The Location of the Thiol Groups of Myosin that are Protected by Pyrophosphate against Reaction with 2,4-Dinitrophenyl β -Hydroxyethyl Disulphide

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Myosin modified in the presence or in the absence of pyrophosphate by 2,4-dinitrophenyl β -hydroxyethyl disulphide was treated with iodo[1- 14 C]acetamide. The residual Ca^{2+} -stimulated adenosine triphosphatase (ATPase) activity of the modified myosin was different depending on the presence or absence of PP_i during modification and the number of 2,4-dinitrophenyl β -hydroxyethyl disulphide-modified thiol groups. The radioactivity incorporated into the light components of myosin correlated with the Ca^{2+} -stimulated ATPase activity of the modified myosin and decreased with decreasing residual Ca^{2+} -stimulated ATPase activity of the modified myosin. When native myosin was treated with low concentrations of iodo[1- 14 C]acetamide the residual Ca^{2+} -stimulated ATPase activity of carboxyamidomethylated myosin was high and the radioactivity incorporated into the light components of myosin was negligible. The thiol groups of the light components of myosin are essential to preserve the ATPase activity of the protein and are close to the pyrophosphate-binding sites.

The inhibitory phase in the biphasic response of Ca^{2+} -stimulated ATPase* activity of myosin, observed during the action of several thiol reagents, is influenced by the presence of ATP and PP_i (Morales & Hotta, 1960; Gilmour & Gellert, 1961; Sekine & Yamaguchi, 1963; Stracher, 1964; Kominz, 1965). Two opposite effects of both ATP and PP_i on the inactivation of Ca^{2+} -stimulated ATPase (ATP phosphohydrolase, EC 3.6.1.3) induced by modification of thiol groups of myosin have been observed, depending on the nature of the blocking agent. If mercurial thiol reagents (Morales & Hotta, 1960; Gilmour & Gellert, 1961; Kominz, 1965), iodoacetamide (Stracher, 1964) or 2,4-dinitrophenyl β -hydroxyethyl disulphide (Kakol & Gruda, 1966) were used for modification of myosin, the inactivation of the Ca^{2+} -stimulated ATPase activity could be decreased by the presence of ATP or PP_i . On the contrary, the inactivation of the Ca^{2+} -stimulated ATPase by *N*-ethylmaleimide is strongly accelerated in the presence of ATP and it was assumed that ATP makes certain thiol groups more reactive to *N*-ethylmaleimide and that their modification results in the loss of the Ca^{2+} -stimulated ATPase activity (Sekine & Yamaguchi, 1963; Yamashita, Soma, Kobayashi & Sekine, 1965).

On the other hand Stracher (1963, 1964) suggested that the thiol groups of myosin, which are protected by ATP during treatment of bis- β -carboxyethyl disulphide-modified myosin with iodoacetamide, are essential in the enzymic activity of myosin. These thiol groups are different from those protected by ATP and PP_i against reaction with bis- β -carboxyethyl disulphide, and are not located in the enzymically active subfragment of myosin obtained by trypsin digestion (Trotta, Dreizen & Stacher, 1968). The findings of Trotta *et al.* (1968) suggest that the thiol groups which remain unmodified after treatment of myosin with bis- β -carboxyethyl disulphide and which are protected by ATP against reaction with iodoacetamide are not actually present at the active site of Ca^{2+} -stimulated ATPase. These thiol groups of myosin do not seem to be the same as those protected by ATP and PP_i against reaction with several thiol reagents, and whose modification is connected with the loss of the Ca^{2+} -stimulated ATPase activity, since bis- β -carboxyethyl disulphide-modified myosin is enzymically inactive, and the disulphide bonds must be reduced if the protein is to recover its catalytic properties.

It was therefore decided to determine the part of the myosin molecule in which are located the thiol groups of myosin that are protected against

* Abbreviation: ATPase, adenosine triphosphatase.

thiol reagents by ATP and PP_i , and whose modification leads to the inactivation of the ATPase activity.

The ability of PP_i to protect the Ca^{2+} -stimulated ATPase activity against inactivation by 2,4-dinitrophenyl β -hydroxyethyl disulphide, and the modification of the remaining thiol groups with iodo[1- ^{14}C]acetamide, were used to study this problem.

MATERIALS AND METHODS

Materials. Myosin was prepared from rabbit skeletal muscle by extraction with Edsall-Weber solution for 10 min and was then purified as described by Portzehl, Schramm & Weber (1950). The myosin was precipitated five times and then dissolved in 0.5 M-KCl-0.02 M-tris-acetate buffer, pH 6.9. This solution was centrifuged at 105 000 g in a Spinco L-50 preparative ultracentrifuge for 1 h. The preparation was not stored for longer than 5-7 days. Some of the myosin preparations were purified by precipitation with ammonium sulphate at 50% saturation. H-Meromyosin was prepared by the method of Lowry & Cohen (1962) and was further purified by fractionation with ammonium sulphate. The fraction precipitated between 45% and 50% saturation was collected. The 2,4-dinitrophenyl β -hydroxyethyl disulphide was prepared by the method of Böhme & Stachel (1957). Iodo[1- ^{14}C]acetamide was obtained from The Radiochemical Centre, Amersham, Bucks., U.K., and unlabelled iodoacetamide was from Koch-Light Laboratories, Colnbrook, Bucks., U.K. The purity of ATP was checked by paper chromatography as described by Bergkvist (1956).

Modification of the thiol groups of myosin by 2,4-dinitrophenyl β -hydroxyethyl disulphide. To prepare myosin with a given number of blocked thiol groups, a myosin solution in 0.5 M-KCl-0.05 M-tris-acetate buffer, pH 6.9, containing either 1 mM- Mg^{2+} or 1 mM- Mg^{2+} with 0.1 mM- PP_i , was incubated with different amounts of the disulphide for 2 h. The final concentration of myosin was 5-10 mg/ml.

After incubation, the modified myosin was separated from the reaction mixture either on a Sephadex G-50 column or by being precipitated by 14-fold dilution with ice-cold deionized water. The precipitate was centrifuged at 6000 g at 0°C, and the pellet washed twice with 0.05 M-KCl and finally dissolved in 0.5 M-KCl.

The number of thiol groups blocked by 2,4-dinitrophenyl β -hydroxyethyl disulphide was calculated as the difference in the number of thiol groups in unmodified protein and in disulphide-modified protein, both determined by the method described by Wroński (1967) or with the use of 2,4-dinitrophenyl β -hydroxyethyl disulphide as described previously (Kakol, Gruda & Bitny-Szlachto, 1964).

Modification of the thiol groups of native and 2,4-dinitrophenyl β -hydroxyethyl disulphide-modified myosin by iodo[1- ^{14}C]acetamide. Native or disulphide-modified myosin solution (approx. 5 mg of protein/ml in 0.5 M-KCl-50 mM-tris-HCl buffer, pH 8.5) was incubated with various amounts of iodo[1- ^{14}C]acetamide at 18°C for 1 h. The reaction mixture was then adjusted to pH 6.9 with 0.01 M-HCl and was diluted 14-fold by dropwise addition of ice-cold deionized water. The precipitate of iodo[1- ^{14}C]acetamide-modified myosin was separated by centri-

fugation at 20 000 g for 30 min, washed three times with 0.03 M-KCl and finally dissolved in 0.5 M-KCl-10 mM-tris-HCl buffer, pH 7.9. The radioactivity of the supernatants from the washings was measured in a Nuclear-Chicago model D47 gas-flow detector. The third wash supernatant was not appreciably radioactive. In some experiments carboxyamidomethylated myosin was purified by dialysis against three changes of 0.5 M-KCl-5 mM-tris-acetate buffer, pH 7.0, at 4°C for 48 h. The radioactivity of the third diffusate was negligible.

The Ca^{2+} -stimulated ATPase activity and radioactivity of iodo[1- ^{14}C]acetamide-modified myosin were measured after dilution to about 0.5 mg of protein/ml with 0.5 M-KCl. The protein content of diluted carboxyamidomethylated myosin was measured spectrophotometrically.

Treatment of myosin with sodium carbonate at pH 11. A portion (5-10 mg) of iodo[1- ^{14}C]acetamide-modified myosin in 1 ml of 0.5 M-KCl-0.1 M- Na_2CO_3 (adjusted to pH 11) was incubated for 30 min at 30°C and then adjusted to pH 6.9 with 0.01 M-HCl and finally diluted 14-fold with ice-cold deionized water. The suspension was centrifuged for 40 min at 20 000 g. The protein content of the supernatant, which contained the liberated light components of the myosin molecules, was measured spectrophotometrically at 280 nm. The radioactivity in the supernatant was measured with a gas-flow detector.

The precipitated high-molecular-weight component was dispersed in 5-10 ml of 0.5 M-KCl-10 mM-tris-HCl buffer, pH 7.9, and the protein content, the Ca^{2+} -stimulated ATPase activity and the radioactivity were measured. The radioactivity of the light alkali component was calculated from the radioactivity of the supernatant, and as the difference between the radioactivity of 1 mol (500 000 g) of carboxyamidomethylated protein before and after treatment with Na_2CO_3 . The u.v.-absorption spectra of native myosin, disulphide-modified myosin, carboxyamidomethylated myosin and carboxyamidomethylated myosin after treatment with Na_2CO_3 were measured at pH 7.9 in a Beckman DB spectrophotometer and were practically the same; therefore the amount of protein in all the myosin solutions was calculated from the E_{280} of the protein solutions by using the extinction coefficients $E_{280}^{1\%}$ 5.6 for myosin, $E_{280}^{1\%}$ 6.47 for H-meromyosin and $E_{280}^{1\%}$ 3.5 for the light component.

Estimation of ATPase activity. Native or modified myosin (0.5-1 mg) was incubated at 25°C for 5 min in 0.15 M-KCl-0.1 M-tris-acetate buffer, pH 6.9, containing 10 mM- $CaCl_2$ and 3 mM-ATP, in a final volume of 2 ml. The reaction was stopped by addition of 1 ml of 10% trichloroacetic acid. Liberated P_i was measured by the method of Fiske & Subbarow (1925).

RESULTS

The effect of PP_i in protecting the Ca^{2+} -stimulated ATPase activity of myosin against inactivation by 2,4-dinitrophenyl β -hydroxyethyl disulphide (Kakol & Gruda, 1966) can also be observed with H-meromyosin. As shown in Fig. 1, modification of H-meromyosin thiol groups by 2,4-dinitrophenyl β -hydroxyethyl disulphide led to a rapid decrease of the Ca^{2+} -stimulated ATPase activity, when more than 1 thiol group equivalent was blocked/ 10^5 g of

Fig. 1. Ca^{2+} -stimulated ATPase activity as a function of the number of thiol groups of H-meromyosin blocked by 2,4-dinitrophenyl β -hydroxyethyl disulphide in the absence (○) or in the presence (●) of PP_i . ATPase activity was measured in 0.5 M-KCl-0.05 M-tris-HCl buffer, pH 7.9, containing 10 mM- CaCl_2 and 3 mM-ATP. ATPase activity of the unmodified myosin was $0.580 \mu\text{mol}$ of P_i /min per mg.

protein. Thus, when PP_i was present during the action of the disulphide, the ATPase activity of H-meromyosin with 4 thiol group equivalents blocked/ 10^5 g of protein was over 80% of the ATPase activity of unmodified H-meromyosin.

Further studies were carried out by incubating H-meromyosin, previously modified by 2,4-dinitrophenyl β -hydroxymethyl disulphide in the presence or in the absence of PP_i , with 0.01 M-iodo[1- ^{14}C]-acetamide at 18°C for 1 h. This led to labelling of the thiol groups that had remained unchanged during modification by the disulphide. The carboxyamido-methylated protein was purified by dialysis for 48 h against three changes of solutions containing 5 mM-tris-acetate buffer, pH 6.9, and was then incubated at 18°C for 60 min in 0.1 M-sodium carbonate or in 4 M-lithium chloride and then subjected to gel filtration on Sephadex G-200 equilibrated with the same solvent mixture. However, the light components could not be quantitatively separated from the core of H-meromyosin. Although the amounts of the separated light components from carboxyamido-methylated H-meromyosin varied, the radioactivity per mg of the light components was always high when disulphide-modified H-meromyosin was enzymically active before treatment with iodo[1- ^{14}C]-acetamide. The radioactivity of the light

components obtained from carboxyamido-methylated disulphide-modified H-meromyosin, inactivated during modification by 2,4-dinitrophenyl β -hydroxyethyl disulphide, was negligible.

Therefore further studies were carried out on disulphide-modified myosin in which the remaining free thiol groups were labelled with iodo[1- ^{14}C]-acetamide. The light components of myosin that had been modified by the disulphide and then carboxyamido-methylated were separated by treatment with sodium carbonate at pH 11 as described in the Materials and Methods section. Protein determinations (Table 1) on light components separated by treatment of both native and modified myosin with sodium carbonate at pH 11 and centrifugation after precipitation of the fibrous component of myosin by 15-fold dilution with ice-cold deionized water at 20 000 g were repeatable.

The the radioactivity of the light components of carboxyamido-methylated myosin, native myosin and of disulphide-modified myosin, and the Ca^{2+} -stimulated ATPase activity of the proteins before and after treatment with various amounts of iodo[1- ^{14}C]-acetamide, were compared. If native myosin was treated with iodo[1- ^{14}C]-acetamide in increasing concentrations, the radioactivity in the complete protein increased, but the light components remained practically unlabelled. The carboxyamido-methylation of native myosin at these concentrations of iodo[1- ^{14}C]-acetamide led in the presence and in the absence of PP_i to a significant increase of Ca^{2+} -stimulated ATPase activity (Table 2). However, the presence of PP_i during carboxyamido-methylation resulted in a greater increase in the ATPase activity, owing to the modification of rapidly reactive thiol groups of myosin.

If disulphide-modified myosin, with about 8 thiol group equivalents blocked/mol of protein, was treated with iodoacetamide at the concentrations described above, the radioactivity incorporated into total protein was greatly diminished.

The treatment of disulphide-modified myosin with a large excess of iodo[1- ^{14}C]-acetamide at pH 8.5 led to inactivation of the Ca^{2+} -stimulated ATPase activity of modified myosin and to a large incorporation of radioactivity into the light components of myosin. There was a correlation between the radioactivity found in light components of myosin and the ATPase activity, and the number of thiol groups blocked by 2,4-dinitrophenyl β -hydroxyethyl disulphide before treatment with iodo[1- ^{14}C]-acetamide. Fig. 2 shows the relation between the radioactivity found in light components of myosin and the ATPase activity of disulphide-modified myosin as well as the number of thiol groups blocked in the protein. Increasing the number of thiol groups blocked by 2,4-dinitrophenyl β -hydroxyethyl disulphide is associated

Table 1. *Separation of light components of myosin by treatment with 0.1 M-sodium carbonate at pH 11*

Abbreviations: M_n , native myosin; M_p , native myosin purified by precipitation with 50% saturated ammonium sulphate; $M_{\text{HEDD-1}}$, myosin modified by 2,4-dinitrophenyl β -hydroxyethyl disulphide in the presence of PP_i (25 thiol group equivalents blocked/mol of protein); $M_{\text{HEDD-2}}$, myosin modified by 2,4-dinitrophenyl β -hydroxyethyl disulphide in the absence of PP_i (25 thiol group equivalents/mol of protein). ATPase activity was completely lost after treatment with sodium carbonate.

Preparation	Amount of protein before treatment with Na_2CO_3		Amount of protein in supernatant		Percentage of total protein
	$E_{1\text{cm}}^{280}/\text{ml}$	mg	$E_{1\text{cm}}^{280}/\text{ml}$	mg	
M_n	14.7	26.2	1.09	3.1	11.8
M_p	14.7	26.2	0.94	2.68	10.3
M_s	10.50	18.8	0.51	1.45	7.7
M_p	10.50	18.8	0.54	1.54	8.2
$M_{\text{HEDD-1}}$	6.65	11.8	0.38	1.08	9.2
$M_{\text{HEDD-1}}$	6.65	11.8	0.40	1.14	9.7
$M_{\text{HEDD-2}}$	8.30	14.8	0.46	1.31	8.8
$M_{\text{HEDD-2}}$	8.30	14.8	0.49	1.39	9.3

Table 2. *Radioactivity of myosin and its light components after treatment of native myosin with iodo[1- ^{14}C]-acetamide*

Myosin was treated with iodo[1- ^{14}C]acetamide in the presence of either (a) 1 mM- Mg^{2+} and 0.1 mM- PP_i or (b) 1 mM- Mg^{2+} (for details see the Materials and Methods section). The specific ATPase activity of native myosin was $0.290 \mu\text{mol}/\text{min}$ per mg of myosin, and the specific radioactivity of the iodo[1- ^{14}C]acetamide was 152 c.p.m./nmol.

	Iodo[1- ^{14}C]acetamide added (mol/mol of myosin)	Radioactivity (c.p.m./nmol of myosin)		Ca^{2+} -stimulated ATPase activity (μmol of P_i/min per mg)
		In the total protein	In the light component	
(a) With 1 mM- Mg^{2+} +0.1 mM- PP_i	2	290	0	1.24
	4	415	0	1.73
	10	550	0	1.44
	20	820	22	1.28
(b) With 1 mM- Mg^{2+}	2	292	0	1.02
	4	460	0	1.1
	10	720	43	1.06
	20	985	45	1.02

with (a) a decrease in the Ca^{2+} -stimulated ATPase activity of the disulphide-modified myosin and (b) a diminished incorporation of radioactivity into the light components of myosin.

If myosin was modified by 2,4-dinitrophenyl β -hydroxyethyl disulphide in the presence of PP_i a similar correlation was observed, except that the Ca^{2+} -stimulated ATPase activity of disulphide-modified myosin was higher than after blocking of the same number of thiol groups in the absence of PP_i (Fig. 2 and Table 3). Table 4 shows the radioactivity found in total protein and in the light components after carboxyamidomethylation of myosin modified by 2,4-dinitrophenyl β -hydroxyethyl disulphide in the presence or in the absence of PP_i . Although carboxyamidomethylation was

carried out under the same conditions, the radioactivity found in total protein and in the light components of myosin was different and there was only a correlation between the enzymic activity of the disulphide-modified myosin and radioactivity of the light components of myosin after treatment with iodo[1- ^{14}C]acetamide. If disulphide-modified myosin was enzymically active before treatment with an excess of iodo[1- ^{14}C]acetamide, then the radioactivity subsequently incorporated into the light components of myosin was high. All preparations of myosin modified by the disulphide in the presence of PP_i retained a high ATPase activity even after loss of 70% of the total thiol groups. These preparations, on further treatment with iodo[1- ^{14}C]acetamide at pH 8.5, lose their ATPase

activity and incorporate radioactivity into the light components of myosin. The greater the residual Ca^{2+} -stimulated ATPase activity of the disulphide-modified myosin then the greater the incorporation of radioactivity into the light components. On the contrary, when preparations of myosin modified by 2,4-dinitrophenyl β -hydroxyethyl disulphide without PP_i , which had lost their ATPase activity, were treated under the same conditions with iodo[1- ^{14}C]-

acetamide only a little radioactivity was incorporated into the light components.

DISCUSSION

The results presented above suggest that the protective effect of PP_i on the inactivation of Ca^{2+} -stimulated ATPase by 2,4-dinitrophenyl β -hydroxyethyl disulphide is connected with the screening by PP_i of the thiol groups of the light components of myosin.

Therefore the thiol groups of the light components react slowly with 2,4-dinitrophenyl β -hydroxyethyl disulphide and with iodoacetamide at low concentrations. The presence of PP_i diminished the reactivity of those thiol groups to 2,4-dinitrophenyl β -hydroxyethyl disulphide and perhaps to other thiol reagents and led to the retention of a high ATPase activity. This diminution of reactivity of the thiol groups of light components of myosin caused by PP_i may be connected with the protective effect of PP_i on inactivation of Ca^{2+} -stimulated ATPase activity by the increasing amount of those reagents.

The number of thiol groups present in light components of myosin that are protected by PP_i cannot be deduced, because the separation of light components of myosin is very difficult and also because the possible presence of the non-essential light components (Weeds, 1967) in myosin preparations could give some errors.

The correlation, however, between the loss of ATPase activity during modification of thiol groups of myosin by 2,4-dinitrophenyl β -hydroxyethyl disulphide and the radioactivity of the light components of myosin seems to indicate an essential role of thiol groups of the light components of myosin in preserving the ATPase activity of the

Fig. 2. Relation between radioactivity found in the light alkali components ($\square$) after treatment of disulphide-modified myosin with iodo[1- ^{14}C]acetamide (specific radioactivity 380 c.p.m./nmol) and the number of blocked thiol groups of disulphide-modified myosin and its ATPase activity ($\circ$).

Table 3. Radioactivity of light components of myosin after treatment of disulphide-modified myosin with iodo[1- ^{14}C]acetamide

Myosin was modified by 2,4-dinitrophenyl β -hydroxyethyl disulphide in the presence of 1 mM- Mg^{2+} and 0.1 mM- PP_i . After separation of disulphide-modified myosin, the ATPase activity and number of free thiol groups were determined. The disulphide-modified myosin was then incubated for 1 h at 18°C with 0.01 M-iodo[1- ^{14}C]acetamide (specific radioactivity 380 c.p.m./nmol) in 0.5 M-KCl-50 mM-tris-HCl buffer, pH 8.5 (for details see the Materials and Methods section).

Thiol groups blocked by disulphide (SH equiv./ mol of myosin)	ATPase activity (μmol of P_i /min per mg of myosin)		Radioactivity of light components (c.p.m./nmol)
	After treatment with disulphide	After subsequent treatment with iodoacetamide	
17	0.600	0.010	665
16	0.525	0.015	485
18	0.450	0.017	620
24	0.289	0.030	230
25	0.110	0.029	145

Table 4. *Radioactivity of myosin and its light components after treatment of disulphide-modified myosin with iodo[1-¹⁴C]acetamide*

Myosin was modified by 2,4-dinitrophenyl β -hydroxyethyl disulphide in the presence of either (a) 1 mM-Mg²⁺ and 0.1 mM-PP_i or (b) 1 mM-Mg²⁺. After separation of disulphide-modified myosin, the ATPase activity and number of free thiol groups were determined. The disulphide-modified myosin was then incubated with radioactive iodoacetamide under the conditions given in Table 3.

	Thiol groups blocked by disulphide (SH equiv./mol of myosin)	ATPase activity (μ mol of P _i /min per mg)		Radioactivity (c.p.m./nmol)	
		After treatment with disulphide	After subsequent treatment with iodoacetamide	Total	In the light components
(a) With 1 mM-Mg ²⁺ + 1 mM-PP _i	20	0.320	0.03	1260	435
	21	0.280	0.02	800	430
	25	0.120	0.01	380	210
	28	0.080	0.01	265	105
(b) With 1 mM-Mg ²⁺	20	0.040	0.02	1080	105
	24	0.020	0.01	410	115
	27	0.010	0.01	270	85

protein. The effect of PP_i in protecting against inactivation of the Ca²⁺-stimulated ATPase activity indicates the presence of thiol groups in the light components of myosin near the binding sites of PP_i. The correlation between the binding of PP_i and the protection of Ca²⁺-stimulated ATPase activity against inactivation by 2,4-dinitrophenyl β -hydroxyethyl disulphide has been shown previously (Kakol, 1969).

The suggestion of Dreizen & Greshman (1969, 1970) that the interaction between the light components and the fibrous component of myosin is stabilized by the terminal phosphate groups of nucleotides and by pyrophosphate alone also indicates the existence of a site on the light component of myosin that participates in the binding of PP_i by myosin. It seems possible that this site is near the thiol groups of the light component.

The findings of Weeds (1967, 1969), suggesting that the peptides of the small subunits produced from myosin by alkaline treatment contain identical thiol sequences, permit the assumption that the differences between the light components, if any, do not involve the site participating in PP_i binding.

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