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STUDIES ON THE PROPERTIES OF CHEMICALLY MODIFIED ACTIN

I. PHOTOOXIDATION, SUCCINYLATION, NITRATION

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SUMMARY

1. The biological properties of photooxidized, succinylated and nitrated F-actin were studied.

2. After 2 min of illumination, when only 2 histidine residues disappeared, the α-actinin sensitivity was completely abolished, the polymerizability and myosin ATPase (ATP phosphohydrolase, EC 3.6.1.3) activation property greatly diminished, and superprecipitation and actomyosin formation slightly diminished.

3. All the studied biological activities but myosin ATPase activation disappeared, if 27 % of the ε-amino groups of actin were succinylated.

4. Tetranitromethane nitrates mostly the tyrosine residues and sulfhydryl groups of actin, tryptophan residues are affected only at high concentration of the reagent. Viscosity and actomyosin formation of actin sharply decreased, and ATPase activation slightly decreased in consequence of nitration, while there was no change in the superprecipitation property of the actomyosin reconstituted from nitrated actin.

INTRODUCTION

Actin belongs to the relatively small group of proteins endowed with several different biochemical activities[1]. One approach to the problem of the active sites of actin is provided by the study of the changes of the activities after applying chemical modification techniques. The role of the sulfhydryl groups has been the subject of many investigations[1–10]. Also a few observations concerning lysine, tyrosine and histidine[11–13] have been reported.

In the present paper, we give an account of the changes in the biochemical activities of photooxidized, succinylated and nitrated actin. The following paper is devoted to the study of the properties of trinitrophenylated actin[14].

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METHODS

Actin was extracted from acetone dry muscle powder prepared according to BÁRÁNY *et al.*[15]. The extraction was carried out at 0°, in order to inhibit the extraction of tropomyosin from the powder[16]. Further purifications were carried out according to MOMMAERTS[17] and the α-actinin contaminations were removed according to EBASHI AND MARUYAMA[18].

Myosin was prepared according to PORTZEHL, SCHRAMM AND WEBER[19].

α-Actinin was prepared according to EBASHI AND EBASHI[20].

ATP was purchased from Sigma Chemical Co., 5-aminotetrazole and tetranitromethane from Fluka. All other chemicals were obtained from Carlo Erba and were of reagent grade.

Viscosity measurements were carried out in an Ostwald viscometer at 0°, the actin concentration being 1–2 mg per ml.

The actomyosin formation at high ionic strength was estimated on the basis of the ATP sensitivity. The measurements were carried out in an Ostwald viscometer at 0° according to BÁRÁNY *et al.*[21] under the following conditions: 1 mg of myosin and 0.25 mg of actin per ml, 0.5 M KCl.

The ATPase activity and superprecipitation measurements were carried out simultaneously. Superprecipitation was measured according to EBASHI[22], on the basis of the turbidity change after addition of ATP, in an Eppendorf photometer at 23°, using a Hg 546 interference filter, and with constant magnetic stirring. The cuvette of the photometer was connected with a combined micro glass-calomel electrode of a Beckman Expandometric Model 76 pH meter, in order to measure the pH change after addition of ATP. The ATPase activity was calculated on the basis of the pH change, because according to NISHIMURA, ITO AND CHANCE[23] the pH change is proportional to the ATPase activity, if it is small enough not to alter the course of the enzymatic activity. In our case, if the whole amount of ATP added was split, the pH change was 0.08, but for the calculations only the first part of the pH curve was used with a pH change not larger than 0.02. The pH and turbidity changes were recorded simultaneously by a Rika-Denki three channel recorder. The conditions for the test were: 0.9 mg of myosin per ml, 0.15 mg of actin per ml, 1 mM ATP, 1 mM $MgCl_2$, 0.02 M Tris–maleate buffer (pH 7.3). The ATPase activity measured by pH change was controlled sometimes by classical P_i liberation method. The same conditions were used in both cases. 5 % trichloroacetic acid (final concentration) was added to stop the reaction and liberated P_i was measured according to FISKE AND SUBBAROW[24]. The results obtained are presented in Table I. The values of $\Delta H^+/\Delta P_i$ ratio are rather constant throughout the time interval used for measurements. These values show the proportionality of H^+ and P_i liberation and are very near to the theoretical values, calculated according to NISHIMURA, ITO AND CHANCE[23]. Different conditions were used if only superprecipitation was measured. In this case we preferred lower ATP and actin concentrations, because the highest degree of precipitation occurs at a much higher myosin to actin ratio than that required for optimum ATPase activity. Therefore a myosin to actin ratio of 24:1 was used: 0.9 mg of myosin and 0.038 mg of actin per ml, in the presence of 0.06 mM ATP, other ingredients were the same as in the former case. Superprecipitation was expressed as the difference of the highest and lowest absorbance observed after addition of ATP. ATPase activity of reconstit-

TABLE I

COMPARISON OF RATES OF ATPASE ACTIVITY DETERMINED BY P_i DETERMINATION AND pH CHANGE

Theoretical value of $\Delta H^+/\Delta P_i$ is 0.812 (calculated according to NISHIMURA, ITO AND CHANCE[23]. Reaction was carried out as described in METHODS.

Time of incubation with ATP (min)	*μatom H^+ liberated/ mg myosin*	*μmole P_i liberated/ mg myosin*	$\Delta H^+/\Delta P_i$
1	0.162	0.20	0.810
2	0.310	0.38	0.817
3	0.455	0.56	0.815
4	0.595	0.74	0.805
5	0.723	0.90	0.802
Mean			0.809 (± 0.008)

uted actomyosin is expressed in percentage of the activity of the pure myosin involved; *i.e.* 100 % of the ordinate is the activity of the myosin in the absence of actin.

The protein content was measured by the biuret method of GORNALL, BARDAWILL AND DAVID[25].

The histidine and tyrosine residues were estimated by the diazotization method of SOKOLOVSKY AND VALLEE[26].

The sulfhydryl groups were determined according to ELLMAN[27]. The amino groups were measured by the ninhydrin method of FRAENKEL-CONRAT[28].

The tryptophan residues were measured according to SPIES AND CHAMBERS[29].

Photooxidation was carried out according to MARTONOSI AND GOUVEA[13]. The F-actin solution, in a final volume of 7 ml, was illuminated at 0° by a 500-W projector lamp placed at a distance of 25 cm. The solution contained 2 mg protein per ml, 0.1 M KCl, 0.012 M Tris–maleate buffer (pH 7.2), 0.12 mM methylene blue and 0.3 mM salyrgan. Several experiments were carried out in the presence of 0.2 mM ATP. Salyrgan was used to inhibit the oxidation of the sulfhydryl groups[13]. At the end of the illumination, the tubes were covered with aluminium foil. Methylene blue and the excess of salyrgan were removed by dialysis against 0.1 M KCl–0.001 M $NaHCO_3$ at 0° (for 36 h with 3 changes of the dialysing solution.)

Succinylation was carried out by mixing different amounts of solid succinic anhydride in a solution containing 1.4 mg of F-actin per ml in 0.1 M KCl with 0.2 mM ATP at 0° (pH was kept constant at 7 by addition of 3 M NaOH as required). After 1 h the reaction mixtures were dialysed at 0° against 0.1 M KCl–0.001 M $NaHCO_3$ for 36 h.

Nitration was carried out according to SOKOLOVSKY, RIORDAN AND VALLEE[30]. Different amounts of tetranitromethane (0.2 M in 95 % ethanol) were added to a solution containing 1.46 mg of F-actin per ml, 0.2 mM ATP, 0.0715 M Tris–HCl buffer (pH 8.1) at room temperature. After 40 min the reaction mixtures were dialysed at 0° against 0.1 M KCl–0.001 M $NaHCO_3$ for 36 h. The number of nitrotyrosine groups formed was determined by the method of SOKOLOVSKY, RIORDAN AND VALLEE[30].

RESULTS

Photooxidation

Very short periods of illumination caused a relatively great change in the properties of F-actin (Figs. 1 and 2). Above all the viscosity and the activation of myosin

ATPase were affected. Both ATPase activation and viscosity decreased to 20% of the original value after 2 min of illumination. The ATP sensitivity and superprecipitation of actomyosin reconstituted from the photooxidized actin were less affected. The changes were less appreciable, if photooxidation was carried out in the presence rather than in the absence of 0.2 mM ATP.

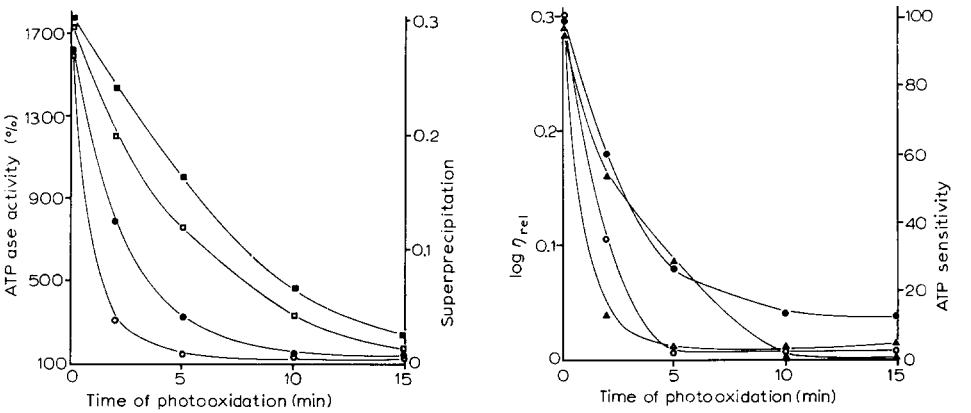


Fig. 1. ATPase activity and superprecipitation of the actomyosin reconstituted from photooxidized F-actin. Details of the procedure see METHODS. Photooxidation carried out in the presence of 0.2 mM ATP (■, ●) and in the absence of ATP (□, ○); ●—●, ○—○, ATPase activity; □—□, ■—■, superprecipitation. Myosin ATPase: 0.012 μatom H^+ liberated per mg myosin per min (100%).

Fig. 2. Effect of photooxidation on the viscosity of F-actin and ATP sensitivity of actomyosin reconstituted from photooxidized F-actin. Details see METHODS. Photooxidation carried out in the presence of 0.2 mM ATP (●, ▲) and in the absence of ATP (○, △). △—△, ▲—▲, viscosity; ○—○, ●—●, ATP sensitivity.

The α-actinin sensitivity, measured as the activation of ATPase of reconstituted actomyosin[14], disappeared completely after 2 min of illumination (Fig. 3). α-Actinin had no activating effect on ATPase of actomyosin reconstituted from photooxidized actin.

Control experiments showed that methylene blue and salyrgan without illumination had no effect on the properties under investigation, in agreement with the results of MARTONOSI AND GOUVEA[13].

TABLE II

PHOTOOXIDATION OF AMINO ACID RESIDUES OF ACTIN

For the details of the procedure see METHODS. Mol.wt. actin: 60 000 (ref. 1).

Time of illumination (min)	*M histidine/M actin*		*M tryptophan/M actin*		*M tyrosine/M actin*	
	ATP present	*ATP absent*	*ATP present*	*ATP absent*	*ATP present*	*ATP absent*
0	11.0	11.3	4.62	4.65	17.4	17.2
2	9.0	9.2	4.27	4.27	17.2	16.9
5	7.8	8.1	3.81	3.95	16.9	17.3
10	7.3	7.5	3.68	3.72	17.1	17.0
15	7.3	7.4	3.64	3.67	17.2	17.1

The estimation of the histidine, tryptophan and tyrosine residues showed that histidine and tryptophan reacted during photooxidation (Table II). The addition of 0.2 mM ATP did not affect the reaction. During the first 2 min of illumination, 2 histidine residues per molecule of actin disappeared, whereas only 1 tryptophan residue disappeared after 15 min. The sulfhydryl groups were not measured because, according to MARTONOSI AND GOUVEA, they are protected from photooxidation in the presence of organic mercurials[13].

TABLE III

EFFECT OF SUCCINIC ANHYDRIDE ON THE AMINO ACID RESIDUES OF ACTIN

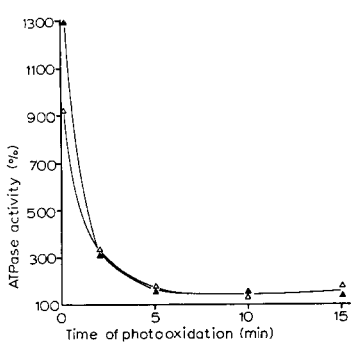
Reaction was carried out as described in METHODS.

M succinic anhydride / *M actin*	*M lysine* / *M actin*	*M–SH* / *M actin*	*M tyrosine* / *M actin*	*M histidine* / *M actin*
840	24	2.72	21.7	11.1
486	27	3.2	21.2	11.3
244	29	3.5	21.9	11.2
122	31	3.9	22.1	10.9
0	34	4.2	21.6	11.4

Succinylation

An 800 times molar excess of succinic anhydride succinylated 30% of the ε-amino groups of lysine residues and 40% of the sulfhydryl groups (Table III). It did not affect the tyrosine and histidine residues.

The activation of myosin ATPase was the least affected of the activities studied (Fig. 4). After treatment with the highest succinic anhydride to actin ratio, it was 77% of the control, whereas after reaction with smaller succinic anhydride concentrations the activation increased. The shape of the ATPase activation curve was similar to



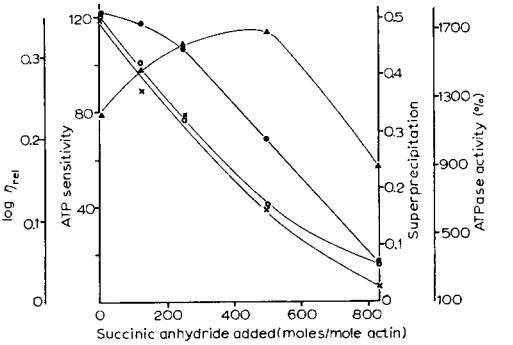


Fig. 3. ATPase activation by the photooxidized F-actin in the presence and absence of α-actinin. ATPase activity in the presence (▲) and in the absence (△) of α-actinin. Details see METHODS. Photooxidation was carried out in the absence of ATP. α-Actinin concentration, 0.19 mg/ml. Myosin ATPase: 0.009 μatom H^+ liberated per mg myosin per min (100%).

Fig. 4. Characteristic properties of succinylated F-actin. Details see METHODS. ▲—▲, ATPase activity; ○—○, viscosity; ×—×, ATP sensitivity; ●—●, superprecipitation. Myosin ATPase: 0.0095 μatom H^+ liberated per mg myosin per min (100%).

that produced by trinitrophenylation[14]. The decrease of the other activities was much more appreciable: they were practically lost when the highest succinic anhydride concentration was used. The loss of the α-actinin sensitivity (*i.e.*, the activation of actomyosin ATPase on addition of α-actinin) was complete after treatment with the lowest anhydride to actin ratio (Fig. 5).

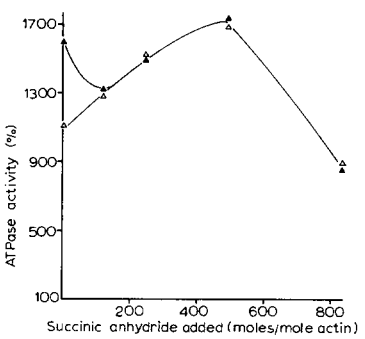


Fig. 5. ATPase activation by succinylated F-actin in the presence and absence of α-actinin. ATPase activity in the presence (▲) and absence (△) of α-actinin. Details see METHODS. α-Actinin concentration 0.17 mg/ml. Myosin ATPase: 0.013 μatom H^+ liberated per mg myosin per min (100%).

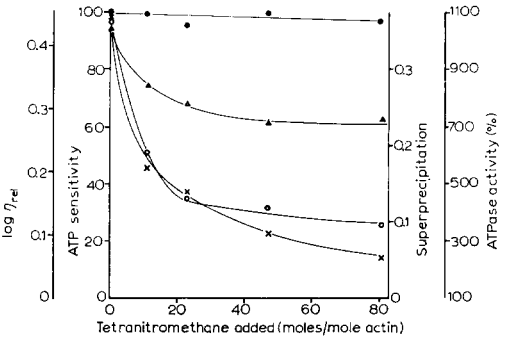


Fig. 6. Characteristic properties of nitrated F-actin. Details see METHODS. △—△, ATPase activity; ○—○, viscosity; ×—×, ATP sensitivity; ●—●, superprecipitation. Myosin ATPase: 0.0087 μatom H^+ liberated per mg myosin per min (100%).

Nitration

Tetranitromethane nitrated a part of the tyrosine residues and oxidized some of the sulfhydryl groups. At higher tetranitromethane concentrations, the number of the nitrated tyrosine residues seemed to be about the same whether it was calculated on the basis of the tyrosine residues which disappeared, or of the nitrotyrosine groups which formed, but, at lower reagent concentrations, the number of the modified tyrosine residues seemed higher on the former basis. It is very probable that the reason for this discrepancy is the uncertainty of the nitrotyrosine determination method of SOKOLOVSKY, RIORDAN AND VALLEE[30].

TABLE IV

EFFECT OF TETRANITROMETHANE ON THE AMINO ACID RESIDUES OF ACTIN

For the details of the procedure see METHODS.

M tetranitromethane / *M actin*	*M nitrotyrosine* / *M actin*	*M tyrosine* / *M actin*	*M histidine* / *M actin*	*M tryptophan* / *M actin*	*M–SH* / *M actin*
82.5	8.2	12.8	12.4	3.80	—*
47.2	4.8	15.9	12.8	3.86	—*
23.6	1.3	17.1	11.9	4.45	0.95
11.8	0.7	19.2	12.4	5.55	1.40
5.9	0.15	19.8	12.6	5.62	1.60
0	0	20.9	12.8	5.60	1.80

* Not measured.

After treatment with comparatively high tetranitromethane concentrations the number of the tryptophan residues decreased appreciably, whereas the number of the histidine residues remained unchanged (Table IV).

There was a sharp decrease in the viscosity and ATP sensitivity even after treatment with the lowest tetranitromethane concentration, when only approx. 1 tyrosine residue per molecule and no tryptophan residue reacted. The decrease of ATP sensitivity values on nitration as well as on succinylation and photooxidation means decreasing actomyosin formation, since the viscosities before the addition of ATP diminish in consequence of the treatments and the log η_{rel} values after the addition of ATP are always the sum of the log η_{rel} of the original actin and myosin solutions. Consequently the treatments did not affect the dissociating effect of ATP on actomyosin as the values of log η_{rel} show that interaction does not take place between the proteins in the presence of ATP[37]. In actomyosin reconstituted from the nitrated actin there was no change in the superprecipitation and only a relatively small reduction in the activation of myosin ATPase (Fig. 6).

DISCUSSION

The properties of photooxidized actin were studied by MARTONOSI AND GOUVEA[13]. According to them, only the number of the histidine residues decreased slightly after 15 min illumination and no change occurred in the tryptophan residues. A possible reason for the variance with our experiments is the difference in the methods of determination. In fact they analysed the amino acid composition after hydrolysis of actin, whereas we estimated the different residues in the protein without hydrolysis. However, since the tryptophan residues were changed only after prolonged illumination, we agree with the conclusions of MARTONOSI AND GOUVEA, that the oxidation of histidine groups is likely to be responsible for the loss of the biochemical activities. Although, one cannot exclude the possibility that the oxidation of other residues (*e.g.* methionine) is responsible for the change in the properties of photooxidized actin. It is worth noting that according to MARTONOSI AND GOUVEA the decrease of polymerizability is much prompter than the activation of myosin ATPase, whereas we did not find any essential difference in the time course of these activities. The reason for this discrepancy may be that their actin to myosin ratio was higher than ours and the amount of actin was in excess over the concentration needed for optimal activation[21].

We confined our studies to F-actin, because the reaction of the sulfhydryl groups of F-actin does not alter the characteristic properties of the protein[2,7–9] as it is the case with G-actin, and we wanted to prevent any effect of the different treatments on sulfhydryl groups from disturbing the evaluation of our results.

Therefore it seems improbable that the reduction of the number of sulfhydryl groups is the reason for the change of the characteristic properties of actin in the succinylation and nitration experiments. In the case of succinylation this assumption is supported by the similarity of the results of the trinitrophenylation experiment[14] in which only the amino groups were reacted.

In our hands tetranitromethane also reacted with the sulfhydryl groups and the tryptophan residues, at variance with the results of SOKOLOVSKY, RIORDAN AND VALLEE[30], but, if the concentration of the reagent was low enough, the reaction occurred only with the tyrosine residues and slightly with the sulfhydryl groups.

Since viscosity and ATP sensitivity decreased very appreciably when only 1 tyrosine residue per molecule and no tryptophan residue reacted, the importance of this tyrosine residue seems obvious. According to MARTONOSI AND GOUVEA, the modification of one tyrosine residue by diazotization led to loss of polymerizability and of the myosin ATPase activating property of actin as well[13]. The data presented here are at variance with these results: in fact the ATPase activation was affected very little by nitration.

The decrease in superprecipitation is relatively small on photooxidation and nitration in spite of the great decrease in viscosity values, which shows the destruction of the actin filaments necessary for superprecipitation. However, superprecipitation can take place, if the myosin/actin ratio is 100:1 (unpublished results) so one may assume that these treatments do not destroy all the F-actin filaments and in the presence of the few remaining intact filaments the superprecipitation takes place.

There are many uncertainties in the literature concerning the role of α-actinin in the actomyosin system. According to EBASHI *et al.* α-actinin enhances the superprecipitation of actomyosin, but does not affect its ATPase activity[31]. MARUYAMA observed only a slight activation[32], whereas according to SERAYDARIAN, BRISKEY AND MOMMAERTS[33], and our results[14] α-actinin, under strictly limited conditions, significantly activates actomyosin ATPase. Of all the characteristic properties studied the α-actinin sensitivity of the actomyosins reconstituted from differently treated actins was the most labile. It was completely lost after a very brief period of photooxidation or trinitrophenylation[14], or after treatment with the lowest succinic anhydride to actin ratio. On the basis of this great lability and of the many uncertainties[31–33] concerning the activating effect of α-actinin on actomyosin ATPase, it is dubious whether α-actinin acts directly through the actin component of actomyosin or through some very labile factor which loses its activity in consequence of the treatment. This factor may sometimes be absent in the actin preparations of different authors and may be the reason why they did not find activation by α-actinin[31,32].

The biochemical activities of actin appeared to change independently after different treatments. ATPase activation decreased sharply after photooxidation, whereas the actomyosin formation was affected mainly by nitration and succinylation. These data seem to support the assumption of MARTONOSI AND GOUVEA[13], PERRY AND COTTERILL[34], and BAILIN AND BÁRÁNY[10], that different, independent active centers are responsible for these properties.

It is quite surprising, however, that actin activates myosin ATPase under such conditions that actin and myosin interaction (measured by viscosity changes) is greatly inhibited, as in the cases of succinylation and nitration. But this contradiction is eliminated, if one considers that (1) the viscosity measurement was carried out at high, while determination of ATPase at low ionic strength, and even if actomyosin formation is prevented at high ionic strength, there is no proof that the same holds true for low ionic strength. (2) There are some data in the literature[34–36] which show that the interaction of actin and H-meromyosin in the presence of ATP, while causing a marked activation of the H-meromyosin ATPase, does not cause an increase in viscosity at low ionic strength. Therefore on the basis of the absence of viscosity effect, the interaction of actin and myosin cannot be excluded. Moreover our observations strongly support the original suggestion of PERRY AND COTTERILL[34] that the part of the actin molecule which exerts its effect on the enzymatic center of myosin

is different from that concerned in the interaction with myosin which produces the viscosity effect.

For the time being it does not seem permissible to assume that the modified residues are actually taking part in active centers, because after the different treatments all activities were changed to some extent. This observation seems to indicate that the modified residues play a role in the maintenance of the active conformation of the actin molecule and that their modification affects the active centers by conformational changes.

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