM) were added to a solution of SH-troponin in the presence of 0.20 mM EGTA. From the ratio of [EGTA] to [Ca^{2+}] it is evident that even when the free Ca^{2+} concentration was considerably less than $1 \cdot 10^{-6}$ M there was a clear reduction in sulfhydryl reactivity. Hence the effects of Ca^{2+} on sulfhydryl reactivity can be demonstrated with the same range of Ca^{2+} concentrations ($1 \cdot 10^{-7}$–$1 \cdot 10^{-6}$ M) which have been shown to be required for binding to troponin[3] and activation of myofibrillar contraction[1]. Sr^{2+}, another cation which can bind to troponin[4], had effects similar to Ca^{2+}. On the other hand, Mg^{2+} was ineffective (Fig. 2), a result consistent with the very weak affinity of this cation for the Ca^{2+} binding site[12].

To see whether the Ca^{2+}-sensitive sulfhydryl groups play an essential role in the inhibition of superprecipitation the SH-troponin was treated with *N*-ethylmaleimide and then tested for Ca^{2+}-sensitizing activity. Following *N*-ethylmaleimide treatment the reactive sulfhydryl content was reduced to less than 1 mole/$1 \cdot 10^5$ g*.

* The reaction with *N*-ethylmaleimide did not appear to be Ca^{2+}-sensitive.

As shown in Fig. 3 the blocking of sulfhydryl groups had no effect on the ability of troponin to inhibit the superprecipitation of desensitized actomyosin in the presence of EGTA. Thus the sulfhydryl groups in question do not appear to be directly involved in the Ca^{2+}-sensitizing activity of troponin. This conclusion is in contrast to that of YASUI *et al.*[5] but in accord with the results of EBASHI *et al.*[4], ARAI AND WATANABE[6], and STAPRANS *et al.*[7].

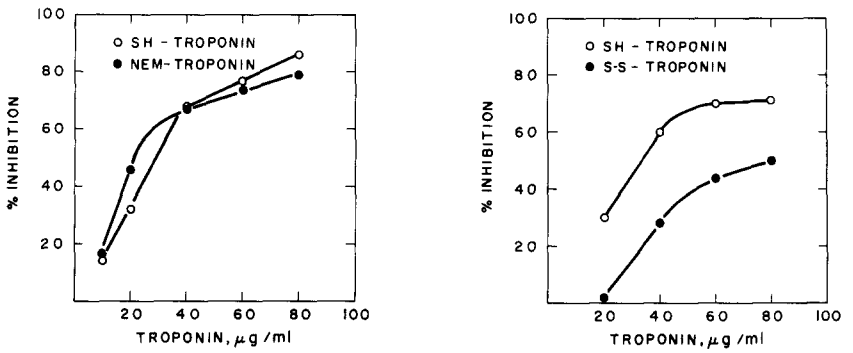

Fig. 3. Inhibition of superprecipitation by SH-troponin and *N*-ethylmaleimide-treated troponin. *N*-Ethylmaleimide (NEM, 5 mM) was added to a solution of SH-troponin (approx. 5 mg/ml) in 50 mM KCl, 10 mM phosphate (pH 7.5), 1 mM dithiothreitol, 0.4 mM EGTA. After incubation at room temperature for 1 h the protein was isolated by gel filtration with Bio-Gel P-10. Superprecipitation was measured in a medium containing 60 mM KCl, 30 mM imidazole (pH 7.0), 2 mM $MgCl_2$, 0.40 mM EGTA, 1 mM ATP, 200 μg/ml desensitized actomyosin, 50 μg/ml tropomyosin, and troponin as indicated. Calculation of per cent inhibition was based on measurement of $A_{660\,m\mu}$ 2 min after the addition of ATP.

Fig. 4. Inhibition of superprecipitation by SH-troponin and S–S-troponin. Reaction conditions same as for Fig. 3.

On the other hand, if dithiothreitol was omitted during preparation (S–S-troponin) the reactive sulfhydryl content was reduced to about 2 moles/$1 \cdot 10^5$ g (mean, 1.75 ± 0.18, $n = 12$) and the Ca^{2+}-sensitizing activity was markedly reduced (Fig. 4), although never completely eliminated (*cf.* ref. 5) Moreover, the reactivity of the sulfhydryl groups of S–S-troponin was not appreciably affected by the presence of Ca^{2+} (Table I). Like the SH-troponin, the S–S-troponin had 3–4 moles/$1 \cdot 10^5$ g of masked sulfhydryl groups, as indicated by sulfhydryl analysis in the presence of 8 M urea.

In accounting for the differences between SH-troponin and S–S-troponin it might be supposed that the presence of dithiothreitol altered the composition of the final preparation rather than the properties of the active component. This possibility is considered unlikely for the following reasons: (1) The two preparations cannot be distinguished electrophoretically, using the polyacrylamide gel technique[5]. (2) Both preparations were found to have identical ultraviolet absorption spectra. For SH-troponin $A_{278\,m\mu}/A_{260\,m\mu}$ was 1.04 and $E^{1\%}_{278\,m\mu}$ was 5.6; the corresponding values for S–S-troponin were 1.05 and 5.5, respectively. (3) On a weight basis the effects of SH-troponin and S–S-troponin on tropomyosin viscosity[13] were identical (unpublished observations). (4) Following reduction of S–S-troponin with excess dithiothreitol or 2-mercaptoethanol its sulfhydryl content and Ca^{2+}-sensitizing activity were approximately the same as those of SH-troponin.

DISCUSSION

The data presented in this paper can be most simply interpreted on the assumption that SH-troponin contains sulfhydryl groups (up to 2 moles/$1 \cdot 10^5$ g) which do not participate directly in the Ca^{2+}-sensitizing activity but which are located at a region which undergoes a conformational change upon the binding of Ca^{2+}. This change is manifested as a loss of reactivity toward the sulfhydryl reagent DTNB. If dithiothreitol is omitted during preparation these groups undergo oxidation to the disulfide form (S–S-troponin) and conformational changes, if any, are not detectable with DTNB. It can be hypothesized that with the incorporation of the Ca^{2+}-sensitive sulfhydryl groups into disulfide bridges there is sufficient conformational strain to account for the partial inactivation of troponin observed experimentally (Fig. 4). Thus the data are consistent with the existence of a flexible region of troponin which is essential for Ca^{2+}-sensitizing activity and the conformation of which is sensitive to Ca^{2+}-binding.

A previous attempt to detect a Ca^{2+}-induced conformational change in troponin was reported by TONOMURA *et al.*[14]. Using spin labels directed to sulfhydryl groups, they found that there was no change in the electron paramagnetic resonance spectrum associated with Ca^{2+}-binding. These workers concluded that the binding of Ca^{2+} caused no change in the microenvironment surrounding the reactive sulfhydryl groups. While this conclusion may not necessarily be incompatible with the results reported in this paper it should be noted that the troponin perparations used by TONOMURA *et al.*[14] were prepared without dithiothreitol. In the light of the data presented here one wonders whether their negative results may only reflect the fact that the relevant sulfhydryl groups were in an oxidized state and therefore unlabeled. It would clearly be of interest to repeat these experiments with troponin preparations in which the sulfhydryl groups are protected.

In contrast to the results with the spin labeling technique, HAN AND BENSON[15] have recently reported that the addition of Ca^{2+} caused a shift in the fluorescence emission maximum of troponin as well as an increase in the fluorescence intensity. They explained these effects in terms of an infolding of tryptophan side chains toward hydrophobic regions. Since the Ca^{2+} concentrations required to elicit fluorescence changes ($1 \cdot 10^5$–$1 \cdot 10^{-4}$ M) were considerably higher than those needed to alter sulfhydryl reactivity it is not clear whether these phenomena are related. Moreover, the experiments reported in this study were also performed with preparations of S–S-troponin.

WAKABAYASHI AND EBASHI[16] have reported that the dissociation of Ca^{2+} from troponin caused a change in the ultracentrifugal sedimentation pattern indicative of an aggregation of troponin. Interestingly, the authors noted that this response was attenuated in the presence of dithiothreitol.

With regard to the mechanism of coupling between Ca^{2+} binding and contraction information is needed on the spatial relationship between the Ca^{2+} receptor site and the Ca^{2+}-sensitive sulfhydryl groups. In approaching this problem recognition must be given to recent studies[17,18] showing that troponin is not a single protein. Rather, it can be fractionated into at least two components, termed troponin A and troponin B by HARTSHORNE AND MUELLER[17]. Troponin B, in conjunction with tropomyosin, inhibits the Mg^{2+}-activated ATPase and superprecipitation of actomyosin whereas

troponin A, the Ca^{2+}-binding moiety of troponin, is required for the Ca^{2+} reversibility of the troponin B inhibition. In preliminary experiments it has not been possible to demonstrate any consistent effect of Ca^{2+} on the sulfhydryl reactivity of biologically active preparations of troponin A obtained by the method of HARTSHORNE AND MUELLER[17]. It would appear that the entire troponin A–troponin B complex is required for this response. Experiments are in progress to determine the location of the Ca^{2+}-sensitive sulfhydryl groups. Such studies, combined with other physicochemical techniques, should provide insight into the molecular events which intervene between the binding of Ca^{2+} to troponin and the onset of tension generation in muscle.

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REFERENCES

1 A. WEBER AND R. HERZ, *J. Biol. Chem.*, 238 (1963) 599.
2 S. EBASHI AND A. KODAMA, *J. Biochem. Tokyo*, 60 (1966) 733.
3 F. FUCHS AND F. N. BRIGGS, *J. Gen. Physiol.*, 51 (1968) 655.
4 S. EBASHI, A. KODAMA AND F. EBASHI, *J. Biochem. Tokyo*, 64 (1968) 465.
5 B. YASUI, F. FUCHS AND F. N. BRIGGS, *J. Biol. Chem.*, 243 (1968) 735.
6 K. ARAI AND S. WATANABE, *J. Biol. Chem.*, 243 (1969) 5670.
7 I. STAPRANS, K. ARAI AND S. WATANABE, *J. Biochem. Tokyo*, 64 (1968) 65.
8 S. EBASHI, *J. Biochem. Tokyo*, 50 (1961) 236.
9 G. L. ELLMAN, *Arch. Biochem. Biophys.*, 82 (1959) 70.
10 O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR AND R. L. RANDALL, *J. Biol. Chem.*, 193 (1951) 265.
11 P. H. W. BUTTERWORTH, H. BAUM AND J. W. PORTER, *Arch. Biochem. Biophys.*, 118 (1967) 716.
12 F. FUCHS, Y. REDDY AND F. N. BRIGGS, *Biochim. Biophys. Acta*, 221 (1970) 407.
13 S. EBASHI AND A. KODAMA, *J. Biochem. Tokyo*, 58 (1965) 107.
14 Y. TONOMURA, S. WATANABE AND M. MORALES, *Biochemistry*, 8 (1969) 2171.
15 M. H. HAN AND E. S. BENSON, *Biochem. Biophys. Res. Commun.*, 38 (1970) 378.
16 T. WAKABAYASHI AND S. EBASHI, *J. Biochem. Tokyo*, 64 (1968) 731.
17 D. J. HARTSHORNE AND H. MUELLER, *Biochem. Biophys. Res. Commun.*, 31 (1968) 647.
18 M. C. SCHAUB AND S. V. PERRY, *Biochem. J.*, 115 (1969) 993.