Cooperation Between the Two Myosin Heads Interacting with Actin in Presence of ADP in Myofibrils

Length changes in muscle take place by a sliding motion of the two sets of filaments which make up the myofibril[1,2], during which production of force is assumed to occur in the overlap regions as a consequence of the formation of myosin-actin links[3,4]. The different conformational states of such links between actin and myosin filaments as can be seen in muscle[5], led to the idea of the moving crossbridge model of contraction[6]. The movement is thought to be generated at the sites of attachment by the myosin heads moving through a series of positions of progressively lower potential energy on actin[7]. One force generating cross-bridge attachment can be produced simply by desorption of an unhydrolyzed ATP analogue such as adenylyl imidodiphosphate (AMPPNP), or even pyrophosphate (PP) alone, from the myosin heads[8]. As ADP generated from ATP hydrolysis is known to remain bound in both myofibrils[9] and relaxed muscle fibres[10], it seems that the ADP-loaded myosin heads ought to have a rather high affinity for the combining sites on actin. We therefore examined conditions which determine whether myosin is able to undergo physical combination with actin, and how this attachment may be regulated by trace amounts of Ca^{2+} via the regulatory proteins (troponin-tropomyosin complex).

Methods. After extraction of myosin from mixed rabbit skeletal muscles[11], 5 m*M* EDTA was included in all solvents for preparation. Myofibrils and purified, desensitized actomyosin were prepared as described earlier[12,13]. Alkylation with N-ethylmaleimide (NEM) was performed on 10 mg protein/ml at 0 °C in 30 m*M* KCl, 25 m*M* *tris*-HCl pH 7.6 and 0.05 m*M* $CaCl_2$ under various conditions as indicated in the text. An 100-fold molar excess of dithiothreitol over NEM was added after 15 min. To remove reagents, the protein was 15 times diluted with 5 m*M* EDTA and 25 m*M* *tris*-maleate pH 7.0, centrifuged at 20,000 *g* and the sedimented protein dissolved in 10 m*M* EDTA, 1 *M* KCl and 25 m*M* *tris*-HCl pH 7.6. Subsequently the K-ATPase activity was measured on aliquots containing 0.2–0.6 mg protein in 2 ml of the same medium in the presence of 2.5 m*M* *tris*-ATP for 5 min at 25 °C. The specific activities of the non-alkylated proteins were on average for myosin 1.61, for desensitized actomyosin 0.74 and for myofibrils 0.49 μmoles of P released/mg protein/min. The initial slope of loss in ATPase activity with increasing extent of alkylation was taken as the inactivation rate of myosin (Figure 1). For desensitized actomyosin and myofibrils the slope of the linear portion of the curve occurring after an initial lag phase, was used (Figure 2). Relative inactivation rates in the Tables are averages of 2–6 experiments each. Protein concentrations were determined by a micromethod involving nesslerization[14].

Results and discussion. From the inactivation rate of the K-ATPase activity it can be calculated that both in the presence of Mg-ADP and of Mg-PP 1.8–2.0 moles of NEM per mole of myosin are sufficient to abolish the activity completely (Figure 1). Then if both myosin heads contain an active site which is able to bind a diphosphate chain[15–17], there is only 1 thiol group per active site exposed that is essential for the K-ATPase activity. One must also conclude that under these conditions the alkylation goes to virtual completion. If alkylation is, however, performed in the absence of bound diphosphate chains (Table I) or at higher temperature (25 °C)[18], the inactivation rate is considerably slower. In desensitized actomyosin in the gel state in which rigor type of interaction

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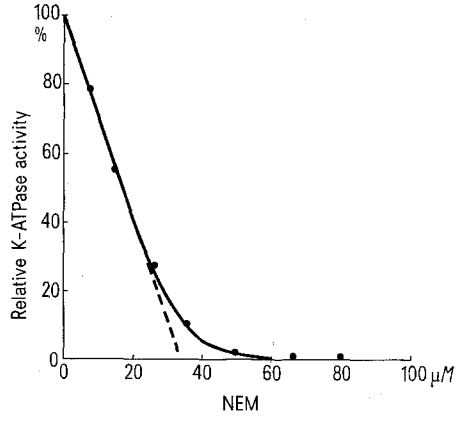

Fig. 1. Inactivation of K-ATPase of myosin. Pretreatment with different NEM concentrations was performed in the presence of 2.5 m*M* Mg-ADP for 15 min at 0 °C and pH 7.6 as described in methods.

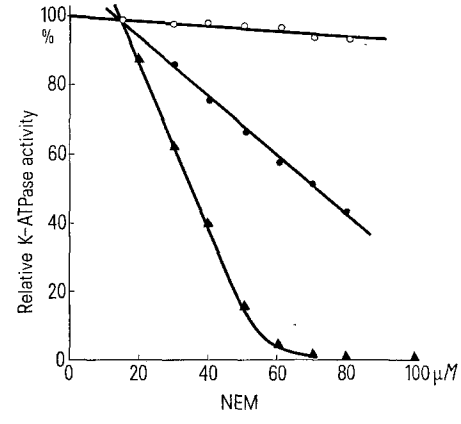

Fig. 2. Inactivation of K-ATPase of myofibrils. Pretreatment with different NEM concentrations was performed for 15 min at 0 °C and pH 7.6 on 10 mg of myofibrils in the presence of ○---○, 2.5 m*M* $MgCl_2$ only; ●---●, 2.5 m*M* Mg-PP; and ▲---▲, 2.5 m*M* Mg-PP plus 250 m*M* KCl.

Table I. Inactivation rates of K-ATPase of myosin by alkylation with NEM under various conditions as described in methods

Conditions	Loss in K-ATPase per μM of NEM (%)	Relative inactivation rate
2.5 mM Mg-ADP	2.95	100
2.5 mM Mg-PP	2.45	83
2.5 mM $MgCl_2$	1.50	51
2.5 mM $MgCl_2$ + 0.5 M KCl	1.47	50
10 mM EDTA	1.69	57

Table II. Relative inactivation rates of K-ATPase of desensitized actomyosin by alkylation with NEM under various conditions as described in methods

Conditions	Relative inactivation rate		
	no KCl	250 mM KCl	0.5 M KCl
2.5 mM Mg-ADP	79	100	98
2.5 mM Mg-PP	81	96	95
2.5 mM $MgCl_2$	21	19	22

Table III. Relative inactivation rates of K-ATPase of myofibrils by alkylation with NEM under various conditions as described in methods

Conditions	Relative inactivation rate	
	no KCl	250 mM KCl
2.5 mM Mg-ADP	49	100
2.5 mM Mg-PP	38	91
2.5 mM $MgCl_2$	6	5
10 mM EDTA	2	2
2.5 mM Mg-ATP	93	100

Table IV. Relative inactivation rates of K-ATPase of myofibrils by alkylation with NEM in the presence of ADP with and without ethanedioxybis (ethylamine) tetra-acetic acid (EGTA)

Conditions		Relative inactivation rate	
mM ADP	mM $MgCl_2$	no EGTA	1 mM EGTA
0	2.5	6	4
0.05	2.5	18	17
0.12	2.5	28	28
0.19	2.5	34	45
0.83	2.5	46	81
2.50	2.5	49	100
5.00	5.0	47	92
10.00	10.0	52	94

prevails, the thiol group connected with K-ATPase activity of myosin is well protected by actin[19]. However 2.5 mM Mg-ADP or Mg-PP which are known to bring about dissociation of actomyosin in solution as judged from viscometric measurements[20,21], lead to almost complete loss of the protective effect of actin in the presence or absence of 250 mM KCl or even dissolved in 0.5 M KCl (Table II). In all cases the rate is as high as for myosin alone in the presence of a bound diphosphate chain. This indicates that at 0 °C in actomyosin the myosin heads may be fully dissociated even at low ionic strength. On the other hand, firm interaction of the heads with actin in the gel, as well as in the dissolved state, is reflected by the slow inactivation rate with Mg^{2+} alone.

In myofibrils Mg-ADP and Mg-PP also seem fully to dissociate the myosin heads in the presence of 250 mM KCl (Table III). But, in the absence of salt, interaction of the myosin heads with actin takes place, although this interaction does not appear to be as protective as when no diphosphate chains were added (Figure 2). ATP, however, which is known to have a high affinity to the active site in the dissolved actomyosin system[22], leads also to full dissociation in myofibrils even in the absence of salt (Table III). The extent of interaction existing in the presence of Mg-ADP exhibits a concentration dependence below 0.2 mM only (Table IV). At ratios of ADP to active sites (assuming myofibrils contain 50% myosin by weight) above 20–30, a stable type of interaction is established which is not further affected by a 50-fold increase of ADP. However, this interaction depends on trace amounts of Ca^{2+}, since in the presence of ethanedioxybis(ethylamine)tetra-acetic acid (EGTA) full dissociation takes place as long as the ADP concentration is higher than 0.2 mM. The lower the ADP concentration below this critical value, the more rigor complexes form. Concomitantly the regulatory proteins of the actin filament lose their ability to prevent the formation of such rigor complexes in the absence of Ca^{2+}. These direct measurements of the interaction between actin and myosin in myofibrils thus lends support to similar conclusions of BREMEL and WEBER[23] based on Mg^{2+}-stimulated ATPase activities of acto-heavy meromyosin-subfragment-1 in solution. This view is also supported by X-ray diffraction studies of HASELGROVE[24] and HUXLEY[25] on whole muscle fibres in which myosin heads, forming rigor links in the absence of Ca^{2+}, change the position of the regulatory protein complex on the actin filament.

In muscle, myosin is known to exist predominantly as myosin product complex[26]. To avoid rigor complex formation in the absence of ATP, high concentrations of ADP have to be used (Table IV). It is concluded that even at such high concentrations of Mg-ADP, at low ionic strength and 0 °C, a specific interaction, distinct from true rigor, occurs in myofibrils, which cannot be produced by $MgCl_2$ and KCl alone. In full rigor, both heads of each myosin

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are supposed to be attached and, as the above results show, all essential thiol groups connected with the K-ATPase of myosin are protected from alkylation. The intermediate inactivation rate, resulting from alkylation in the presence of high Mg-ADP concentrations, implies some extent of ADP binding to myosin. It is unlikely that this effect is due to the existence of different myosin species (e.g. some with both heads protected by rigor attachment and others inactivated because of dissociation of both heads from actin) since it is not influenced by a 50-fold change in the ADP cencentration. Hence it may be concluded that all myosin molecules are in the same state, binding at least one ADP and interacting at the same time with actin. The additional evidence that when myosin-ADP complexes combine with actin, ADP becomes liberated[26], suggests that this specific interaction occurring in myofibrils comprises one ADP-free rigor-like interacting, and one ADP-loaded non-interacting head. Furthermore this particular type of cooperative interaction is regulated by the troponin-tropomyosin complex. In the context of active muscle, this view is in line with the suggestion of VINIEGRA-GONZALEZ and MORALES[27] that the myosin heads of one cross-bridge bind alternately on, and exert force at, two different monomers of the actin filament.

Zusammenfassung. Alkylierung von 2 Thiolgruppen pro Myosin mit NEM bei 0 °C in Gegenwart einer Diphosphatkette inaktiviert die K-ATPase vollständig. In Myofibrillen werden diese Thiolgruppen durch Rigor-«interaction» beider Myosinköpfchen mit Actin vor Alkylierung geschützt. In Gegenwart von Mg-ADP tritt eine spezifische vom Rigor verschiedene «interaction» zwischen Myosin und Actin auf. Man muss annehemen, dass dabei nur 1 Myosinköpfchen ans Actin bindet und dass das andere Köpfchen ein ADP gebunden hat.

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Storage of Insecticides in the Fat Body of *Spodoptera littoralis* (Boisd.) as a Possible Mechanism of Resistance

Storage of insecticides in the insect fat body has been shown by many workers to be a contributing factor in developing resistance. Lipids may act as competitive sites for toxic agents, especially if the latter are lipid soluble compounds (MUNSON et al.[1]). The fat body is also considered to be one of the most active tissues in the metabolism of some insecticides (FEWICK[2]; KUHR[3]).

Resistance of *Spodoptera littoralis* against insecticides is now a common phenomenon in Egypt. Among the factors that might contribute to the total resistance of this pest is the enhanced rate of storage of insecticides in the fat body. This possibility was investigated.

Materials and methods. Three R-strains and 1 S-strain of *S. littoralis* were used. The R-strains were reared under continuous pressure of the corresponding insecticides, DDT, methyl parathion and/or carbaryl. By injection, all the R-strains had an almost identical resistance level, i.e., 3-fold. By topical application, however, the DDT R-strain was 25-fold, the carbaryl R-strain was 13-fold, and the methyl parathion R-strain was 3-fold. Total fat content was determined by extracting the dried larvae according to BENNET and THOMAS[4].

Analytical grade samples of DDT, methyl parathion and carbaryl were used. All strains were injected with the LD_{50} of the S-strain. Batches of 50 full grown larvae were dissected to remove the fat body at various intervals after injection, and 0.2–0.3 g. samples were extracted by homogenizing in the appropriate solvent.

The extraction and clean up procedures of KLEIN et al.[5], MOLLHOFF[6] and JOHANSON et al.[7] and those of SCHECHTER et al.[8], COFFIN and MCKINELY[9], and MISKUS et al.[10] for the colourimetric determination, were adopted for the samples containing DDT, methyl parathion and carbaryl, respectively.

Storage of insecticides in the fat body of susceptible and resistant strains of the 6th instar larvae of *S. littoralis*

Insecticides applied	Time after treatment (h)	Amount of insecticide stored	
		S-strain (ppm)	R-strain (ppm)
DDT	0	Negligible	Negligible
(Amount injected	1.5	36	47
200 µg/g)	3	280	533
	6	167	208
	12	144	197
	18	150	131
	24	150	125
Methyl parathion	0	Negligible	Negligible
(Amount injected	1.5	17	43
30 µg/g)	3	28	48
	6	9	34
	12	7	15
	18	5	3
	24	0	0
Carbaryl	0	Negligible	Negligible
(Amount injected	1.5	5	8
400 µg/g)	3	16	15
	6	10	11
	12	9	8
	24	7	8

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