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Similar effects on enzymic activity due to chemical modification of either of two sulfhydryl groups of myosin

It is well known that increasing concentrations of *p*-mercuribenzoate or *N*-ethylmaleimide[1] increase Ca^{2+}-activated ATPase activity and inhibit both the K^+-activated ATPase and the Ca^{2+}-activated ITPase activities of myosin. Higher concentrations of reagent inhibit all activity[1-3]. These results have been interpreted in terms of two functionally different classes of sulfhydryl groups in myosin, the first (S_1) containing rapidly reacting groups and the second (S_2) containing slowly reacting groups. Two peptides, each containing single cysteine residues believed to correspond to S_1 and S_2, have been isolated from myosin and their amino acid sequences determined[4-7].

In previous work myosin has been chemically modified by the reaction of only S_1 or of both S_1 and S_2, but no attempt has been made to specifically modify S_2 leaving S_1 unchanged. The present experiments have been designed to allow S_2 to react with *N*-ethylmaleimide but to leave S_1 unchanged. This has been achieved by the reaction of S_1 with a disulfide (5,5'-dithio-bis-(2-nitrobenzoate) (DTNB)), the reaction of S_2 with *N*-ethylmaleimide, and finally, regeneration of the thiol group at S_1 by reduction with 1,4-dithiothreitol. The experimental procedure and nomenclature used are illustrated in Table I.

TABLE I

METHOD OF MODIFICATION OF SH GROUPS OF MYOSIN

Reaction	*SH groups modified*
Myosin + DTNB → DTNB–S_1–myosin + 5-thio-2-nitrobenzoate	S_1
DTNB–S_1–myosin + *N*-ethylmaleimide → DTNB–S_1, *N*-ethylmaleimide–S_2–myosin	S_1 and S_2
DTNB–S_1, *N*-ethylmaleimide–S_2–myosin + 1,4-dithiothreitol → *N*-ethylmaleimide–S_2–myosin + 5-thio-2-nitrobenzoate + oxidized 1,4-dithiothreitol	S_2

The effects of each of these reactions on the ATPase and ITPase activities of myosin are shown in Table II. Modification with DTNB resulting in the formation of DTNB–S_1–myosin produces 80–90% loss of K^+-activated ATPase and Ca^{2+}-activated ITPase, accompanied by a small (50% in 0.6 M KCl) increase in Ca^{2+}-ATPase activity. DTNB appears to be less effective than other sulfhydryl reagents in activating Ca^{2+}-ATPase of myosin; the largest activation observed with DTNB was 100% in contrast to a 500–1000% activation produced by *N*-ethylmaleimide. Modification of DTNB–S_1–myosin with *N*-ethylmaleimide produces complete loss of enzymic activity. The addition of 1,4-dithiothreitol, which leads to the removal of 5-thio-2-nitrobenzoate from S_1 leaving *N*-ethylmaleimide–S_2–myosin, restores Ca^{2+}-activated ATPase activity but not K^+-ATPase or Ca^{2+}-ITPase activities. Ca^{2+}-ATPase activity of *N*-ethylmaleimide–S_2–myosin at low salt concentrations is 50% of the activity of native myosin, while at higher salt concentrations the activity is 260%

Abbreviation: DTNB, 5,5'-dithio-bis-(2-nitrobenzoate).

TABLE II

EFFECT OF SEQUENTIAL REACTION OF MYOSIN WITH DTNB, *N*-ETHYLMALEIMIDE AND 1,4-DITHIOTHREITOL ON ENZYMIC ACTIVITIES

Myosin (20 mg/ml) was treated with 1 mM DTNB in a solution containing 0.5 M KCl, 0.04 M Tris (pH 8.0). After 5 min at 0° the pH was reduced to 7.0 by the addition of 0.4 M imidazole (pH 6.0) and the myosin passed through a 1 cm × 10 cm column of Biogel P-60 which had been equilibrated with a solution containing 0.5 M KCl and 0.002 M *N*-tris-(hydroxymethyl)methyl-2-aminoethanesulfonic acid at pH 7.0. Myosin at this step is referred to as DTNB–S_1–myosin. DTNB–S_1–myosin (8.3 mg/ml) was treated with 2 mM *N*-ethylmaleimide in a solution containing 5 mM $CaCl_2$, 5 mM ADP, 0.35 M KCl and 0.02 M imidazole (pH 7.0) for 30 min at 0° and again passed through the Biogel column. Myosin at this step is referred to as DTNB–S_1, *N*-ethylmaleimide–S_2–myosin. 0.1 vol. of 0.1 M 1,4-dithiothreitol was added and the solution incubated for 30 min at 0°. Myosin at this step is referred to as *N*-ethylmaleimide–S_2–myosin. ATPase and ITPase activities were measured by determination of P_i released at 25° in 0.05 M Tris (pH 7.5). For K^+-ATPase the assay systems also contained: 0.6 M KCl, 5 mM EDTA and 5 mM ATP; for Ca^{2+}-ATPase, 10 mM $CaCl_2$ and 5 mM ATP; and for Ca^{2+}-ITPase, 10 mM $CaCl_2$ and 5 mM ITP.

Myosin	*Activity (μmoles P_i/mg myosin per min)*			
	K^+-ATPase 0.6 M KCl	*Ca^{2+}-ATPase*		*Ca^{2+}-ITPase 0.05 M KCl*
		0.05 M KCl	*0.6 M KCl*	
Native myosin	1.85	0.74	0.29	1.02
DTNB–S_1–myosin	0.09	0.94	0.44	0.13
DTNB–S_1, *N*-ethylmaleimide–S_2–myosin	0.00	0.02	0.09	0.00
N-Ethylmaleimide–S_2–myosin	0.27	0.38	0.75	0.06

of the activity of native myosin. The activation by KCl of the Ca^{2+}-ATPase of *N*-ethylmaleimide–S_2–myosin is similar to that of *N*-ethylmaleimide–S_1–myosin[8]. In contrast the Ca^{2+}-ATPase activity of native or DTNB–S_1–myosin is inhibited by increasing KCl concentrations[9,10]. From these results it appears that reaction of either S_1 or S_2 with *N*-ethylmaleimide has essentially the same effect on enzymatic activity, *viz.* the loss of K^+-ATPase and Ca^{2+}-ITPase activities and the increase of Ca^{2+}-ATPase activity at high KCl concentrations The question could be raised whether the product of the treatment with DTNB, *N*-ethylmaleimide and 1,4-dithiothreitol, as described above has been correctly identified as *N*-ethylmaleimide–S_2–myosin. If DTNB–S_1–myosin underwent a disulfide exchange involving the translocation of 5-thio-2-nitrobenzoate from S_1 to S_2 before or during modification with *N*-ethylmaleimide, the procedure designed to produce *N*-ethylmaleimide–S_2–myosin might actually lead to the formation of *N*-ethylmaleimide–S_1–myosin. To confirm that our procedure does indeed produce a reaction of *N*-ethylmaleimide with S_2, the effect of ADP on the inactivation of DTNB–S_1–myosin and *N*-ethylmaleimide–S_2–myosin by further addition of *N*-ethylmaleimide was investigated, since in contrast to S_1, S_2 is known to react slowly with *N*-ethylmaleimide, and the reaction is accelerated by ADP[11]. If S_1 were blocked and S_2 had not reacted, the reaction with *N*-ethylmaleimide should lead to a slow loss of Ca^{2+}-ATPase activity which should be accelerated by ADP; on the other hand, if S_2 were blocked and S_1 free, the reaction with *N*-ethylmaleimide should produce a rapid loss of Ca^{2+}-ATPase even in the absence of ADP. In fact, reaction of DTNB–S_1–myosin with *N*-ethylmaleimide, as indicated by the loss of Ca^{2+}-ATPase activity, is slow and markedly accelerated by ADP (Fig. 1A), while the reaction of *N*-ethylmaleimide–S_2–myosin with *N*-ethylmaleimide is rapid in the ab-

sence of ADP (Fig. 1B). These facts strongly suggest that it is indeed S_2 which is modified by *N*-ethylmaleimide during the preparation of what we describe as *N*-ethylmaleimide–S_2–myosin.

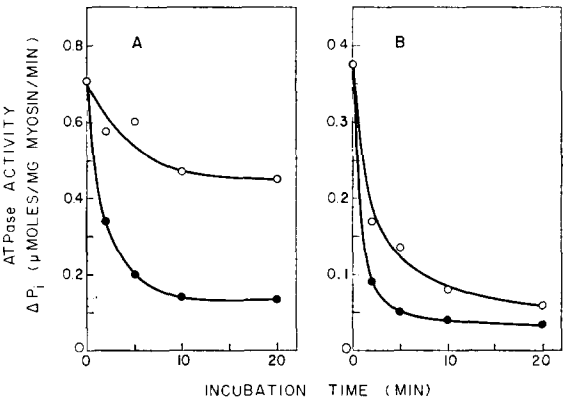

Fig. 1. Effect of Ca^{2+} and ADP on the rate of inactivation by *N*-ethylmaleimide of Ca^{2+}-activated ATPase activity of DTNB–S_1–myosin and *N*-ethylmaleimide–S_2–myosin. The preparation of DTNB–S_1–myosin and *N*-ethylmaleimide–S_2–myosin is described in the legend to Table II. The conditions for final reaction with *N*-ethylmaleimide were 0.39 M KCl, $1.5 \cdot 10^{-4}$ M *N*-ethylmaleimide, 0.015 M imidazole (pH 7.0) and, when added, 4 mM $CaCl_2$ and 4 mM ADP: (A) DTNB–S_1–myosin (5 mg/ml); (B) *N*-ethylmaleimide–S_2–myosin (3.2 mg/ml). ATPase activity was measured at 25° in a mixture containing 0.025 M KCl, 10 mM $CaCl_2$, 5 mM ATP, 0.05 M Tris (pH 7.5) and 0.1 mg myosin/ml. ○, reaction with *N*-ethylmaleimide in the absence of Ca^{2+} and ADP; ●, reaction in the presence of Ca^{2+} and ADP.

In the interpretation of these results, it is assumed that the sulfhydryl groups we refer to as S_1 and S_2 are the same as S_1 and S_2 referred to by other workers[4,5,11,12]. Whether or not this assumption is correct, it is clear that there are two different classes of sulfhydryl groups of myosin having different reactivities toward sulfhydryl reagents, and chemical modification of either class has similar effects on enzymic activity. Modification of either S_1 or S_2 is sufficient to abolish K^+ activation of ATPase and Ca^{2+} activation of ITPase activity and to increase Ca^{2+}-ATPase activity. Modification of S_2 alone does not abolish Ca^{2+}-ATPase; thus this group, as such, is not essential for Ca^{2+} activation of ATPase activity. Activation of ATPase activity by Ca^{2+} requires the presence of one intact sulfhydryl group which may be either S_1 or S_2.

The recent work of TROTTA *et al.*[12] suggests that S_1 and S_2 are not directly involved in the binding or hydrolysis of ATP by myosin since they apparently are not present in Subfragment 1 prepared by tryptic digestion of myosin. The sulfhydryl groups may be regarded as being present in a regulatory region whose conformation is changed by thiol reagents. Our findings that modification of either S_1 or S_2 produces similar effects on ATPase activity suggest that modification of either group alone alters activity by producing similar changes in the conformation of the regulatory region accompanied by a change in conformation at the active site such that K^+ activation of ATPase activity and Ca^{2+} activation of ITPase activity are lost, and Ca^{2+} activation of ATPase activity at high salt concentration is increased. In terms of this model modification of both sulfhydryl groups leads to a conformational change in the regulatory region that produces loss of all activity.

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