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THE REACTION OF MYOSIN WITH *N*-ETHYLMALEIMIDE IN THE PRESENCE OF ADP

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SUMMARY

Myosin reacted with *N*-ethylmaleimide in the presence of ADP lost its ability to be activated by actin. Subfragment 1 behaved similarly. About 2 moles of *N*-ethylmaleimide per mole of Subfragment 1 were required to eliminate actin activation of the Mg^{2+}-ATPase activity. At the point at which actin activation was lost the K^+-EDTA-ATPase activity was also lost, but the Ca^{2+}-activated ATPase activity was increased. Kinetic measurements indicated that the labelling with *N*-ethylmaleimide in the presence of ADP reduced V (the ATPase activity at infinite actin concentration) but did not effect K_{app} (which is related to the dissociation constant of the actin–Subfragment 1 complex). The Mg^{2+}-activated activity of the reacted myosin alone remained unaltered and the ability to bind actin was retained. We propose that the *N*-ethylmaleimide labelling blocked the actin activation by preventing the accelerated release of hydrolysis products from the myosin.

INTRODUCTION

Since the discovery by Kielly and Bradley [1] of the biphasic response of the Ca^{2+}-ATPase activity of myosin as a result of sulfhydryl modification it has been conventional to classify the sulfhydryl groups of myosin into two classes. This classification refers only to those sulfhydryls which affect the ATPase activity of myosin. Reaction of the first class of sulfhydryls (S_1) leads to an activation of Ca^{2+}-ATPase activity, and the subsequent reaction of the second class (S_2) results in an inhibition of activity. Following this discovery a considerable literature has accumulated dealing with various aspects of the reaction of the sulfhydryl groups of myosin.

One variable which has been investigated, and in which we recently became interested, was the effect of ATP and its analogs on sulfhydryl modification. Initially it was discovered by Sekine and Yamaguchi [2] that ATP accelerated the reaction of *N*-ethylmaleimide with the S_2 groups of myosin which had previously been blocked

Abbreviation: EGTA, 2,2′-ethylenedioxybis[ethyliminodi(acetic acid)].

at the S_1 sites. This work was later extended [3] to include the effects of other analogs, and it was found that ADP had a similar, although more marked effect, whereas AMP had no effect.

There is general agreement that S_1-labelled myosin still retains activation of ATPase activity by actin [4–7]. However, Silverman et al. [8] using heavy meromyosin reacted with *N*-ethylmaleimide at the S_1 site found a loss of actin activation. We have recently been able to combine both effects, that is to prepare myosin or Subfragment 1 labelled with *N*-ethylmaleimide which exhibited some characteristics of S_1 blocking and which also had lost activation by actin. These experiments are described in the following communication.

EXPERIMENTAL PROCEDURE

The preparation of proteins was as previously described [5].

Assay of ATPase activity

Mg^{2+}-activated ATPase activity was assayed in the following medium: 2.5 mM $MgCl_2$, 2.5 mM ATP and 25 mM Tris–HCl (pH 7.6). The Ca^{2+} concentration was approx. $5 \cdot 10^{-5}$ M 2,2′-ethylenedioxybis [ethyliminodi(acetic acid)] (EGTA) (1 mM) was added to this solution to remove free Ca^{2+}. Ca^{2+}-activated ATPase activity was assayed in 2.5 mM $CaCl_2$, 2.5 mM ATP, 0.5 M KCl, 25 mM Tris–HCl (pH 7.6). K^+-EDTA activities were measured in 0.4 M KCl, 5 mM EDTA, 2.5 mM ATP and 25 mM Tris–HCl (pH 7.6). Other conditions were as given previously [5].

Reaction with N-ethylmaleimide

Proteins were reacted with *N*-ethylmaleimide in 10 mM Tris–HCl (pH 7.6) at 25 °C for 30 min and the reaction was stopped by the addition of at least a 10-fold excess of dithiothreitol. The amount of *N*-ethylmaleimide used was varied and is indicated on the figures. In all cases the amount of *N*-ethylmaleimide refers to that added and the actual extent of labelling will therefore be less.

Actin binding

A modification of the method of Bárány et al. [9] was used to measure the binding of myosin and actin. Myosin in 0.5 M KCl was mixed with appropriate amounts of actin, and dialysed versus 0.035 M KCl, 1 mM $MgCl_2$ and 0.01 M imidazole (pH 6.5). The resultant suspension was centrifuged at $30\,000 \times g$ for 5 min. The absorbance at 280 nm of the supernatant indicated the amount of actin which was not bound to myosin. Bovine serum albumin added to myosin was used as a control.

Initial rate of H^+ liberation

This was measured using a Gibbs–Durrum stopped-flow apparatus following the procedure of Finlayson and Taylor [10]. The absorbance changes were monitored on a Tektronics 564 storage screen oscilloscope. The liberation of protons was followed at 572 nm using the indicator, cresol red. The experiments were carried out in 0.5 M KCl. ATP and Mg^{2+} concentrations were chosen so that first-order kinetics would be followed.

RESULTS

Myosin in 10 mM Tris–HCl (pH 7.6) and 2 mM ADP was reacted with varying amounts of *N*-ethylmaleimide. After 30 min at 25 °C the reactions were stopped by the addition of excess dithiothreitol and then actin, troponin and tropomyosin were added. The Mg^{2+}-activated ATPase activities of the formed actomyosins were assayed in the presence or absence of Ca^{2+} (i.e. in the presence or absence of EGTA), with the results as shown in Fig. 1. The Mg^{2+}-activated activity was lost in an almost linear fashion as the extent of sulfhydryl modification increased. In contrast very little alteration of the Mg^{2+}-EGTA-moderated activity was apparent, and Ca^{2+} sensitivity was lost only at very low levels of actin activation. To achieve this point about 5 moles of added *N*-ethylmaleimide per mole of myosin were required. It should be emphasized that these results are quite different from those obtained when myosin was reacted with *N*-ethylmaleimide in the absence of ADP [5]. (In the latter case, although the Ca^{2+} sensitivity of the actomyosins (made with the labelled myosins) was lost, a considerable extent of actin activation was retained. The Mg^{2+}-EGTA-activated ATPase activity was also significantly activated.)

In the experiments described in Fig. 1, relatively high levels of ADP were used. If the effects of the nucleotide on sulfhydryl modification were due to binding at the active site of myosin, it was reasoned that lower concentrations of ADP should prove equally effective. This was tested at 0.1 mM ADP and essentially the same results were obtained. At this concentration of ADP the active site would be about 90% saturated (using the binding constant of Lowey and Luck [11]).

The addition of Ca^{2+} or Mg^{2+} (2 mM) to 2 mM ADP did not affect the results significantly.

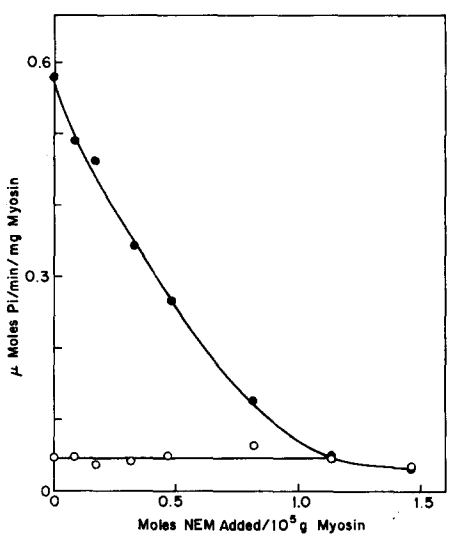
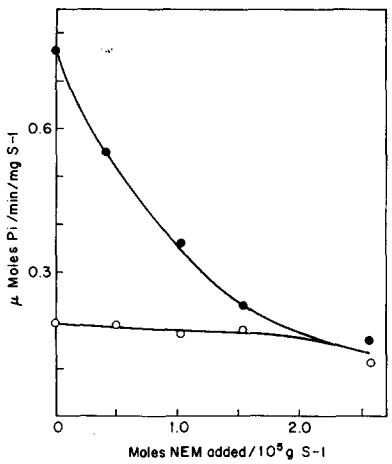


Fig. 1. Reaction of myosin with *N*-ethylmaleimide (NEM) in the presence of ADP. Myosin (2.46 mg/ml) was reacted with *N*-ethylmaleimide in the presence of 2 mM ADP. The reaction was stopped after 30 min by the addition of excess dithiothreitol. The myosin (0.49 mg) was combined with actin (0.12 mg), troponin (0.05 mg), and tropomyosin (0.05 mg) and assayed in the Mg^{2+} (●) and Mg^{2+}-EGTA (○) assay solutions.



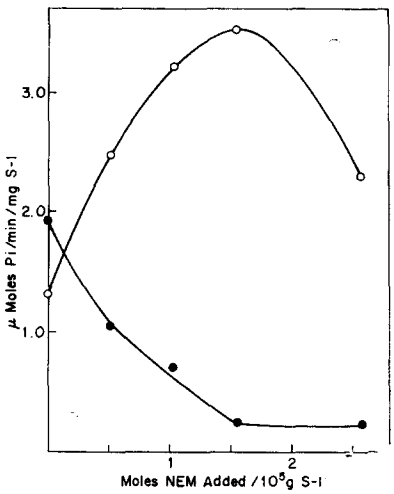


Fig. 2. Reaction of Subfragment 1 (S-1) with *N*-ethylmaleimide (NEM) in the presence of ADP. Subfragment 1 (2.68 mg/ml) was reacted with *N*-ethylmaleimide in the presence of 2 mM ADP. The reaction was stopped after 30 min by the addition of excess dithiothreitol. Subfragment 1 (0.40 mg) was combined with actin (0.17 mg), troponin (0.10 mg), and tropomyosin (0.10 mg) and assayed in the Mg^{2+} (●) and Mg^{2+}-EGTA (○) media.

Fig. 3. The Ca^{2+}- and K^{+}-EDTA-ATPases of Subfragment 1 (S-1) reacted with *N*-ethylmaleimide (NEM) in the presence of ADP. Subfragment 1, reacted as in Fig. 2, was assayed in the K^{+}-EDTA (●) and Ca^{2+} (○) media. All assays contained 0.067 mg of Subfragment 1.

In an experiment analogous to that presented for myosin, the effect of *N*-ethylmaleimide labelling on Subfragment 1 in the presence of ADP was investigated. As shown in Fig. 2 a similar pattern emerged. The Mg^{2+}-ATPase activity was lost without any appreciable activation of the Mg^{2+}-EGTA level. Thus, in this case the myosin and Subfragment 1 systems behaved identically (cf. the effect of sulfhydryl modification on Ca^{2+} sensitivity [12]).

In Fig. 3 is illustrated the Ca^{2+}- and EDTA-activated ATPase activities of each of the Subfragment 1 samples (i.e. at different degrees of sulfhydryl reaction). The EDTA-activated level rapidly decreased in a manner similar to the Mg^{2+}-activated activity. In contrast the Ca^{2+}-ATPase activity (measured in 0.5 M KCl) was activated. A maximum level of activity was obtained at approximately the same extent of *N*-ethylmaleimide labelling where the EDTA and Mg^{2+} activities were minimal. Again these effects were duplicated when myosin instead of Subfragment 1 was used.

The actin activation of *N*-ethylmaleimide-labelled Subfragment 1 was studied next. Subfragment 1 was reacted with varying levels of *N*-ethylmaleimide (0, 0.62 and 1.24 moles *N*-ethylmaleimide/10^5 g Subfragment 1) in the presence of 2 mM ADP. The Mg^{2+}-activated ATPase activity of each sample was measured as a function of actin concentration, and the data was expressed in the form of Eadie plots (Fig. 4). The intercept on the *y* axis indicated *V* (μmoles P_i liberated $\cdot$ min^{-1} $\cdot$ mg^{-1}, at infinite actin concentration) and the slope gave the value of the Michaelis–Menten constant, K_{app}, which is closely related to the dissociation constant of the actin–Subfragment 1 complex. Clearly K_{app} remained relatively constant and *V* decreased as the level of

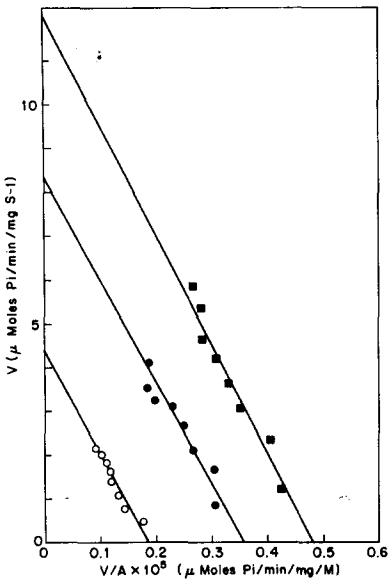


Fig. 4. Actin activation of Subfragment 1 (S-1) reacted with *N*-ethylmaleimide (NEM) in the presence of ADP. Control Subfragment 1 (■), Subfragment 1 reacted with 0.62 mole *N*-ethylmaleimide/10^5 g protein (●) and Subfragment 1 reacted with 1.24 moles *N*-ethylmaleimide/10^5 g protein (○). Samples were assayed with varying concentrations of actin in the Mg^{2+} medium. Each assay contained 0.067 mg of Subfragment 1.

TABLE I

CHANGES OBSERVED IN THE STEADY-STATE PARAMETERS WHEN MYOSIN OR SUBFRAGMENT 1 WAS REACTED WITH *N*-ETHYLMALEIMIDE IN THE PRESENCE OF ADP

The Subfragment 1 (S-1) was assayed as in Fig. 4. Myosin samples (0.21 mg) were assayed in the Mg^{2+} medium in the presence of varying levels of actin.

(A) Subfragment 1

Moles *N*-ethylmaleimide added / 10^5 g S-1	*V* (μmoles P_i/min per mg S-1)	K_{app}
0	12.43±1.06	2.56 (±0.35) · 10^{-5}
0.62	7.87±0.83	2.17 (±0.38) · 10^{-5}
1.24	4.80±0.27	2.69 (±0.23) · 10^{-5}

(B) Myosin

Moles *N*-ethylmaleimide added / 10^5 g myosin	*V* (μmoles P_i/min per mg myosin)	K_{app}
0	1.32±0.02	2.76 (±0.16) · 10^{-7}
0.4	1.00±0.05	2.63 (±0.42) · 10^{-7}
0.6	0.84±0.03	2.99 (±0.37) · 10^{-7}
0.8	0.43±0.03	2.86 (±0.53) · 10^{-7}

N-ethylmaleimide labelling was increased. The values of K_{app} and V from Fig. 4, and also from a similar experiment with myosin, are given in Table I. The trend for myosin and Subfragment 1 was similar in that V decreased and K_{app} was not markedly altered. Plots of V versus the extent of *N*-ethylmaleimide labelling were in fact similar to the Mg^{2+}-ATPase curves shown in Figs 1 and 2. About 2 moles of *N*-ethylmaleimide per mole of Subfragment 1 were required to give $V = 0$.

The above results could be explained either by a loss of actin binding or by a loss of activation following actin binding. Therefore, an experiment designed to test the binding of actin to *N*-ethylmaleimide-labelled myosin was carried out. Control and *N*-ethylmaleimide-labelled myosin were mixed with actin and centrifuged. The amount of actin not precipitated as actomyosin was estimated in the supernatant. It was found that both myosin samples bound about the same amount of actin (the results showed that between 93 to 100% of the added actin was bound to myosin). Although this experiment was rather crude, it illustrated that the *N*-ethylmaleimide labelled myosin did bind actin. The effect can then be regarded as a loss of actin activation and in order to test this hypothesis further we studied the transient and steady-state kinetics of the Mg^{2+}-activated ATPase activity of myosin alone. The steady-state rates of ATP hydrolysis for myosin at different levels of *N*-ethylmaleimide (labelled in the presence of ADP) are given in Table II. For comparison the actin-activated ATPase activities are also shown. Whereas the actin activation was progressively reduced as the extent of *N*-ethylmaleimide labelling increased, the Mg^{2+}-ATPase activity of myosin alone remained constant. These results support the contention that only the actin activation of myosin-ATPase activity was inhibited by the *N*-ethylmaleimide reaction.

TABLE II

THE STEADY-STATE RATE OF Mg^{2+}-ATPase ACTIVITY OF MYOSIN LABELLED WITH *N*-ETHYLMALEIMIDE IN THE PRESENCE OF ADP

Myosin (5 mg/ml) was reacted with *N*-ethylmaleimide in the presence of ADP (2 mM). The reaction was stopped with excess dithiothreitol. Myosin (0.65 mg) was assayed in the Mg^{2+} medium. Actin (0.20 mg) was added to determine the actin-activated ATPase activity.

Moles *N*-ethylmaleimide added / 10^5 g myosin	Actin-activated Mg^{2+}-ATPase (μmoles P_i/min per mg)	Myosin-Mg^{2+}-ATPase (μmoles P_i/min per mg)
0	0.527	0.023
0.30	0.445	0.023
0.60	0.327	0.026
0.90	0.223	0.026
1.20	0.120	0.025
1.50	0.092	0.022

The transient kinetics of myosin-ATPase activity were investigated by monitoring the rate and magnitude of the initial burst of H^+ liberation. The reaction was followed in a stopped-flow apparatus by measuring the change in absorbance ($A_t - A_\infty$) of the indicator dye as a function of time. The data expressed as a semi-logarithmic plot is shown in Fig. 5. First-order kinetics were followed. A summary of the rate constants and the magnitudes for the initial H^+ liberation are given in Table III.

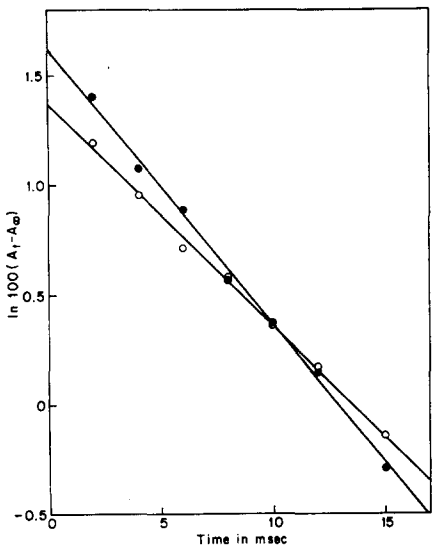


Fig. 5. First-order plots of the initial liberation of H^+. Control myosin (●); myosin reacted with 1.18 moles *N*-ethylmaleimide/10^5 g protein (○). The conditions for these experiments are given in Table III.

No significant changes were detected either in the rate constants or the size of the initial phase of H^+ liberation. These results confirm the initial hypothesis: namely, that the mechanism of Mg^{2+}-ATP hydrolysis by myosin was unaffected by the *N*-ethylmaleimide labelling and it was only the ability of actin to activate the Mg^{2+}-ATPase activity of labelled myosin that was altered.

TABLE III

THE INITIAL BURST OF H^+ LIBERATION FOR MYOSIN LABELLED WITH *N*-ETHYLMALEIMIDE IN THE PRESENCE OF ADP

All determinations were made in 0.5 M KCl with myosin at approx. 2.5 mg/ml, ATP at 0.25 mM, $MgCl_2$ at 2.5 mM and cresol red at $3.76 \cdot 10^{-6}$ M. The specific activity of actin-activated Mg^{2+}-ATPase was reduced from 0.615 to 0.086 (μmoles P_i/min per mg) as a result of the reaction.

Moles *N*-ethylmaleimide added / 10^5 g myosin	Rate of initial H^+ liberation (s^{-1})	Magnitude of initial H^+ liberation (mole/mole myosin)
0	120 ± 16 ($n = 9$)	0.60 ± 0.07
1.18	128 ± 15 ($n = 7$)	0.54 ± 0.06

DISCUSSION

The difference between the modification reaction described above and those carried out by Sekine and Yamaguchi [2] is that in our case the reaction with *N*-ethylmaleimide was done with native myosin in the presence of ADP and not with S_1-labelled myosin, as in the earlier work. Therefore, in the procedure which we have

described both the S_1 and S_2 groups were available for reaction. The fact that the Ca^{2+}-ATPase activity was increased suggests that the S_1 sites were in fact modified. However, there are some differences between classical S_1-labelled myosin (i.e. reacted at high salt concentration and in the absence of nucleotide) and our preparation. For example, in most reports [4–7] the Mg^{2+}-ATPase activity of S_1-modified myosin was activated by actin; in our case little if any activation was observed and in this respect is similar to that described by Silverman et al. [8]. Another difference is that the Mg^{2+}-ATPase activity of myosin alone was increased on reaction of the S_1 groups [13], while ours remained constant.

We feel that despite these differences we reacted the S_1 site of myosin and that it was the binding of ADP which effected the alteration of the ATPase characteristics. We have shown previously [5] that the solvent conditions prevailing during the sulfhydryl reaction are important in determining to some extent the ATPase properties of the product. For example, it was shown [5] that reaction of myosin with *N*-ethylmaleimide at low and high salt concentrations produced quite distinct results. Also, it has been demonstrated [12] that acto–subfragment 1 behaved differently from actomyosin following sulfhydryl modification at low ionic strength, where one system is soluble and the other insoluble. Thus it is reasonable to suppose that the conformation of myosin is dependent to some extent on its environment. Reaction of the sulfhydryl groups of myosins in various conformations would then result in products with different ATPase characteristics. If the above premise is accepted, namely, that the conformation of myosin during sulfhydryl modification can affect the ATPase properties of the product, then it is easier to understand the effect of ADP. It has been demonstrated [6, 14–16] that ADP does alter the conformation of myosin. Thus we propose that ADP binds to the active site and effects a conformational change which is reflected on sulfhydryl modification. Whether the cysteine residues involved are close to the nucleotide binding site or at some distance away cannot be decided without additional data.

As an alternative explanation it is also possible that the effect of the ADP is due to a physical blocking of certain cysteine residues, possibly S_2, thus allowing reaction only at the S_1 site. The final choice between the two possibilities must await further experimental evidence, e.g. the specificity of our labelling must be established, and the primary structure around any specific cysteine residues should be compared to that determined previously for S_1 [17].

It is interesting that the *N*-ethylmaleimide-labelled myosin which showed no activation by actin did hydrolyze Ca^{2+}-ATP. This "uncoupling" of the two activities merited a more detailed investigation and we felt that it would be relevant to consider the effect with reference to the kinetic scheme proposed by Taylor and Lymn [18]. Initially it was established that the decrease of actin activation was due to a reduction of V rather than an alteration of K_{app}. This could be attributed to a decrease in the effective enzyme concentration, which in this case was actomyosin (myosin alone contributing very little to the steady-state rate of hydrolysis). Next it was shown that the hydrolysis of Mg^{2+}-ATP by the labelled myosin was similar to control myosin. This was demonstrated by measuring the steady-state hydrolysis rate and the initial rate of H^+ liberation (assuming a correlation between the liberation of H^+ and phosphate). Thus, the results indicated that it was the mechanism of actin activation which was blocked. In the simplest terms this can be considered as a two-step

process: (i) the binding of actin to myosin, (ii) the accelerated release of products from myosin. We have shown that *N*-ethylmaleimide-reacted myosin will form rigor bonds with actin and although this is not conclusive, it does suggest that the alteration caused by *N*-ethylmaleimide is somehow linked to the second of the two phases. Let us assume that actin binding to the myosin–products complex, under normal conditions, effects a slight change (presumably in conformation) of myosin such that the affinity of the products to the active site is reduced. The effect of the *N*-ethylmaleimide labelling would then be to block the actin-induced change, or to "freeze" the myosin conformation in that characteristic of the myosin–products stage. It should be emphasized that the hydrolysis site was still functional and the labelled myosin retained the ability to bind actin. The effect of the *N*-ethylmaleimide therefore must be restricted to that event which occurs in the normal cycle as a consequence of the binding of actin and may be visualized as suggested above.

ACKNOWLEDGEMENTS

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