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RADIOACTIVE LABELING AND LOCATION OF SPECIFIC THIOL GROUPS IN MYOSIN FROM FAST, SLOW AND CARDIAC MUSCLES

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Summary

1. Based on incorporation of radioactively labeled *N*-ethylmaleimide, the readily reactive thiol groups of isolated myosin (EC 3.6.1.3) from fast, slow and cardiac muscles could be classified into 3 types. All 3 myosins contain 2 thiol-1, 2 thiol-2 and a variable number of thiol-3 groups per molecule. Both thiol-1 and thiol-2 groups which are essential for functioning of the K^+-stimulated ATPase, are located in the heavy chains in all 3 myosin types.

2. The variation in the incorporation pattern of *N*-ethylmaleimide over the 3 thiol group classes under steady-state conditions of $Mg^{2+} \cdot$ ATP hydrolysis allowed different conformations of some reaction intermediates to be characterized. In all 3 types of myosin the hydrolytic cycle of $Mg^{2+} \cdot$ ATP was found to be controlled by the same step at 25°C. In all three cases, this rate-limiting step is changed in the same way by lowering temperature.

3. Using the chemically determined molecular weights for myosin light chains, their stoichiometry was found on the basis of sodium dodecyl sulfate electrophoresis to be 1.2 : 2.1 : 0.8 for light chain-1:light chain-2:light chain-3 per molecule of fast myosin, 2.0 : 1.9 for light chain-1:light chain-2 per molecule of slow myosin and 1.9 : 1.9 for light chain-1:light chain-2 per molecule of cardiac myosin. This qualitative difference in light subunit composition between the fast and the two types of slow myosin is not reflected in the small variations of the characteristics exhibited by the isolated myosins, but rather seems to be connected with their respective myofibrillar ATPase activities.

Introduction

Myosin (EC 3.6.1.3) isolated from different types of striated muscles, such as fast-contracting (fast myosin), slow-contracting (slow myosin) and cardiac muscles, are known to differ in their specific monovalent and divalent

cation stimulated ATPase activities [1]. Chemical analyses showed that myosin from slow muscles as well as from the heart does not contain 3-methylhistidine as does fast myosin [2—4]. Intact fast myosin migrates faster in the presence of Mg^{2+} · ATP in polyacrylamide gel electrophoresis than slow myosin [5], and their subunit composition also differs, fast myosin containing 3 types of light chains in the 20 000 molecular weight range whereas myosins from slow and caridac muscles contain only 2 in sodium dodecyl sulfate electrophoresis [6].

Studies on thiol group reactivities, which may be carried out on intact myosin at low ionic strength in the presence of high concentrations of nucleotides, allow characterization of some of the intermediates occurring during the hydrolytic cycle of Mg^{2+} · ATP [7]. Under mild alkylation conditions only a limited number of thiol groups are accessible, but their blockage has a marked effect on its enzymic properties [8,9]. Changes in the reactivity of these available thiol groups reflect changes in their microenvironment, thereby yielding information on the conformational state of the protein [10]. In this way the conformation of the predominant species existing under steady-state conditions in the presence of either substrate or product was investigated. Since the accumulation of this species indicates the rate-limiting step in the process it may be concluded whether the hydrolytic cycle of fast and slow myosins is controlled by the same step.

The results presented here, concerning the reactivity of those thiol groups which affect the enzymic properties, indicate that the rate of hydrolysis of Mg^{2+} · ATP is limited by the same elementary step in both fast and slow myosins. At the same time all three types of myosin examined exhibit differences with respect to the reactivity of their non-essential thiol groups, reflecting differences in the composition of their respective heavy chains. These variations do not, however, parallel the large difference found in myofibrillar ATPase activities from fast muscles on the one hand and the two types of slow muscles on the other. It seems rather to be related to the subunit composition of the fast and slow myosins, respectively.

Methods

Preparation of myofibrils and myosin. Myofibrils and myosin were prepared from rabbit skeletal muscles (fast-contracting muscles: outer white parts of longissimus dorsi, quadriceps femoris and adductor group of the hindlegs; slow-contracting muscles: soleus and ischiotibialis) and from hearts of rabbits and pigs. Myofibrils were prepared according to Perry and Zydowo [11]. Myosin was extracted with a buffer system containing 150 mM potassium phosphate, 10 mM sodium pyrophosphate, 1 mM $MgCl_2$ and 300 mM KCl at pH 6.5 and 0°C [12]. In the following 3—4 precipitation cycles and in the medium for storage, 5 mM EDTA was found to be needed, in order to obtain consistent results of the degree of incorporation [13] of radioactive *N*-ethylmaleimide. The purity of all myosin preparations was monitored by electrophoresis in sodium dodecyl sulfate on polyacrylamide gels and it was found necessary to perform the final centrifugation for 2 h at 100 000 × *g* in the presence of 300—350 mM KCl in order to ensure that the actin contamination did not exceed about 1%.

Alkylation of myosin. Alkylation was carried out with a 2—100 molar excess of radioactively labeled *N*-[^{14}C]ethylmaleimide on 2—8 mg myosin/ml in 25 mM Tris · HCl buffer at pH 7.6 containing 20—50 mM KCl for 15—30 min under various conditions as indicated in the text. When alkylation was performed in the presence of Mg^{2+} · ATP, not more than 5% of it was hydrolyzed during the reaction time. The alkylation reaction was stopped by the addition of dithiothreitol, excess reagents removed by exhaustive dialysis, incorporated radioactivity assessed by liquid scintillation techniques and the K^+-stimulated, the Ca^{2+}-stimulated and the Mg^{2+}-stimulated ATPase activities of the alkylated myosin were measured as described in detail elsewhere [7]. To calculate the number of incorporated *N*-ethylmaleimide molecules per total myosin, a molecular weight of 470 000 was used for the heavy subunit 200 000 and for the light subunits the chemically-deduced molecular weight given in Table II.

Electrophoresis. Electrophoresis of myosin on 10% and 5% polyacrylamide gels in the presence of sodium dodecyl sulfate was carried out essentially according to Dunker and Rueckert [14] using 1% amidoblack 10B in 45% methanol and 9.2% acetic acid for staining. After exhaustive destaining in 7% acetic acid, the gels were scanned for staining intensity of the protein bands on a densitometer (Integraph CH, Bender and Hobein AG, Zürich, Switzerland). The peak areas of the heavy chains in 5% gels and of light chain-2 in 10% gels were calibrated for mass against tropomyosin which was then used as a marker in all electrophoretic runs. The amount of light chain-2 was calculated for calibration using the fact that it accounts for 8.14% by weight of total myosin [15,16].

For determination of the light subunit stoichiometry it was assumed that all light chains are stained with equal intensity. Usually 40—80 μg myosin were loaded on 10% gels and 5—15 μg on 5% gels. For assessment of radioactivity incorporated into the individual subunits, after scanning the gels the stained protein bands were cut out, the slices dissolved in 0.3 ml of 30% hydrogen peroxide for 15 h at 50° C [17] and counted for radioactivity after addition of 10 ml scintillation fluid. No additional quenching arising from the dissolved gel slices was observed.

ATPase assays. Myofibrillar ATPase activity was measured [18] on 0.15—0.60 mg protein/ml in 25 mM Tris · HCl buffer pH 7.6 containing 2.5 mM $MgCl_2$, 2.5 mM ATP, 0.05 mM $CaCl_2$ and 30 mM KCl at 25° C. In the cases of myofibrils from slow and cardiac muscles, 10 mM NaN_3 was included during the incubation. ATPase activities of myosin were measured at 25° C in 25 mM Tris · HCl pH 7.6 and 2.5 mM ATP on 0.1—0.2 mg protein/ml in the presence of 10 mM EDTA and 1 M KCl (K^+-stimulated ATPase) or on 0.5—0.8 mg protein/ml in the presence of 10 mM $CaCl_2$ and 0.5 M KCl (Ca^{2+}-stimulated ATPase) or on 0.8—1.4 mg protein/ml in the presence of 5 mM $MgCl_2$ and 20—50 mM KCl (Mg^{2+}-stimulated ATPase).

Buffers. Tris · HCl buffers were used in the pH range 7.6—9.5 and Tris · maleate for 5.5—7.1. All buffers were brought to the required pH at the temperature at which each incubation was carried out.

Protein concentration. Protein concentrations were determined by the biuret reaction which was standardized by ultramicro-Kjeldahl estimation of nitrogen [19].

Chemicals. Disodium ATP was converted into Tris · ATP by ionic exchange [20]. *N*-ethyl-1[^{14}C]maleimide of specific activity 8.4 mCi/mmol was obtained from New England Nuclear, Boston, U.S.A. All reagents were of analytical grade and all water was distilled and deionized on a mixture of Amberlite IR-120 and IRA-14 resins (1 : 2, w/w).

Results

1. Enzymic activities and myosin light chain composition

The well-known differences in the physiological properties between fast-contracting and slow-contracting muscles are reflected more pronouncedly in the Mg^{2+}-stimulated actomyosin ATPase of the structurally intact myofibrils than in the enzymic activities of isolated myosin from the respective types of muscle (Table I). The Mg^{2+}-stimulated ATPase of actomyosin isolated from fast muscles is reported to be twice [1,21] and about 4 times [22,23] as active as that of actomyosin from slow muscles. Weeds et al. [24] have shown, however, that this factor may be as high as 10, provided the 2 types of myosin are tested in the presence of high enough concentrations of actin. The factor ranges from 5—15 when the enzyme activity of myofibrils from fast muscles are compared to that of myofibrils from slow and heart muscles (Table I). Values of 68 for the Mg^{2+}-stimulated ATPase of rabbit heart myofibrils [25] and of 80—100 nmol P_i/min/mg for dog heart myofibrils [26], similar to that given here for rabbit heart myofibrils, have been reported.

With isolated myosin such large differences in the enzymic activities were not found. Again similar values ranging from 540—650 nmol P_i/min/mg for the K^+-stimulated ATPase activity of isolated myosin from dog, mouse, rabbit and rat hearts have appeared in the literature [27—29]. To characterize the divalent cation-stimulated ATPase of myosin, Mg^{2+} is preferred to Ca^{2+}, because in the latter case a wide variety of conditions are used by different workers. More importantly, the unavoidable contamination by Mg^{2+} which has a strong influence on the kinetics of the ATP hydrolysis cycle [30], cannot effectively be controlled. Of the scarce reports of values for the Mg^{2+}-stimulated ATPase,

TABLE I

SPECIFIC ATPase ACTIVITIES OF MYOFIBRILS AND MYOSINS FROM DIFFERENT MUSCLES

ATPase tests were carried out as described in Methods (number of experiments in brackets).

Muscle type	Specific ATPase activity in nmol P_i/min/mg protein			
	Myofibrils	Myosin		
	Mg^{2+}-stimulated ATPase	K^+-stimulated ATPase	Mg^{2+}-stimulated ATPase	Mg^{2+}-stimulated ATPase*
Rabbit fast	410 ± 80 (18)	1850 ± 250 (23)	8.2 ± 2.0 (23)	9.7 ± 1.7
Rabbit slow	81 ± 23 (8)	1260 ± 360 (9)	5.3 ± 2.1 (9)	2.3 ± 1.2
Rabbit heart	85 ± 19 (6)	600 ± 130 (6)	3.2 ± 1.3 (6)	2.4 ± 0.9
Pig heart	25 ± 4 (7)	630 ± 120 (11)	2.7 ± 0.6 (11)	4.1 (dog heart)

* Taken from ref. 31.

those taken from Katz et al. [31] show good agreement for the various muscle types.

Electrophoresis on polyacrylamide gels in sodium dodecyl sulfate revealed the 3-band pattern typical for fast muscle myosin and the 2-band pattern typical for slow and cardiac muscle myosin. The molecular weights calculated, using the migration distances on the gels, yielded values lying in the range of those reported by various authors [6,32–36]. However, for evaluation of the light chain stoichiometry in the cases of fast and heart muscle myosin, the molecular weights determined on the basis of their chemical composition (also shown in Table II) were used, since the electrophoretically estimated values are consistently too high. As the light chains of slow muscle myosin co-migrated with those of heart myosin when loaded on the same gels, the same values for molecular weights were used for determination of the light-chain stoichiometry for slow muscle myosin. However, in mixtures of fast muscle myosin with either slow or cardiac muscle myosin, the light chains resolved themselves into 5 distinct bands each at its appropriate molecular weight position.

The molar relationship clearly indicates that there are 2 mol of light chains of each type per mol of both slow and cardiac muscle myosin. In the case of fast muscle myosin, the results imply a light chain composition, when taken to the nearest integer, of 1 : 2 : 1 mol of light chain-1, light chain-2 and light chain-3 per myosin, corresponding to the so-called alkali-1, DTNB and alkali-2 light chains, respectively. The slight indication that there may be more of light chain-1 in comparison to light chain-3 could reflect heterogeneity in the fast muscles this myosin was prepared from. In fact, it has been claimed that in some preparations from fast muscles there are as many as 2 mol of light chain-1 per mol of light chain-3 [16,39].

Quantitative evaluation of the densitometric traces of the gels of all 3 types of myosin revealed the contamination by actin and C-protein [40] to be

TABLE II

MOLECULAR WEIGHTS AND STOICHIOMETRY OF LIGHT CHAIN COMPONENTS OF DIFFERENT MYOSINS

These were calculated from relative mobilities and staining intensities on sodium dodecyl sulfate electrophoretic gels as described in Methods. The chemically determined molecular weight values were used for the stoichiometry. In the case of slow myosin, they are assumed to be equal to those of cardiac myosin (see text).

Light chain	Calculated molecular weight	Chemical molecular weight	Reference	Staining intensity (%)	Mol per mol myosin
Fast myosin					
LC-1	24 750 ± 1 660	20 700	[37]	31.7 ± 3.6	1.15
LC-2	17 540 ± 1 820	19 000	[38]	52.2 ± 3.5	2.07
LC-3	13 280 ± 2 010	16 500	[37]	16.8 ± 2.4	0.77
Slow myosin					
LC-1	27 620 ± 1 420	21 000	—	55.7 ± 1.2	2.00
LC-2	22 050 ± 1 560	18 000	—	44.4 ± 1.2	1.85
Cardiac myosin					
LC-1	26 630 ± 1 130	21 000	[15]	53.4 ± 2.5	1.91
LC-2	20 570 ± 1 960	18 000	[15]	46.5 ± 2.5	1.94

below 1%. Between C-protein and the heavy chain positions, another faint band was occasionally observed. The myosin from pig heart showed an additional band in the 16 000 molecular weight position, which appeared after 5—10 h from the beginning of preparation and which increased in staining intensity over the following 3 days. It was established that this band represents a fragment of the heart light chain-2 since the latter decreased in proportion with the increase of this new band, while that of light chain-1 remained constant. Only a negligible amount of light chain-2 plus its degraded fragment is lost within a period of 3 weeks. Over the same period the K^+-stimulated ATPase falls only by about 15—20%. The degradation of light chain-2 into this distinct electrophoretic band at the 16 000 molecular weight position occurs in the same way whether the preparation and storage of pig cardiac myosin was done in the presence of either 5 mM EDTA or 1 mM $MgCl_2$. The occurrence of a third band in sodium dodecyl sulfate electrophoresis of cardiac myosins has also been reported by Swynghedauw et al. [35]. All further studies on thiol group reactivity and enzymic properties of cardiac myosin were carried out using preparations from pig hearts.

2. Incorporation of N-[^{14}C] ethylmaleimide

As the time course shows, the incorporation of radioactive *N*-[^{14}C]ethylmaleimide into cardiac myosin is almost complete after 20 min in the presence of Mg^{2+} · ADP at 25°C and low ionic strength, whereby 5 of the 23 *N*-ethylmaleimide molecules offered per myosin react (Fig. 1). Concomitantly with the increase of blockage of thiol groups by *N*-ethylmaleimide the K^+-stimulated ATP decreases and is virtually abolished when about 3 *N*-ethylmaleimide molecules are incorporated. The plateau exhibited by the extent of incorporation which develops after about 20 min suggests that only 5 thiol groups are readily available for alkylation. That the number of readily reacting thiol groups is rather a small one in view of the total of around 40 thiol groups in myosin [41] and that it is depending on temperature and the type of myosin,

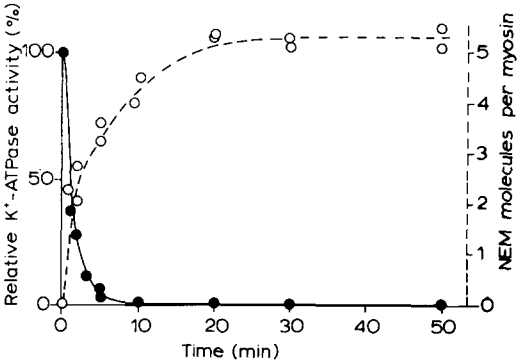

Fig. 1. Time course of incorporation of *N*-[^{14}C]ethylmaleimide into cardiac myosin and of the concomitant inhibition of the K^+-stimulated ATPase. Prior to the ATPase tests, alkylation was performed on 9.8 μM myosin in the presence of 5 mM $MgCl_2$, 5 mM ADP and 225 μM *N*-ethylmaleimide at 25°C and pH 7.6 for different times. (●), K^+-stimulated ATPase; (○), *N*-ethylmaleimide molecules (NEM) incorporated per myosin.

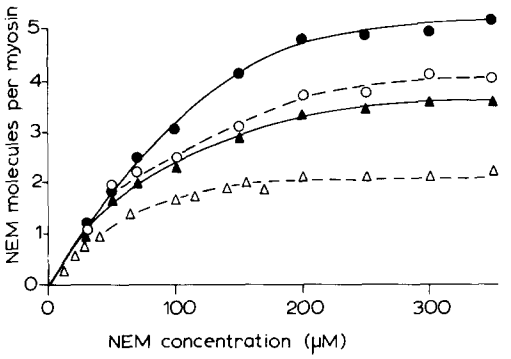

Fig. 2. Incorporation of *N*-[^{14}C]ethylmaleimide into fast and cardiac myosin plotted against *N*-ethylmaleimide (NEM) concentration. Alkylation was performed on 13—14 μM myosin in the presence of 5 mM $MgCl_2$ and 5 mM ADP for 20 min at pH 7.6 at 25° C (●,○) and 0° C (▲,△). Closed symbols, cardiac myosin; open symbols, fast myosin.

is supported by the pronounced level of extent of incorporation which also develops when the number of reacted *N*-ethylmaleimide molecules per myosin are plotted against concentration of the offered alkylation reagent to the protein (Fig. 2). In both types of myosin a significantly greater number of thiol groups react readily with *N*-ethylmaleimide at 25° C than at 0° C in the presence of Mg^{2+} · ADP. At both temperatures, however, about one more thiol group becomes readily blocked in cardiac as compared to fast muscle myosin. The extent of incorporation of *N*-ethylmaleimide into slow muscle myosin at high and low temperature under comparable conditions was similar to that found in fast muscle myosin.

It has been reported [42] that the degree of incorporation of *N*-[^{14}C]-ethylmaleimide into myosin of frog striated muscles was somewhat lower (at most 30%) when alkylation was performed at 1° C and low ionic strength in the presence of Mg^{2+} · ATP during hydrolysis than in the presence of Mg^{2+} · ADP. However, in these experiments the myosin was sequentially treated with dithiothreitol and iodoacetate, which may have led to some modification of the protein prior to the incorporation studies with *N*-[^{14}C]ethylmaleimide. Our results stemming from a total of 45 experiments on all 3 types of untreated myosin showed no significant difference in incorporation with Mg^{2+} · ADP or Mg^{2+} · ATP neither at 0° C nor at 25° C at low ionic strength (Table III). In fact the general trend is that in the presence of Mg^{2+} · ATP the degree of incorporation was a little higher.

3. Effect of thiol group blockage on ATPase activities

The 2 thiol groups of fast and slow muscle myosins which readily react with *N*-ethylmaleimide at 0° C in the presence of magnesium pyrophosphate, Mg^{2+} · ADP or Mg^{2+} · ATP during hydrolysis, are essential for the functioning of the K^+- stimulated ATPase because their blockage leads to complete loss of activity in both types of myosin (Fig. 3). In the case of cardiac myosin, however, the blockage of 3 thiol groups is required. The initial lag in the ATPase activity curve in Fig. 3C would indicate that the first reacting thiol group is

TABLE III

COMPARISON OF THE NUMBER OF *N*-[^{14}C]ETHYLMALEIMIDE MOLECULES PER MYOSIN INCORPORATED IN THE PRESENCE OF ADP OR ATP INTO DIFFERENT MYOSINS

Alkylation was carried out on 3—15 μM myosin for 1—20 min with *N*-[^{14}C]ethylmleimide ranging from 50—300 μM at 0°C and 25°C and pH 7.6 in the presence of 5 mM $MgCl_2$ and 5 mM nucleotide. Each pair of numbers was obtained under identical conditions of alkylation. Bottom row gives mean and standard deviation of ratio of values for ATP to ADP.

Mol *N*-[^{14}C]ethylmaleimide incorporated per mol myosin

Fast myosin				Slow myosin				Cardiac myosin			
0°C		25°C		0°C		25°C		0°C		25°C	
ADP	ATP	ADP	ATP	ADP	ATP	ADP	ATP	ADP	ATP	ADP	ATP
0.37	0.45	0.28	0.30	0.62	0.72	0.42	0.40	0.76	0.82	1.71	1.29
0.59	0.62	0.82	0.88	0.80	1.29	0.73	0.88	1.35	1.77	3.28	2.83
0.60	0.78	1.13	1.97	1.46	1.68	0.97	0.91	1.55	2.22	4.02	3.83
0.84	1.04	1.57	1.74	1.42	1.88	1.24	2.08	2.20	2.38	4.90	4.69
0.88	1.09	2.09	2.13	1.18	2.13	1.39	2.03	2.67	3.20	5.68	6.50
1.16	1.49	2.37	2.28			1.83	2.11	3.09	3.25	6.18	6.27
1.35	1.31	3.42	4.07			2.04	2.79			7.16	7.80
1.57	1.48	3.77	3.76			2.11	3.50				
1.81	1.80	4.99	5.42			2.79	3.15				
1.14 (±0.15)		1.14 (±0.24)		1.41 (±0.29)		1.28 (±0.28)		1.19 (±0.15)		0.97 (±0.13)	

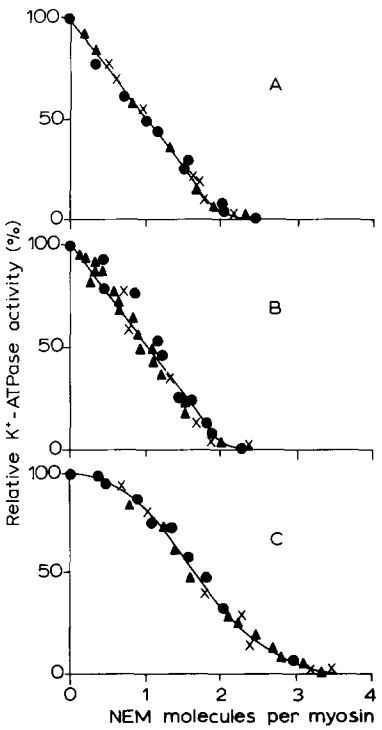

Fig. 3. Effect of incorporation of *N*-[^{14}C]ethylmaleimide (NEM) into different myosins on the K^+-stimulated ATPase. Before ATPase tests alkylation was carried out on 5—15 μM myosin at 0°C and pH 7.6 in the presence of 5 mM $MgCl_2$ and: (●), 2.5 mM pyrophosphate; (▲), 5 mM ADP; (X), 5 mM ATP. (A), fast myosin; (B), slow myosin and (C), cardiac myosin.

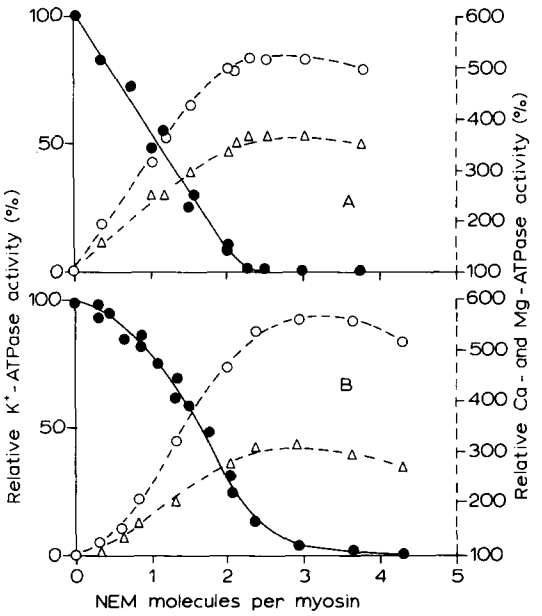

Fig. 4. Effect of incorporation of *N*-[^{14}C]ethylmaleimide (NEM) into slow and cardiac myosin on the mono- and divalent cation stimulated ATPases. Prior to the ATPase tests alkylation was performed on 5—15 μM myosin at 25° C and pH 7.6 in the presence of 5 mM $MgCl_2$ and 2.5 mM pyrophosphate. (●), K^+-stimulated ATPase; (○), Ca^{2+}-stimulated ATPase and (△), Mg^{2+}-stimulated ATPase. (A), slow myosin and (B) cardiac myosin.

not essential for the enzymic function. This non-essential thiol group which is the most reactive in cardiac myosin increases the number of readily available thiol groups to 3, in agreement with the level of incorporation reached at 0° C shown in Fig. 2.

Blockage of the 2 thiol groups essential for the K^+-stimulated ATPase in the presence of magnesium pyrophosphate at 25° C leads also, in slow and heart muscle myosin, to a concomitant increase of the divalent cation-stimulated ATPase activities when tested at high ionic strength and 25° C (Fig. 4), as has been shown for fast muscle myosin [13].

Both the Ca^{2+}- and Mg^{2+}-stimulated ATPases reach their optimum activities when the K^+-stimulated ATPase is abolished. Again in the case of cardiac myosin, the initial lag clearly visible in all 3 curves indicates that the first thiol group to react must be a non-essential one, just as when alkylation was carried out at 0° C (compare Fig. 3C). If higher concentrations of *N*-ethylmaleimide are offered to cardiac myosin in the presence of Mg^{2+} · ADP at 0° C, so that more than the 3 readily available thiol groups become blocked, the divalent cation-stimulated ATPases are fully inactivated after incorporation of 5 *N*-ethylmaleimide molecules per myosin. Since one of these groups is a non-essential one, there seem to be 4 thiol groups essential for the divalent cation-stimulated ATPases of cardiac myosin. Indeed it has been established that there are just 4 essential thiol groups in fast muscle myosin [13]. When alkylation is carried out under identical conditions but at 25° C, 6—8 *N*-ethylmaleimide molecules need to be incorporated before the Ca^{2+}-stimulated ATPase is abolished in both

slow and cardiac muscle myosins. Thus it has to be assumed that more than one non-essential thiol groups react interspersedly with the 4 essential ones at 25° C as opposed to 0° C. In the case of slow myosin at 0° C, 4—5 *N*-ethylmaleimide molecules per myosin need to be incorporated before the Ca^{2+}-stimulated ATPase is abolished, indicating that also in this type of myosin there are probably a total of 4 thiol groups essential for its enzymic functioning.

In contrast to the case at 0° C, a large difference in the pattern of incorporation into all 3 types of myosin emerges, resulting from the presence of the substrate Mg^{2+} · ATP or the product Mg^{2+} · ADP during the alkylation reaction at 25° C. At this temperature, several thiol groups which are not essential for the functioning of the K^+-stimulated ATPase are as reactive to *N*-ethylmaleimide as the essential ones in the state of the protein prevailing during ATP hydrolysis (Fig. 5). In the presence of Mg^{2+} · ADP, 2 essential thiol groups are the most reactive in fast muscle myosin as at 0° C, whereas one additional non-essential group must be simultaneously blocked before the enzymic activity of the slow and heart muscle myosins is abolished. Unlike the case of magnesium pyrophosphate (Fig. 4B), the lack of initial lag phase in the activity curves indicates that the non-essential thiol group does not have a higher reactivity than the 2 essential ones (Fig. 5B and C). Raising the temperature from 0° C to 25° C in the case of Mg^{2+} · ATP for all 3 types of myosin, causes an

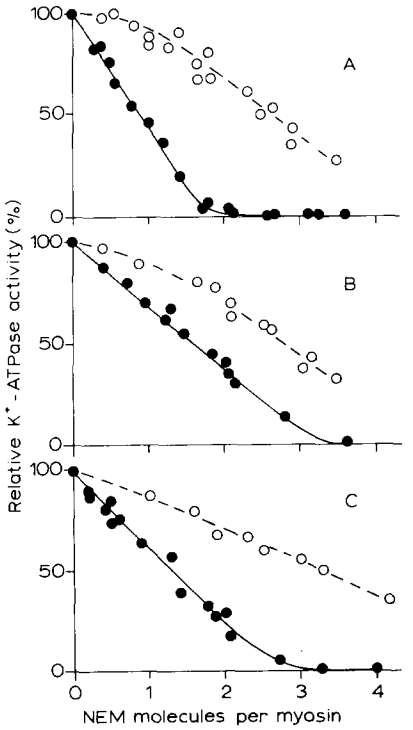

Fig. 5. Effect of incorporation of *N*-[^{14}C]ethylmaleimide (NEM) into different myosins on the K^+-stimulated ATPase. Prior to ATPase tests, alkylation was carried out at 25° C and pH 7.6 in the presence of 5 mM $MgCl_2$ and: (●), 5 mM ADP or (○), 5 mM ATP. (A), fast myosin; (B), slow myosin and (C), cardiac myosin.

increase in reactivity of non-essential thiol groups which must be accompanied by a decline in that of essential groups, rather than simply the exposure of more thiol groups additional to those exposed in the presence of Mg^{2+} · ADP. This follows since, as Table III shows, the degree of incorporation depending on whether substrate or product is present in the alkylating medium is always similar.

The number of molecules of *N*-[^{14}C]ethylmaleimide incorporated per mol of myosin subunit was calculated from the radioactivity recovered in the corresponding protein bands in sodium dodecyl sulfate gel electrophoresis. The total incorporation recovered from the elctrophoretic bands in the 6 experiments shown in Table IV amounted to 98.2% ± 6.3.

In all 3 types of myosin virtually no radioactivity was found in light chain-2 and light chain-3 and only less than 0.3 mol of *N*-ethylmaleimide were bound per mol of light chain-1. Therefore, most of the radioactivity was incorporated in the heavy chains indicating that the thiol groups essential for the K^+-stimulated ATPase preferentially blocked in the presence of Mg^{2+} · ADP as well as the non-essential ones readily blocked in the presence of Mg^{2+} · ATP are all located in the heavy chains. Even when the total degree of incorporation achieved in the presence of Mg^{2+} · ADP was increased to 5—6 *N*-ethylmaleimide molecules per myosin so that the divalent cation-stimulated ATPase activities were inhibited, there was no specific incorporation into any single light chain. Thus all 4 thiol groups essential for the activity must be located in the heavy chains.

4. Effect of temperature

The temperature dependence of the Mg^{2+}-stimulated ATPase activities of the fast muscle myosin on the one hand and the slow and heart muscle myosins

TABLE IV

DISTRIBUTION OF INCORPORATED *N*-[^{14}C]ETHYLMALEIMIDE MOLECULES OVER THE HEAVY AND LIGHT SUBUNITS OF FAST, SLOW AND CARDIAC MYOSINS RESOLVED IN SODIUM DODECYL SULFATE GEL ELECTROPHORESIS

Alkylation was performed on 12—15 μM myosin for 20 min at 25°C and pH 7.6 in the presence of 5 mM $MgCl_2$ and 5 mM nucleotide.

Nucleotide	*N*-[^{14}C]ethylmaleimide molecules incorporated per myosin	*N*-[^{14}C]ethylmaleimide molecules per myosin recovered from gel	*N*-[^{14}C]ethylmaleimide molecules recovered per subunit			
			Heavy chain	Light chain-1	Light chain-2	Light chain-3
Fast myosin						
ADP	2.93	2.75	1.13	0.20	0.08	0.14
ATP	3.57	3.45	1.39	0.29	0.10	0.18
Slow myosin						
ADP	3.42	3.14	1.28	0.24	0.05	—
ATP	3.62	3.40	1.34	0.29	0.07	—
Cardiac myosin						
ADP	3.30	3.56	1.38	0.29	0.11	—
ATP	3.35	3.48	1.39	0.27	0.08	—

on the other, exhibit somewhat different characteristics. The two distinct changes of slope in the Arrhenius plot of the former myosin type are virtually absent in those of the latter types whose slightly curved plots deviate only a little from linearity (Fig. 6). Because of the distinct regions visible in the Arrhenius plot of fast muscle myosin, the activation energies have been calculated for the corresponding temperature ranges of all 3 types of myosin (Table V). In view of the fact that values of the activation energy for enzyme catalyzed reactions are usually around 40—50 kJ/mol [43], all values shown for the Mg^{2+}-stimulated myosin ATPase at low ionic strength are relatively high with the exception of that of fast muscle myosin in the middle temperature range whose implications are discussed elsewhere [7]. In the high range near the physiological temperature there is a tendency towards higher activation energies the higher the specific activities are.

The curvature occurring over the temperature range of 5° C to about 12° C in the case of fast muscle myosin has been shown to correspond with the appearance of the difference in reactivity of essential thiol groups depending on the presence of Mg^{2+} · ADP or Mg^{2+} · ATP as the temperature of the alkylation reaction was raised [7]. Since this difference is obviously sensitive to temperature also in the cases of the slow and heart muscle myosins (compare Figs 3 and 5) but, however, is not reflected in their Arrhenius plots, the behaviour of the K^+-stimulated ATPase of cardiac myosin after alkylation in the presence of Mg^{2+} · ADP and Mg^{2+} · ATP at various temperatures was compared. From the linear portion of the plots of K^+-stimulated ATPase activity against increasing degree of incorporation achieved at various temperatures as in Figs 3 and 5, the percentage loss in activity per *N*-ethylmaleimide molecule incorporated was calculated. As soon as the temperature during alkylation was raised above 5° C, the percentage loss in activity began to decline in the case with Mg^{2+} · ATP whereas it remained at approximately the same level with Mg^{2+} · ADP (Table VI).

The hydrogen ion dependence of the steady state rate of the Mg^{2+}-stimu-

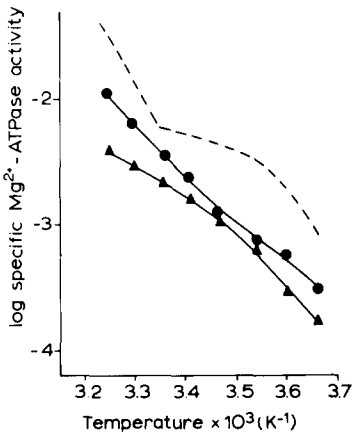

Fig. 6. Plot of logarithm of specific Mg^{2+}-stimulated ATPase activity against reciprocal absolute temperature. ATPase tests were carried out as described in Methods, using (●), slow myosin and (▲), cardiac myosin. Broken line for fast myosin is taken from ref. 7.

TABLE V

ACTIVATION ENERGY OF THE Mg^{2+}-STIMULATED ATPase REACTION OF FAST, SLOW AND CARDIAC MYOSIN OVER DIFFERENT TEMPERATURE RANGES EXPRESSED IN KJ/MOL

Myosin	Temperature range (°C)		
	0—5	10—20	25—35
Fast	119	17	153
Slow	80	71	80
Cardiac	96	71	45

lated ATPase at low ionic strength of all 3 types of myosin also exhibits a dependence on temperature, whereby the minimum occurring at around pH 8 at 25° C is transformed into an optimum in the pH range 7—8 at 0° C (Fig. 7). The well-known monophasic activation of the K^+-stimulated ATPase activity with decreasing hydrogen ion concentration of fast muscle myosin is also exhibited by the myosins from slow contracting and heart muscles. As has been shown earlier for fast muscle myosin this ATPase has the same pH dependence at 0° C and at 25° C. The pH dependence of the K^+-stimulated ATPase activity of cardiac and slow muscle myosins was also found to be insensitive to temperature.

Discussion

The light chain pattern in sodium dodecyl sulfate electrophoresis of both slow and cardiac myosins were found to be identical. This result is in good agreement with those from chemical analyses of the isolated light chains which yielded identical amino acid sequences of their thiol peptides [44]. In particular, the deduced stoichiometry strongly indicates that there are just 2 of each type of light chain in both types of myosin. There was no indication in the electrophoretic pattern in any preparation of slow muscle myosin of contamina-

TABLE VI

EFFECT OF TEMPERATURE ON THE INHIBITION OF THE K^+-STIMULATED ATPase OF CARDIAC MYOSIN EXPRESSED AS PERCENT LOSS IN ACTIVITY PER INCORPORATED *N*-[^{14}C]-ETHYLMALEIMIDE MOLECULE

Prior to ATPase tests, alkylation was performed on 4.7 μM myosin for 20 min at pH 7.6 and at different temperatures in the presence of 5 mM $MgCl_2$ and 5 mM nucleotide.

Temperature (°C)	% loss of K^+-stimulated ATPase per *N*-[^{14}C]ethylmaleimide incorporated per myosin		
	Mg^{2+}·ADP	Mg^{2+}·ADP	Ratio
0	41.2	41.2	1.0
5	31.6	29.4	1.1
10	36.1	27.4	1.3
15	46.6	26.8	1.7
20	46.7	27.6	1.7
25	39.1	16.7	2.3
32	34.5	18.3	1.9

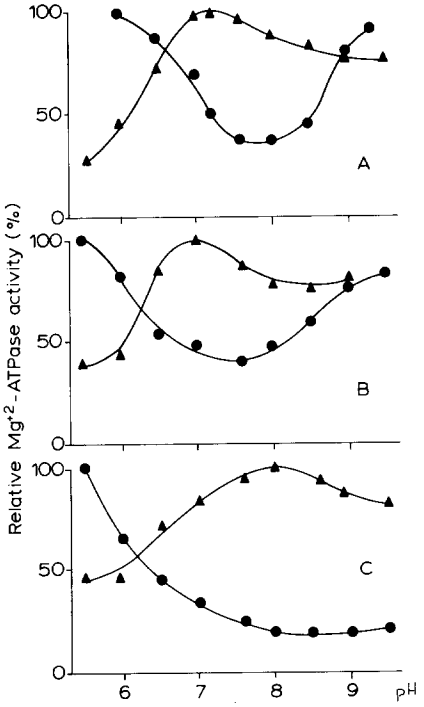

Fig. 7. Relative Mg^{2+}-stimulated ATPase activities of different myosins plotted against pH. ATPase tests were carried out as described in Methods at (●), 25°C and (▲), 0°C. (A), fast myosin; (B), slow myosin and (C), cardiac myosin.

tion with light chains typical for fast muscle myosin. Nevertheless both the K^+- and the Mg^{2+}-stimulated ATPase activities of isolated slow muscle myosin lie closer to those of fast muscle myosin than to those of cardiac myosin. Thus there seems to be no correlation between the light subunit composition and the enzymic activities of isolated myosins. It has to be emphasized, however, that the steady state rates of mono- and divalent cation-stimulated ATPases do only vary within a range of about 3-fold. The much larger difference in hydrolysis rates of the actin-activated ATPase of intact myofibrils from fast muscles on the one hand and from slow and cardiac muscles on the other, may indicate that the light chain composition of these 2 classes of myosins is involved rather in the interaction with actin as has recently been suggested [45,46].

Myosin from fast and slow muscles is reported to contain a total of 41—42 thiol groups [47]. In the case of fast muscle myosin the major portion, i.e. 32, is known to be located in the 2 globular head parts of the molecule [48], and that 6 of these are contained in the light chains [38]. Also the set of light chains in heart and slow muscle myosins contain 6 thiol groups [44], though the total number of thiol groups in cardiac myosin seems to be only about 31—36 [49,50; Léger, J.J., personal communication]. However, of this large number of thiol groups in all 3 types of myosin, only 4—6 react readily with *N*-ethylmaleimide in the presence of Mg^{2+} and polyphosphate ligands at low ionic strength. These reactive groups have been divided into 3 classes in fast myosin [13]. Thus it has been established that there are per myosin 2 thiol-1

groups, whose blockage activates the divalent cation-stimulated ATPase, 2 thiol-2 groups, whose subsequent blockage then inhibits this ATPase, and that all 4 of these thiol groups are required for the functioning of the K^+-stimulated ATPase. The third class contains several groups whose blockage does not affect the enzymic properties of myosin. The results presented here show that slow and cardiac myosins also contain 4 essential thiol groups, two of which display the properties of the thiol-1 class and the other two those of the thiol-2 class of fast myosin. A common feature of both slow types of myosin as opposed to fast, is that some thiol groups of class-3 always exhibit an equal or even higher reactivity towards *N*-ethylmaleimide than those of class-1 and class-2 in the presence of Mg · ADP. It has been reported that the thiol-2 groups essential for the Ca^{2+}-stimulated ATPase may be in the light chains of myosin [51], however, as many as 18—22 thiol groups had to be blocked with 2,4-dinitrophenyl β-hydroxyethyl disulfide before this ATPase was abolished, indicating that this reagent has a low specificity for the thiol-2 groups. Using *N*-ethylmaleimide for alkylation, a much lower degree of blockage is sufficient to fully inactivate the divalent cation-stimulated ATPase and the results clearly show that the most readily reacting thiol groups, in particular the 4 essential ones, are located in the heavy chains in all 3 types of myosin.

Kinetic studies on fast myosin subfragment-1 have established that the rate-limiting step in the hydrolytic cycle of Mg^{2+} · ATP at temperatures above 15°C is a conformational change of the myosin product complex from M^{**}ADPP to M^{*}ADPP [52,53]. In other words M^{**}ADPP is the predominant species under steady state conditions. It has been confirmed that M^{*}ADPP exists in series between M^{**}ADPP and M^{*}ADP [53]. It is very short lived and indistinguishable in its conformation from M^{*}ADP which can be generated by the binding of Mg^{2+} · ADP to myosin. Whereas isomerization of the myosin product complex from M^{**}ADPP to M^{*}ADPP is rather insensitive to temperature, the dissociation step of the product from myosin becomes markedly slower with decreasing temperature [53]. Thus at 0°C during turnover the product dissociation is rate limiting and the species M^{**}ADPP is short lived relative to M^{*}ADP which is now the predominant species under steady state conditions.

The 2 different conformational states, M^{**}ADPP formed on addition of Mg^{2+}· ATP to myosin at 25°C and M^{*}ADP formed on addition of Mg^{2+} · ADP to myosin, have been characterized by the readily reacting thiol groups and their distribution over the 3 defined thiol group classes [13]. Although a thiol-3 group reacts simultaneously with the first 2 essential ones in both types of slow myosins already in the presence of Mg^{2+} · ADP at 25°C, more groups of the third class become just as reactive as the essential ones in the presence of Mg^{2+} · ATP. Thus the slow myosins must adopt distinct conformations depending on whether substrate or product is present as is the case with fast myosin. Since the degree of incorporation of *N*-ethylmaleimide is the same whether substrate or product is present under identical conditions in all 3 types of myosin, the 2 conformations observed are characterized by a switch in the relative reactivity of thiol groups belonging to different classes. Moreover this difference in conformation disappears with lowering temperature, implying that in all 3 types of myosin, the conformation of the species prevailing during

hydrolysis at 0° C is identical to that formed by addition of Mg^{2+} · ADP. The overall parallel behaviour with respect to thiol group reactivity between both types of slow myosins on the one hand and the fast myosin on the other, indicates that despite the differences in specific ATPase activities, the isomerization of the myosin product complex is the rate-limiting step at 25° C and the product dissociation the rate-limiting step at 0° C in all 3 types of myosin. In fact it has recently been shown that the rate-limiting step in the hydrolytic cycle of cardiac myosin at 25° C is the isomerization of the myosin product complex and that its rate constant is about half that for fast myosin [54].

This change in the rate-limiting step is accompanied by a pronounced curvature in the Arrhenius plot of steady state activities occurring below 15° C in fast myosin [7]. The sharp break in slope around 18° C is not paralleled by changes in conformation as reflected in thiol group reactivity and so may result from a transition in the molecule not directly related to the hydrolytic site. No such marked deviations from linearity are to be seen in the Arrhenius plots of slow and cardiac myosin. Nevertheless, the fact that the incorporation pattern of *N*-ethylmaleimide obtained with Mg^{2+} · ATP is identical to that obtained with Mg^{2+} · ADP at 0° C in all 3 types of myosin, indicates that in the slow types of myosin this shift in the rate-limiting step also occurs by lowering the temperature. Further evidence that the kinetics of the hydrolysis cycle changes with temperature in fast and slow myosins alike, is given by the inversion of the shape of the steady-state rate dependence of the Mg^{2+}-stimulated ATPase on hydrogen ion concentration.

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