The Action of Thiol Inhibitors on the Interaction of F-Actin and Heavy Meromyosin

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It is now well known that actin and myosin possess thiol groups of functional significance. In the original investigations (Bailey & Perry, 1947) the actomyosin-forming and ATPase* activities of myosin were shown to be inhibited by thiol reagents, but it appeared that the ability of F-actin to form actomyosin under similar conditions was much less sensitive to these inhibitors. Kuschinsky & Turba (1951) confirmed the latter finding, but also established that if G-actin were treated with the thiol reagent Salyrgan it would no longer be polymerized to F-actin. The present investigation is concerned with the effects of thiol reagents on the interaction of F-actin with heavy meromyosin (H-meromyosin) under conditions of low ionic strength which are more comparable with those occurring *in vivo*. As the complex formed is soluble under these conditions the interaction can be studied viscometrically without the complicating effects of the relatively high ionic strength required to keep actomyosin in solution. The findings presented below indicate that at low ionic strength the acto-H-meromyosin-forming ability of F-actin is sensitive to low concentrations of certain thiol reagents. Evidence is also provided that suggests that the active centres of H-meromyosin involved in enzymic activity can be distinguished from those involved in the interaction with F-actin, which is responsible for the formation of the viscous complex. Certain aspects of this work have already been briefly reported (Perry & Leadbeater, 1962; Perry & Cotterill, 1963).

METHODS

Preparation of muscle proteins. L-Myosin, actin and the H-meromyosins were prepared as described by Leadbeater & Perry (1963). Unless otherwise stated, H-meromyosin obtained by chymotryptic digestion was used for most of the work described below because its ATPase activity was most consistently strongly activated by Mg^{2+} ions in the presence of actin (Leadbeater & Perry, 1963). The normal range of specific ATPase activity ($\mu g.$ of P_i liberated in 5 min. by 1 ml. of protein solution with $E_{280}^{1\text{ cm}}$ 1.0) was 130–160 for L-myosin and 180–270 for chymotrypsin-prepared H-meromyosin.

Viscosity. Relative viscosities were determined at 0° on 3.0 ml. samples in Ostwald viscometers of flow times for water at 40–60 sec. Viscosity in the absence of ATP was determined from the average of three consecutive readings made before and immediately after the addition, in most cases, of 0.075 ml. of 100 mM-ATP to bring the final ATP concentration to the same level as that used for the enzymic experiments, i.e. 2.5 mM. In other cases 0.03 ml. of 50 mM-ATP was added.

Procedure for poisoning proteins and for acto-H-meromyosin formation. A 2 ml. sample of either F-actin (2.25 mg./ml.) or L-myosin (4.65 mg./ml.) was diluted to 4 ml. by the addition of inhibitor to the appropriate concentration and tris-HCl buffer, pH 7.6, to give a final concentration of 25 mM. The sample was usually allowed to stand for 10 min. at 0°, the other (unpoisoned) protein added and the volume made up to 6 ml., so that the final concentrations were 0.75 mg. of F-actin/ml., 1.55 mg. of H-meromyosin/ml., 25 mM-tris-HCl buffer, pH 7.6, and 2.5 mM-MgCl₂. Of this solution 3 ml. samples were used for viscometry and 0.2–0.4 ml. samples for enzymic assay. Both after the addition of inhibitor and the second protein to the system the solutions were carefully mixed with a pipette to avoid frothing and undue aeration, which appeared to inactivate the actin as reflected in its ability to combine with H-meromyosin. The length of time in contact with inhibitor was found to be important, and samples at each concentration of inhibitor were therefore treated separately and immediately assayed for complex-formation and ATPase activity.

Enzymic assays. ATPase determinations were carried out under the ionic conditions described in the text in a final volume of 2 ml. for 5 min. at 25°. The reaction was stopped with 1 ml. of 15% (w/v) trichloroacetic acid and the P_i liberated estimated by the method of Fiske & Subbarow (1925). In some cases specific activity was determined by averaging the initial rates determined at three different concentrations of enzyme; otherwise all estimations were carried out in duplicate. The pH was measured as described by Mueller & Perry (1961).

Materials. ATP and proteolytic enzyme preparations were those described by Leadbeater & Perry (1963). *N*-Ethylmaleimide was supplied by L. Light and Co. Ltd., phenylmercuric acetate by Hopkin and Williams Ltd. and *p*-chloromercuribenzoate by British Drug Houses Ltd.

RESULTS

Effect of thiol reagents on the ability of actin to interact with H-meromyosin. Treatment of F-actin with low concentrations of phenylmercuric acetate markedly inhibited its ability to form a viscous

* Abbreviation: ATPase, adenosine triphosphatase.

soluble complex with H-meromyosin at low ionic strength. At acto-H-meromyosin concentrations of approx. 1–3 mg. of total protein/ml., containing myosin and actin in a 2:1 ratio, 30–50 μ M-phenylmercuric acetate decreased the fall in relative viscosity obtained on the addition of ATP to about 20–30 % of the value obtained in the absence of the mercurial (Fig. 1). Increase of the phenylmercuric acetate concentration to 100 μ M did not have any further marked effect on the viscosity of the acto-H-meromyosin complex. The viscosity in the presence of ATP was similar in the control and poisoned systems, indicating that the mercurial was inducing changes in the viscosity of the complex rather than that of the individual proteins from which it was composed. If enzymic measurements were made immediately after the viscosity determinations and over the range of phenylmercuric acetate concentration producing marked inhibition of acto-H-meromyosin formation, very little change occurred in the ATPase activity of the system measured in the presence of Mg^{2+} ions (Fig. 1). The data illustrated in Fig. 1 were obtained with an H-meromyosin:actin ratio of 2:1, but Mg^{2+} ion activation of the ATPase could still be demonstrated if the concentration of poisoned actin was decreased to give a ratio of 10:1. Under these conditions the extent of activation by Mg^{2+} ions was appreciably less but very similar to that obtained with the same concentration of unpoisoned actin.

At higher phenylmercuric acetate concentrations, the Mg^{2+} ion-stimulated ATPase activity fell off, but the enzymic activity measured in 0.2M-potassium chloride and 2.5 mM-calcium chloride also fell correspondingly. This observation suggested that the fall in activity was associated with changes in the myosin (see below) itself rather than in further modification of the interaction of actin with it, for the latter protein is not essential for Ca^{2+} ion-stimulated myosin ATPase activity.

The effect on the actin was apparently instantaneous (see Fig. 2). Slow secondary changes, however, affected the enzymic behaviour of the complex, for, in cases where the enzymic activity, in contrast with the viscosity determination, showed little change on determination immediately after treatment with mercurial, subsequent storage overnight at 0° resulted in appreciable loss of both Ca^{2+} ion- and Mg^{2+} ion-stimulated ATPase activity.

The activation by Mg^{2+} ions of the ATPase of the poisoned acto-H-meromyosin was clearly due to the presence of the poisoned actin and not the mercurial alone. The ATPase activity of H-meromyosin alone in the presence of 2.5 mM-magnesium chloride and 25 mM-tris-hydrochloric acid buffer, pH 7.6, is only about 3 % of that obtained when actin is also present in the system. A concentration of phenylmercuric acetate producing 70 %

Fig. 1. Adenosine-triphosphatase activity and viscometric behaviour with ATP of acto-H-meromyosin formed from F-actin treated with phenylmercuric acetate. Activities are represented as percentages of the value obtained in the unpoisoned control. $\square$, Fall in relative viscosity on the addition of ATP; $\circ$, ATPase activity in 2.5 mM- $MgCl_2$ -2.5 mM-ATP-30 mM-tris-HCl buffer, pH 7.6; $\bullet$, ATPase activity in 2.5 mM-ATP-30 mM-tris-HCl buffer, pH 7.6; $\triangle$, ATPase activity in 5 mM- $CaCl_2$ -0.2 M-KCl-5 mM-ATP-approx. 29 mM-tris-HCl buffer, pH 7.6.

Fig. 2. Effect of time of exposure of F-actin and H-meromyosin to phenylmercuric acetate on the ability to form acto-H-meromyosin. $\blacksquare$, Viscosity of acto-H-meromyosin formed from F-actin treated with 30 μ M-phenylmercuric acetate; $\square$, control with untreated F-actin. The final measurement was taken after 245 min. in this case. $\bullet$, Effect of ATP on the viscosity of acto-H-meromyosin formed from H-meromyosin treated with 30 μ M-phenylmercuric acetate; $\circ$, control with untreated H-meromyosin.

inhibition of complex-formation decreased the enzymic activity of H-meromyosin still further.

The effects described above were not unique to treatment with phenylmercuric acetate: very similar behaviour was obtained also with *p*-chloromercuribenzoate. Some of the results obtained with the latter reagent are presented in Table 1 and generally the findings were similar to those with phenylmercuric acetate. Even though the ATPase activity in the presence of Mg^{2+} ions fell off with higher concentrations of *p*-chloromercuribenzoate, the percentage activation produced by the addition of magnesium chloride increased (column 3 in Table 1).

In contrast with the results with organomercurials, treatment of F-actin with *N*-ethylmaleimide under similar conditions did not produce inhibition of the actin-H-meromyosin interaction (Fig. 3).

Site of mercurial action. In the experiments described above, F-actin was poisoned before combination with the meromyosin, but, as it was not practical to remove any uncombined inhibitor before complex-formation, it could not be decided unequivocally from these experiments on which protein the mercurial was acting to produce the viscosity effects observed. Experiments in which H-meromyosin rather than F-actin was treated with organomercurials before the proteins were combined indicated, however, that the ability of actin to form acto-H-meromyosin was much more sensitive to the mercurials than was that of H-meromyosin (Fig. 4). For example, concentrations of phenylmercuric acetate that markedly inhibited the acto-H-meromyosin formation when actin was poisoned did not decrease the complex-forming ability of H-meromyosin as measured by the viscosity fall on the addition of ATP. In fact, in the latter case there was often a slight increase in the viscosity fall obtained compared with the control values (Fig. 4). At higher phenylmercuric acetate concentrations the ability of phenylmercuric acetate-treated H-meromyosin to form acto-H-meromyosin also began to fall off. *p*-Chloromercuribenzoate produced effects similar to those of phenylmercuric acetate.

In experiments of the type illustrated in Fig. 4 the protein was usually exposed to the mercurial for 10 min. at 0°. Increasing the time of exposure of H-meromyosin to 30 μM -phenylmercuric acetate up to 150 min. did not produce any change in its ability to interact with actin compared with the unpoisoned control. A similar concentration of mercurial produced an immediate effect on the acto-H-meromyosin-forming ability of actin (Fig. 2).

It was noted that often the effect of thiol reagents was greater if magnesium chloride was not

present in the buffer in which the viscosity of the acto-H-meromyosin was measured. Viscosities were also somewhat variable when measured in the absence of magnesium chloride and therefore, unless specifically stated otherwise, all viscosity

Table 1. *Adenosine-triphosphatase activity and the effect of adenosine triphosphate on the viscosity of acto-H-meromyosin formed from F-actin treated with p-chloromercuribenzoate*

Enzymic assays were carried out in 2.5 mM- $CaCl_2$ -2.5 mM-ATP-0.2 M-KCl-25 mM-tris-HCl buffer, pH 7.6, or in 2.5 mM- $MgCl_2$ -2.5 mM-ATP-25 mM-tris-HCl buffer, pH 7.6. The values in parentheses indicate percentage increases in activity produced by the activating bivalent cation.

Concn. of <i>p</i> -chloromercuribenzoate (μM)	Viscosity fall with ATP (%)	Mg^{2+} ion-activated ATPase (%)	Ca^{2+} ion-activated ATPase (%)
0	100	100 (333)	100
60	31	97 (467)	92
200	15	34 (870)	62
0	100	100 (321)	100
80	31	101 (417)	88
100	30	81 (406)	88

Fig. 3. Comparison of the effects of ATP on the viscosity of acto-H-meromyosins formed from F-actin treated with phenylmercuric acetate and *N*-ethylmaleimide. For conditions see the Methods section. ●, F-actin poisoned with *N*-ethylmaleimide; ○, F-actin poisoned with phenylmercuric acetate.

determinations on acto-H-meromyosin were carried out in 2.5 mM-magnesium chloride.

Nature of interaction of actin and H-meromyosin at low ionic strength. The fall in viscosity obtained on the addition of ATP to actomyosin in 0.4M-potassium chloride-2.5 mM-magnesium chloride-25 mM-tris-hydrochloric acid buffer, pH 7.6, was of the same order as that obtained with a similar concentration of acto-H-meromyosin in the same medium with the 0.4M-potassium chloride omitted. The presence of 0.4M-potassium chloride in the medium in the latter case, however, decreased the relative fall in viscosity obtained on the addition of ATP by 70-75 %, although the viscosity of acto-H-meromyosin in the presence of ATP was only slightly affected by this increase in ionic strength.

The interaction at low ionic strength between actin and H-meromyosin, despite its sensitivity to increasing ionic strength, appeared to be of the specific type associated with actomyosin at higher ionic strength. On the addition of ATP to the acto-H-meromyosin in 25 mM-tris-hydrochloric acid

buffer, pH 7.6, the ATP was rapidly hydrolysed even in the presence of magnesium chloride (cf. Leadbeater & Perry, 1963), and after the initial sharp fall in viscosity associated with the dissociation of the complex the viscosity returned to values equal to and often exceeding the original level. On the further addition of ATP the cycle could be repeated, as can be demonstrated for actomyosin (Needham *et al.* 1942).

To relate the present findings to the previous results (Bailey & Perry, 1947), which suggested that the ability of F-actin complex with myosin at ionic strength about 0.5 was relatively insensitive to thiol reagents, the effect of ionic strength on the interaction of poisoned F-actin with H-meromyosin was studied. Fig. 5 shows that the inhibitory effect of the mercurials is much decreased as the potassium chloride concentration of the medium, in which the viscosity of the acto-H-meromyosin was determined, is increased.

Effect of higher concentrations of thiol reagents on the ability of H-meromyosin to interact with F-actin. The experiments with F-actin indicated that its property of forming a viscous complex with H-meromyosin was more sensitive to organomercurials than were its effects on the myosin ATPase. So far as H-meromyosin itself was concerned there were, however, suggestions that, as the concentrations of inhibitor increased, the converse was the case. To examine this aspect in more detail H-meromyosin was treated with thiol reagents over the range

Fig. 4. Effect of ATP on the viscosity of acto-H-meromyosin formed from actin and myosin independently treated with phenylmercuric acetate. MgCl_2 was not present in the medium used for viscometric assay. ●, H-meromyosin treated with inhibitor; ○, F-actin treated with inhibitor.

Fig. 5. Effect of ionic strength on the fall in viscosity produced by ATP on acto-H-meromyosin formed from F-actin treated with 50 μM -phenylmercuric acetate. ●, F-actin treated with inhibitor; ○, untreated control. The poisoning was carried out as described in the Methods section. The fall in viscosity obtained with untreated protein in 2.5 mM- MgCl_2 -25 mM-tris-HCl buffer, pH 7.6, was taken as 100 (actual fall in relative viscosity was 1.74).

10 μM –1 mM, and its ability to interact with F-actin and catalyse the hydrolysis of ATP in the presence of Ca^{2+} ions were compared. At the higher concentrations of inhibitor the enzymic activity was either diminished to a very low level or destroyed completely, but significant interaction as assessed by the viscometric effects survived. Although in general similar results were obtained with *p*-chloromercuribenzoate, *N*-ethylmaleimide and phenylmercuric acetate, the last-named appeared to distinguish more clearly between the two properties. A typical example is shown in Fig. 6, where under conditions in which the ATPase was completely inhibited the fall in relative viscosity obtained on the addition of ATP was 60 % of the control value. At low phenylmercuric acetate concentrations the ATPase was not activated. For this effect (Kielley & Bradley, 1955) to be demonstrated the ionic strength of the enzyme medium must be greater than 0.1 (S. V. Perry & J. Cotterill, unpublished work). In the experiment illustrated in Fig. 6 the viscosity was determined after the enzymic activity to confirm that the observation indicated a real difference rather than an apparent one due to progressive action of the poison which

might occur if the enzymic experiments were carried out some time after the viscometric determination. Similar studies in which H-meromyosin was replaced by L-myosin and in which assays were carried out in 0.4 M-potassium chloride showed, as has been reported by Bailey & Perry (1947), that the viscosity and enzymic effects fell off roughly in parallel with increasing inhibitor concentration.

DISCUSSION

If the findings reported above on the interaction of actin with H-meromyosin can be applied to the interaction with myosin, and it is reasonable to suppose that they can, it appears that certain factors are involved in the interaction which have not previously been noted in studies on actomyosin solutions.

In the first place, although the interaction is specifically broken by low concentrations of ATP, the effects of increasing ionic strength indicate that the interaction must have considerable electrostatic character. Barany & Barany (1959) have also commented on this aspect for actomyosin itself. Clearly the interaction is not of the normal electrostatic kind, for even at ionic strength greater than 1.5 the interaction of actin and myosin is not completely broken (Perry, 1948).

So far as the F-actin is concerned low concentrations of organomercurials markedly inhibit but do not completely eliminate the interaction with H-meromyosin at low ionic strength. The effect is less marked at an ionic strength of 0.4 or greater, and this explains why thiol reagents were originally reported to have relatively little effect on the ability of F-actin to interact with myosin when studied in the presence of 0.5 M-potassium chloride (Bailey & Perry, 1947; Kuschinsky & Turba, 1951).

If thiol groups on the actin molecule are required to remain intact for normal interaction with myosin at low ionic strength, they are much less reactive to *N*-ethylmaleimide than to mercaptide-forming organomercurials. The rate of reaction with the latter is rapid and, in view of the fact that there is no fall in viscosity of the dissociated system on treatment of the F-actin with mercurials, it is unlikely that the reagent is bringing about depolymerization. This conclusion is supported by the observation that the bound nucleotide of F-actin is not readily displaced by Salyrgan (Martonosi & Gouvea, 1961).

Even though there is significant inhibition of the interaction as measured by the viscosity response to ATP, the activation of the system by Mg^{2+} ions is not significantly changed. This is in agreement with the findings reported by Leadbeater & Perry (1963) that, although acto-H-meromyosin can be completely dissociated as judged by viscometric

Fig. 6. Acto-H-meromyosin formation and ATPase activity of H-meromyosin treated with higher concentrations of phenylmercuric acetate. ●, Fall in relative viscosity on the addition of 2.5 mM-ATP. The conditions were as described in the Methods section. ○, ATPase activity in 2.5 mM- CaCl_2 –2.5 mM-ATP–approx. 29 mM-tris-HCl buffer, pH 7.6.

studies, the hydrolysis of ATP by the system remains strongly activated by Mg^{2+} ions.

Whereas the ability of the F-actin to form a viscous complex with H-meromyosin at low ionic strength is sensitive to organomercurials, the property of H-meromyosin in interacting with actin is relatively insensitive. Indeed, with phenylmercuric acetate it was possible to inhibit completely the ATPase activity yet preserve in a large measure the interaction between the proteins as indicated by viscometric measurements. This indicates that different centres, presumably involving thiol groups, are associated with enzymic activity and actin combination. It is of interest in this respect that Stracher (1964) has produced evidence that the thiol residues involved in interaction of myosin with F-actin are localized in a different part of the molecule from those involved in ATP binding. The present study also confirms that, by direct poisoning of myosin itself and studying the interaction with F-actin at an ionic strength of 0.4 or greater, the enzymic and actin-combining properties are less easily differentiated (Bailey & Perry, 1947; Barany & Barany, 1959; cf. Gilmour & Calaby, 1949). Whereas with myosin the enzymic centres can be selectively poisoned with iodoacetamide only when the enzyme is previously combined with actin (Barany & Barany, 1959), this end can be achieved with H-meromyosin in the absence of actin. Since this work was completed Barany, Barany & Oppenheimer (1963) have reported that the actin-combining properties of L-myosin and H-meromyosin are less sensitive to ultrasonic vibration than is the Ca^{2+} ion-activated ATPase activity. In view of our finding that these two properties are more readily differentiated by the action of thiol reagents on H-meromyosin, it is of interest that a similar situation applies with ultrasonic treatment.

SUMMARY

1. Low concentrations of phenylmercuric acetate and *p*-chloromercuribenzoate inhibit the ability of F-actin to form a viscous complex with H-meromyosin at low ionic strength. *N*-Ethyl-

maleimide was not effective under similar conditions.

2. The adenosine triphosphatase of H-meromyosin is strongly activated by Mg^{2+} ions in the presence of F-actin poisoned with concentrations of phenylmercuric acetate and *p*-chloromercuribenzoate, which markedly inhibit interaction as measured by viscosity changes with adenosine triphosphate.

3. The ability of H-meromyosin to form a complex with F-actin is not inhibited by concentrations of thiol reagents that inhibit the complex-forming properties of the latter protein.

4. At higher concentrations of organomercurials and *N*-ethylmaleimide the enzymic activity of H-meromyosin is destroyed but substantial interactions with F-actin as measured viscometrically survive.

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