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CHARACTERISTICS OF MYOSIN MODIFIED BY β-HYDROXYETHYL-2,4-DINITROPHENYL DISULPHIDE IN THE PRESENCE OF PYROPHOSPHATE

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SUMMARY

The influence of pyrophosphate during modification of myosin by β-hydroxyethyl-2,4-dinitrophenyl disulphide (HEDD) was studied.

Pyrophosphate influenced the inhibitory phase of the action of HEDD on myosin Ca^{2+}-ATPase (ATP phosphohydrolase, EC 3.6.1.3), and protected the ability of myosin to form the actomyosin complex. Superprecipitation of actomyosin obtained from native actin and myosin, modified in the presence of pyrophosphate, was delayed.

Other HEDD-induced changes of properties of Ca^{2+}-ATPase were not influenced by pyrophosphate. Independent of the presence or absence of pyrophosphate during modification, the Ca^{2+}-ATPase activity of HEDD-modified myosin, in contrast to the Ca^{2+}-ATPase activity of native myosin (1) increased with increasing pH, (2) decreased only slightly with increasing concentration of KCl, (3) was inhibited at higher concentrations of Mg^{2+}. The actomyosin complex obtained from myosin, modified by HEDD in the presence or absence of pyrophosphate, dissociated at higher concentrations of ATP than the actomyosin obtained from native myosin and actin.

INTRODUCTION

Since KIELLEY AND BRADLEY[1] observed the biphasic effect of organic mercurials on Ca^{2+}-ATPase of myosin (ATP phosphohydrolase, EC 3.6.1.3) the influence of thiol reagents has been studied in more detail. It is now well known that blocking of the rapidly reacting myosin sulphydryl groups leads to the activation of myosin Ca^{2+}-ATPase, which is consistent with the characteristic changes in the response of the enzyme to variations in pH, temperature and ionic strength[2-5]. However, if myosin was modified by iodoacetamide, the pH–activity curve of carboxyamidomethyl myosin was very similar to that of untreated myosin, although the Ca^{2+}-ATPase activity increased considerably as compared with native myosin[6].

The effect of asymmetric β-hydroxyethyl-2,4-dinitrophenyl disulphide (HEDD) on Ca^{2+}-ATPase of myosin is similar to that of other thiol reagents. This effect, however, could be changed by the presence of pyrophosphate[7]. The aim of the present

Abbreviation: HEDD, β-hydroxyethyl-2,4-dinitrophenyl disulphide.

study was to examine in detail the influence of pyrophosphate on the modification of myosin by HEDD and on the properties of such modified myosin.

MATERIALS AND METHODS

Myosin was obtained from skeletal rabbit muscle by 10-min extraction with Edsall–Weber solution, and purified as described by PORTZEHL *et al.*[8]. After 5-fold precipitation, myosin was dissolved in 0.5 M KCl and 0.02 M Tris–acetate buffer (pH 6.9), centrifuged at 150000 × *g* in a Spinco L-50 preparative ultracentrifuge for 1 h, and stored at 0°. The myosin preparation used for experiments was never older than 5–7 days.

Actin was extracted from acetone-dry muscle powder prepared by the method of BÁRÁNY *et al.*[9]. Extraction was performed at 0°, and the product was purified further by the method of MOMMAERTS[10].

HEDD was obtained according to the method of BÖHME AND STACHEL[11]. The 0.25 mM standard solution of HEDD was freshly prepared before each experiment in the following way: 1 ml of 0.025 M HEDD solution in ethanol was added to 100 ml of 0.05 M Tris–acetate buffer (pH 6.9) containing 0.5 M KCl. The 0.25 mM HEDD solution was then filtered through VEB 388 h filter paper.

The ATP preparations were checked for purity by paper chromatography using ethanol–1 M acetic acid–1 M NH_4OH (75:28:2, by vol.) as described by BERGKVIST[12], and the preparations of pyrophosphate by paper chromatography according to the method of VOLMAR *et al.*[13].

Modification of thiol groups

In order to obtain myosin with different numbers of blocked thiol groups, a myosin solution in 0.5 M KCl, 0.05 M Tris–acetate buffer (pH 6.9) was incubated at 18° for 2 h with varying amounts of HEDD. Pyrophosphate, $MgCl_2$, or other reagents were added to the myosin solution 5 min before treatment with HEDD; the concentrations of these reagents are given in the table and figures. Modified myosin was separated from the reaction mixture on a Sephadex G-50 column.

The number of thiol groups of myosin blocked by HEDD was calculated as the difference in the amount of sulphydryl groups of native and of HEDD-modified myosin, both determined by the method described by WROŃSKI[14] or with the use of HEDD as described previously[15].

Estimation of ATPase activity

0.5–1 mg of myosin was incubated at 25° in a solution containing, if not otherwise stated, 0.17 M KCl, 0.1 M Tris–acetate buffer (pH 6.9) and 5 mM $CaCl_2$ and 3 mM ATP. The ATP solution and the incubation mixture before addition of ATP were adjusted to a definite pH with Radiometer PHM-22 (Kopenhagen). The reaction was stopped after 5 min by adding 1 ml of 10 % trichloroacetic acid.

The activating effect of actin on the ATPase activity of native or modified myosin was estimated in a medium containing 0.05 M KCl, 1 mM $MgCl_2$, 2 mM ATP, 20 mM Tris–acetate buffer (pH 6.9) and about 1 mg/ml of the mixture of myosin and actin. The incubation time was 5 min, and the reaction was stopped by addition of trichloroacetic acid. Myosin and actin were mixed at 0° in a ratio of 3:1 in 0.5 M KCl solution 1 h before ATPase estimation.

Orthophosphate liberated by myosin or actomyosin from ATP was determined either by the method of FISKE AND SUBBAROW[16] or that of MARTIN AND DOTY[17].

Determination of actomyosin formation

1.5–2.5 mg/ml native or modified myosin were mixed with 0.6–1.0 mg/ml of actin in 0.5 M KCl and 0.05 M Tris–acetate buffer (pH 6.9). After a 1-h incubation at 0°, the viscosity of the complex formed and that of the myosin and actin solutions used were measured in an Ostwald viscosimeter at 0°. Flow time for buffer was 40–60 sec. For the determination of viscosity three to six readings were taken if the difference between them was not more than 1 sec (to eliminate the effect of thixotropy). The ability of modified myosin to form a complex with actin was expressed as percentage of the ability of native myosin to form actomyosin. Calculation was done according to the equation given by BÁRÁNY AND BÁRÁNY[18].

Superprecipitation (syneresis) of actomyosin

This was determined by following the absorbance changes at 660 nm as described by EBASHI[19]. The change in absorbance of 2 mg of actomyosin gel suspended in a solution containing 0.05 M KCl, 0.05 M Tris–acetate buffer (pH 6.9), 1 mM $MgCl_2$; the final volume (4 ml) was measured after addition of ATP up to a concentration of 2 mM.

Protein estimation

The concentration of actin, native and modified myosin was determined either by protein estimation by the biuret method[20] or, in the case of native and modified myosin, by measuring their absorbance at 280 nm. The extinction coefficient $E^{1\%}_{280\,nm} = 5.60$.

RESULTS

Fig. 1 shows the ATPase activity of myosin with a varying equivalent of sulphydryl groups blocked by increasing amounts of HEDD in the absence and presence of pyrophosphate. As in the case of other thiol reagents, the Ca^{2+}-ATPase of myosin is first activated and then inhibited during the action of HEDD. The influence of pyrophosphate during the modification of myosin sulphydryl groups by HEDD is significant in the inhibitory phase of HEDD action. The Ca^{2+}-ATPase activity decreased slowly with increasing amounts of HEDD during modification when pyrophosphate was present, as compared with the rapid decrease of enzymic activity of myosin modified in the absence of pyrophosphate. ATPase activity of myosin determined at pH 6.9 and 0.5 M KCl increased during the activating phase of HEDD action only twice.

The protective effect of pyrophosphate was also observed when modification occurred in its presence without added Mg^{2+} (Table I). On the other hand, the protective effect of pyrophosphate was almost completely absent when myosin was modified in the presence of pyrophosphate and EDTA.

The modification of myosin by varying amounts of HEDD was carried out in a medium containing 0.5 M KCl and 50 mM Tris–acetate buffer (pH 6.9), 0.1 mM pyrophosphate at 18° for 2 h. The amount of sulphydryl groups blocked in the presence

or absence of pyrophosphate was found to be similar. The difference was ± 0.3 equiv of sulphydryl groups per 10^5 g of myosin.

The catalytic properties of myosin modified by HEDD in the presence of pyrophosphate were also studied, and it was found that some of the HEDD-induced changes in properties of Ca^{2+}-ATPase were not influenced by pyrophosphate.

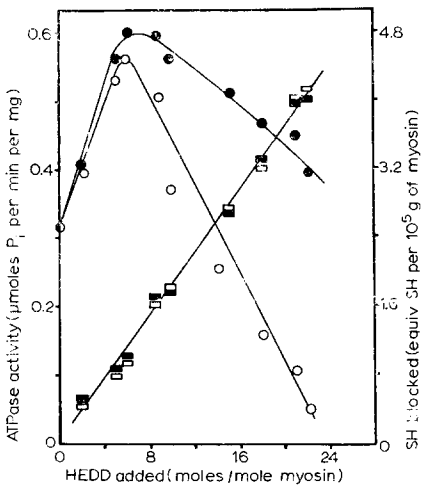

Fig. 1. Ca^{2+}-ATPase activity and number of blocked sulphydryl groups of myosin modified by varying amounts of HEDD. Myosin was modified in the presence of 1 mM Mg^{2+} and 0.1 mM pyrophosphate or without pyrophosphate. ATPase activity was measured at pH 6.9 and in the medium containing 0.5 M KCl, 10 mM Ca^{2+} and 2 mM ATP. ATPase activity of myosin modified by HEDD in the presence (●—●) and in the absence of pyrophosphate (○—○). The number of sulphydryl groups blocked by HEDD in the presence (■—■) and in the absence of pyrophosphate (□—□).

TABLE I

ATPase ACTIVITY OF MYOSIN MODIFIED BY HEDD UNDER VARIOUS CONDITIONS

20 mg of myosin solution in 0.5 M KCl and 50 mM Tris–acetate buffer (pH 6.9) was incubated for 2 h at 18° with 0.72 μmoles HEDD and with the additions given in the table. ATPase activity was measured in the presence of 0.5 M KCl at pH 6.9 after separation of modified myosin on a Sephadex G-50 column (for details see MATERIALS AND METHODS). The ATPase activity of native myosin determined under the same conditions was 0.320 μmole P_i per min per mg myosin.

Additions during modification	*Blocked sulphydryl groups (equiv/10^5 g myosin)*	*ATPase activity (μmoles P_i per min per mg myosin)*
1 mM pyrophosphate	3.4	0.540
1 mM pyrophosphate and 1 mM Mg^{2+}	3.1	0.675
1 mM pyrophosphate and 1 mM EDTA	3.3	0.242
1 mM Mg^{2+}	3.2	0.192
1 mM EDTA	3.2	0.208

Only a negligible decrease of Ca^{2+}-ATPase activity of HEDD-modified myosin can be noted with increasing KCl concentration, whereas the ATPase activity of native myosin decreased rapidly and that of *N*-ethylmaleimide-modified myosin

increased at higher KCl concentration. This effect of KCl was the same in all examined preparations of HEDD-modified myosin independent of the absence or presence of pyrophosphate during modification.

The specific Ca^{2+}-ATPase activity of HEDD-modified myosin at pH 7.6 was higher than that determined at pH 6.9, despite the fact that native myosin has the lowest activity at pH 7.6. In this respect, myosin modified by HEDD in the presence or absence of pyrophosphate was found to be similar to myosin treated with low concentrations of some other thiol reagents[3-5,21,22], and different from that modified by iodoacetamide[6].

Independent of the presence or absence of pyrophosphate during modification of myosin sulphydryl groups, the Mg^{2+}-ATPase activity of modified myosin increased in a manner similar to that described by SEKINE AND KIELLEY[5] and by GAETJENS *et al.*[23].

The antagonistic effect of Mg^{2+} and Ca^{2+} on the catalytic activity of native myosin observed by MOMMAERTS AND GREEN[24] was reduced when myosin was modified by HEDD. The ATPase activity of native myosin determined in the presence of 0.5 mM Mg^{2+} and 10 mM Ca^{2+} decreased to a value of 30 % of the specific ATPase activity determined in the presence of Ca^{2+} only. The decrease of Ca^{2+}-ATPase activity of myosin modified by HEDD in the presence or absence of pyrophosphate caused by the presence of 0.5 mM Mg^{2+}, was only about 10 % of the value obtained when ATPase activity of HEDD-modified myosin was determined in the presence of 10 mM Ca^{2+} only.

The ability of myosin modified by varying amounts of HEDD in the presence of pyrophosphate to form an actomyosin complex was compared with that of myosin modified by HEDD without pyrophosphate (Fig. 2). The values are expressed as percent of the ability of native myosin to form an actomyosin complex. They were calculated using the equation of BÁRÁNY AND BÁRÁNY[18] after measuring the viscosity of the mixture of actin and native or HEDD-modified myosin, and of actin and native and modified myosin solutions separately, in the concentrations used in the mixture. As seen from Fig. 2, the ability to form an actomyosin complex decreased slowly with increasing amount of blocked sulphydryl group equivalents if modification occurred in the presence of pyrophosphate, as compared with the rapid decrease of the ability of myosin, the sulphydryl groups of which were blocked in the absence of pyrophosphate.

Actomyosin formed from actin and HEDD-modified myosin dissolved in 0.5 M KCl undergoes dissociation at higher ATP concentrations than actomyosin formed from actin and native myosin. This occurred whether myosin was modified in the presence or in the absence of pyrophosphate.

It is well known that actin significantly enhances the ATPase activity of myosin in the presence of Mg^{2+} at low ionic strength. The activation is reduced by various agents which interfere with the interaction of myosin and actin. GAETJENS *et al.*[23] have shown, that the activating effect of actin on myosin modified by dithioglycolic acid dimethyl ester is partially abolished.

Fig. 3 shows the ATPase activity of myosin modified by HEDD in the presence or absence of pyrophosphate determined at low ionic strength in the presence of Mg^{2+} and actin. As can be seen, the activating effect of actin decreased rapidly if myosin was modified in the absence of pyrophosphate, and the value of ATPase activity

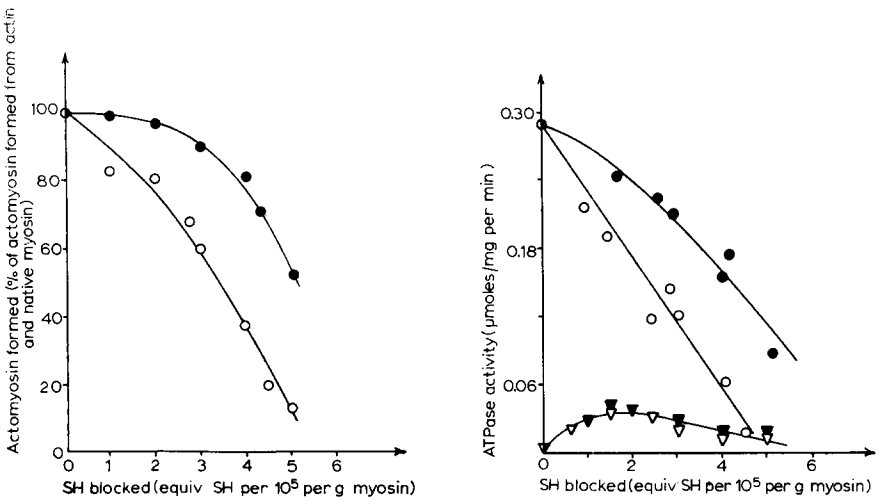

Fig. 2. Actomyosin formation from actin and myosin modified by HEDD in the presence or absence of pyrophosphate. The ability to form an actomyosin complex is expressed as percentage of the ability of native myosin (see MATERIALS AND METHODS). Actomyosin formation by myosin modified by HEDD in the presence (●—●) and in the absence of pyrophosphate (○—○).

Fig. 3. Mg^{2+}-ATPase activity of HEDD-modified myosin determined in the presence and absence of actin at low ionic strength. ATPase activity was measured at pH 6.9 in the presence of 0.05 M KCl, 1 mM $MgCl_2$ and 2 mM ATP (for details see MATERIALS AND METHODS). ATPase activity of myosin modified by HEDD in the presence of pyrophosphate determined in the presence of actin (●—●) and without actin (▲—▲). ATPase activity of myosin modified by HEDD without pyrophosphate determined in the presence of actin (○—○) and without actin (△—△).

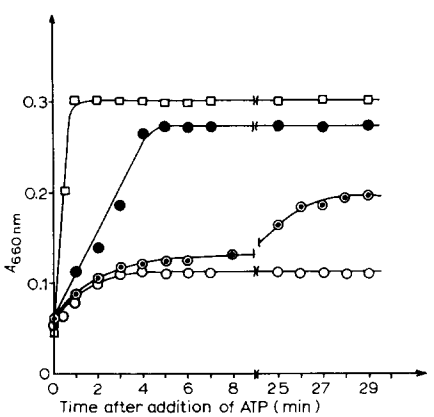

Fig. 4. Superprecipitation of actomyosin formed from native or HEDD-modified myosin and actin. Superprecipitation of actomyosin was determined by following the absorbance change at 660 nm after addition of ATP (for details see MATERIALS AND METHODS). Actomyosin obtained from actin and myosin modified by HEDD in the presence of pyrophosphate: ●—●, 1.6 equiv of sulphydryl groups blocked per 10^5 g of myosin; ⊙—⊙, 4.2 equiv of sulphydryl groups blocked per 10^5 g of protein. Actomyosin obtained from actin and myosin modified by HEDD without pyrophosphate: ○—○, 1.6 equiv of sulphydryl groups blocked; □—□, actomyosin obtained from actin and native myosin.

became of the same order as that of myosin modified by HEDD and estimated at low ionic strength in the presence of Mg^{2+} without actin. These results are in good agreement with those obtained with myosin modified by dithiodiglycolic acid dimethyl ester[24]. The activating effect of actin on ATPase activity of myosin modified in the presence of pyrophosphate decreased with increasing number of blocked sulphydryl group equivalents, but this decrease was not rapid.

Fig. 4 shows the superprecipitation of actomyosin complexes obtained from native myosin and from myosin modified in the presence or absence of pyrophosphate.

Superprecipitation of actomyosin obtained from myosin with 1.6 equiv of sulphydryl groups per 10^5 g protein blocked by HEDD in the presence of pyrophosphate, occurs later than that of actomyosin formed from native myosin and actin. Syneresis of actomyosin from myosin modified by HEDD without pyrophosphate with the same number of blocked sulphydryl group equivalents, was much lower. The ATPase activity of both modified myosins estimated in the presence of Ca^{2+} was higher than that of native myosin.

An actomyosin complex obtained from myosin with about 4.5 equiv of sulphydryl groups per 10^5 g protein blocked by HEDD in the presence of pyrophosphate superprecipitated 30 min later than actomyosin from native myosin and actin, and the absorbance increase measured at 660 nm was lower. The ATPase activity of HEDD-modified myosin used to form this actomyosin complex, estimated in the presence of Ca^{2+}, was about 50 % of the ATPase activity of native myosin.

DISCUSSION

Pyrophosphate present during modification of myosin by HEDD mainly influences the inhibitory phase of the action of HEDD. The effect of pyrophosphate protecting Ca^{2+}-ATPase activity against the inactivation by HEDD is correlated with the binding of pyrophosphate by myosin, as previously described[25].

The presence of pyrophosphate during the action of HEDD does not prevent changes resulting in the modification of the more reactive sulphydryl groups of myosin by HEDD.

Myosin in which about 2 equiv of sulphydryl groups per 10^5 g of protein have been blocked shows maximum activation of Ca^{2+}-ATPase, irrespective of whether the reaction with HEDD occurred in the presence or in the absence of pyrophosphate.

The characteristic response of the enzymic activity of myosin modified by HEDD to variations in KCl concentration and pH is independent of the absence or presence of pyrophosphate during modification.

The inhibitory effect of Mg^{2+} on ATPase activity of both HEDD-modified myosins (in the presence or absence of pyrophosphate) is diminished as compared with native myosin.

All these facts seem to suggest that the changes in the characteristic response of enzymic activity to the pH and KCl concentration as well as to the presence of Mg^{2+} are connected with the changes induced by sulphydryl reagents around a part of the ATP-binding sites near the adenine moiety of ATP if we assume that the ATP and pyrophosphate binding sites of myosin are the same.

The presence of pyrophosphate during modification of myosin sulphydryl groups by HEDD does not prevent the change connected with the influence of Mg^{2+}

on the Ca^{2+}-ATPase activity of myosin and the decrease of the dissociating effect of ATP on the actomyosin formed from actin and HEDD-modified myosin.

On the other hand, the presence of pyrophosphate during modification of myosin protects the ability of myosin to form an actomyosin complex. The actomyosin complex formed from myosin modified in the presence of pyrophosphate undergoes delayed superprecipitation in the presence of ATP and Mg^{2+} at low ionic strength.

These observations suggest that the change induced by modification of the more reactive myosin sulphydryl groups leads to alteration of the characteristic properties of the actomyosin complex, and that the decrease of ATPase activity of actomyosin is connected with the modification of myosin sulphydryl groups which can be protected by pyrophosphate against reaction with HEDD. The nature of these alterations requires further detailed study of this problem.

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