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The Action of Thiol Reagents on the Adenosine-Triphosphatase Activities of Heavy Meromyosin and L-Myosin

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1. The Ca^{2+}-activated adenosine triphosphatase of heavy meromyosin is maximally stimulated by lower relative molar concentrations of phenylmercuric acetate than are required with myosin. 2. Stimulation of the Ca^{2+}-activated adenosine triphosphatase of both heavy meromyosin and myosin by thiol reagents is markedly affected by ionic strength, the effects being greater with the former than with the latter. In particular, *N*-ethylmaleimide strongly inhibits the Ca^{2+}-activated adenosine triphosphatase of heavy meromyosin at ionic strength below about 0·2. 3. The precise behaviour of the thiol reagents at low ionic strength is slightly modified by the age of the heavy meromyosin and myosin preparations. 4. Stimulation of the Mg^{2+}-activated adenosine triphosphatase of heavy meromyosin by thiol reagents is relatively insensitive to ionic strength. 5. The adenosine triphosphatases of heavy meromyosin and myosin activated by potassium chloride in the absence of bivalent activators are inhibited by thiol reagents over the range of ionic strength at which stimulation occurs in the presence of calcium chloride as activator. 6. The modifying effects of potassium chloride and sodium chloride are qualitatively different when heavy-meromyosin adenosine triphosphatase is stimulated with phenylmercuric acetate. No such difference is observed when the enzyme is stimulated with *N*-ethylmaleimide.

There is now good evidence that myosin consists of an asymmetric molecule about 1600Å long with the centres associated with its biological activities localized in a thickened region at one end (Mueller & Perry, 1962; Rice, 1964; Huxley, 1963). By controlled enzymic digestion sub-fragments can be prepared which represent different proportions of the whole molecule yet which retain the ability to split ATP and combine with actin (Perry, 1950, 1951; Gergely, 1950, 1953; Mihály & Szent-Györgyi, 1953; Mueller & Perry, 1962). Study of the biological activity of these fragments in relation to those of the original myosin offers a unique opportunity for investigating the influence of the apparently inert part of the molecule on the centres concerned with biological activity. In previous papers (Leadbeater & Perry, 1963; Perry & Cotterill, 1963, 1964) investigations on the interaction of actin with heavy meromyosin have been reported, and the present investigation compares the effects of thiol reagents on the ATPase* activities of the heavy meromyosins and L-myosin itself. Unlike the latter protein heavy meromyosin is soluble in the absence of salt and study of its enzymic properties has special advantages. The investigation has revealed a complex dependence of the effects of thiol reagents on the nature and concentration of the inorganic cations present during enzymic assay and suggests that present views on the role of thiol groups at the active centre of myosin ATPase may be too simple to explain the facts.

* Abbreviation: ATPase, adenosine triphosphatase.

METHODS

Preparation of muscle proteins. L-myosin and the heavy meromyosins were prepared as described by Leadbeater & Perry (1963) and Perry & Cotterill (1964). Unless otherwise stated heavy meromyosin refers to the preparation made by chymotryptic digestion, and stock solutions of about 11–15mg./ml. were prepared in 25mM-tris–HCl buffer, pH7·6. Normally, L-myosin stock solutions (approx. 23–46mg./ml.) were prepared in 0·5M-KCl, pH7·0, but for experiments at low ionic strength protein solutions (20–30mg./ml.) were prepared in 0·3M- or 0·5M-tris–HCl buffer, pH7·6, and diluted with water to give a suspension at the approximate protein concentration for enzymic study. Virtually salt-free myosin preparations were also obtained by diluting stock solutions to 16mM-KCl with cold glass-distilled water and centrifuging for 10min. at 12000*g*. The precipitate was resuspended in a hand-operated homogenizer in about 6vol. of water. After centrifuging down again the myosin was resuspended in water to give the required concentration.

Although some studies were carried out on enzyme preparations 2–3 weeks after preparation, for most of the work the proteins were less than 2 weeks old. During the latter period L-myosin ATPase changed very little, whereas the specific activity (in terms of μg. of P liberated in 5 min. by 1 ml. of enzyme solution of a protein concentration such that $E_{280}=1$) of heavy meromyosin often decreased by up to 25%.

Enzyme assay. Enzyme preparations were in some cases pretreated with the indicated concentration of inhibitor under the conditions specified for 10 min. at 0°. Samples (0·2–0·3 ml.) were then used immediately for assay in a final volume of 2 ml. for 5 min. at 25°. This method is referred to in the text as the 'pretreatment' procedure. For some experiments the thiol reagent was introduced directly into the incubation medium, and in these cases its concentration was lower, but the molar ratio of reagent to enzyme was the same as in the 'pretreatment' procedure. The enzyme was preincubated for 10 min. at 0° in the complete incubation mixture less 0·2 ml. of ATP solution. The reaction was started by the addition of the substrate. In all cases enzymic reactions were stopped with 1 ml. of 15% (w/v) trichloroacetic acid. The conditions and the materials used were otherwise as described by Perry & Cotterill (1964). Tris–ATP was prepared by the method of Schwartz, Bachelard & McIlwain (1962).

RESULTS

Comparison of effect of thiol reagents on the Ca^{2+}*-activated adenosine triphosphatases of heavy meromyosin and myosin.* (*a*) Phenylmercuric acetate. The effects of phenylmercuric acetate on the Ca^{2+}-activated ATPases of the heavy meromyosin and L-myosin were similar, although distinct quantitative differences in response could be observed between the fragment and the whole molecule. At similar protein concentrations and ionic strength, maximum activation of the ATPase was obtained with lower phenylmercuric acetate concentrations for heavy meromyosin prepared either with trypsin or chymotrypsin. The experiment illustrated in Fig. 1 was carried out by treating proteins with phenylmercuric acetate at the concentrations indicated and adding a portion of the treated protein to the incubation medium ('pretreatment' procedure; see the Methods section). In this kind of experiment an average maximum activity was obtained with 3·0 moles of phenylmercuric acetate/mole of heavy meromyosin (mol. wt. 350 000) and 9·4 moles/mole of myosin (mol. wt. 524 000). When the proteins were treated with the same relative amount of thiol reagent in the incubation medium similar results were obtained to those obtained with the 'pretreatment' procedure.

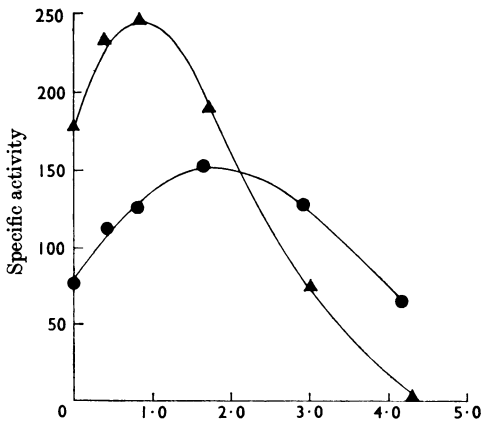

Fig. 1. Effect of phenylmercuric acetate on the Ca^{2+}-activated ATPase of L-myosin and heavy meromyosin. Protein (1·6 mg./ml.) was treated with the reagent for 10 min. at 0° in 0·3 M-KCl in 25 mM-tris–HCl buffer, pH 7·6. The enzymic assays were carried out in 0·2 M-KCl–5 mM-sodium ATP–5 mM-$CaCl_2$ in approx. 20 mM-tris–HCl buffer, pH 7·6, for 5 min. at 25°. ●, L-myosin; ▲, heavy meromyosin.

It was noted during the present study on heavy meromyosin, as has been reported for myosin by other workers (Greville & Needham, 1954; Greville & Reich, 1956; Blum, 1960), that the stimulation of myosin ATPase activity produced by phenylmercuric acetate was sensitive to ionic conditions. The behaviour at very low ionic strength has not been described because almost all the data in the literature apply to myosin, which is insoluble under these conditions and is usually added in solutions of 0·5 M-potassium chloride. In consequence, incubation conditions have rarely been obtained in the absence of this salt. This difficulty does not arise with heavy meromyosin; the data represented in Fig. 2 obtained in a 'pretreatment' type of experiment show that, in the absence of ions other than those due to the buffer, substrate and bivalent activator, concentrations of phenylmercuric acetate either had little effect or slightly inhibited the ATPase up to about 4 moles/mole of heavy meromyosin. As the relative amount of thiol reagent rose, inhibition increased progressively. Over the range of ionic strength studied there was little change in the concentration of phenylmercuric acetate required for maximum stimulation. As the concentration of phenylmercuric acetate was increased the action of potassium chloride changed from one of suppression to stimulation of the ATPase of heavy meromyosin. Comparable studies on trypsin-prepared meromyosin and L-myosin revealed similar results to those presented in Fig. 2. With myosin, however, higher phenylmercuric acetate concentrations were required to produce maximal stimulation of the ATPase, and the thiol reagent produced stimulation in the absence of ions other than those due to buffer, substrate and bivalent activator. In this behaviour it clearly differed from

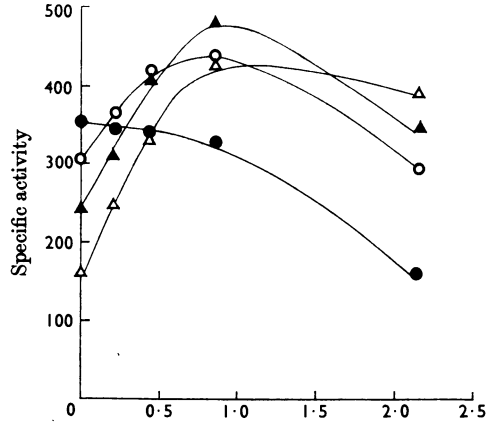

Fig. 2. Effect of phenylmercuric acetate on the Ca^{2+}-activated ATPase of heavy meromyosin in the presence of KCl. Protein (2·3 mg./ml.) was treated with the reagent for 10 min. at 0° in 25 mM-tris–HCl buffer, pH 7·6. The enzymic assays were carried out in 2·5 mM-sodium ATP–2·5 mM-$CaCl_2$ in 54 mM-tris–HCl buffer, pH 7·6. ●, No added KCl; ○, 0·1 M-KCl; ▲, 0·2 M-KCl; △, 0·4 M-KCl.

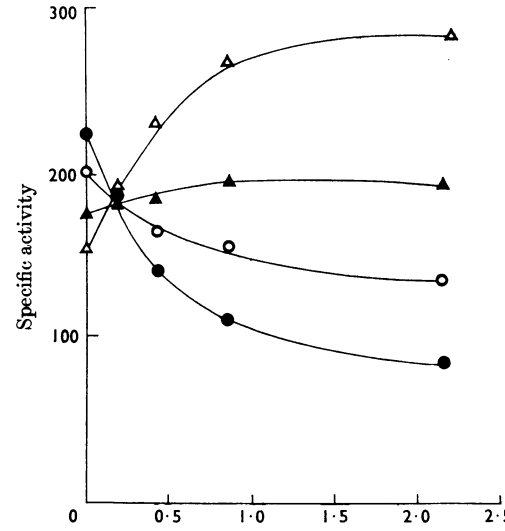

Fig. 3. Effect of *N*-ethylmaleimide on the Ca^{2+}-activated ATPase of heavy meromyosin in the presence of KCl. Protein (2·3 mg./ml.) was treated with the reagent, and enzymic assays were carried out as indicated in Fig. 2. ●, No added KCl; ○, 0·1 M-KCl; ▲, 0·2 M-KCl; △, 0·4 M-KCl.

heavy meromyosin in the 'pretreatment' type of experiment. In some cases when heavy meromyosin was treated with phenylmercuric acetate slight activation in the absence of added potassium chloride was obtained. This activation was relatively small, rarely amounting to more than 10% and much less than that obtained as the ionic strength of the system increased. This stimulation was usually only obtained with freshly prepared heavy meromyosin and disappeared after a few days' storage at 0°.

Limited studies with *p*-chloromercuribenzoate on heavy meromyosin indicated that this mercurial behaved in a similar way to phenylmercuric acetate.

(*b*) *N*-Ethylmaleimide. *N*-Ethylmaleimide modified the ATPase activity of heavy meromyosin in a qualitatively similar way to phenylmercuric acetate. The effect of the two reagents differed, however, in that in the absence of added potassium chloride *N*-ethylmaleimide sharply inhibited over the range 0–2·5 moles/100 000 g. of protein, and stimulation by this thiol reagent was not appreciable until the salt concentration was greater than 0·2 M (Fig. 3). This potassium chloride concentration was significantly higher than that required to get comparable effects with phenylmercuric acetate. Comparison of the behaviour of *N*-ethylmaleimide with myosin indicated, as with phenylmercuric acetate, that the behaviour of the two enzymic systems was similar. Nevertheless, it was also apparent that, to obtain activation effects with heavy meromyosin comparable with those obtained with myosin, higher concentrations of potassium chloride were necessary. The marked difference in behaviour of the two enzymic systems in the absence of added potassium chloride is illustrated in Fig. 4.

Thiol reagents and the adenosine-triphosphatase activity of heavy meromyosin in the presence of magnesium chloride. In the absence of potassium chloride, 2·5 mM-magnesium chloride stimulated the ATPase of chymotrypsin- and trypsin-prepared heavy meromyosins only very slightly at a substrate concentration of 2·5 mM. The enzymic activity amounted to about 3% of that obtained with calcium chloride under otherwise identical conditions (Leadbeater & Perry, 1963). The addition of phenylmercuric acetate to the Mg^{2+}-activated system caused a steady increase in activity until at 1·7 moles/100 000 g. of heavy meromyosin it was 2–3 times that obtained in the absence of inhibitor (Fig. 5). Similar results were obtained with *N*-ethylmaleimide, although maximal activation was obtained with 0·9 mole/100 000 g. of heavy meromyosin. In contrast with the effects of both thiol reagents in the presence of calcium chloride, with magnesium chloride as activator stimulation of enzymic activity occurred in the absence of salt and was relatively insensitive to sodium chloride or potassium chloride.

Thiol reagents and the adenosine triphosphatase of

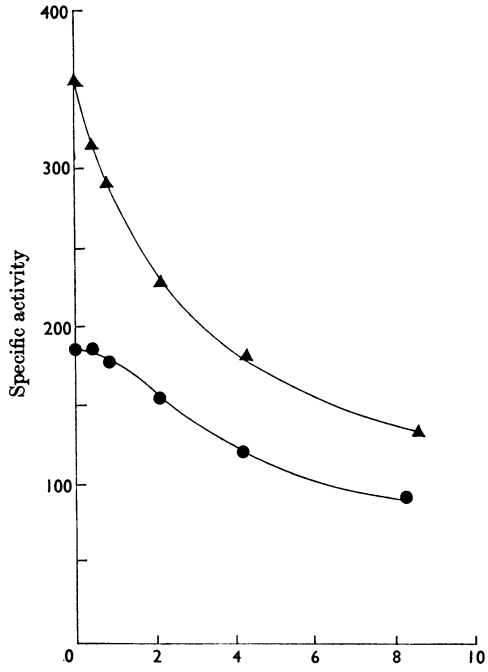

Fig. 4. Comparison of the action of *N*-ethylmaleimide on Ca^{2+}-activated ATPase of myosin and heavy meromyosin prepared from it. Protein was treated with the reagent for 10 min. at 0° in 1·8 ml. containing 0·2 ml. of 54 mM-tris–HCl buffer, pH 7·6, and 0·2 ml. of 2·5 mM-$CaCl_2$. The incubation was started by the addition of 0·2 ml. of 25 mM-tris–ATP. The assays were carried out 14 days after heavy-meromyosin preparation was completed. ●, Myosin; ▲, heavy meromyosin.

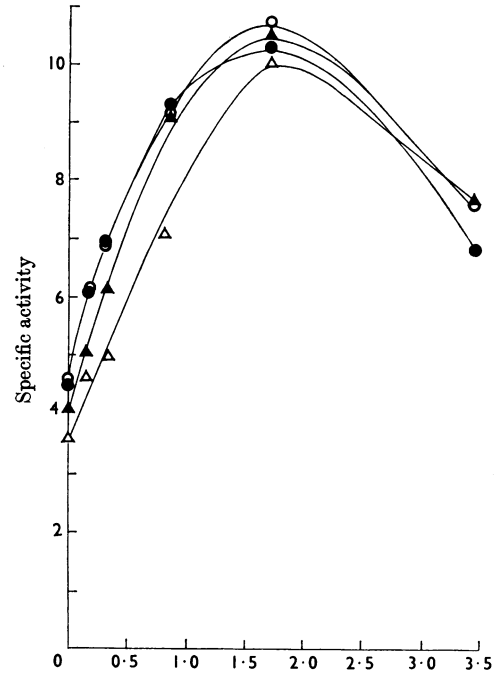

Fig. 5. Effect of phenylmercuric acetate on the Mg^{2+}-activated ATPase of heavy meromyosin in the presence of KCl. The thiol reagent was added to the assay tubes as indicated in Fig. 4, and the final concentrations were 2·5 mM-$MgCl_2$ and 2·5 mM-tris–ATP in 50 mM-tris–HCl buffer, pH 7·6. ●, No added KCl; ○, 0·1 M-KCl; ▲, 0·2 M-KCl; △, 0·4 M-KCl.

myosin and heavy meromyosin in the absence of bivalent cations. In the absence of any activating bivalent cation the heavy-meromyosin preparations had no significant ATPase activity. Potassium chloride activated the enzyme and, although it was more effective than magnesium chloride, the levels of ATP hydrolysis obtained were less than those with calcium chloride. For example, the enzymic activity with 0·6 M-potassium chloride was usually about 10–20% of that obtained with 2·5 mM-calcium chloride measured at pH 7·6 and with 2·5 mM-ATP in both cases. Increasing phenylmercuric acetate concentrations inhibited the ATPase activity over the range 0·1–0·6 M-potassium chloride (Fig. 6), and at none of the conditions studied could significant activation of the ATPase by phenylmercuric acetate be demonstrated. Similar results were obtained with *N*-ethylmaleimide. Likewise myosin behaved similarly with both thiol reagents, although, with this protein at least, some activation was obtained with the reagents at potassium chloride concentrations greater than 1·5 M. At these salt concentrations, however, the specific activity of myosin in the absence of thiol reagent was much reduced. In contrast with potassium chloride, in the range 0–0·5 M sodium chloride produced no activation of heavy-meromyosin ATPases either in the absence or presence of phenylmercuric acetate (3·0 moles/mole of enzyme).

Comparison of effects of sodium chloride and potassium chloride on heavy-meromyosin adenosine triphosphatase in the presence of thiol reagents. In view of the marked differences in the response of heavy-meromyosin ATPase to sodium chloride and potassium chloride in the absence of bivalent cations, the effects of these salts on the stimulation of the Ca^{2+}-activated ATPase produced by thiol reagents were compared. Fig. 7 shows that, with 2·5 mM-calcium chloride and 0·86 mole of phenylmercuric acetate/100 000 g. of protein, as the potassium chloride concentration was increased to 0·3 M the ATPase activity rose. With increasing sodium

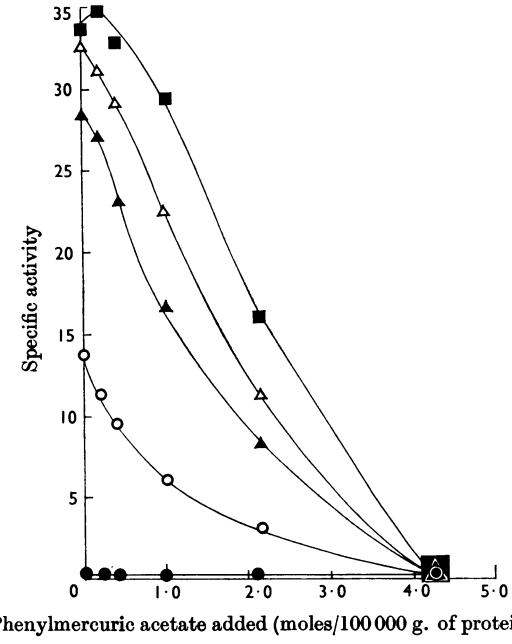

Fig. 6. Effect of phenylmercuric acetate on the ATPase of heavy meromyosin in the presence of KCl and the absence of activating bivalent cations. Protein (4·6 mg./ml.) was treated with the reagent for 10 min. at 0° in 25 mM-tris–HCl buffer, pH 7·6. The assays were carried out in 2·5 M-sodium ATP in 54 mM-tris–HCl buffer, pH 7·6. ●, No added KCl; ○, 0·1 M-KCl; ▲, 0·2 M-KCl; △, 0·4 M-KCl; ■, 0·6 M-KCl.

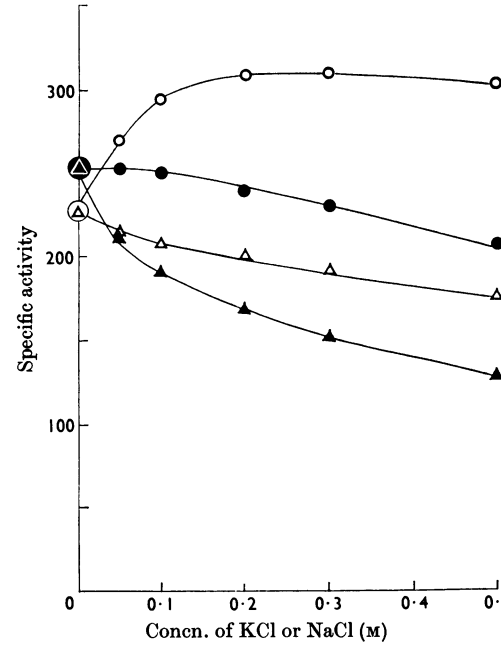

Fig. 7. Comparison of the effects of phenylmercuric acetate on the Ca^{2+}-activated heavy-meromyosin ATPase in the presence of KCl and NaCl. Protein (2·3 mg./ml.) was treated with 20 μM-phenylmercuric acetate for 10 min. at 0° in 25 mM-tris–HCl buffer, pH 7·6. The assays were carried out in 2·5 mM-sodium ATP in 54 mM-tris–HCl buffer, pH 7·6. ●, KCl; ○, KCl and phenylmercuric acetate; ▲, NaCl; △, NaCl and phenylmercuric acetate.

chloride concentrations, but with otherwise similar conditions, the ATPase activity fell off progressively. With both salts, however, at ionic strength below 0·05 M, phenylmercuric acetate inhibited, but stimulated the enzymic activity at higher ionic strength. Ammonium chloride behaved in a similar manner to potassium chloride.

With *N*-ethylmaleimide there was no qualitative difference in response to sodium chloride and potassium chloride as was obtained with phenylmercuric acetate. Fig. 8, which illustrates a similar experiment to that represented in Fig. 7, shows that, when heavy meromyosin is treated with *N*-ethylmaleimide, increasing ionic strength, whether it is obtained by adding sodium chloride or potassium chloride, causes an increase in activity, although the increase with potassium chloride is somewhat more pronounced. Both trypsin- and chymotrypsin-prepared meromyosins showed this effect. When the inhibitor was omitted, increasing the ionic strength caused a progressive inhibition, the activity falling off more sharply with sodium chloride than with potassium chloride.

With inosine triphosphate as substrate and 3·0 moles of phenylmercuric acetate/mole of enzyme present the inosine triphosphatase of heavy meromyosin was inhibited at all concentrations of sodium chloride or potassium chloride tested (Fig. 9). In the absence of phenylmercuric acetate maximum ATPase activity occurred at an ionic strength of about 0·3 M, and a somewhat lower value when the mercurial was present. In contrast with the effects obtained with ATP, with inosine triphosphate as substrate there was no qualitative difference in the effects of sodium chloride or potassium chloride.

DISCUSSION

The enzymic behaviour of the heavy meromyosins prepared by tryptic and chymotryptic digestion is remarkably similar to that of myosin in its response to a variety of complex ionic conditions. This suggests that the part of the myosin molecule represented by the light-meromyosin fragment, and largely determining the solubility properties of L-myosin, has little influence on the enzymic properties, which are localized at the thickened end

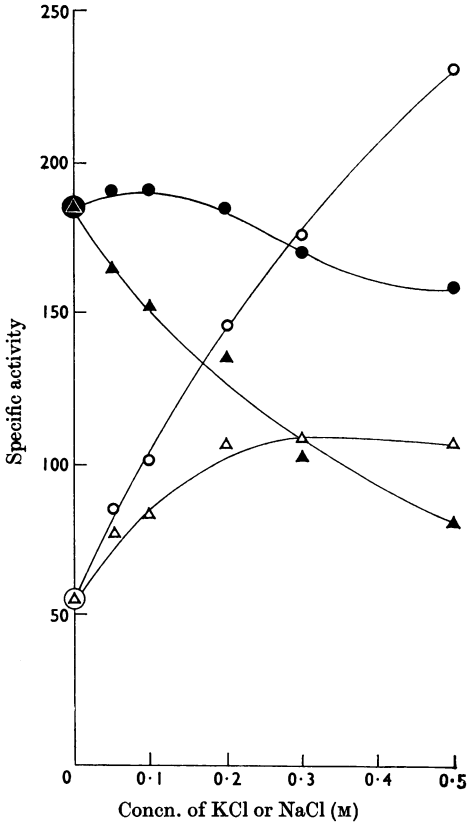

Fig. 8. Comparison of the effects of *N*-ethylmaleimide on the Ca^{2+}-activated heavy-meromyosin ATPase in the presence of KCl and NaCl. Protein was treated with *N*-ethylmaleimide, and enzyme assays were carried out as indicated in Fig. 7. ●, KCl; ○, KCl and 20 μM-*N*-ethylmaleimide; ▲, NaCl; △, NaCl and 20 μM-*N*-ethylmaleimide.

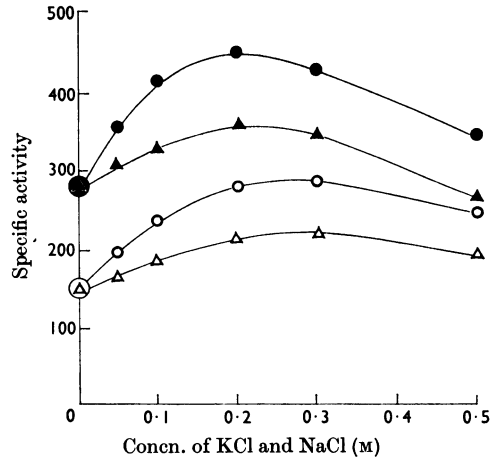

Fig. 9. Comparison of the effects of phenylmercuric acetate on the Ca^{2+}-activated heavy-meromyosin inosine triphosphatase in the presence of KCl and NaCl. Conditions are as indicated in Fig. 7. ●, KCl; ○, KCl and 20 μM-phenylmercuric acetate; ▲, NaCl; △, NaCl and 20 μM-phenylmercuric acetate.

of the asymmetric myosin molecule. There are indications, nevertheless, that the tryptic digestion accompanying the formation of heavy meromyosin can influence the ability of actin to confer on the myosin ATPase the ability to split substrate in the presence of Mg^{2+} (Nagai, Konishi, Yutasaka, Takahashi & Makinose, 1957; Leadbeater & Perry, 1963). Indeed, digestion to the sub-fragment I stage (Mueller & Perry, 1962) results in the almost complete loss of the latter property (J. Jones & S. V. Perry, unpublished work). Likewise, the present study indicates that the maximum stimulation of enzymic activity produced by phenylmercuric acetate occurs with significantly lower inhibitor concentration than with an equal amount of myosin, estimated either on a molar or nitrogen basis.

The current explanation of the stimulatory action of thiol inhibitors (Gilmour & Gellert, 1961; Blum & Felauer, 1959; Blum, 1960) is that it is due to the combination of the inhibitor with a thiol group at or close to the enzymically active site. As all the enzymically active sites of myosin are considered to be localized on the heavy-meromyosin fraction it is of interest that myosin, which has a lower total thiol content than heavy meromyosin, requires about three times the amount of phenylmercuric acetate/mole of protein to produce maximum stimulation compared with heavy meromyosin. The observed increased sensitivity to phenylmercuric acetate would, however, be explained if the thiol groups concerned on heavy meromyosin were relatively more active to the reagent. This increased sensitivity may help to explain why it is easier to inhibit selectively the ATPase rather than the actin-combining property of heavy meromyosin than it is with L-myosin (Perry & Cotterill, 1964).

The fact that the ionic conditions of assay determine whether the organomercurials and *N*-ethylmaleimide stimulate or suppress the enzymic activity of heavy meromyosin does not appear to have been considered hitherto in discussions on the mechanism of action of these thiol reagents. The results obtained in the present investigation particularly apply to heavy meromyosin, but similar trends are also apparent with myosin, although the effects are not so striking with this enzymic system.

The requirement of a higher ionic strength for the stimulation of the enzymic activity of heavy meromyosin by thiol reagents is another example of the modification of the properties of the active site by

the removal of a portion of an enzymically inert part of the myosin molecule. It is possible, however, that some direct enzymic attack occurs at or close to the enzymic centre during meromyosin formation.

The stimulation obtained with ATP, but not with inosine triphosphate, as substrate is considered to be due to the absence of some interaction between a thiol group associated with the active centre and the 6-amino group of the purine ring of the substrate. This interaction which occurs with substrates possessing a 6-amino group is considered to impede in some way the enzymic hydrolysis of ATP (Blum & Felauer, 1959; Gilmour & Gellert, 1961).

As a result of our observations, it is necessary to explain why the system should be sensitive to ionic strength, particularly for heavy meromyosin. It is unlikely that combination of the inhibitor with the appropriate thiol group on the active centre has a very pronounced dependence on the ionic strength. Indeed, the present work indicates that only the ionic conditions of the enzymic assay are important. It follows therefore that the most probable effect of the change in ionic strength is to invoke configurational changes, and only in certain conformations is the enzyme with the blocked thiol group of greater enzymic activity than the untreated enzyme. Hence, so far as primary structure is concerned, the thiol group involved is sufficiently distant from the enzymically active centre to enable the conformational modification resulting from ionic change to have a marked influence on the active centre. It is also necessary to account for the special features of ATP hydrolysis in the absence of bivalent cations that differ from those associated with the presence of Ca^{2+} and Mg^{2+} in that stimulation by thiol reagents is obtained only at very high concentrations of potassium chloride.

A K^+-specificity for certain enzymic systems involving nucleoside triphosphates has long been recognized (see Lowenstein, 1960). The present investigations indicate that Na^+ and K^+ have different effects on an ATPase that is not associated with a membrane structure, and they support the view that differences in behaviour between the ions are a general feature of enzyme systems involving ATP as substrate. The effects produced by these two ions are in some way related to the presence of the 6-amino group in the purine ring of nucleoside triphosphate and, for heavy-meromyosin ATPase at least, can be significantly affected by the type of thiol inhibitor reacting with the enzyme.

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