BBA 46314

ALKYLATION OF UREA-DENATURED ACTIN WITH IODOACETATE: FUNCTIONAL REORGANIZATION OF ACTIN

KOSHIN MIHASHI

Department of Physics, Faculty of Science, Nagoya University, Chikusa-ku, Nagoya 464 (Japan)

(Received December 24th, 1971)

SUMMARY

1. Starting with actin alkylated with monoiodoacetate in the presence of 4 M urea, the functional reorganization of actin, namely polymerization, its interaction with myosin, and the binding of nucleotide, was examined.

2. After removal of urea by dialysis, the alkylated actin was in a monomeric state in a low ionic strength solution at neutral pH. Nucleotide was completely removed from the solution. Addition of neutral salt did not cause polymerization of alkylated actin. A polymer of alkylated actin was formed when the protein was precipitated at pH 5.5 and redissolved into a low ionic strength solution of neutral pH. Polymerization occurred without participation of nucleotide. The sedimentation pattern of alkylated actin polymer gave a single peak of 10 S.

3. Both increasing ionic strength and raising temperature (above 20 °C) caused a change in polymer conformation which was reversible.

4. Myosin and heavy meromyosin bound to alkylated actin polymer and the ATPase of heavy meromyosin were activated through the interaction with alkylated actin polymer. Specifically, alkylated actin polymer incubated at pH 6.5 for several days showed a Mg^{2+}-sensitized ATPase of heavy meromyosin. The extent of the Mg^{2+}-sensitized ATPase was close to that found with normal F-actin.

5. The monomer of alkylated actin exhibited a specific receptivity to ATP with a dissociation constant of the order of 10^{-5} M. 1-Anilinonaphthalene-5-sulfonic acid also bound to alkylated actin competitively with ATP. Fluorescence of the bound 1-anilinonaphthalene 5-sulfonic acid suggested that the binding site forms a hydrophobic cleft.

INTRODUCTION

Actin, when extracted from acetone-dried powder of muscle mince in distilled water, is in a monomeric state (G-actin) to which ATP binds (1 mole per mole of actin[1,2]). The binding is specific and the dissociation constant is of the order of 10^{-6} M. Addition of an appropriate amount of neutral salt (*e.g.* 0.1 M) induces the polymerization of actin to a characteristic form (F-actin) which displays the biological activity of actin. However, when the solution of G-ATP-actin is left standing for a long time,

Abbreviation: ANS, 1-anilinonaphthalene-5-sulfonic acid.

actin begins to lose both ATP receptivity and polymerizability spontaneously. A removal of free ATP from the solution speeds up this process of denaturation[3,4]. Although addition of an excess amount of ATP retards the denaturation, if actin is once denatured ATP is not able to renature actin, thus indicating that molecular state of denatured actin is more stable energetically than G-ATP-actin. This implies that native G-actin takes a metastable state which favours the binding of ATP. Ultraviolet absorption of actin showed that the conformation of actin is different among G-ATP-actin, F-ADP-actin and denatured actin, indicating that G-ATP-actin assumes an intermediate conformation between F-actin and denatured actin[5].

The above conclusion leads us to suppose that if we activate the denatured actin to an intermediate conformation, it may regain its receptivity to nucleotide and polymerizability as well. Further, its functional ability to interact with related proteins, *e.g.* myosin, tropomyosin and troponin, may be reorganized. Thus the theory of the renaturation of actin has to include activation of the tertiary structure of denatured actin. We considered that chemical modification of denatured actin would result in a modification of the tertiary structure and an attempt was made to modify denatured actin by alkylation with monoiodoacetate. The following three aspects of effect of the modification were examined: (*i*) the polymerizability, (*ii*) the ability to interact with related proteins, (*iii*) the receptivity of the ATP-binding site.

EXPERIMENTAL

Preparation of actin

An acetone-dried powder of rabbit skeletal muscle mince was prepared according to the method of Straub[2]. A slight modification of the procedure was made as follows. After the extraction of myosin A, the minced muscle was washed in 0.4 % $NaHCO_3$ solution and in distilled water successively. Then the minced muscle was suspended in 3 vol. of 0.3 mM $NaHCO_3$ for a few hours. The suspension was centrifuged at 5×1000 rev./min for 20 min. The sediment of minced muscle was dried with acetone as in the method of Straub. This was first proposed by Ebashi for extraction of native tropomyosin (or troponin and tropomyosin)[6]. Actin was extracted from the acetone-dried muscle powder in cold distilled water. Polymerization of actin was induced by addition of 30 mM KCl. An aliquot of the solution was sonicated and the sonicated solution was mixed with the rest of the actin solution. This procedure accelerated actin polymerization[7]. About half an hour after sonication, F-actin was centrifuged and the pellet of F-actin was dispersed in cold distilled water containing 350 μM ATP and 0.3 mM bicarbonate, and was dialyzed against a large volume of cold distilled water containing 40 μM ATP and 0.3 mM bicarbonate. Actin was purified with a cycle of polymerization and depolymerization in the same manner. Finally actin was polymerized in 0.1 M KCl, 0.3 mM bicarbonate.

Denaturation and alkylation of actin

F-actin was denatured in concentrated urea in the following way. The pellet of F-actin was suspended in 3 vol. of 5 M urea solution containing 10 mM Tris–glycine buffer (pH 9.0). The solution was stirred in an ice-bath until the actin was fully dissolved. Then, 0.1 M of β-mercaptoethanol was added and the pH was adjusted to 9.0. The solution was left standing at least 2 h in the cold room. Alkylation was carried out

by the addition of a conc. aq. solution of monoiodoacetic acid (about 3 M) with a micropipet. The final concentration of monoiodoacetate was 0.2 M. The reaction was followed for 3–5 h in an ice-bath and the pH of the solution was adjusted to 9.0 continuously with conc. KOH. Then the solution was dialysed against a large volume of cold distilled water to remove urea and other reagents. The dialysate contained 2 mM or 5 mM Tris–HCl (pH 8.5). The original solution of alkylated actin was thus prepared.

Myosin and heavy meromyosin

Myosin and heavy meromyosin was prepared by standard methods[9,10].

Physicochemical measurements

Viscosity was measured with an Ostwald-type viscometer of which the water flow time at room temperature was about 30 s. The temperature at which measurements were made was regulated within 0.05 °C. Sedimentation analysis was done with a Spinco Model E analytical centrifuge in which an automatic temperature control system was installed. An Yphantis-type multi-channel cell was used for molecular weight determination.

ATPase activity

The ATPase activity of myosin and heavy meromyosin was measured by the method of Tausky and Shorre[11]. The reaction temperature was controlled within 0.05 °C. ATP was purchased from Sigma Chemical Co.

Column chromatography

A column of Sephadex G-50 equilibrated with 10 mM Tris-acetate buffer (pH 7.5) was used for the examination of the binding of 1-anilinonaphthalene-5-sulfonic acid to alkylated actin. Elution was performed at room temperature.

Reagents

The ammonium salt of ANS (8-anilino-1-naphthalene sulfonic acid) was purchased from Sigma Co. and purified according to the method of Weber and Young[6]. The concentration of ANS was determined by its absorbance at 350 nm with molar extinction $5 \cdot 10^3$. Monoiodoacetic acid was from Tokyo–Kasei and Merck. Other reagents were all analytical grade.

Fluorometric titration of ANS

A Hitachi MPF-2A was used for fluorescence measurement. The temperature of sample was controlled by circulation of water in a cell holder which was connected to a Haake thermostat. For the fluorometric titration, the excitation wavelength was 360 nm and fluorescence intensity was measured at 475 nm, and the fluorescence intensity was conventionally normalized by dividing the observed fluorescence intensity by the absorbance at the excitation wavelength.

The equilibrium constants of the ANS binding were determined in the following way. Assuming that there are n binding sites per molecule of alkylated actin which are identical and independent of each other, the observed fluorescence intensity (F) is proportional to the ratio of the total bound ANS to the total moles of protein.

This ratio ($\bar{\lambda}$) is related to the dissociation constant (K_{ANS}) and the concentration of free ANS (ANS_f) by the expression

$$\bar{\lambda} = \frac{n \cdot \frac{ANS_f}{K_{ANS}}}{1 + \frac{ANS_f}{K_{ANS}}} \tag{1}$$

Introducing the total concentration of ANS (ANS_0) and total protein concentration (P_0)

$$ANS_f = (1 - x) \cdot ANS_0$$

$$\bar{\lambda} = x \cdot ANS_0/P_0$$

where x is the fraction of the bound ANS and is equal to F/F_0 (F_0 is the fluorescence intensity when all ANS is bound to the protein), Eqn 1 becomes

$$\frac{P_0}{x \cdot ANS_0} = \frac{1}{n}\left\{1 + \frac{K_{ANS}}{(1-x) \cdot ANS_0}\right\} \tag{2}$$

Thus the plot of $P_0/x \cdot ANS_0$ as a function of $1/(1-x) \cdot ANS_0$ gives n and K_{ANS}. In the following experiments, the concentration of alkylated actin was of the order of 10^{-6} M and that of ANS was from $5 \cdot 10^{-6}$ to 10^{-4} M. The fluorescence spectrum of the bound ANS did not alter within the concentration range. Fluorescence measurement was made at least 12 h after mixing ANS with alkylated actin solutions.

RESULTS

Original solution of alkylated actin

The sedimentation pattern of the original solution of alkylated actin in 10 mM Tris–acetate buffer (pH 8.5) showed a single peak whose sedimentation constant was about 3.0 at a protein concentration of 2.5 mg/ml, indicating that most of the alkylated actin under these conditions was in a monomeric state. In some preparations, however, a rapidly sedimenting material (presumably an aggregate of alkylated actin) was observed and the aggregate was removed by centrifugation at $4 \cdot 10^4$ rev./min for 2 h. The addition of neutral salt, *e.g.* 0.1 M KCl, to the original solution in 10 mM Tris–acetate (pH 8.5) did not cause polymerization of the protein. An ultraviolet absorption spectrum of the original solution around 260 nm showed that nucleotide (ATP or ADP) was entirely absent (Fig. 1).

Polymerization of alkylated actin

Since alkylated actin did not polymerize on addition of KCl, various attempts were made to obtain a polymer. An alkylated actin solution, after dialysing out urea and other reagents, was brought to low pH by the addition of HCl in an ice-bath. The protein began to precipitate around pH 6. The precipitate at pH 5.5 was collected by centrifugation at a speed of $5 \cdot 10^3$ rev./min for 10 min. When a few drops of 0.1 M Tris–HCl buffer (pH 8.5) were added, the precipitate became a clear gel. The gel was then dialysed against a large volume of cold distilled water containing 2 mM Tris–HCl

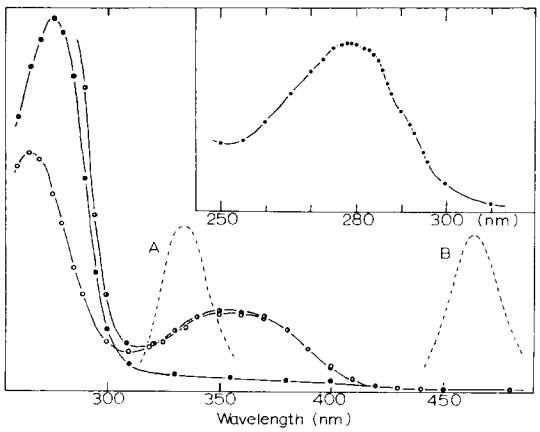


Fig. 1. Absorption and emission spectra of alkylated actin, ANS and alkylated actin *plus* ANS at pH 7.5 (Tris–acetate, 10 mM). ●—●, absorption spectrum of alkylated actin; ○—○, absorption spectrum of ANS; ◑—◑, absorption spectrum of alkylated actin *plus* ANS. – – – –, fluorescence spectra; (A) fluorescence emission from alkylated actin (excitation at 280 nm), (B) fluorescence emission from ANS bound to alkylated actin (excitation at 360 nm). It should be mentioned that when the solution of alkylated actin *plus* ANS was irradiated at 280 nm, an emission maximum was observed not only at 334 nm but also at 475 nm, the latter indicating an excitation energy transfer from the protein to ANS. (The ordinate is chosen arbitrarily.) The insertion is a fine structure of the absorption spectrum of alkylated actin.

(pH 8.5). The high viscosity of alkylated actin was not lost but enhanced during dialysis. Since alkylated actin before acid-precipitation had low viscosity and the high viscosity appeared only after acid precipitation, it was concluded that the acid precipitation induced polymerization of alkylated actin. Actually, the solution of high viscosity showed flow birefringence and the sedimentation pattern gave a single peak of about 10 S (Fig. 2), while alkylated actin without acid treatment gave a single peak of 3 S in the same solvent conditions (5 mM, Tris–HCl, pH 8.5). There was no trace of 3-S component in the alkylated actin solution after acid precipitation; therefore, almost all alkylated actin was converted into the 10-S polymer. Since the

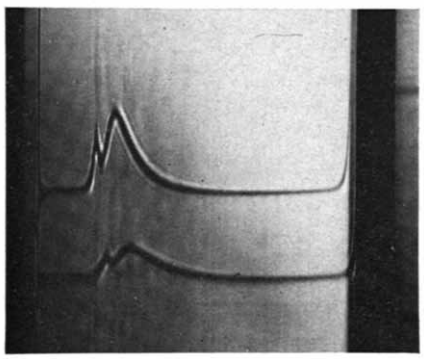
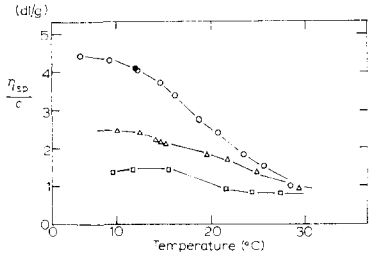


Fig. 2. Sedimentation pattern of polymer of alkylated actin which was prepared in the following way: The original solution of alkylated actin was precipitated at pH 5.5 and the precipitate, *i.e.* polymer of alkylated actin, was dissolved in 10 mM Tris–HCl (pH 8.5) and dialysed against the same solution. Concentration of polymer of alkylated actin; upper 5.0 mg/ml, lower 3 mg/ml; at 24.0 °C; sedimentation from left to right; bar angle 70°. Note a minor component of 8 S (see text).

original alkylated actin solution is free from ATP or ADP, polymerizability is wholly attributable to the polypeptide chain of alkylated actin. In some preparations, a minor component of about 8 S was observed and this kind of heterogeneity was also observed on chromatography in a Sephadex G-100 column. The specific viscosity of



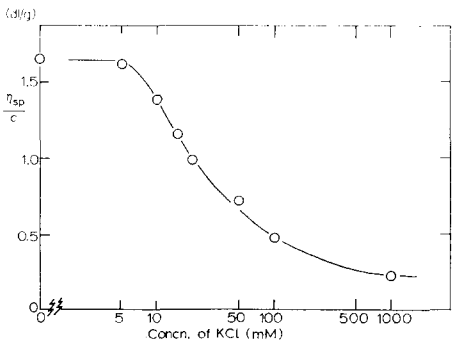


Fig. 3. Viscosity of alkylated actin polymer as a function of temperature. ○—○, alkylated actin polymer, 3.7 mg/ml; 5 mM Tris–HCl (pH 8.5); the temperature was changed from lower to higher and the closed circle (●) corresponds to the reversion of temperature from 23.5 °C to 12.0 °C. △—△, polymer of alkylated actin, 2.0 mg/ml; 20 mM Tris–HCl (pH 8.5); □—□, polymer of alkylated actin, 4.4 mg/ml; 10 mM Tris–maleate (pH 7.0).

Fig. 4. Viscosity of polymer of alkylated actin as a function of KCl concentration. Various amounts of KCl were added to alkylated actin polymer and the solution was left standing over night at 10.0 °C and then viscosity measurement was made at the same temperature. Concentration of alkylated actin polymer, 2.5 mg/ml; Tris–HCl, 25 mM (pH 8.5). When KCl was removed by dialysis, the viscosity of each solution was essentially the same as that of the original KCl-free solution.

alkylated actin polymer varied among different preparations, the reason of which being unknown. As is shown in Fig. 4, an increase of ionic strength of the solution of alkylated actin polymer (to, for example, 100 mM KCl) caused a reduction of viscosity. However, this probably does not indicate depolymerization of the polymer, because the sedimentation constant was not largely altered. It was rather due to a change in polymer conformation. The reduction of viscosity was completely reversed after removal of KCl by dialysis. A similar viscosity change was demonstrated when the temperature of the solution was raised above 20 °C (Fig. 3). The transitional decrease in viscosity was observed under various solvent conditions. Depending on the viscosity of the original solution of alkylated actin polymer, the viscosity at high temperature varied from sample to sample. As long as temperature did not exceed 30 °C, the viscosity change was reversible with respect to temperature. The change took place in a few minutes, suggesting also that it is mostly due to intrapolymer change. In fact, the molecular weight determined by equilibrium sedimentation in a short column at 10.5, 16.0 and 25.5 °C gave essentially the same value of $(470 \pm 50) \cdot 10^4$, although at 25.5 °C, the concentration gradient in the cell during centrifugation showed the presence of a small amount of a low molecular weight polymer probably formed by depolymerization.

Binding of myosin to polymer of alkylated actin

There arises an interesting question whether the polymer of alkylated actin possesses the ability to bind to myosin. Both the polymer of alkylated actin and

myosin were dialysed separately against a Tris–maleate buffer (10 mM, pH 7.0) at 0 °C. The two solutions were mixed at room temperature to give final concentrations of 2.5 mg/ml alkylated actin and 0.7 mg/ml myosin. Soon after mixing, the solution became turbid and in about half an hour the protein precipitated. In the control solutions, neither F-actin nor myosin precipitated. This clearly indicates that alkylated actin formed a complex with myosin giving large aggregates (Fig. 5). Addition of ATP at the time of mixing of the two proteins accelerated the precipitation, suggesting a specific effect of ATP on the formation of the aggregate as in the case of superprecipitation. Direct observation of the binding of the two proteins in the absence of ATP was made in the sedimentation experiment, where heavy meromyosin instead of myosin was used. A control run of the alkylated actin polymer showed a peak of 10 S and heavy meromyosin gave a peak of 6 S (ref. 10). The mixed solution gave a new peak of about 14 S at the expense of both the alkylated actin and heavy meromyosin peaks.

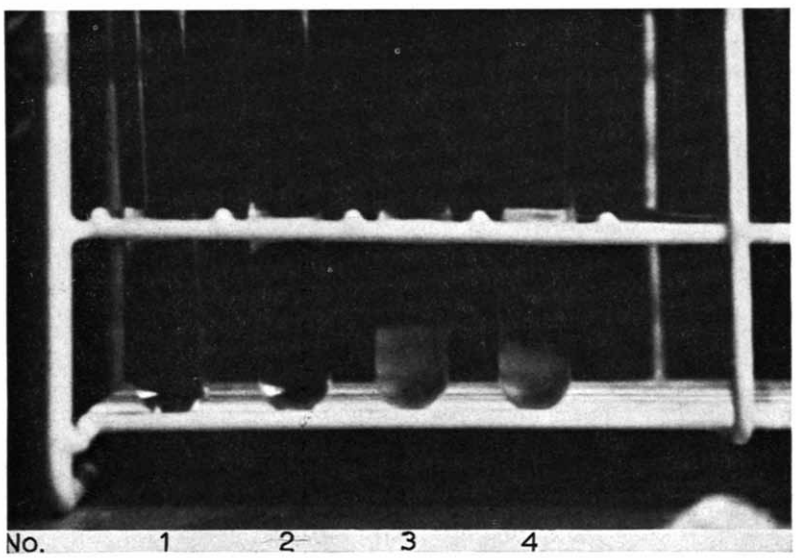


Fig. 5. Coprecipitation of polymer of alkylated actin with myosin. Polymer of alkylated actin and myosin were dialysed in 0.1 M KCl, 10 mM Tris–maleate (pH 7.0) in the cold room overnight. Final concentrations of proteins were as follows: No. 1, polymer of alkylated actin 0.5 mg/ml; No. 2, myosin 0.2 mg/ml; No. 3, polymer of alkylated actin 0.5 mg/ml, myosin 0.2 mg/ml. No. 4. ATP was added to solution No. 3 (finally 2 mM) at the time of mixing of alkylated actin polymer and myosin. The four solutions were left standing at room temperature and the picture was taken 3 h after preparation.

Activation of ATPase of heavy meromyosin by polymer of alkylated actin

Since ATP affected the interaction of alkylated actin with myosin, we investigated whether heavy meromyosin ATPase is influenced by the presence of alkylated actin. When alkylated actin was added to heavy meromyosin at the ratio of 1:1 by wt, a small but distinct activation of the heavy meromyosin ATPase was observed. For assurance, measurements were made at least three times under the same conditions. On an average, activation was about 30 % in excess of that of heavy mero-

myosin only, which is far beyond possible experimental errors. The extent of activation was a little dependent on preparation of the alkylated actin polymer. In the results summarized in Table I, two points should be noted; the different species of divalent cation present in the solution (Ca^{2+} or Mg^{2+}) gave essentially the same activation, and the extent of activation was practically the same at 10.2 °C and at 25.0 °C, indicating that in this case the polymer conformation had no influence.

TABLE I

ACTIVATION OF HEAVY MEROMYOSIN ATPase BY ALKYLATED ACTIN POLYMER IN THE PRESENCE OF Ca^{2+} OR Mg^{2+}

Heavy meromyosin 0.3 mg/ml, polymer of alkylated actin 0.5 mg/ml; solvent contained 10 mM Tris–maleate buffer (pH 7.0); Mg^{2+}-ATP or Ca^{2+}-ATP, 2.0 mM. Prior to the addition of Mg^{2+}-ATP (or Ca^{2+}-ATP), the protein solution was incubated at the specified temperature for 15 min. The initial rate of inorganic phosphate liberation was measured, and the figures listed in this table are the ratio of the two initial rates, one in the presence and the other in the absence of alkylated actin polymer. Initial rates ratio = v_i (heavy meromyosin + alkylated actin polymer)/v_i (heavy meromyosin).

Type of alkylated actin polymer	*Initial rates ratio*		*Temperature (°C)*
	$+Ca^{2+}$	$+Mg^{2+}$	
Before incubation at pH 6.5	1.33	1.20	10.2
	1.28	1.15	25.0
After incubation at pH 6.5 for several days	1.12	18.5	10.2
	1.07	27.4	25.0

During the course of these experiments, we happened to find that the alkylated actin polymer solution which had been incubated at pH 6.5 in a cold room for several days showed very high viscosity and remarkable elasticity, which had not been found before the incubation. The solution was mixed with heavy meromyosin to see the effect of the polymer on heavy meromyosin ATPase. As shown in Table I, the ATPase of the mixture in the presence of Mg^{2+} was remarkably enhanced, 20 times as high as that of heavy meromyosin alone at 10.2 °C. It was almost 30 times greater than heavy meromyosin alone at 25.0 °C; such activation is comparable with the activation by ordinary F-actin. In contrast, ATPase in the presence of Ca^{2+} was low and practically the same as with non-incubated alkylated actin polymer. Thus, discrimination of divalent cations (between Mg^{2+} and Ca^{2+}) by the polymer of alkylated actin appeared. Since this is a unique character of F-actin, it is suggested that the basic structure of the polymer of alkylated actin was changed to that of ordinary F-actin during the incubation at weakly acidic pH. Actually, preliminary electron microscopic observation of polymers of alkylated actin both before and after acidic incubation showed a conspicuous difference between them; namely, the polymer of alkylated actin before acidic incubation assumed amorphously entangled polymers, while the solution of polymer of alkylated actin after acidic incubation was enriched with two stranded helical polymers which were indistinguishable from ordinary F-actin. Very compatible with this was the result of flow birefringence measurement. That is, the extinction angle of alkylated actin polymer before acidic incubation was 22° and that after acidic incubation 5°. (Details of the physical properties of this polymer will be

reported elsewhere.) So far, the necessary conditions for this conversion of the polymer of alkylated actin have not been thoroughly investigated. Also the extent of activation of the heavy meromyosin ATPase varied among different preparations of the alkylated actin polymer.

Binding of ANS and ATP to alkylated actin

The above results clearly indicate that the functional ability of actin to polymerize and to interact with myosin specifically, which was once lost by the urea treatment, is restored through the process of alkylation and subsequent removal of the reagents, though the resultant polymer of alkylated actin is not exactly the same as normal F-actin[1,2]. Since the polymerization of alkylated actin and its binding with myosin occurred without participation of ATP, an interesting question arises as to the binding affinity of alkylated actin to ATP. As shown below, alkylated actin can bind ATP. In order to examine the property of the ATP-binding site of alkylated actin, we introduced a hydrophobic probe, ANS, which had proved to be an excellent tool for obtaining information about the ligand-binding site of several proteins[12,13]. When ANS was dissolved in a 10 mM Tris–acetate buffer (pH 7.5), an irradiation at 350 nm gave a weak fluorescence at 515 nm (ref. 12). Upon addition of a small amount of the original solution of alkylated actin to the ANS solution, an intense fluorescence appeared with a maximum at 475 nm (Fig. 1). This wavelength scarcely changed with variation of the excitation wavelength from 340 nm to 390 nm. The increase of the intensity of fluorescence with the concentration of the alkylated actin indicated the binding of the dye to the protein. In a column chromatography of the protein–dye solution on a Sephadex G-50 column, ANS appeared as two fractions, one at the same position as the protein and the other as a free fraction. As shown in Fig. 6, it was the former fraction which was responsible for the intense fluorescence

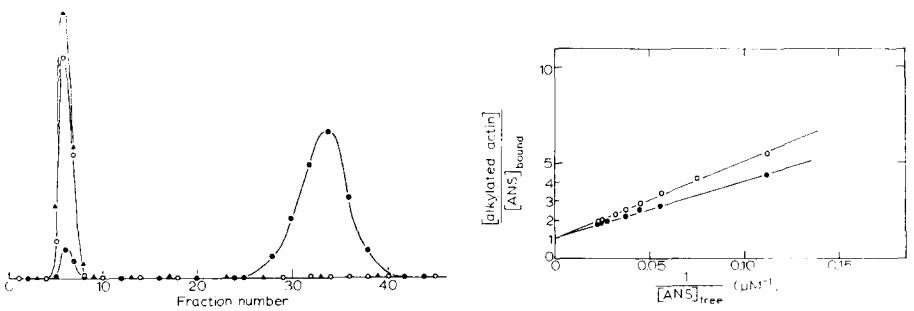


Fig. 6. Column chromatogram of the mixture of alkylated actin monomer and ANS on Sephadex G-50 column (1.5 cm × 50 cm). Alkylated actin 2.0 mg/ml, ANS 460 μM, 10 mM Tris–acetate (pH 7.5). Elution was performed at room temperature. ▲—▲, alkylated actin (content of the protein was determined fluorometrically with excitation at 280 nm and emission at 340 nm). ●—●, ANS (concentration was determined from absorption at 360 nm); ○—○, fluorescence emission at 470 nm with excitation at 360 nm. Note that a sensitized fluorescence of ANS at 470 nm was observed only in the Fractions 6, 7 and 8, while even a high concentration of ANS at fraction Nos 28–38 gave substantially no fluorescence at 470 nm.

Fig. 7. Specific binding of ANS to alkylated actin monomer and the effect of Mg^{2+}-ATP. Concentration of alkylated actin, 1.55 mg/ml; 10 mM Tris–acetate (pH 7.5). ○—○, *plus* 10 μM Mg^{2+}-ATP; ●—●, ATP free. After mixing alkylated actin with ANS (and ATP) the solutions were left standing at room temperature overnight. Fluorescence measurement was made at 15.0 °C. For details of analysis, see text.

at 475 nm. The latter fraction gave only a weak fluorescence at 515 nm. Thus the binding of ANS to alkylated actin was confirmed. The absorption spectrum of ANS did not change appreciably upon binding to alkylated actin (Fig. 1). In order to examine the stoichiometry of the binding, the amount of bound ANS was determined at various concentrations of the total ANS. The concentration of alkylated actin was taken to be of the order of 1 μM and that of ANS from 1 μM to 100 μM. The fluorescence spectrum of bound ANS did not alter in this range of ANS concentration. From the result in Fig. 7, by using Eqn 2 the dissociation constant K_{ANS} in the solvent of 10 mM Tris–acetate (pH 7.5) was estimated to be 32 μM. The number of the binding sites on the monomer of the protein was 1.09, assuming the molecular weight of the protein[14,15] to be $4.7 \cdot 10^4$. The effect of neutral salts (KCl, $MgCl_2$, $CaCl_2$) was also examined and the results were summarised in Table II. Increase in the ionic

TABLE II

APPARENT DISSOCIATION CONSTANT OF ANS WITH ALKYLATED ACTIN MONOMER UNDER VARIOUS SOLVENT CONDITIONS

Binding of ANS to alkylated actin monomer was measured fluorometrically in the same way as in Fig. 7 at 15.0 °C.

Solvent conditions		K_{app} (μM)
Tris–acetate	10 mM (pH 7.5)	32
Tris–HCl	10 mM (pH 8.5)	
+ KCl	10 mM	34
	60 mM	20
	100 mM	18
+ $MgCl_2$	1 mM	18
+ $CaCl_2$	1 mM	20

strength by the addition of KCl favoured the dye–protein binding, but the number of the binding sites did not deviate from unity. Therefore, the effect of the salt can be understood as being due to shielding. However, this does not mean that the binding is simple ionic adsorption of ANS to the protein, since Mg^{2+} and Ca^{2+} of a concentration as low as 1 mM exhibited a remarkable effect. The dissociation constant of the order of 10 μM also suggests that the binding between ANS and alkylated actin is specific. In connection with the specificity of the binding site, the binding of ANS in the presence of ATP was examined. Addition of a small amount of ATP to the mixture of alkylated actin and ANS reduced the fluorescence intensity of ANS. Neither the fluorescence spectrum nor the wavelength giving maximum fluorescence was changed by ATP; that is, there is no direct interaction between ATP and ANS. The observed reduction of fluorescence intensity upon the addition of ATP therefore indicates a decrease in the number of bound ANS. As shown in Fig. 7, the number of the binding sites was not changed from unity. Therefore, it is reasonable to consider that ATP and ANS competed for binding to alkylated actin. A competitive binding of ATP and ANS to a single site on alkylated actin may be expressed by the relation

$$\frac{ANS_{max}}{ANS_{bound}} = \frac{K_{ANS}}{ANS_0}\left(1 + \frac{1}{K_{ATP}} ATP_0\right) + 1 \qquad (3)$$

where ANS_{max}/ANS_{bound} is the ratio of the amount of the maximum binding of ANS to the amount of ANS actually bound at a given concentration of the protein, which is proportional to F_0/F at a given condition. As shown in Figs 7 and 8 this relation was satisfied in the experiment. The dissociation constant of ATP, K_{ATP}, can be determined by plotting $(F_0/F) - 1$ against ATP_0 (the concentration of ATP added); that is, the intercept on the abscissa, where F_0/F equals unity, gives the relation $-ATP_0 = K_{ATP}$. Fig. 8 gave K_{ATP} equal to 12.4 μM. Since K_{ANS} in the same conditions was 42.8 μM, it was concluded that ATP bound to alkylated actin more strongly than ANS. It is to be noted that the dissociation constant of ATP with alkylated actin is close to that of ATP with natural F-actin[3,4].

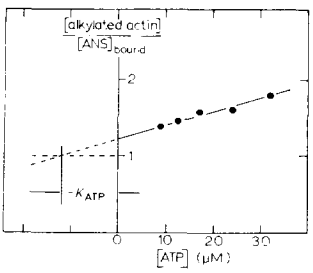


Fig. 8. Competitive binding of ATP and ANS to the single site on alkylated actin. Alkylated actin, 0.75 mg/ml; ANS, 61.5 μM; other conditions were the same as in Fig. 7. For details, see text.

From measurements of the binding constant of ANS at various temperatures, the enthalpy change upon the binding of ANS to alkylated actin ΔH_b was determined. The value of ΔH_b was about 12 kcal/mole; the binding was exothermic. Similarly, the free energy change and the entropy change both due to the binding were also determined and summarized in Table III.

TABLE III

THERMODYNAMIC PARAMETERS OF THE BINDING OF ANS WITH ALKYLATED ACTIN MONOMER

Tris–acetate buffer, 10 mM (pH 7.5). For comparison, corresponding parameters of the binding of ATP with G-actin which were determined by Asakura[4] were listed.

Thermodynamic parameters	*ANS ~ alkylated actin (kcal/mole)*	*ATP ~ G-actin[4] (kcal/mole)*
ΔH_b	−11.7	−24
ΔF_b*	− 7.1	−11
$T\Delta S_b$*	− 4.7	−12

* At 25.0 °C.

DISCUSSION

Actin spontaneously denatures when bound nucleotides are lost. The polypeptide chain of actin can not maintain its functional site by itself. In the absence of nucleotides the unfolded structure seems to be more stable than the native structure. Therefore, renaturation of unfolded actin without nucleotides is impossible.

Even in the presence of nucleotides renaturation has never been performed successfully. However, it was shown previously[16] that nucleotides are not absolutely necessary to form F-actin and its polypeptide chain is responsive to the binding between them. The present experiment proved that unfolded actin can recover its function after chemical modification.

In a high concentration of urea (5 M) the G-actin molecule is unfolded (except its central core[17]) and the α-helical structure is almost lost[18], according to the measurements of optical rotation and ultraviolet absorption spectra[5]. At this condition almost all sulfhydryl groups of actin are probably carboxymethylated by the reaction with iodoacetate[19], although chemical analyses were not made for confirmation. The modified actin is not exactly the same protein molecule as native actin. Actually it has properties different from the original actin. Polymerization of ordinary actin is, as is well known, induced by the addition of neutral salts[2]. Alkylated actin, however, is in the polymer state when dissolved in a salt-free solvent after acid precipitation. Since before the precipitation it was in the monomer state in the same solvent conditions, it is likely that some intramolecular conformational change took place during the treatment at acidic pH. This polymer formed by dissolution of the precipitate at neutral pH can bind with myosin, but has viscosity, sedimentation constant and flow birefringence lower than those of ordinary F-actin. Then, it was transformed into the other type of polymer, having higher viscosity, during prolonged dialysis at weakly acidic pH. This transformation also is considered to be associated with further intramolecular conformational change of alkylated actin. The most remarkable property of the last polymer is a very high ability to activate the ATPase of myosin or heavy meromyosin depending on the species of divalent cations. Its structure is very similar to ordinary F-actin according to the electron micrograph.

Thus, the most important functions of original actin were restored after unfolding and it was definitely concluded that those functions are wholly attributable to the polypeptide chain of actin.

So far as we have examined, actin without alkylation after urea denaturation was by no means renatured, as judged by polymerizability and the ability of activation of myosin ATPase. The alkylation was essential for renaturation.

Judging from the change in the emmission maximum of ANS upon the binding of alkylated actin (from 510 nm to 475 nm), ANS is apparently transferred to a hydrophobic region of the protein[13]. The bound ANS is likely surrounded by a group of hydrophobic side chains. This group of side chains may form a kind of cleft giving a remarkable receptivity to ligands of a hydrophobic nature. It was shown in the present study that the dissociation constant of alkylated actin with ATP was of the order of 10 μM, which was not so greatly different from that of ordinary actin[6] (which is of the order of 1 μM[3,4]). Hence, the possibility of the identity of the ATP-binding site of actin and that of alkylated actin is suggested. Although the present work did not give direct evidence for such a possibility, it seems natural to consider that the ATP-binding site of actin, first destroyed by the action of urea, was reorganized in the course of alkylation in urea and succeeding dialysis. The difference in the dissociation constant mentioned above may indicate incompleteness of the reorganization of the binding site, which is inevitable in the case of irreversible chemical modification. Relating to the possible identity of the ATP-binding site, we have examined the binding of ANS to G-actin. Addition of ANS to a G-actin solution at

neutral pH brought about distinct sensitization of the fluorescence of ANS, indicating the binding of ANS to actin; the blue shift in the emmission maximum was quite similar to that with alkylated actin. Increase in the amount of free ATP suppressed the binding. However, a gradual and spontaneous denaturation of actin occurred at a low concentration of free ATP. It was also observed that the addition of a large amount of ANS induced a gelation of actin. These occurrences made it difficult to analyse the system of native G-actin.

Finally, it is to be noted that analysis of the properties of alkylated actin is helpful for comparison among naturally occurring actin-like proteins. If natural modification of the primary structure of actin occurs, for instance by the process of mutation, the resultant actin might differ functionally or conformationally from actin of skeletal muscle. The variety and polymorphism of so-called actin-like proteins[20-22], or the observed similarity and difference among a family of actin and actin-like proteins, may be understood in terms of natural modification of the primary structure of actin.

REFERENCES

1 F. B. Straub, *Studies Inst. Med. Chem., Univ. Szeged*, 3 (1943) 23.
2 F. B. Straub and G. Feuer, *Biochim. Biophys. Acta*, 4 (1950) 455.
3 A. Martonosi, M. Gouvea and J. Gergely, *J. Biol. Chem.*, 235 (1960) 1700.
4 S. Asakura, *Arch. Biochem. Biophys.*, 92 (1961) 140.
5 S. Higashi and F. Oosawa, *J. Mol. Biol.*, 12 (1965) 843.
6 S. Ebashi, A. Kodama and F. Ebashi, *J. Biochem. Tokyo*, 64 (1968) 465.
7 K. Mihashi, *Arch. Biochem. Biophys.*, 107 (1964) 441.
8 G. Weber and L. B. Young, *J. Biol. Chem.*, 239 (1964) 1415.
9 A. Szent-Györgyi, *Chemistry of Muscular Contraction*, Academic Press, New York, 1947.
10 A. G. Szent-Györgyi, *Arch. Biochem. Biophys.*, 42 (1953) 131.
11 H. H. Tausky and E. Shorre, *J. Biol. Chem.*, 202 (1953) 675.
12 G. Weber and D. J. R. Laurence, *Biochem. J.*, 56 (1954) 319.
13 L. Stryer, *J. Mol. Biol.*, 13 (1965) 482.
14 E. Godfrey and W. W. Kielley, *Biochem. Biophys. Res. Commun.*, 12 (1963) 34.
15 M. K. Rees and M. Young, *J. Biol. Chem.*, 242 (1967) 4449.
16 M. Kasai, E. Nakano and F. Oosawa, *Biochim. Biophys. Acta*, 94 (1965) 494.
17 K. Mihashi and T. Ooi, *Biochemistry*, 4 (1965) 805.
18 B. Nagy and W. P. Jencks, *Biochemistry*, 1 (1962) 987.
19 M. E. Carsten, *Biochemistry*, 2 (1963) 32.
20 S. Hatano, T. Totsuka and F. Oosawa, *Biochim. Biophys. Acta*, 140 (1967) 109.
21 T. Totsuka and S. Hatano, *Biochim. Biophys. Acta*, 223 (1970) 189.
22 T. Miki-Noumura and H. Kondo, *Exp. Cell Res.*, 61 (1970) 31.