STUDIES ON "ACTIVE CENTERS" OF L-MYOSIN*

M. BÁRÁNY AND K. BÁRÁNY

Department of Biophysics, The Weizman Institute of Science, Rehovoth (Israel) and Institut für Physiologie im Max-Planck-Institut für medizinische Forschung, Heidelberg, (Deutschland)

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SUMMARY

1. The effects of various –SH group reagents and of some other agents on characteristic properties of L-myosin and actomyosin has been studied.

2. Different –SH group reagents, when blocking the ATPase activity of L-myosin, also destroy its reaction with actin.

3. Actomyosin, treated with the same reagents under the same conditions, loses its ATPase activity even when it is not dissociated into L-myosin and actin.

4. L-Myosins which react perfectly with actin but show no ATPase activity either in the absence or presence of actin can be isolated from iodoacetamide-treated actomyosins.

5. These findings suggest that the combining sites of L-myosin with ATP and with actin are not the same.

6. The protection of some –SH group(s) of L-myosin by actin indicates that sulfhydryl groups are somehow involved in the myosin-actin binding.

7. When blocking the ATPase activity of actomyosin, the –SH group reagents also diminish the dissociating effect of ATP on actomyosin; this inhibition of the dissociation is exhibited by the L-myosin component of actomyosin.

8. It is shown that blocking some –SH groups of L-myosin in actomyosin reduces the high affinity of actomyosin for ATP to the low level for pyrophosphate.

9. It is assumed that the "pyrophosphate-binding part" of L-myosin participates in the interaction with ATP and with actin.

10. Binding the $-NH_2$ groups of actomyosin—with N-carboxy-cysteine anhydride—inhibits completely its ATPase activity, although the net anionic charge of the protein is not increased.

11. Neutralization of the free carboxylic groups of actomyosin or L-myosin—by excess polylysine—does not affect the Ca activated ATPases; Mg activated actomyosin ATPase in gel is, however, strongly inhibited by polylysine.

INTRODUCTION

Since the important discoveries by ENGELHARDT AND LJUBIMOVA that the main

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structural muscle protein L-myosin splits ATP[1], and by STRAUB that the 2 structure proteins L-myosin and actin react with one another to form actomyosin[2], most of the theories of muscle contraction are based on these 2 characteristic properties of the L-myosin molecule. The old view of SZENT-GYÖRGYI—that resting muscle contains actin and L-myosin side by side, and that contraction leads to actomyosin formation, while the ATP interacts with the L-myosin during the whole contraction cycle[3]—became extremely important after the revolutionary discovery by HUXLEY AND HANSON that muscle fibrils are composed of 2 sets of filaments[4,5], identified chemically as L-myosin filaments and actin filaments[5–8]. In their new concept of contraction, the English workers propose a sliding of the actin filaments past the L-myosin filaments[5,9], and WEBER presented recently a chemical theory of the molecular mechanism of muscle contraction based on this idea[10].

It is reasonable to assume that the chemical reactions of the L-myosin filaments with the actin filaments on the one hand and with the ATP on the other hand take place at the "active centers" of the L-myosin molecule. The chemical nature of these "centers" is, however, still obscure in spite of the great effort which has been made in this particularly interesting field. Of the many papers dealing with this problem[11–13] that of BAILEY AND PERRY is especially important[14]. These authors have shown that a large number of –SH group reagents which destroy the ATPase activity of L-myosin also destroy its affinity for actin. They found a close correlation between the loss of the ATPase activity of L-myosin and its ability to form actomyosin, "indicating that the same –SH groups are concerned in both the colloid and the enzyme reactions". TURBA AND KUSCHINSKY reported later, however, that treatment of L-myosin with Oxarsan inhibits subsequent actomyosin formation, though the reagent has little influence on the ATPase activity of actomyosin sols and gels[15]. These findings, therefore, would suggest that different –SH groups of L-myosin are involved in ATP splitting and actomyosin formation. In order to elucidate the role of sulfhydryl groups and some other groups in the activity of L-myosin, the effect of various –SH group reagents and of some other agents on characteristic properties of L-myosin and actomyosin has been studied.

MATERIALS AND METHODS

L-Myosin: was prepared by the procedure of PORTZEHL, SCHRAMM AND WEBER[16]; the crude L-myosin extract was filtered through a paper-pulp pad to remove the fatty materials, as recommended by PERRY[17]. After being reprecipitated three times, the protein was stored in 0.5 *M* KCl at 0° in concn. of 20–30 mg/ml. These stock preparations were used up within 10–12 days.

Actin: was obtained as follows. The muscle residue, after extraction of L-myosin, was reextracted with 2 vols. of WEBER-EDSALL solution (0.6 *M* KCl, 0.04 *M* $NaHCO_3$ and 0.01 *M* Na_2CO_3) at room temp. for 5 min, diluted with an equal vol. of distilled water and pressed through a thin cloth; the residue was washed with 5 vols. of a 0.4 % $NaHCO_3$ solution for 30 min, with 10 vols. of distilled water for 5 min and 3 times with acetone—2.5, 1 and 1 vol. respectively—for 10 min (all these operations were carried out at room temp. with mechanical stirring). Actin was extracted from the acetone dried powder with 25 vol. of dist. water, concentrated by isoelectric precipitation, stored at 0°, pH 7.0, in 0.1 *M* KCl for about 5 days.

Natural actomyosin: was prepared by extracting fresh rabbit muscle mince with

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WEBER-EDSALL soln. at 0°. In order to extract the total actomyosin of muscle, 5–6 vols. of WEBER-EDSALL soln. was added gradually to the mince over a 3 days period. (Bacterial contamination was avoided by using a few crystals of thymol.) When converted to a gel the mixture was diluted with an equal vol. of 0.6 *M* KCl, and pressed through a gauze-like cloth which retained connective tissue pieces. Any insoluble material was removed by high speed centrifugation in a Servall centrifuge and the fatty materials collected at the top were discarded. After repeated filtration through dense gauzes to remove fats, the actomyosin was precipitated by 20-fold dilution with glass-distilled water, left to settle overnight, centrifuged and reprecipitated twice.

–SH groups: were titrated in conc. guanidine hydrochloride, with nitroprusside as end-point indicator according to KATCHALSKI *et al.*[18]; 20–30 mg of proteins were used for each determination. 3 parallel titrations were carried out; the average deviation for a given sample was not more than 5 %.

Freshly prepared L-myosin or actomyosin contain about 7.5 *M* –SH groups/10^5 g protein. This value does not decrease appreciably within one week, but some decrease was found during prolonged incubation and additional reprecipitations.

Protein: was determined by biuret reaction as described by GORNALL *et al.*[19]; the calibration curve was based on the nitrogen content of L-myosin, and purified actin[20] estimated by the micro Kjeldahl technique; the absorption of the violet colour was measured at 320 mμ[21]. Under these conditions 0.1 mg/ml of L-myosin or purified actin have an O.D. of 0.119.

ATPase activity: was measured in a water bath of 25° with magnetic stirring in the test tubes. Incubations were carried out for 5 min with a final protein concn. of 100–500 μg/ml; not more than 20 % of the total terminal P of ATP was split during this time.

At pH 9.1, $5 \cdot 10^{-3}$ *M* ATP, $1 \cdot 10^{-2}$ *M* $CaCl_2$, 0.2 *M* KCl and 0.1 *M* glycine buffer were employed; this test applies to L-myosin ATPase, since actomyosin is dissociated under these conditions. At pH 6.7 the system contained $1–3 \cdot 10^{-3}$ *M* ATP, $1–3 \cdot 10^{-3}$ *M* $MgSO_4$ or $1 \cdot 10^{-2}$ *M* $CaCl_2$, 0.02 *M* histidine and KCl in a final ionic strength of about 0.08; this test applies to actomyosin ATPase activity; one weight-part of F-actin was added to 2 parts of L-myosin when L-myosin was used as a starting material.

Percentage activity was defined as: $v_i/v \times 100$ where v_i = μmole P/mg prot./min with inhibitor treated samples, and v = μmole P/mg prot./min without inhibitor treated samples.

Superprecipitation: was observed in small test tubes in a final vol. of 2 ml; 1 mg/ml protein and $5 \cdot 10^{-4}$ *M* ATP were used at pH 6.7, μ = 0.1 and room temperature; of the parallel systems one contained $1 \cdot 10^{-2}$ *M* Ca, the other $1 \cdot 10^{-3}$ *M* Mg, and both included the ATP to ensure the specificity of the reaction.

Actomyosin formation: was measured in Ostwald type viscometers with outflow times of 30–40 sec at 0°; 1.5–2.5 mg/ml L-myosin was mixed with 0.6–1.0 mg/ml F-actin in 0.5 *M* KCl, 0.05 *M* phosphate buffer pH 7.0 and 0.001 *M* $MgSO_4$. The same solvent was used when the viscosity changes of the actomyosin were studied. For determination of viscosity, at least 3 readings were taken; if thixotropy was observed the readings were continued until a "constant" value was attained, that is until 2 subsequent readings did not differ by more than 1–2 sec.

Actomyosin formation is usually calculated from the difference of the viscosities

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of actomyosin before and after the addition of ATP[11,14]. In this calculation it is tacitly assumed that the dissociation of actomyosin brought about by ATP is the reverse reaction of actomyosin formation. In the course of these studies we have found, however, that some reagents do not influence equally the reaction of L-myosin with actin and the reaction of the formed actomyosin with ATP. We have therefore separated actomyosin formation and ATP sensitivity in the calculations. Percentage activity of actomyosin formation has been defined as:

$$\left(\frac{\log\eta_{\mathrm{rel}\,i} - (\log\eta_{\mathrm{rel}\,i\,M} + \log\eta_{\mathrm{rel}\,A})}{\log\eta_{\mathrm{rel}} - (\log\eta_{\mathrm{rel}\,M} + \log\eta_{\mathrm{rel}\,A})}\right) \times 100$$

where $\log\eta_{\mathrm{rel}\,i}$ = viscosity of actomyosin solutions prepared from L-myosin samples treated with inhibitor; $\log\eta_{\mathrm{rel}}$ = viscosity of actomyosin solutions prepared from L-myosin samples treated without inhibitor; $\log\eta_{\mathrm{rel}\,i\,M}$ = viscosity of the L-myosin samples treated with inhibitor; $\log\eta_{\mathrm{rel}\,M}$ = viscosity of the L-myosin samples treated without inhibitor, and $\log\eta_{\mathrm{rel}\,A}$ = viscosity of the F-actin solution which was added to the L-myosin solutions for determination of the actomyosin formation.

The term $(\log\eta_{\mathrm{rel}\,i\,M} + \log\eta_{\mathrm{rel}\,A})$ was chosen to ensure that any viscosity increase represents a specific reaction between the inhibitor treated L-myosin and actin (see ref.[11], p. 226).

A similar expression was used for calculating the influence of the reagents employed on the viscosity of the actomyosin. Percentage activity of the viscosity of actomyosin was defined as:

$$\left(\frac{\log\eta_{\mathrm{rel}\,i} - \log\eta_{\mathrm{rel\,ATP}}}{\log\eta_{\mathrm{rel}} - \log\eta_{\mathrm{rel\,ATP}}}\right) \times 100$$

where $\log\eta_{\mathrm{rel}\,i}$ = viscosity of actomyosin solutions treated with inhibitor; $\log\eta_{\mathrm{rel}}$ = viscosity of actomyosin solutions treated without inhibitor, and $\log\eta_{\mathrm{rel\,ATP}}$ = viscosity of actomyosin solutions treated without inhibitor in presence of $2.5\cdot10^{-4}\,M$ ATP.

In the nominator of this expression the viscosity of the inhibitor-treated actomyosin is compared with the viscosity of the completely dissociated control actomyosin. The difference is compared to the maximum dissociating effect of ATP on the control actomyosin given in the denominator. The choice of the viscosity of the control actomyosin in presence of ATP as a reference point is the result of our observation that in certain cases the viscosity of the inhibitor-treated actomyosin in presence of ATP is not reduced to that of the control value.

ATP sensitivity was calculated according to WEBER AND PORTZEHL[11] as:

$$\left(\frac{\log\eta_{\mathrm{rel}} - \log\eta_{\mathrm{rel\,ATP}}}{\log\eta_{\mathrm{rel\,ATP}}}\right) \times 100$$

where $\log\eta_{\mathrm{rel}}$ = viscosity of actomyosin solutions (inhibitor-treated or control) before addition of ATP, and $\log\eta_{\mathrm{rel\,ATP}}$ = viscosity of actomyosin solutions (inhibitor-treated or control) after addition of $2.5\cdot10^{-4}\,M$ ATP.

The value of $\log\eta_{\mathrm{rel\,ATP}}$ has been calculated as the first viscosity value obtained after addition of ATP, since it was shown that the immediate drop in the viscosity is most characteristic for the ATP sensitivity (see Fig. 10). Percentage activity of ATP sensitivity was defined as:

$$\left(\frac{\text{ATP sensitivity of actomyosin soln. treated with inhib.}}{\text{ATP sensitivity of actomyosin soln. treated without inhib.}}\right) \times 100$$

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Modification of the *-NH_2 groups of actomyosin:* was carried out by adding 1 vol. of N-carboxy-L-cysteine-anhydride[22] dissolved in anhydrous dioxane to 5 vols. of actomyosin in 0.5 *M* KCl, pH 7.0, and leaving to stand at 0° over night*; about 100 equiv. excess anhydride was added to the concn. of the $-NH_2$ groups of actomyosin. Since in this reaction the number of the total $-NH_2$ groups does not decrease, the degree of modification was checked by determining the introduced cysteine residues in the altered actomyosin molecule; after exhaustive dialysis more than a tenfold increase in the number of -SH groups was found, as compared with the 15 % aqueous dioxane-treated control. Despite this large alteration of the actomyosin molecule, the protein remained for the most part soluble.

Iodoacetamide: was prepared from chloroacetamide and NaI by the method of ANSON[24]; it was recrystallized twice and its melting point was checked.

Dithioglycolic acid: was a generous gift of Dr. M. SELA prepared from sodium thioglycollate by oxidation with iodine; the purified material gave no nitroprusside reaction.

Polylysine and the OLA copolymer: composed of ornithine, leucine and alanine residues, were obtained from the Department of Biophysics of the Weizman Institute of Science[25,26]. The average chain length of polylysine was 57, and the OLA copolymer contained an average of 15 of each of the amino acid residues/molecule.

Methyl mercury nitrate: solution was also received from the Biophysics Department[18]; pure *p*-chloromercuribenzoate was kindly given by Dr. M. SELA. Oxarsan (*m*-amino-*p*-oxyphenyl- As III-oxide) was the product of Parke Davis & Co., Detroit, Michigan, USA, Salyrgan (Salicyl-(γ-hidroxy-β-methoxypropyl)amide-O-acetate), free of theophylline, that of Farbwerke Hoechst A.G., Frankfurt-Main (Germany), N-Ethylmaleimide of Nutritional Biochemicals Corporation, Cleveland, Ohio, USA, and ATP was the crystalline sodium salt preparation of the Sigma Chemical Co., St. Louis, Missouri, USA.

RESULTS

The effect of various -SH group reagents on L-*myosin and actomyosin*

Oxarsan: Fig. 1 shows that all effects of Oxarsan on characteristic properties of L-myosin depend in the same way on the Oxarsan concn. All the curves—representing Ca-activated L-myosin ATPase, Ca- and Mg-activated actomyosin ATPase and actomyosin formation—run fairly parallel; thus it can be concluded that Oxarsan does not separate the ATPase activity from the actin-combining properties of L-myosin.

A similar expt. performed with actomyosin instead of L-myosin is given in Fig. 2. Oxarsan does not split actomyosin into L-myosin and actin, as observed already by TURBA AND KUSCHINSKY[15], but the loss of ATPase activity by actomyosin in presence of Oxarsan is very similar to that of the L-myosin shown in Fig. 1. It is of interest that the dissociating effect of ATP on actomyosin also diminishes when the ATPase activity of actomyosin decreases.

These data show that the sulfhydryl groups of L-myosin, which seem necessary

* The reactions of proteins with N-carboxyamino acid anhydrides at neutral pH and 0° appear specific for $-NH_2$ groups of proteins[23].

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for the interaction with ATP and with actin, are equally sensitive to the attack of Oxarsan when the L-myosin is "free", *i.e.*, when it is not combined with actin, since ATPase activity and actomyosin formation have been lost simultaneously. Some of the –SH groups of L-myosin seem, however, to be protected by the actin in actomyosin from the dissociating effect of Oxarsan, for actomyosin remains unimpaired even at high Oxarsan concn. It is reasonable to assume that the protected –SH groups of L-myosin are those which would participate in the actin–myosin binding, since the ATPase activity of L-myosin is not protected by the actin in actomyosin. In order to obtain more detailed information about this assumption, we have tried to measure the number of unreacted –SH groups in Oxarsan-treated L-myosin and actomyosin.

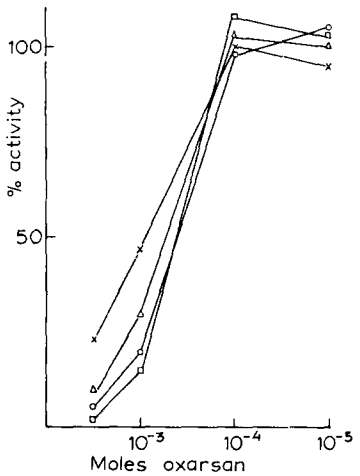

Fig. 1. The effect of Oxarsan on the ATPase activity and the actomyosin formation of L-myosin as a function of Oxarsan concn. The L-myosin samples, 10 mg/ml, were preincubated in 0.5 *M* KCl, at pH 6.7, at room temp., for 10 min with the Oxarsan concn. indicated at the abscissa. This Oxarsan concn. was kept constant when the L-myosins were diluted in course of the expts. □—□ ATPase activity of L-myosin at pH 9.1; △—△ ATPase activity of synthetic actomyosin at pH 6.7 in the presence of Ca; ○—○ ATPase activity of synthetic actomyosin at pH 6.7 in the presence of Mg; ×—× actomosyin formation.

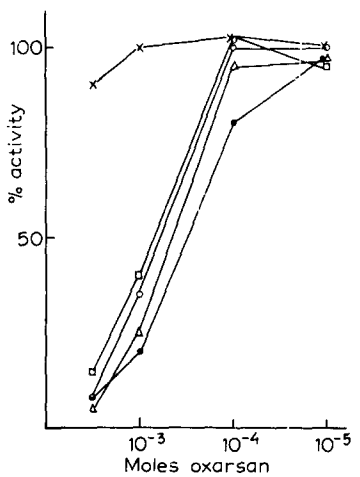

Fig. 2. The effect of Oxarsan on the ATPase activity, the viscosity and the ATP sensitivity of actomyosin as a function of Oxarsan concn. Natural actomyosin, 10 mg/ml, was treated with Oxarsan according to the conditions of Fig. 1. □—□ ATPase activity at pH 9.1; △—△ ATPase activity at pH 6.7, in the presence of Ca; ○—○ ATPase activity at pH 6.7, in the presence of Mg; ×—× viscosity of actomyosin; ●—● ATP sensitivity.

Unexpectedly, however, all the –SH groups were recovered when, after incubation with $5 \cdot 10^{-3}$ *M* Oxarsan, the myosins were reprecipitated 3 times. All activity lost upon addition of Oxarsan was regained at the same time (Table I)*.

Disulfide compounds: We had observed, in collaboration with J. SPIRÓ some years ago, that half of the total –SH groups of L-myosin react with cystine without any loss of any activity[28]. In continuation of this work, the effect of another disulfide com-

* Since these results disagree with those of STAIB AND TURBA[27], it should be noted that full reversibility of inactivation could be achieved by 50-fold dilution of the Oxarsan containing reaction mixture as well.

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TABLE I

REVERSAL EFFECT OF OXARSAN ON CHARACTERISTIC PROPERTIES OF L-MYOSIN AND ACTOMYOSIN BY THREEFOLD PRECIPITATION

L-myosin and natural actomyosin, 10 mg/ml, dissolved in 0.5 *M* KCl were incubated at pH 6.7 with $5 \cdot 10^{-3}$ *M* Oxarsan at room temp. for 10 min and precipitated by addition of 20 vols. of 0.02 *M* KCl solution at 0°. The precipitated proteins were brought into solution by adding 1/7 vol. of saturated KCl to the precipitate and were reprecipitated twice, as before, by 20-fold dilution.

	Moles SH/10^5g prot.	*Enzymic activity µMoles P/mg protein/min*			*Actomyosin formation $\log \eta_{rel}$*		*Super precipitation*
		pH 9.1	*pH 6.7*				
		Ca	*Ca*	*Mg*	*Before ATP*	*After ATP*	
L-myosin control	6.5	1.22	0.56*	0.18*	0.568	0.255	+
L-myosin treated with Oxarsan	6.3	1.12	0.50*	0.20*	0.579	0.271	+
Actomyosin control	6.3	0.64	0.42	0.30	0.456	0.235	+
Actomyosin treated with Oxarsan	6.2	0.65	0.40	0.31	0.454	0.244	+

* Synthetic actomyosin.

pound—sodium dithioglycollate—has been studied because its solubility is better than that of cystine. The reactions between L-myosin and the disulfide compounds were carried out at 0°, pH 9.3–9.5*, with concentrated protein solutions.

By varying the reagent concn. and the incubation time, we were able to eliminate gradually the –SH groups of L-myosin (Fig. 3). After removal of about 2/3 of its initial –SH groups, the L-myosin still retains all its characteristic properties (including its capacity for superprecipitation). Below this –SH level ATPase activity and actomyosin formation decrease; both are completely abolished at about 1.0 *M* SH/10^5 g L-myosin. Addition of excess cysteine (in a final concn. of 0.03 *M*) reverses these inhibitions.

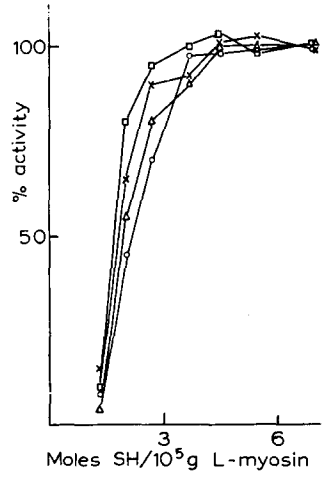

Fig. 3. The effect of disulfide compounds on the ATPase activity and the actomyosin formation of L-myosin as a function of the remaining –SH groups of L-myosin. The L-myosin solutions (~ 25 mg/ml) were treated with graded amounts — 20–100 equiv. concn. to the –SH groups of L-myosin — of cystine or sodium dithioglycollate for 1–15 h at 0°, pH 9.3–9.5. Excess reagent was removed by threefold reprecipitation in the pH range 6.5 to 7.0. □—□ ATPase activity of L-myosin at pH 9.1; △—△ ATPase activity of synthetic actomyosin at pH 6.7 in the presence of Ca; ○—○ ATPase activity of synthetic actomyosin at pH 6.7 in the presence of Mg; ×—× actomyosin formation.

* Below pH 9.0 the reaction does not proceed with an appreciable rate, as was shown with sodium dithioglycollate, which reagent—unlike cystine—is soluble in the neutral pH range also.

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The rate of reaction between actomyosin and the disulfide compounds under the same conditions is much slower. Half of the initial –SH groups could be eliminated without loss of any activity; further eliminations were handicapped by the depolymerization of the F-actin component of actomyosin during the prolonged incubation.

Mercurials: Several authors have reported recently that low concns. of organic mercurials did not alter the ATPase activity of L-myosin; an increase was even found when Ca was employed as activator[29–32]. According to our data, the full enzymic activity of L-myosin is preserved when more than half of its –SH groups are blocked with mercurials (Fig. 4). The parallelism of ATP splitting and actomyosin formation, found with the disulfide compounds, however, could be reproduced only with methyl mercury nitrate; *p*-chloromercuri-benzoate and more effectively Salyrgan, which in

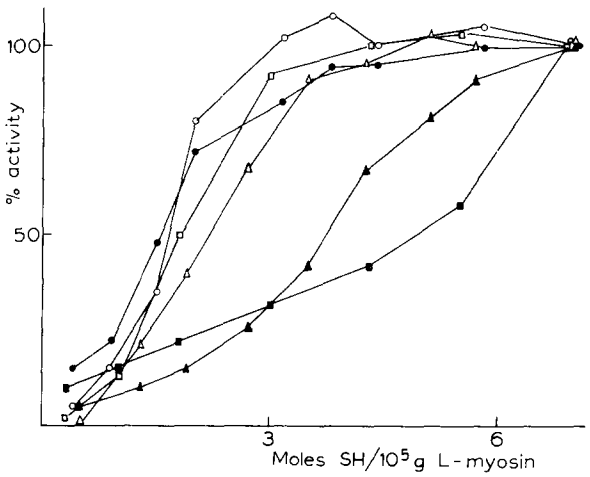

Fig. 4. The effect of mercurials on the ATPase activity and the actomyosin formation of L-myosin as a function of the remaining –SH groups of L-myosin. The L-myosin solns., 10 mg/ml, were incubated at pH 7.0 with 0–8 *M* mercurial/10^5 g L-myosin at room temp. for 10 min, when samples were taken for determination of enzymic activity and viscosity; the stock solutions were precipitated by 20 vols. of glass-distilled water to remove any unreacted mercurials; the precipitate was centrifuged immediately at 0° and –SH groups were determined after dissolution of the L-myosins. ○—○ ATPase activity of methyl mercury nitrate treated L-myosin at pH 9.1*; ●—● actomyosin formation of methyl mercury nitrate treated L-myosin; △—△ ATPase activity of *p*-chloromercuribenzoate treated L-myosin at pH 9.1*; ▲—▲ actomyosin formation of *p*-chloromercuribenzoate treated L-myosin; □—□ ATPase activity of Salyrgan treated L-myosin at pH 9.1*; ■—■ actomyosin formation of Salyrgan treated L-myosin.

addition to the benzene ring also contains a long side chain, inhibit actomyosin formation although the ATPase activity is still unimpaired. This inhibition seems to be caused by some steric hindrance, for each of the 3 different mercurials react stoichiometrically with the –SH groups of L-myosin.

These inhibitions are fully reversed by excess cysteine, provided that the mercurial treatment of L-myosin has not lasted more than 10 min at room temp.

The results of investigations on the reaction of mercurials with actomyosin resemble those achieved with Oxarsan. Fig. 5 shows that Salyrgan (similar results

* The Ca- and Mg-activated synthetic actomyosin ATPases were also tested; the results are similar to those of Ca-activated L-myosin ATPases; their presentation is omitted to get a better survey in the figure.

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were obtained with methyl mercury nitrate) affects first the ATPase activity of actomyosin and only afterwards the dissociation of actomyosin; this supports the idea that the "ATPase" –SH groups of the myosin component react first, followed later by those which, protected by actin, take part in the actomyosin formation. It has been established again that the extent to which ATP causes dissociation of actomyosin in solution depends on the ATPase activity of actomyosin sols or gels. We could show, furthermore, that this inhibition of dissociation of actomyosin by ATP can be interrupted if sodium pyrophosphate is added along with ATP as dissociating agent to the actomyosin solutions (*e.g.* the Salyrgan-treated actomyosin (4.0 mg/ml) had a viscosity of $\log\eta_{rel} = 0.696$ before addition of ATP; after addition of $2.5 \cdot 10^{-4}$ *M* of ATP the viscosity dropped to 0.651; after adding pyrophosphate (final concn. $2.5 \cdot 10^{-3}$ *M*) to the same mixture the viscosity decreased to 0.400).

Iodoacetamide: The data of BAILEY AND PERRY[14] being taken into account, this reagent was used in 0.1 *M* final concn. at pH 8.0 and 0–25° for not longer than 2 h. Under these conditions iodoacetamide can be considered as a specific –SH group blocking agent[33]. The rate of reaction is plotted in Fig. 6, which compares the effect of iodoacetamide and iodoacetate on L-myosin. The latter reacts more slowly with the –SH groups of L-myosin than iodoacetamide; the delay is evidently due to the negative charge of iodoacetate which hinders its reaction with the similarly charged protein. This finding explains why monoiodoacetate up to a concn. of $5 \cdot 10^{-2}$ *M* does not influence ATP splitting and contraction[34].

Iodoacetamide-treated L-myosin loses most of its activity before half of its initial –SH groups has been blocked (Fig. 7). This finding differs from that with disulfide compounds and mercurials. (It was thought that this difference results from the reaction of iodoacetamide with some other amino acid residue such as cysteine. No change in the absorption spectra of the iodoacetamide-treated L-myosin was found in the range of 250–300 mμ which could indicate reaction with the benzene ring containing amino acid residues. How far any other side-reaction occurs during the alkylation of the cysteine residues of L-myosin may be decided with the help of the method recently reported by MORE *et al.*[35] for the separation of carboxymethylated amino acids from proteins treated with iodoacetic acid.) However, the parallelism between the loss of ATPase activity and actomyosin formation is maintained.

Iodoacetamide-treated actomyosin produces effects very similar to the Oxarsan or Salyrgan-treated actomyosins (Fig. 8). The difference between the splitting of actomyosin into L-myosin and actin and the blocking of its ATPase activity as a function of the reacted –SH groups is considerably greater than in the former cases; as a matter of fact this can be expected on the basis of the different features of the L-myosin ATPase curves. This is a clear indication that actin protects in actomyosin those –SH groups of the myosin component which would participate in the actin–myosin bonding, but does not protect those which would be responsible for ATP splitting.

Evidence for this hypothesis was brought about by isolating the L-myosin component from the iodoacetamide-treated actomyosins represented in Fig. 8. The preparation of the actin-free L-myosin was carried out by the method of A. G. SZENT-GYÖRGYI[36], *i.e.*, the actomyosins were precipitated by tenfold dilution, dissolved in 0.5 *M* KI, left to stand at room temp. for 25 min and reprecipitated twice. The isolated L-myosins were again tested for the interaction with ATP and with actin.

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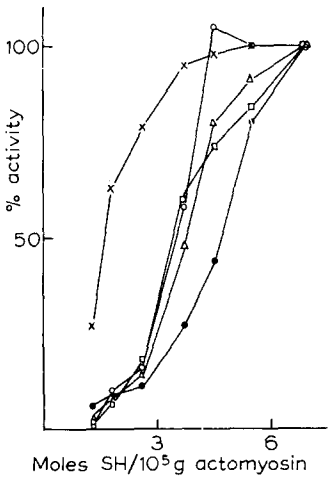

Fig. 5. The effect of Salyrgan on the ATPase activity, the viscosity and the ATP sensitivity of actomyosin as a function of the remaining –SH groups of actomyosin. Synthetic actomyosin solns., 10 mg/ml, composed of 2 part of L-myosin and one part of F-actin, were used under the same conditions as noted in Fig. 4. □—□ ATPase activity at pH 9.1; △—△ ATPase activity at pH 6.7, in the presence of Ca; ○—○ ATPase activity at pH 6.7, in the presence of Mg; ×—× viscosity of actomyosin; ●—● ATP sensitivity.

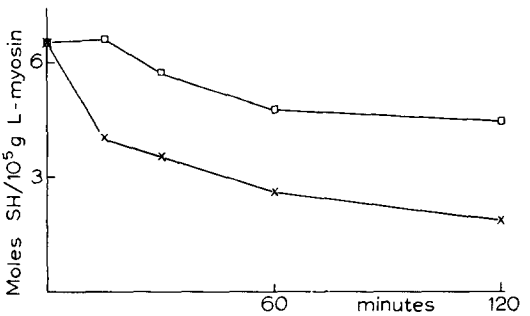

Fig. 6. Reaction of the –SH groups of L-myosin with iodoacetamide and iodoacetate as a function of time. L-myosin solns., 12 mg/ml, were incubated with 0.1 *M* reagent in 0.5 *M* KCl, 0.02 TRIS buffer pH 8.0, at 0°; samples were removed from the incubation mixtures at the times indicated at the abscissa and the –SH groups were determined after threefold reprecipitation of the proteins. ×—× iodoacetamide; ○—○ iodoacetate.

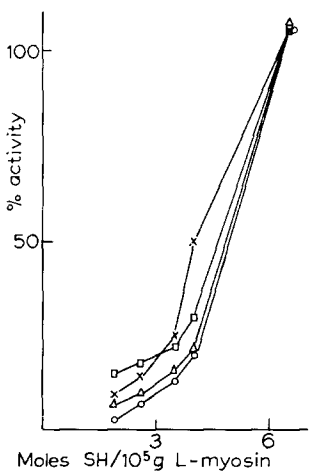

Fig. 7. The effect of iodoacetamide on the ATPase activity and the actomyosin formation of L-myosin as a function of the remaining –SH groups of L-myosin. Preparation of iodoacetamide-treated L-myosin is presented in Fig. 6. □—□ ATPase activity of L-myosin at pH 9.1; △—△ ATPase activity of synthetic actomyosin at pH 6.7 in the presence of Ca; ○—○ ATPase activity of synthetic actomyosin at pH 6.7 in the presence of Mg; ×—× actomyosin formation.

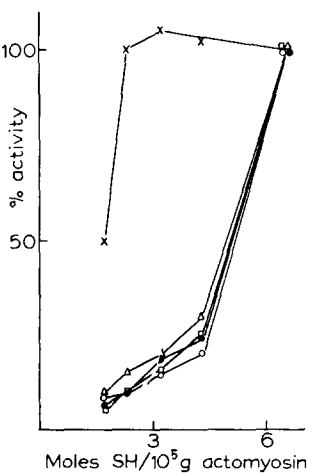

Fig. 8. The effect of iodoacetamide on the ATPase activity, the viscosity and the ATP sensitivity of actomyosin as a function of the remaining –SH groups of actomyosin. Synthetic actomyosin, 15 mg/ml, composed of 2 parts L-myosin and one part of F-actin, was incubated with 0.1 *M* reagent, in 0.5 *M* KCl, 0.02 *M* TRIS buffer pH 8.0 at room temp.; samples were taken at 15, 30, 60 and 120 min and reprecipitated three times. □—□ ATPase activity at pH 9.1; △—△ ATPase activity at pH 6.7 in the presence of Ca; ○—○ ATPase activity at pH 6.7 in the presence of Mg; ×—× viscosity of actomyosin; ●—● ATP sensitivity.

Fig. 9 shows that these L-myosins form a perfect actomyosin after the addition of a new sample of actin, but they have practically no ATPase activity whatsoever either in the absence or presence of actin. L-Myosin which reacts with actin but does not split ATP was isolated from iodoacetamide-treated synthetic and natural actomyosins as well. The superprecipitation reaction was never observed with these materials.

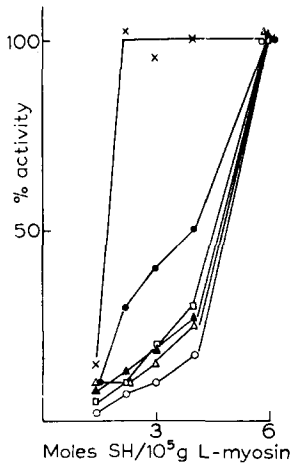

Fig. 9. Separation of the ATPase activity and actomyosin formation of L-myosin. The L-myosins were isolated from the iodoacetamide-treated actomyosins, presented in Fig. 8; the figure shows the ATPase activity, actomyosin formation and ATP sensitivity as a function of the remaining –SH groups of L-myosin. □—□ ATPase activity of L-myosin at pH 9.1 in the presence of Ca; ▲—▲ ATPase activity of L-myosin at pH 6.7 in the presence of Ca; △—△ ATPase activity of synthetic actomyosin at pH 6.7 in the presence of Ca; ○—○ ATPase activity of synthetic actomyosin at pH 6.7 in the presence of Mg; ×—× actomyosin formation; ●—● ATP sensitivity.

The ATP sensitivity of the iodoacetamide-treated actomyosin, like that of the Oxarsan or Salyrgan-treated, is considerable reduced when its ATPase activity is decreased (Fig. 8). Fig. 9 shows that this inhibition of the dissociating effect of ATP is due to the myosin component of the iodoacetamide-treated actomyosin, for the effect can also be found after removal of the actin and formation of a new actomyosin from the remaining L-myosin and fresh actin. It was found, furthermore, that the iodoacetamide-treated actomyosin can be readily dissociated by $2.5 \cdot 10^{-3}$ *M* sodium pyrophosphate, in the same way as the Salyrgan-treated actomyosin mentioned before. The comparison of the dissociating effect of pyrophosphate and ATP on an actomyosin which has been prepared from the L-myosin component of the iodoacetamide-treated actomyosin and from a new sample of actin is given in Fig. 10. The addition of pyrophosphate in a final concn. of $2.5 \cdot 10^{-3}$ *M* to this actomyosin solution produced an immediate drop in the viscosity. When $2.5 \cdot 10^{-4}$ *M* ATP was used instead of pyrophosphate, however, the sudden drop was only 30 % of the maximum effect; during the following 10 min an additional 15 % viscosity decrease was observed but when the ATP concn. was increased to $1.5 \cdot 10^{-3}$ *M* the total effect was over 90 %; the process was completed by subsequent pyrophosphate addition. It follows, therefore, that this actomyosin, the L-myosin component of which has been modified, has approx. equal affinity for ATP and for pyrophosphate; the decrease of affinity for ATP is somehow related to the loss of ATPase activity of the myosin component. No such effect could be found in the similarly prepared but untreated control.

N-Ethylmaleimide (NEM): This reagent was first applied by KIELLEY AND BRADLEY for study of the behaviour of L-myosin ATPase[32]. Of all the –SH group reagents NEM needs the lowest concn. to affect the characteristic properties of L-

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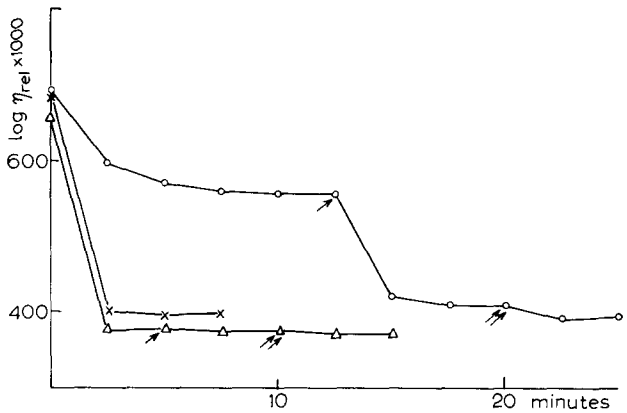

Fig. 10. The comparison of the dissociating effect of pyrophosphate and ATP on an actomyosin, the L-myosin component of which has been modified, as a function of time and ATP concn. The L-myosin component was prepared from a iodoacetamide-treated actomyosin; it contained 2.2 M SH/10^5 g L-myosin and reacted perfectly with actin but had only 10% of the ATPase activity of the control. In the system 2.0 mg/ml L-myosin, 0.8 mg/ml F-actin, 0.5 M KCl, 0.05 M phosphate buffer pH 7.0 and 0.001 M $MgSO_4$ were employed at 0°. ×—× modified actomyosin, at 0 min sodium pyrophosphate was added in a final concn. of $2.5 \cdot 10^{-3}$ M; ○—○ modified actomyosin, at 0 min ATP was added in a final concn. of $2.5 \cdot 10^{-4}$ M; at the arrow the ATP concn. was increased to $1.5 \cdot 10^{-3}$ M; at the double arrow in addition $2.5 \cdot 10^{-3}$ M of sodium pyrophosphate was added; △—△ control actomyosin treated at 0 min with $2.5 \cdot 10^{-4}$ M of ATP, at the arrow with $1.5 \cdot 10^{-3}$ M of ATP, at the double arrow with $2.5 \cdot 10^{-3}$ M of $Na_4P_2O_7$.

myosin and actomyosin. The result is very similar to that of iodoacetamide. In short, after about 1/3 of the total –SH groups of L-myosin has been blocked, the ATPase activity and actomyosin formation is decreased to about 25% of the control. NEM-treated actomyosin likewise loses its ATPase activity when only slightly dissociated by the reagent. L-Myosin reacting completely with actin but having only 20% of the ATPase activity was also prepared from a NEM-treated natural actomyosin.

The effect of some other reagents on L-myosin and actomyosin

Evidently not only the cysteine residues are involved in the combination of L-myosin with ATP and with actin. It has been known for a long time that strong salt solutions favour the dissociation of actomyosin[16,37] and also inhibit its ATPase activity[38,39]. It seems possible, thus, that ionized groups also take part in these specific interactions. KUSCHINSKY AND TURBA reported that binding the amino groups of actomyosin with benzaldehyde blocks the enzymic property[40]; we observed the same effect with formaldehyde-treated L-myosin[28]. Since in these cases the net anionic charge of the protein molecule increases enormously, the specificity of these reactions can be doubted. In order to overcome this difficulty in the interpretation of the results, we have prepared a N-carboxy-L-cysteine anhydride[22] modified derivative of actomyosin; the free amino groups of the protein serve as initiators of a polymerization that proceeds according to the scheme on next page.

Hence each amino group that has reacted is replaced in the end-product by a new one formed in the reaction. The modified actomyosin retained only 3% of the Ca-activated ATPase activity of the control (treated with 15% aqueous dioxane), despite the preservation of –SH and $-NH_2$ groups in the altered actomyosin molecule.

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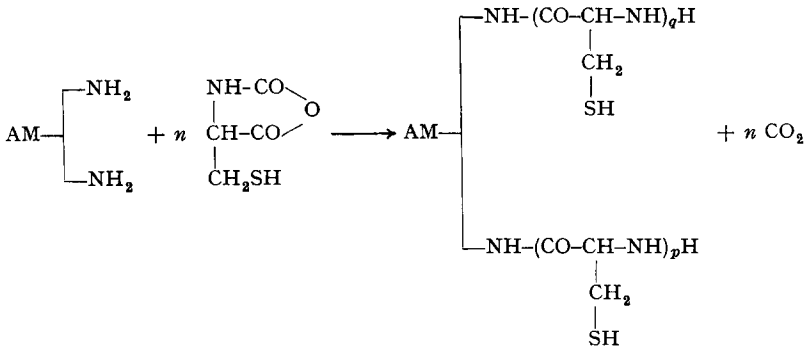

Fig. 11 shows that, in contrast to the binding of $-NH_2$ groups, neutralization of the free carboxylic groups of L-*myosin* by excess polylysine (The basic polyamino acid, when mixed with L-myosin or actomyosin solutions, precipitates the proteins) does not result in any loss of the ATPase activity in the presence of either Ca or Mg (The specific activities are, of course, very different — at pH 6.7 0.7 μmole P/mg L-myosin/minute with Ca and 0.03 μmole P/mg/min with Mg.) The polylysine-treated *actomyosin* ATPase, on the other hand, is preserved when activated with Ca but abolished when activated with Mg. It seems that this inhibition of the basic polypeptide in presence of Mg is caused by dissociation of the actomyosin gel into actin and L-myosin, for the ATPase activity of actomyosin is reduced to the level of the L-myosin ATPase.

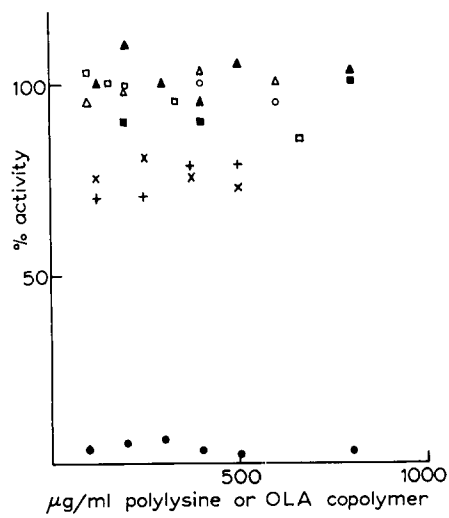

Fig. 11. The effect of polylysine and OLA copolymer on the ATPase activity of L-myosin and natural actomyosin as a function of the reagent concn. The myosins, 0.1–1.0 mg/ml, were preincubated with the polymers in the presence of KCl, buffer and alkaline earth ions at room temp. for 15 min; the reaction was started with the ATP. □ polylysine-treated L-myosin at pH 9.1 in the presence of Ca; ionic strength 0.3; ■ polylysine-treated actomyosin at pH 9.1 in the presence of Ca; ionic strength 0.3; △ polylysine-treated L-myosin at pH 6.7 in the presence of Ca; ionic strength 0.1; ▲ polylysine-treated actomyosin at pH 6.7 in the presence of Ca; ionic strength 0.1; ○ polylysine-treated L-myosin at pH 6.7 in the presence of Mg; ionic strength 0.1; ● polylysine-treated actomyosin at pH 6.7 in the presence of Mg, ionic strength 0.1; × OLA-treated L-myosin at pH 6.7 in the presence of Ca; ionic strength 0.1; + OLA-treated actomyosin at pH 6.7 in the presence of Ca; ionic strength 0.1.

It is remarkable that even the OLA copolymer (composed of ornithine, leucine and alanine residues) which forms a heavy precipitate with these proteins has only a small effect on the Ca-activated ATPase of L-myosin or actomyosin; this suggests that a great deal of the protein surface can be covered without affecting the specific sites.

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DISCUSSION

The data presented above confirm the results of BAILEY AND PERRY[14] that when different –SH group reagents block the ATPase activity of L-*myosin*, they also destroy its reaction with actin (Figs. 1, 3, 7). It was shown, however, that when L-myosin combined with actin in *actomyosin* is treated with the same reagents under the same conditions, its affinity for actin is preserved though its reaction with ATP is lost. The following observations support this view: (1) actomyosin whose ATPase activity has been blocked by treatment with Oxarsan, Salyrgan, iodoacetamide or NEM is not, or only slightly, dissociated by these reagents (Figs. 2, 5, 8); (2) the L-myosin component isolated from the iodoacetamide- and NEM-treated actomyosin reacts perfectly with actin but has no ATPase activity either in the absence or presence of actin (Fig. 9). The separation of the interaction of L-myosin with ATP and with actin suggests therefore that its "ATPase center" and "actin-binding center" are not the same.

There are indications, furthermore, that the –SH groups actually take part in the formation of these "centers". Thus it would be very difficult to explain why some –SH group(s) of L-myosin are protected by actin from –SH group blocking agents. This protection favours the idea that actin combines with these –SH groups of L-myosin. In the field of enzyme chemistry or immunology the ability of the substrate or the hapten to preserve the activity of the enzyme or antibody in the presence of an inhibiting protein reagent is generally taken as a definite proof in support of the location of the protein groups involved in the active site[41]. Since the L-myosin–actin interaction is a very specific one, like that between enzyme and substrate or between antigen and antibody, the ability of actin to protect certain –SH group(s) of L-myosin indicates that these –SH groups participate in the myosin-actin binding.

Sulfhydryl group blocking agents influence not only the interaction of L-myosin with actin but also the reverse reaction, *i.e.* the dissociation of the formed actomyosin brought about by ATP[15]. As was shown above, $2.5 \cdot 10^{-4}$ *M* ATP, which is greatly in excess of the minimum amount necessary to dissociate normal actomyosin, does not produce a maximum effect on the modified actomyosins. The following expts. may help in understanding the mechanism of this inhibition: –SH group reagents which do not dissociate preformed actomyosin nevertheless considerably decrease its ATP sensitivity (Figs. 2, 5, 8); when the actin is removed from this actomyosin, the L-myosin component, after addition of a new sample of actin, produces an actomyosin which, like the original, is also not dissociated by ATP (Fig. 9). It follows, therefore, that the –SH groups, which are somehow necessary for the dissociation, belong to the L-myosin and are not those which participate in the myosin–actin bond. Some further information about the function of these –SH groups in the dissociating reaction is given by the following observations: (1) Salyrgan or iodoacetamide-treated actomyosin can be readily dissociated if, instead of $2.5 \cdot 10^{-4}$ *M* of ATP, $2.5 \cdot 10^{-3}$ *M* of sodium pyrophosphate is used as dissociating agent (pages 301, 303). (2) Pyrophosphate has the same effect on an actomyosin formed from the L-myosin component of the iodoacetamide-treated actomyosin and fresh actin (Fig. 10). (3) The same dissociation can be reproduced by $1.5 \cdot 10^{-3}$ *M* ATP, *i.e.* with a concn. near to that of pyrophosphate (Fig. 10).

These findings indicate that the –SH groups of L-myosin are necessary only

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when dissociation of actomyosin is carried out by ATP; they play no part when pyrophosphate is used. In other words, blocking some –SH groups of L-myosin in actomyosin reduces the high affinity of actomyosin for ATP to the low level for pyrophosphate.

These data give exptl. evidence for the old view that the high affinity of ATP for actomyosin is based on the simultaneous contribution of the nucleoside and the pyrophosphate parts to the ATP binding by actomyosin*. They show, furthermore, that what the nucleoside moiety of ATP contributes to its affinity has as a prerequisite the intactness of certain –SH groups of L-myosin. About the location of these –SH groups in the L-myosin, the following should be pointed out. When –SH group reagents block the dissociating effect of ATP on actomyosin, the ATPase activity of actomyosin is abolished in a parallel fashion (Figs. 2, 5, 8). Thus both kinds of –SH groups may be identical and may participate in the ATP-binding site(s) of the L-myosin molecule.

The groups of actomyosin necessary for the dissociation interact with pyrophosphate but are not affected by –SH group blocking agents. It may be supposed that they are the –SH groups of L-myosin[44] which are protected by actin in actomyosin. One may assume, on the other hand, that the "pyrophosphate-binding part" of actomyosin consists of positively charged amino acid residues or of a firmly bound alkaline earth metal[45], for abundant evidence indicates that cationic side-chains of proteins participate in interactions with anions[46]. Apart from these different interpretations, it is reasonable to assume that the pyrophosphate part of ATP combines with the same site of the L-myosin as actin in actomyosin. It would be difficult to understand otherwise the dissociating effect of pyrophosphate or ATP on normal actomyosin or on that treated with –SH group reagents. That means that the "pyrophosphate-binding part" of L-myosin participates in the interaction with ATP and with actin.

Concerning the chemical nature of the "active centers" of L-myosin, it is important to recall the observations of KUSCHINSKY AND TURBA that binding the amino groups of actomyosin with benzaldehyde inhibits its ATPase activity[40]. The treatment of actomyosin with aldehyde reagents has two disadvantages; the net anionic charge of the protein is enormously increased and actomyosin becomes insoluble[28]. Both of these handicaps were overcome in our expt. by preparing a N-carboxy-L-cysteine anhydride modified derivative of actomyosin. Even so, however, ATPase activity was lost.

When the free carboxylic groups of actomyosin or L-myosin are treated with the basic polylysine, the ATPase activity is not affected at all as long as Ca is used as activator. This finding is valid both in sol and in gel. If the actomyosin gel is activated with Mg, however, polylysine, strongly decreases its ATPase activity; the exact mechanism of this inhibition is still awaiting elucidation.

NOTE ADDED IN PROOF

Shortly after this work has been submitted for publication it came to our notice

* It may be that in the binding of ATP the —SH groups of the L-myosin partner and the adenine moiety of the ATP partner are interacting. Examples of this binding have been suggested[42,43].

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that SNELLMAN[47] isolated a peptid material from L-myosin, which presumably forms a part of its "ATPase center". The sequence of this peptide is Asp·CySH·Tyr·Arg·Lys·Val·Gly·Glu·; this fits well with the data presented in this paper.

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ACKNOWLEDGEMENTS

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DEOXYRIBOSENUCLEIC ACIDS

X. THE SHAPE OF THE SEDIMENTING BOUNDARY OF SODIUM DEOXYRIBONUCLEATE

J. H. COATES AND D. O. JORDAN

Department of Physical and Inorganic Chemistry, The Johnson Laboratories, University of Adelaide (South Australia)

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SUMMARY

The formation of a hypersharp boundary during the sedimentation of sodium deoxyribonucleate in electrolyte solutions has been investigated using a synthetic boundary cell. Hypersharp boundaries are formed between solutions possessing not greatly different sedimentation coefficients and it is thus concluded that the formation of a hypersharp boundary cannot be solely attributed to the concentration dependence of the sedimentation coefficient as generally assumed. A tentative explanation based on the variation of charge with concentration is put forward.

INTRODUCTION

One of the most striking features of the sedimentation behaviour of sodium deoxyribonucleate (DNA) is the hypersharp boundary that is always observed for a sample which is relatively undenatured, both in the presence and absence of added electrolyte. A typical hypersharp boundary for DNA is shown in Fig. 1. The phenomenon of boundary sharpening is well known in both electrophoresis and sedimentation. In sedimentation, the boundaries are found to be less spread than would be expected from a knowledge of the sedimentation coefficient distribution and the diffusion coefficient and this behaviour has usually been regarded as a consequence of the concentration dependence of the sedimentation coefficients of the solutes. BALDWIN[1]

References p. 316.