under the same conditions[2]. Therefore the turbidity, flow birefringence and ultraviolet absorption spectrum of F-actin–heavy meromyosin solutions were measured to reveal static aspects of the interaction between these proteins in the absence of ATP as a function of the molar ratio, keeping the concentration of F-actin constant. Variant behaviour in the flow birefringence and difference spectrum of F-actin–heavy meromyosin solutions was observed at a low degree of saturation of combining. After a careful study of these variations, we suggest that there are at least two types of states in the binding between F-actin and heavy meromyosin.

MATERIALS

Heavy meromyosin was prepared by the method of LOWEY AND COHEN[3]. The crude heavy meromyosin thus obtained was fractionated further by treatment with $(NH_4)_2SO_4$. The fraction that was 42 to 55% saturated with $(NH_4)_2SO_4$ was dissolved in 5 mM Tris–HCl buffer (pH 8.0), then extensively dialysed against the same solvent to remove $(NH_4)_2SO_4$. The solution was centrifuged at 50 000 $\times$ g for 2 h. 0.1 M sucrose was added to the heavy meromyosin solution. The heavy meromyosin in the sucrose solution was lyophilized. Before the experiments, the lyophilized heavy meromyosin was dissolved in 60 mM KCl solution containing 10 mM Tris-HCl buffer (pH 8.0) and dialysed against the same solvent to remove sucrose. The lyophilization of heavy meromyosin in sucrose did not affect its ATPase activity or its ability to bind to F-actin; therefore, lyophilized heavy meromyosin proved effective in our experiments.

Dry muscle was prepared by almost the same method as that of STRAUB[23], except that new proteins discovered recently by EBASHI and co-workers[4–6] were carefully removed before the acetone treatment of the myosin-extracted minced muscle by its incubation in distilled water for 3 h at room temperature. Purification of the actin solution extracted from dry muscle was performed as described previously[7]. At the final stage of purification, the F-actin pellet was dispersed in 60 mM KCl containing 10 mM Tris–HCl (pH 8.0). To completely disperse F-actin, this solution was stirred for a few hours at room temperature before heavy meromyosin was added.

To obtain nearly homogeneous solutions, 1-vol. portions of heavy meromyosin solutions of various concentrations were mixed slowly with 1-vol. portions of F-actin solutions having constant concentrations. The mixing was done in the absence of ATP, except where otherwise noted. The composite ratio of F-actin–heavy meromyosin solutions (heavy meromyosin to F-actin) was expressed as a molar ratio. The molecular weight of actin was taken as 57 000 (refs. 8,9) and that of heavy meromyosin, as 320 000 (ref. 3).

ATP was obtained from Sigma Chemical. Other chemicals used were of reagent grade and obtained from Katayama Chemical (Osaka, Japan).

METHODS

The protein concentration was determined by the biuret reaction standardized with Kjeldahl nitrogen determinations. The concentration of F-actin was determined by measuring the degree of flow birefringence of its solutions[10]. Flow birefringence and the extinction angle were determined by a Rao-type home-made apparatus. A Shimazu multipurpose spectrophotometer (Model MPS-50 L) was used to measure the

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PHYSICOCHEMICAL STUDIES OF F-ACTIN–HEAVY MEROMYOSIN SOLUTIONS

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SUMMARY

In the absence of ATP an F-actin–heavy meromyosin complex was prepared at room temperature and in a solution of 60 mM KCl containing 10 mM Tris–HCl buffer (pH 8.0). A constant concentration of F-actin was mixed with various concentrations of heavy meromyosin.

1. Centrifugation experiments revealed that one heavy meromyosin molecule can bind to one actin monomer in F-actin.

2. The relationship of the turbidity change of F-actin–heavy meromyosin solution to the ratio of components is linear until the binding between heavy meromyosin and F-actin becomes saturated. The turbidity experiment gave the same value of the stoichiometric binding ratio for heavy meromyosin and actin as given in (1).

3. The degree of flow birefringence of the F-actin–heavy meromyosin solution decreased as the concentration of heavy meromyosin in the F-actin–heavy meromyosin solution increased. A minimum point was reached at a molar ratio of 1/6, and then the flow birefringence increased again, reaching a plateau beyond the saturation point of binding where the degree of flow birefringence is about 50% larger than that of F-actin. This abnormal behaviour of the flow birefringence was not dependent upon either the protein concentration or the shear rate of flow. The ionic strength of the solvent also had little effect on this abnormality. The extinction angle also showed similar features but reached a maximum at a molar ratio of 1/6.

4. The degree of flow birefringence and the extinction angle of the F-actin–heavy meromyosin solution at every ratio studied, when dissociated by Mg^{2+}-ATP, took the value corresponding to the pure F-actin solution before mixing with heavy meromyosin.

5. The ultraviolet absorption of the F-actin–heavy meromyosin solution was hypochromically smaller than the sum of the ultraviolet absorptions of heavy meromyosin and F-actin only when the molar ratio lay between 0 and 1/2. This difference was also greatest at a molar ratio of 1/6.

6. From these findings it is suggested that there are at least two types of binding between heavy meromyosin and F-actin.

INTRODUCTION

Under physiological salt conditions actomyosin is insoluble in the absence of ATP[1]. But the complex between actin and heavy meromyosin is completely soluble

ultraviolet absorption of even turbid solutions. (In this model a photomultiplier, separated from the cell by frosted glass, received the forward-scattered light.) The ultraviolet absorption value of opaque F-actin–heavy meromyosin solutions at 280 mμ was just proportional to the protein concentration. In a reference box two cells were placed in series; one contained F-actin, and the other contained heavy meromyosin solution. In a sample box two cells containing F-actin–heavy meromyosin solution were also placed in series. In this way it is possible to detect the small difference in the absorption spectra of the two systems[11]. Turbidity of the F-actin–heavy meromyosin solution was measured by a Zeiss spectrophotometer (Model PMQ III) in a cell with an optical path length of 1 cm at a wavelength of 350 mμ where no absorption of the proteins is observed.

RESULTS

Binding of heavy meromyosin to F-actin

We wanted first to determine the number of actin monomers that can be bound by one heavy meromyosin molecule under the following conditions: 60 mM KCl, 10 mM Tris–HCl (pH 8.0) and room temp. (Fig. 1). For this purpose, F-actin–heavy meromyosin solutions of various concentrations at each ratio were prepared by the same method as described in the previous section. Three series of such solutions were prepared in which the concentrations of actin were 0.1, 0.3 and 0.5 mg/ml. A few hours later these F-actin–heavy meromyosin solutions were centrifuged at 80000 × *g* for 3 h in order to sediment the heavy proteins, *e.g.*, F-actin and heavy meromyosin bound to F-actin. The free heavy meromyosin and G-actin, if present, must be in the supernatant. Because of the contamination of denatured G-actin in actin solutions, the biuret reaction was even observed in the supernatant of the preparation without heavy meromyosin. The amount of denatured G-actin was determined to be about 38 % of the total actin concentration in the original solutions. Of course, the presence of denatured G-actin does not affect our experiments because the F-actin content in the sediment was separately determined. When the molar ratio was smaller than 1, the protein content in the supernatant remained equal to the total amount of denatured G-actin; that is, no trace of heavy meromyosin was left in the supernatants after centrifugation. When the molar ratio was larger than 1, the excess heavy meromyosin always existed in supernatants. Therefore, for the complete formation of one F-actin–heavy meromyosin complex, one molecule of heavy meromyosin binds to one monomer of actin in the F-actin filament.

Turbidity and flow birefringence of F-actin–heavy meromyosin solutions

The turbidity of F-actin–heavy meromyosin solutions prepared by the method mentioned above was measured at 350 mμ a few hours after preparation, as shown in Fig. 2. The turbidity increases linearly with the molar ratio until the ratio reaches 1. Thus the turbidity change reflects the process of binding of heavy meromyosin molecules to F-actin. Even when the mixing of heavy meromyosin and F-actin was done in the presence of a sufficient amount of ATP to make the solution more homogeneous, the same turbidity change was seen after ATP was hydrolysed by heavy meromyosin, and F-actin–heavy meromyosin complexes were re-formed.

The flow birefringence, however, shows an abnormal change. As the heavy

meromyosin concentration in the F-actin–heavy meromyosin solution increased, the degree of flow birefringence decreased at first and then reached a minimum point at a molar ratio of 1/6; it then increased again. The degree of flow birefringence ceased to increase beyond the saturation point of binding. The extinction angle showed features similar to the degree of flow birefringence; namely, it became larger at first, reaching a maximum at a molar ratio of 1/6, and then gradually decreased. The extinction angle also ceased to change at saturation of binding, as did the turbidity and flow birefringence. Such small values for the degree of flow birefringence and large values

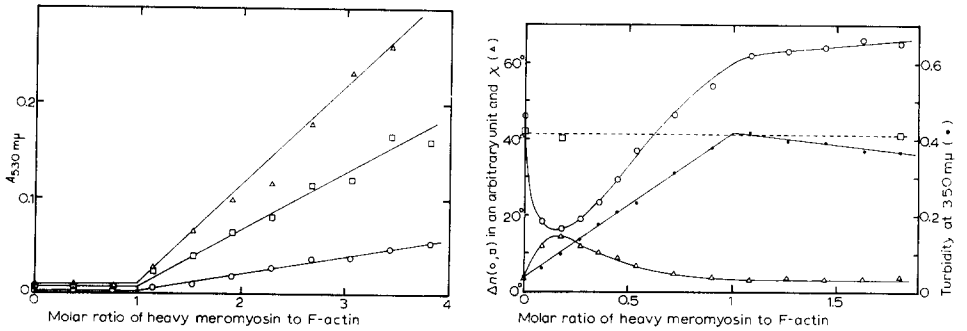


Fig. 1. Binding capacity of F-actin to heavy meromyosin. F-actin concentration: 0.36 mg/ml (△), 0.21 mg/ml (□), 0.07 mg/ml (○). Solvent conditions: 60 mM KCl, 10 mM Tris–HCl buffer (pH 8.0). The concentration of the protein left in the supernatant after spinning off F-actin and F-actin–heavy meromyosin complex was determined by the biuret reaction at 530 mμ.

Fig. 2. Degree of flow birefringence (○), (□) in 4 mM Mg^{2+}-ATP, extinction angle (△) and turbidity (●) of the F-actin–heavy meromyosin solution. F-actin concentration: 0.5 mg/ml. Solvent conditions: 60 mM KCl, 10 mM Tris–HCl buffer (pH 8.0). Shear rate of flow: $G = 260\ sec^{-1}$. At room temperature. The turbidity of the solution was measured at 350 mμ in a cell of 1-cm path length.

for the extinction angle at the intermediate degree of saturation might be due to the depolymerization or fragmentation of F-actin during the binding process of heavy meromyosin molecules to F-actin. But this is not the case because on the addition of Mg^{2+}-ATP to F-actin–heavy meromyosin solutions, the degree of flow birefringence and the extinction angle returned instantaneously to the values corresponding to pure F-actin before the mixing with heavy meromyosin. (Mg^{2+}-ATP is known to dissociate F-actin–heavy meromyosin complexes into F-actin and heavy meromyosin[12–15].) Electron microscopic pictures taken after the uranyl acetate staining of these solutions in the presence or absence of ATP also suggested that the size of F-actin filaments did not change appreciably. This abnormal flow birefringence behaviour of F-actin–heavy meromyosin solutions is determined only by the ratio and is not dependent upon the protein concentration, as shown in Fig. 3. The degree of flow birefringence of F-actin–heavy meromyosin solutions changed with the shear rate of flow (Fig. 3). With the increase of the shear rate, the degree of flow birefringence at each ratio increased and then tended to become saturated. However, the molar ratio at which the degree of flow birefringence of the F-actin–heavy meromyosin solution was minimal was not altered, being 1/6 at every shear rate of flow. Therefore, it is concluded that the abnormal behaviour of flow birefringence and the extinction angle of F-actin–heavy meromyosin solutions is due to the intrinsic structure of F-actin–heavy meromyosin filaments.

Salt concentration also had little effect on the abnormal nature of F-actin–heavy meromyosin solutions (Fig. 4). F-actin–heavy meromyosin solutions were prepared at a salt concentration of 0.06 M KCl, and the salt concentration was then raised to 0.3 or 0.6 M KCl. The higher the salt concentration, the smaller the degree of flow birefringence of F-actin–heavy meromyosin solutions. In high salt concentrations, F-actin–heavy meromyosin or F-actin may be broken into fragments. (The measurements were taken 12 h after the mixing.) The minimum point of the degree of flow birefringence was, however, the same at every salt concentration studied.

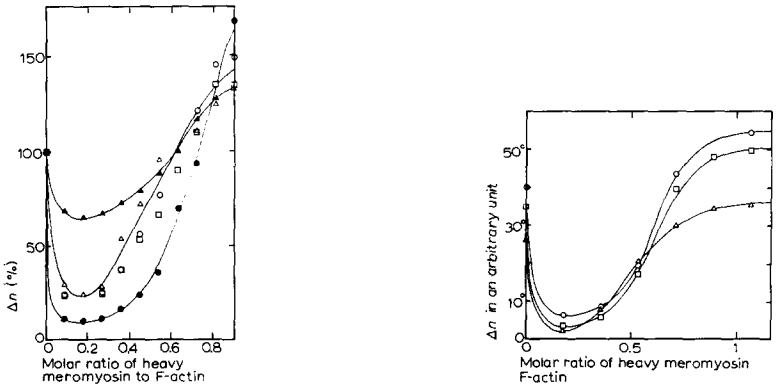


Fig. 3. Effect of total protein concentration and the shear rate of flow on the degree of flow birefringence of F-actin–heavy meromyosin solutions. 100% in the ordinate corresponds to the degree of flow birefringence of the solution of F-actin only. F-actin concentration: 0.5 mg/ml (□), 1.0 mg/ml (○, ●), 1.5 mg/ml (△, ▲). Solvent conditions: 60 mM KCl, 10 mM Tris–HCl buffer (pH 8.0). Shear rate of flow: $G = 17$ sec^{-1} (●), $G = 260$ sec^{-1} (□, ○, △), saturated degree of flow birefringence (▲), *e.g.*, at a molar ratio of 1/6, $G = 1700$ sec^{-1}. At room temperature.

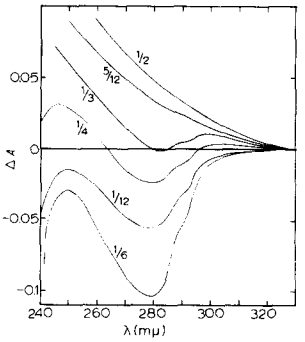
Fig. 4. Effect of salt concentration on the degree of flow birefringence of F-actin–heavy meromyosin solutions. F-actin concentration: 0.5 mg/ml. Salt concentration: 0.06 M KCl (○), 0.3 M KCl (□), 0.6 M KCl (△). 10 mM Tris–HCl buffer (pH 8.0). At room temperature. $G = 260$ sec^{-1}.

As was shown previously, one molecule of heavy meromyosin binds to one monomer of actin. This is true when the complete saturation of binding occurs. It is still uncertain how many monomers of actin in the F-actin filament can be bound by one molecule of heavy meromyosin before saturation occurs. The degree of flow birefringence reaches a minimum at such an intermediate degree of saturation of binding; namely at a molar ratio of 1/6. One pitch of a strand of one F-actin double helix consists of 13 monomers[16]. Therefore, when one molecule of heavy meromyosin binds to about half a pitch of one strand of the F-actin filament, the degree of flow birefringence of the F-actin–heavy meromyosin complex is minimum.

The ultraviolet absorption spectra of F-actin–heavy meromyosin solutions

The ultraviolet absorption spectra of F-actin–heavy meromyosin solutions were compared with the sum of ultraviolet absorptions of separate solution of heavy meromyosin and F-actin by the technique of differential spectrophotometry. The result is shown by difference spectra at different molar ratios (Fig. 5). A minimum of the trough in the difference spectrum always appeared at a wavelength of 280 mμ and a shoulder appeared at 291 mμ. The former may be due to tyrosyl residues, and the latter to tryptophanyl residues. Fig. 5 shows that the decrease of absorption at 280 mμ

is associated with the formation of the F-actin–heavy meromyosin complex. A Zeiss spectrophotometer gave nearly the same spectrum, which, however, lies on a steep base line due to scattering.



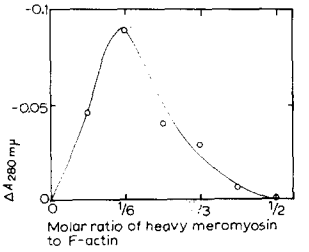


Fig. 5. Difference spectra of F-actin–heavy meromyosin solutions. The technique of difference spectrophotometry was applied. The cuvettes contained F-actin–heavy meromyosin solutions. Each reference cell contained heavy meromyosin and F-actin solutions. F-actin concentration: 0.5 mg/ml. The optical path length: 4 mm. Solvent condition: 60 mM KCl, 10 mM Tris–HCl buffer (pH 8.0). The number in the figure indicates the molar ratio.

Fig. 6. Difference in the optical absorption at 280 mμ as a function of the composite molar ratio. See the explanation in the text.

The turbidity of F-actin–heavy meromyosin solutions increases linearly with the composite ratio, as shown previously in Fig. 2. Therefore, the difference absorption spectrum always lies on a base line due to scattering. From Fig. 5, it is apparent that when the molar ratio increases to more than 1/6, the difference in absorption tends to decrease; at a molar ratio of 1/2 there remains only a base line, probably due to the difference in the scattering. By assuming that the difference spectrum at this molar ratio (1/2) represents the difference in the scattering only, the scattering at every ratio can be estimated. Thus the true difference in ultraviolet absorbance at 280 mμ was tentatively estimated at each ratio. The true difference at a wavelength of 280 mμ, defined in this way, was plotted as a function of the ratio (Fig. 6). The maximum of the difference was found at a molar ratio of 1/6, as in the case of the flow birefringence. The maximum value was usually 15% of the sum of absorptions of heavy meromyosin and F-actin, which is nearly of the same order of magnitude as in the case of the ultraviolet absorption spectrum change of actin associated with its polymerization[17].

The spectrum change associated with the formation of the complex indicates more clearly the structural change of the heavy meromyosin and/or F-actin due to binding. This structural change is greatest when one heavy meromyosin molecule binds to about half a pitch of one strand of the F-actin filament.

DISCUSSIONS

From the centrifugation and turbidity experiments of F-actin–heavy meromyosin solutions, it was found that at saturation, one heavy meromyosin molecule can bind to one actin monomer in F-actin. We obtained the same value for the stoichiometric binding ratio of heavy meromyosin and actin polymerized in $MgCl_2$

instead of KCl. Our results concerning the stoichiometric binding ratio is in accord with that reported recently by YOUNG, who used sedimentation analysis[18].

The most remarkable findings in our experiments are the abnormal behaviour of the flow birefringence and the difference spectrum of ultraviolet absorption of F-actin–heavy meromyosin solutions. The degree of flow birefringence of F-actin–heavy meromyosin solutions reaches a minimum at the composite molar ratio of 1/6; the extinction angle reaches a maximum at the same molar ratio. This phenomenon is not explained by the fragmentation of F-actin bound with heavy meromyosin nor by the interaction between filaments, but is the manifestation of the intrinsic structure of F-actin–heavy meromyosin complex, as proved by a few experiments described above. Flow birefringence is indicative of size and shape of macromolecules in solution[19], and difference spectrum is indicative of their conformational changes[11,17]. The large hypochromic effect due to the binding of heavy meromyosin to F-actin disappeared when the binding of heavy meromyosin to F-actin was half saturated. Since the more saturated the binding of heavy meromyosin to F-actin the more decreased the change in ultraviolet absorption, this fact is accounted for by the assumption that both two subunits of one heavy meromyosin molecule[20] can bind to two neighbouring actin monomers in F-actin. If so, every actin monomer in F-actin is bound by heavy meromyosin molecules even when the stoichiometric molar ratio is 1/2. Therefore, no change in ultraviolet absorption is observed at this ratio. (A more quantitative theoretical explanation of this experimental fact will be published elsewhere by OOSAWA[21].) The conformation of aromatic groups (tyrosyl and tryptophyl) in the F-actin–heavy meromyosin complex at a molar ratio of 1/6 is very different from that in free heavy meromyosin and free F-actin. (Detection of the change in ultraviolet absorption of F-actin and heavy meromyosin after their association has received some attention, but has hitherto been unsuccessful[17,22]. A possible explanation may be that the hypochromic effect due to the binding of to F-actin can only be observed at a very low degree of saturation.)

Thus it is suggested that there may be two types of binding states of the F-actin–heavy meromyosin complex; one type is predominant at a molar ratio of 1/6, and the other becomes predominant at the saturation of binding. At molar ratio of 1/6, one molecule of heavy meromyosin binds to about half a pitch of one strand of the F-actin double helix. In this state, the conformation of the F-actin–heavy meromyosin complex is greatly different from that of the saturated complex. For example, the difference in the geometrical relationship of the bound heavy meromyosin to the F-actin filament* (as was indicated by the low value of the degree of flow birefringence) and/or the distortion of the filament due to the binding (as was shown by the hypochromic effect in the ultraviolet absorption) can be supposed. The transition of the binding state from one type to the other, supposedly regulated by ATP, may be involved in the local mechanisms of muscle contraction.

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* Preliminary electron microscopic observations of F-actin–heavy meromyosin complex substantiate this possibility.

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