CONFORMATIONAL DIFFERENCES IN THE MYOSIN–ADP COMPLEX IN MYOFIBRILS AND ISOLATED MYOSIN

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1. Introduction

The existence of two distinct conformational states of the myosin enzyme (EC 3.6.1.3) depending on the presence of either ATP or ADP, has been demonstrated with the use of UV absorption [1], tryptophan fluorescence [2] and ESR [3] spectral techniques.

Transient kinetic investigations have further revealed that during the hydrolytic cycle a long-lived intermediate complex of myosin with the product which is rate-limiting must occur [4]. While the above-mentioned techniques require that the protein be in solution, we have been able to observe these conformational differences on myosin in the gel state and even in the myofibrils by following the extent of inactivation by alkylation of those thiol groups influencing the active sites.

2. Methods

Myofibrils [5] and myosin [6] were prepared from mixed rabbit skeletal muscles. For myosin preparations 5 mM EDTA were included in all solvents. Heavy meromyosin (HMM) was obtained from myosin by tryptic digestion [7]. Alkylation was performed with 40–400 molar excess of *N*-ethylmaleinimide (NEM) over active sites of myosin on 0.1–0.3 mg protein per ml in 30 mM KCl and 25 mM Tris-HCl pH 7.6 under conditions as indicated in the text. Usually NEM was added 1 min after addition of the nucleotide and the reaction terminated 5 min later by addition of a 100-fold molar excess of dithiothreitol over NEM. The samples were then 15 times diluted with 5 mM EDTA and 25 mM Tris-maleate pH 7.0, centrifuged at 20 000 *g* and the sedimented protein dissolved in 10 mM EDTA, 1 M KCl and 25 mM Tris-HCl pH 7.6 for assessment of the K-ATPase activity [8]. Protein concentrations were determined by the biuret method standardized on nitrogen estimations [9]. For calculations molecular weights for myosin and HMM were taken as 500 000 and 340 000, respectively, both containing two active sites per molecule [10]. The myosin content in myofibrils was assumed to be 50% of total protein.

3. Results and discussion

The detection of different conformational states of myosin–ADP complexes by ESR as used by Seidel and Gergely [3] involves attachment of spin labeled iodoacetamide to thiol groups (S_1 thiol group as defined by Kielley and Bradley [11]) on myosin which itself alters the transient kinetics of the enzyme [12, 13]. However, the reactivity towards alkylation of those thiol groups essential for the K-stimulated ATPase activity is strongly dependent on the conformation of the myosin–ADP complex resulting from either hydrolysis of ATP (M*ADP) or addition of ADP to myosin only (MADP). Therefore, in the present work, the inactivation of the K-ATPase by alkylation with NEM was used to differentiate between these two conformational states. Although with a 70-fold excess of NEM over myosin inactivation occurs

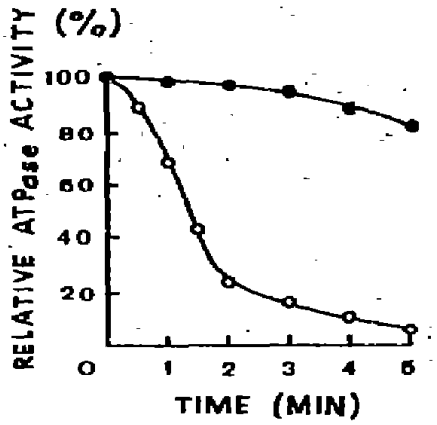

Fig. 1. Inactivation of K-ATPase of myosin by alkylation. Previous to the ATPase test alkylation was performed as described in Methods for different periods of time with 30 μM *N*-ethylmaleimide on 0.44 μM myosin at 25° in 30 mM KCl, 25 mM Tris-HCl pH 7.6 and 2.5 mM Mg-ATP (•—•—•) or Mg-ADP (o—o—o).

mainly within the first 2 min in the presence of Mg-ADP, alkylation was usually performed for 5 min revealing the largest difference to the case with Mg-ATP (fig. 1). If the alkylation reaction is performed after different incubation times of myosin with ATP, the extent of inactivation increases as the ATP becomes depleted by the ATPase activity (fig. 2). With a 28

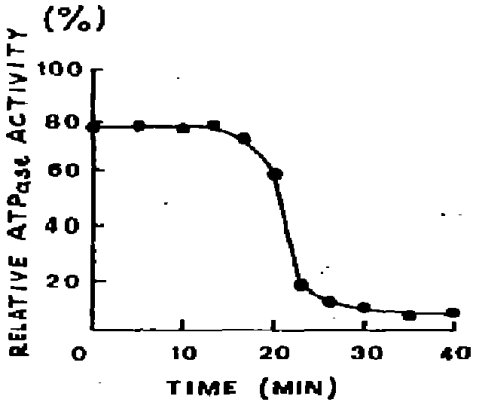

Fig. 2. Time course of change in inactivation of K-ATPase of myosin following addition of Mg-ATP. Previous to the ATPase test alkylation was performed with 30 μM *N*-ethylmaleimide for 5 min at 25° as described in Methods after incubation of myosin for different periods of time with Mg-ATP. 12.5 μM Mg-ATP were added at 0 time to 0.44 μM myosin in 30 mM KCl and 25 mM Tris-HCl pH 7.6 at 25°.

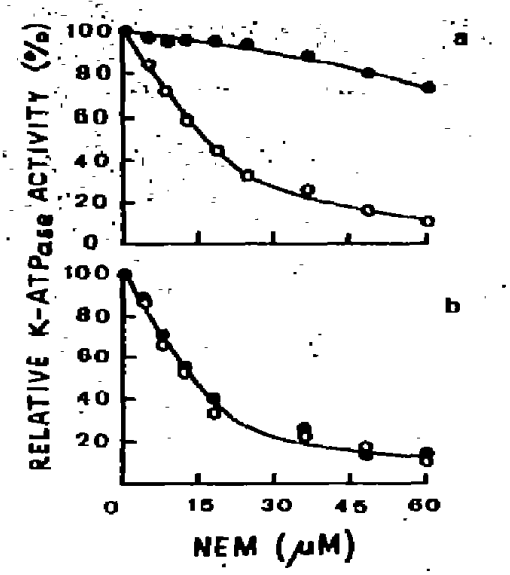

Fig. 3. Inactivation of K-ATPase of myofibrils by alkylation at 25° and 0°. Previous to the ATPase test alkylation was performed with different amounts of *N*-ethylmaleimide for 5 min as described in Methods on 0.25 mg myofibrils/ml in 30 mM KCl, 25 mM Tris-HCl pH 7.6 and 2.5 mM Mg-ATP (•—•—•) or Mg-ADP (o—o—o) at 25° (a) and at 0° (b).

molar excess of ATP over myosin it takes more than 20 min until fast inactivation of the K-ATPase occurs, indicating that one complete hydrolytic cycle lasts on average for about 100 sec. This value should be halfed, however, if only one of the two sites per myosin molecule is active at any instant.

It was moreover possible to use this method whether myosin was in the dissolved or gel state or even within the structural texture of the myofibril (fig. 3). The initial slope of loss in ATPase activity with increasing extent of alkylation was taken as the inactivation rate. In table I the inactivation rates in the presence of ATP are given as relative values compared to those which were obtained in the presence of ADP but otherwise identical conditions. Since the progress curve of ATP splitting by fully activated myofibrils at 37° tends to fall off rapidly with time [14] alkylation was started 3 sec after addition of ATP and terminated 30 sec later. As under these conditions an equally slow inactivation rate occurs in the presence of ADP due to partial transformation of ADP to ATP by myokinase, the inactivation rate in the presence of ATP had to be compared to that in the presence of 2.5 mM Mg-pyrophosphate and 250 mM KCl [15].

Table 1

Inactivation rates of K-ATPase of heavy meromyosin, myosin and myofibrils due to alkylation under various steady state conditions of hydrolysis.

Protein	Addition	Temperature in °C	Substrate hydrolysed in the steady state (μmole/min/mg protein)	Life time of hydrolytic cycle per active site (sec)	Relative inactivation rate in presence of ATP (rate in presence of ADP = 100)
Heavy meromyosin	Mg-ATP	25	0.00991	36	33 (2)
Myosin	Mg-ATP + 0.6 M KCl	25	0.000791	303	12 (2)
Myosin	Mg-ATP	25	0.00316	76	20 (9)
Myosin	Mg-ATP	0	0.000524	457	92 (3)
Myosin	Ca-ATP + 0.6 M KCl	25	0.112	2.1	7 (2)
Myosin	Ca-ATP	25	0.177	1.4	12 (4)
Myosin	Ca-ATP	0	0.0678	3.5	100 (3)
Myofibrils	Mg-ATP	25	0.421	0.28	25 (4)
Myofibrils	Mg-ATP	0	0.0173	6.9	97 (2)
Myofibrils	Mg-ATP	37*	1.47	0.082	29 (4)

* At 37° experiments were performed as indicated in the text.

Values in column 5 are calculated on the basis that both hydrolytic sites of myosin are simultaneously active [20, 21]. In each experiment, previous to the K-ATPase tests, alkylation with different amounts of *N*-ethylmaleimide was performed as described in Methods in the presence of 2.5 mM ATP and ADP in parallel. In column 6 inactivation rates in presence of ATP are given in arbitrary units relative to those obtained in presence of ADP, the latter being taken as 100 (number of experiments in parentheses).

From the results of Trentham and coworkers [4] it can be deduced that the M*ADP conformation of HMM exists at least for 97% of the complete hydrolytic cycle time. Their value for the catalytic centre activity of HMM of 0.041 sec^{-1} obtained from stopped-flow kinetics would indicate a life time of the rate-limiting M*ADP conformation of about 24 sec and corresponds well to the value given in table 1 of 36 sec derived from steady state measurements. Its duration for intact myosin in the gel state is also of the same order of magnitude whereas in solution in high salt it is even much longer lived. That the inactivation rate due to alkylation is persistently low under various conditions in which the turnover rate per active site is increased up to 4000-fold, indicates that the M*ADP conformation always remains the predominant species at 25°. If the M*ADP conformation does also occur at low temperature following hydrolysis, it is shorter lived relative to the subsequent MADP complex which is thus sufficiently long lived to allow full alkylation (table 1). In this case the MADP complex must become the rate-limiting step as recently suggested by Malik and Martonosi [13].

Our results on myofibrils indicate that not only in resting muscle [4] but also in the activated state M*ADP conformation exists as the prevailing form. Actin is thought to activate by shortening the lifetime of M*ADP through interaction with it and concomitantly accelerating the speed of ADP desorption [4], although actin is also able to undergo interaction with the MADP complex in myofibrils [15]. On the other hand Ca-ATP which does not elicit contraction [16], also leads to formation of M*ADP at 25°, a result also reported by Werber and coworkers [2] using fluorescence techniques. It therefore appears that this conformation of myosin is not directly linked to the force-generating part of the contraction cycle. In addition it has recently been shown that this part of the cycle can be produced without preceding hydrolytic step, simply by desorption of pyrophosphate or of the ATP analogue adenylyl imidodiphosphate from the myosin heads [17, 18]. According to Lymn and

Taylor [19] in the following step of the contractile cycle, ATP accomplishes displacement of the myosin heads from actin also without hydrolysis. Hydrolysis takes place after and leads to the long-lasting M*ADP conformation. One may conclude therefore, that this conformation ensures a slow turnover rate in order to avoid energy loss during rest. It also ensures the direct coupling of the hydrolytic with the working cycle on the molecular level because ATP can combine with the active site only after desorption of the product through actin intervention.

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