Interactions Between Contractile and Regulatory Proteins of the Myofibril

The mechanico-chemical energy transduction occurring during muscle contraction is known to be regulated by changes in the concentration of trace amounts of Ca^{2+} within the myofibril[1]. These ions control the Mg^{2+}-stimulated ATPase of actomyosin via the relaxing protein system (RPS)[2]. This system includes tropomyosin, inhibitory factor (troponin B) and calcium-sensitizing factor (troponin A)[2–4]. In the absence of Ca^{2+}, the RPS effectively inhibits the Mg-ATPase of the myofibril. Natural actomyosin extracted as a complex from myofibrils still contains RPS. It has been shown that washing natural actomyosin (NAM) at low ionic strength ($\mu = 0.002$) removes the RPS producing desensitized actomyosin[5]. Its Mg-ATPase is no longer able to be controlled by Ca^{2+}. If, however, natural actomyosin is washed repeatedly at moderate concentrations of salt ($\mu = 0.02$– 0.10), the RPS remains associated with the actomyosin complex preserving its Ca^{2+}-sensitivity. We attempted to recombine the RPS with desensitized actomyosin and to establish under which conditions effective binding takes place to restore the Ca^{2+}-sensitivity.

Methods. Natural and desensitized actomyosin, tropomyosin and RPS fractions were prepared from washed myofibrils of white skeletal muscles of rabbits as previously reported[3,4]. The Ca^{2+}-sensitivity was assayed by measuring the ATPase activity of 0.2–0.5 mg of actomyosin in a volume of 2 ml containing 2.5 m*M* $MgCl_2$, 2.5 m*M* *tris*-ATP, 25 m*M* *tris*-HCl, pH 7.6, at 25 °C in the presence and absence of 1 m*M* ethanedioxybis (ethylamine) tetra-acetic acid (EGTA)[5]. Protein samples were radioactively labelled after reduction in the presence of 10 m*M* dithiothreitol in 6 *M* guanidine-HCl by the addition of about 10 moles of ^{14}C-iodoacetic acid to 10^5 g of protein at pH 8.4. Subsequently the samples were exhaustively dialysed against 10 m*M* *tris*-HCl, pH 7.6. Radioactivity was determined by liquid scintillation counter techniques as earlier[6] and related to protein content on the basis of nitrogen estimations carried out by nesslerization after digestion[7].

Experiments and results. Since it has been previously shown that individual components of the RPS can be carboxymethylated without loss of their regulatory function[6,8,9], such radioactively labelled crude preparations of RPS were used in this study. Mixtures of 2.48 mg of desensitized actomyosin and 20.4 µg of labelled RPS were centrifuged for 20 min at 1700 *g* and the radioactivity of the non-bound RPS was determined in the supernatants. At the relatively low buffer concentration of 25 m*M*, substantial amounts of RPS were bound to the insoluble actomysin only at such a low pH where isoelectric precipitation of this protein fraction started (Table I). At pH 7.6 higher buffer concentrations, as well as $MgCl_2$, $CaCl_2$ or ATP in concentrations as used in enzymic tests, significantly reduced the extent of binding. The similar effect produced by EDTA indicates that the bivalent cations are not involved in the association in a specific way. The general effect of all added salts in reducing the extent of binding seems to contradict that, during the washing process of natural actomyosin in the presence of salt, RPS remains firmly bound to the actomyosin complex. In RPS preparations tropomyosin

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Table I. Extent of association of regulatory proteins with suspensions of desensitized actomyosin

Conditions	Fraction bound of total added (%) RPS fraction	Tropomyosin
25 m*M* *tris*-maleate pH 5.6	63	92
25 m*M* *tris*-maleate pH 6.5	11	78
25 m*M* *tris*-maleate pH 7.6	14	55
25 m*M* *tris*-maleate pH 8.3	9	23
25 m*M* *tris*-maleate pH 9.4	~1	~1
2 m*M* *tris*-HCl pH 7.6	43	4
13 m*M* *tris*-HCl pH 7.6	20	36
25 m*M* *tris*-HCl pH 7.6	14	55
50 m*M* *tris*-HCl pH 7.6	~1	76
80 m*M* *tris*-HCl pH 7.6	~1	70
125 m*M* *tris*-HCl pH 7.6	~1	66
2 m*M* *tris*-HCl pH 7.6 + 2.5 m*M* $MgCl_2$	15	75
2 m*M* *tris*-HCl pH 7.6 + 2.5 m*M* $CaCl_2$	12	75
2 m*M* *tris*-HCl pH 7.6 + 5 m*M* EDTA	11	70
2 m*M* *tris*-HCl pH 7.6 + 2.5 m*M* ATP	11	—
2 m*M* *tris*-HCl pH 7.6 + 2.5 m*M* Mg-ATP	15	—

Table II. Extent of firm binding of RPS fraction to desensitized actomyosin after 3 times washing with different buffer concentrations at pH 7.6 and recovery of Ca^{2+}-sensitivity of the Mg-ATPase

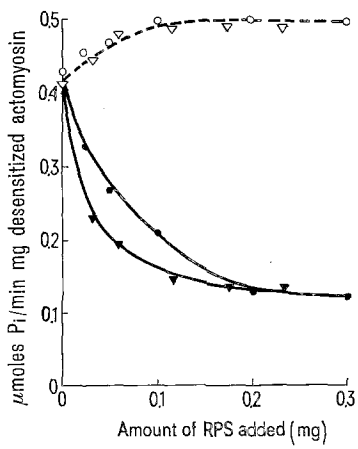
Washing medium	RPS (μg) bound per 10 mg of desensitized actomyosin	Amount bound of total added (%)	μmoles of Pi/min/mg of desensitized acto-myosin	Inhibition of Mg-ATPase by 1 m*M* EGTA (%)
2 m*M* *tris*-HCl	2.80	0.62	0.486	5.5
10 m*M* *tris*-HCl	11.7	2.6	0.510	28
80 m*M* *tris*-HCl	23.7	5.2	0.474	47

is that individual protein component present in largest amount[10]. It is thought to act as a link responsible for the binding of the inhibitory and calcium-sensitizing factors to the actomyosin system[11,12]. Therefore similar experiments were conducted by mixing 116 μg of labelled tropomyosin with 1.62 mg of desensitized actomyosin (DAM). In contrast to the case of RPS, substantial amounts of tropomyosin bind only in the presence of some salt. The apparently high binding ratio at pH 5.6 again may be due to isoelectric precipitation.

Mixtures of desensitized actomyosin and labelled RPS were also subjected to the washing procedure with different buffer concentrations. 452 μg of radioactive RPS were added to 10 mg of desensitized actomyosin, 3 times washed by centrifuging for 20 min at 20,000 *g*, the final pellet was then resuspended in 2 m*M* *tris*-HCl, pH 7.6, and assessed for radioactivity as well as for Ca^{2+}-sensitivity of the Mg-ATPase. Table II shows that the extent of firm binding is very small but increases with increasing salt concentrations. Although the amount of the bound RPS is a factor of about 1000 less than that of desensitized actomyosin, it significantly restored Ca^{2+}-sensitivity.

In control experiments which did not involve the washing process prior to the enzymic tests, the Ca^{2+}-sensitivity of desensitized actomyosin was tested with increasing amounts of carboxymethylated as well as untreated RPS fractions. Carboxymethylation did not affect the specific regulatory activity of the RPS preparations (Figure). On average 0.45 mg and 1.10 mg of RPS per 10 mg of desensitized actomyosin produced 30% and 50% inhibition of the Mg-ATPase in the presence of EGTA, respectively. Hence about 40 times more RPS was required to achieve the same degree of Ca^{2+}-sensitivity as in the experiments referred to in Table II. Since only 1–5% of the added RPS become firmly bound in the presence of some salt, but, on the other hand, are sufficient to restore the Ca^{2+}-sensitivity in the enzymic tests, this material must be specifically bound to the actomyosin. RPS associated with actomyosin in larger amounts seems to represent a non-specific binding. This second type of association is affected in the opposite manner by salt (Table I). The extent of the specific binding could be increased, however, when RPS was added to desensitized actomyosin which had been solubilized in 0.6 *M* KCl. After reducing the salt concentration to 80 m*M* by dialysis in order to precipitate the actomyosin complex, and after 3 times washing of the suspension, up to 30% of the added radioactive RPS could be recovered as firmly bound to the actomyosin. In similar experiments done with tropomyosin, the extent of binding was not increased in comparison with that found in the experiments referred to in Table I, implying that there is only one type of association.

The results indicate that the presence of the other proteins in RPS interfere with the affinity of tropomyosin to desensitized actomyosin. In spite of this fact, the small proportion of the crude RPS fraction which binds firmly in the presence of salt, exhibits a specific regulatory activity much higher than currently appreciated. It suggests that in the myofibril very small amounts of the regulatory proteins are sufficient for the regulation of the interaction between myosin and actin and hence for the regulation of contraction.



Recovery of Ca^{2+}-sensitivity of desensitized actomyosin by carboxymethylated and untreated RPS fractions. Assay conditions as described in Methods. Open symbols, without EGTA; full symbols, with EGTA; circles, untreated RPS; triangles, carboxymethylated RPS.

Zusammenfassung. Die Regulationseiweisse (Tropomyosin und Troponinkomplex), welche die Wechselwirkung der kontraktilen Eiweisse in der Myofibrille steuern, zeigen eine spezifische und unspezifische Bindung an den Actomyosinkomplex. Die spezifisch gebundene Menge der Regulationseiweisse beträgt nur etwa 1/100 derjenigen von Actomyosin, genügt aber trotzdem, um die Steuerung der Mg-ATPase und damit der Muskelkontraktion durch Ca^{2+} zu gewährleisten.

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