The Dissociation and Reassociation of the Subunit Polypeptide Chains of Myosin*

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Recent studies on the behavior of myosin in solutions of guanidine·HCl (1) and urea (2) have shown this protein to consist of three subunit chains of apparently identical mass and electrophoretic mobility. Moreover, results obtained from paper chromatographic and electrophoretic separation of the tryptic peptides of myosin suggest that the three chains are closely similar, if not identical, in their primary structures (3).

The present study is directed toward an understanding of the molecular dissociation observed in concentrated aqueous guanidine·HCl solutions as assessed by optical rotation, viscosity, sedimentation, and adenosine triphosphatase activity. The reverse of this dissociation-unfolding process, i.e. removal of guanidine·HCl from guanidine·HCl-myosin solutions has also been examined with a view to understanding the requirements for reformation of the native secondary and tertiary structures of complex, multistranded protein molecules.

EXPERIMENTAL PROCEDURE

Reagents—Rabbit skeletal myosin A was prepared as previously described (4), and was stored as a 0.5 M KCl solution at 4° until used. No preparation older than 1 week was employed in these studies.

Reagent grade guanidine·HCl was purchased from Eastman Organic Chemicals and Matheson-Coleman-Bell. It was twice recrystallized from methanol, dried overnight at 40°, and stored in a desiccator over P2O5. Concentrations of this material in aqueous solution were determined by index of refraction, employing the data of Kielley and Harrington (1).

β-Mercaptoethanol was the Eastman Organic Chemicals product and was used without further purification.

Analytical Methods—Optical rotatory studies were performed with the Rudolph model B photoelectric spectropolarimeter employing an oscillating polarizer prism and the xenon, mercury, and sodium light sources supplied with the instrument. A 1-decimeter, jacketed polarimeter tube was used for all measurements and temperature was controlled (±0.01°) by circulating water from an external water bath. Specific rotations were normalized to equivalent values in water by employing the refractive indices of the solvents at the sodium D line, assuming a negligible correction for dispersion (1, 5).

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Velocity sedimentation studies were made with the Spinco model E ultracentrifuge operating at 50,780 r.p.m. Temperature control was maintained to within 0.05° by means of the RTIC unit supplied with the instrument. A standard single-sector Kel-F centerpiece was employed for all measurements. Photographic plates were analyzed by means of a two-dimensional Bausch and Lomb micro comparator equipped with a specially constructed stage for vertical and horizontal alignment of the plate and calculated sedimentation coefficients were corrected to a standard state of water at 20°. The partial specific volumes of myosin in 0.5 M KCl and in 5 M guanidine·HCl were taken to be 0.728 ml per g (6) and 0.720 ml per g, respectively (1).

A 2-ml Ostwald-type viscometer was employed for viscosity measurements at 25.0 ± 0.04°. The water outflow time at this temperature was 70 seconds. Viscosities were calculated relative to the solvent neglecting the kinematic corrections.

Ultraviolet absorption spectra were obtained by means of a Cary model 14 recording spectrophotometer.

Protein concentration was estimated with the Beckman model DU spectrophotometer. The extinction coefficients of myosin in 0.5 M KCl and in 5 M guanidine·HCl were taken to be 5.60 × 10² cm² per g (λ = 279 mp) (2) and 5.07 × 10² cm² per g (λ = 276 mp) (1), respectively. No correction was made for light scattering in either solvent.

ATPase activity was measured in the presence of 0.001 M ATP, 0.02 M histidine (pH 7.0), and either 0.005 M CaCl₂ or 0.001 M EDTA (4). Inorganic phosphate was estimated by the Fiske-SubbaRow procedure.

The sulfhydryl groups of myosin were determined spectrophotometrically by titration with p-mercuribenzoate (7).

RESULTS

Dissociation Process—When solutions of native myosin are transferred into progressively increasing concentrations of guanidine·HCl, the specific levorotation increases abruptly (in less than 1 minute) to a terminal level which is characteristic for each guanidine·HCl concentration (Fig. 1). This behavior is in contrast to that observed in aqueous urea solutions in which time-dependent optical rotatory changes are seen at concentrations of urea as high as 2 M (2).

Paralleling this abrupt increase in levorotation, the reduced viscosity of the system drops immediately to a markedly lower value, then gradually increases over a period of several hours to a final, invariant level. The viscosity changes are summarized in Fig. 2, in which it will be noted that up to a concentration of 3
Guanidinium Chloride (Moles/Liter)

FIG. 1. Specific rotation of myosin as a function of guanidine-HCl concentration at 546 μc and 25°C. O—O, as a function of increasing guanidine-HCl concentration; △—△, progressive dilution of a 5 M guanidine-HCl-myosin solution with 0.5 M KCl. △ at 0 M obtained by dialysis to remove guanidine-HCl, followed by concentration of the protein as described in the text.

M guanidine-HCl, the magnitude of the initial viscosity drop increases with increasing concentration of guanidine-HCl, whereas both the rate and extent of the time-dependent viscosity changes are gradually diminished over this range. At higher levels of guanidine-HCl concentration, two features of the viscosity behavior are notable: (a) an inversion occurs in the magnitude of the primary, time-independent phase of the reaction; and (b) the reduced viscosity becomes virtually constant with respect to time.

These phenomena are best appreciated from a study of Fig. 3, which depicts the initial and final values of the intrinsic viscosity, [η]₀ and [η]₉, respectively, as a function of guanidine-HCl concentration. Values of [η]₀ were obtained at each guanidine-HCl concentration by extrapolation of a series of reduced viscosity-versus-time curves to zero time and these values were in turn extrapolated to zero protein concentration. Similarly, [η]₉ at each guanidine-HCl concentration was obtained when the reduced viscosity became constant with respect to time. In the range 1 to 6 mg per ml of protein, the reduced viscosity in all instances was a linear function of protein concentration.

When the viscosity had reached its final value for each of the several guanidine-HCl and protein concentrations, the solutions were examined in the ultracentrifuge. In every instance, a single sedimenting boundary was observed, although at the lower guanidine-HCl concentrations, boundary spreading suggested that these systems were somewhat polydisperse. The reciprocals of the sedimentation coefficients, 1/εₛₒ, were at each guanidine-HCl concentration by extrapolation of a series of reduced viscosity-versus-time curves to zero time and these values were in turn extrapolated to zero protein concentration. Similarly, [η]₉ at each guanidine-HCl concentration was obtained when the reduced viscosity became constant with respect to time. In the range 1 to 6 mg per ml of protein, the reduced viscosity in all instances was a linear function of protein concentration.

Reversal Studies—It was of interest to examine the possibility of reversing the complex unfolding-dissociation reactions described above. Accordingly, myosin in 5 M guanidine-HCl was diluted stepwise by addition of 0.5 M KCl, and the specific rotation for each dilution was determined within 1 minute. No time-

Reduced viscosity (100 ml per g) of myosin as a function of time after addition of guanidine-HCl to final concentrations indicated for each curve. Protein concentration, 2 mg per ml.

Values of η_red, at 360 minutes represent the maximal increase in viscosity observed.

Fig. 2. Reduced viscosity (100 ml per g) of myosin as a function of guanidine-HCl concentration. O—O, intrinsic viscosity at zero time, [η]₀; △—△, intrinsic viscosity obtained after time-dependent viscosity changes were complete, [η]₉ (see text).
GUANIDINIUM CHLORIDE (MOLES/LITER)

![Graph](image)

**FIG. 4.** Sedimentation coefficient of myosin at infinite dilution as a function of guanidine-HCl concentration.

**Table I**

Comparison of some physical properties of native and guanidine-HCl-myosin in 0.5 M KCl and myosin subunit in 5 M guanidine-HCl

<table>
<thead>
<tr>
<th>Preparation</th>
<th>[a]_20^°</th>
<th>λ_0</th>
<th>λ_∞</th>
<th>S</th>
<th>[α]_1%</th>
<th>SH</th>
</tr>
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<tr>
<td>Native myosin</td>
<td>-27.0°</td>
<td>290</td>
<td>279</td>
<td>6.2-6.4</td>
<td>2.3</td>
<td>0.074</td>
</tr>
<tr>
<td>Guanidine-HCl-myosin</td>
<td>-28.0°</td>
<td>285</td>
<td>279</td>
<td>6.2'</td>
<td>2.4</td>
<td>0.040</td>
</tr>
<tr>
<td>Subunit</td>
<td>-90.0°</td>
<td>218</td>
<td>276</td>
<td>4.2</td>
<td>0.93</td>
<td></td>
</tr>
</tbody>
</table>

* Specific rotation at 589 mm, 20°.
* Rotatory dispersion Drude parameter.
* Wave length of maximal extinction.
* Intrinsic viscosity determined from plot of reduced viscosity versus concentration.
* Sulphhydryl content per mg of myosin determined by p-mercuribenzoate titration.
* Sedimentation coefficient of slowest sedimenting component of guanidine-HCl-myosin.

Results of this study are summarized in Fig. 1, which shows that the optical rotatory changes produced by guanidine-HCl are completely reversible. At protein concentrations above 4 mg per ml and guanidine-HCl concentrations below 1 M, either precipitation, gelation, or both processes, of the protein occurred. However, when myosin at concentrations of 0.1 to 1.0 mg per ml in 5 M guanidine-HCl was dialyzed exhaustively at 4° against 0.5 M KCl, 0.05 M Tris buffer, pH 7.6 to 9.5, to remove guanidine-HCl, there was no evidence of precipitation and the resulting specific rotation was essentially identical with that of native myosin (Fig. 1). Moreover, the value of the Drude parameter, λ_0, obtained from a plot of λ [α] versus [α] in the range 320 to 600 μg was found to be essentially identical with that of native myosin (Table I).

The resulting protein was precipitated either by addition of ammonium sulfate (to 50% saturation at 4°) or by dialysis against 0.1 M KCl. Qualitatively, its solubility behavior with respect to ionic strength was identical with that of native myosin.

Sedimentation patterns of this material, hereafter referred to as guanidine-HCl-myosin, are shown in Figs. 5A and B, indicating an initially hypersharp sedimenting boundary, which on further centrifugation splits into at least three components. The slowest of these components sediments with s_{20,w} = 6.17 S, closely approximating the sedimentation coefficient of native myosin (6.2 to 6.4) (8, 9). However, the concentration of this slower component is artificially enhanced in Fig. 5B as a result of the Johnston-Ogston effect (10). On dilution of such guanidine-HCl-myosin solutions with 0.5 M KCl, the slower peak was found by area analysis to comprise between 10 and 20% of the total protein. The similarity in the sedimentation behavior of this component to that of native myosin may be judged from an experiment summarized in Fig. 6. Here it will be seen that addition of a small amount of native myosin to a solution of guanidine-HCl-myosin results in a striking enhancement in the most slowly sedimenting boundary, together with a decrease in area of the more quickly sedimenting species. Such behavior is to be expected on the basis of the Johnston-Ogston effect (11).

Although a significant fraction of guanidine-HCl-myosin approached the sedimentation behavior of native myosin, no measurable calcium or EDTA-activated ATPase activity was detected after removal of guanidine-HCl by dialysis. Several attempts were made to reduce the polydispersity of this preparation such that some enzymatic activity might be regenerated. In view of the known protective effect of substrate against denaturation of ribonuclease (12), 0.01 M ATP was included in several of the regeneration experiments in the hope that this nucleotide would facilitate specific reordering of the secondary and tertiary structure of myosin. In addition, the effect of 0.01 M Ca, 0.01 M Mg, and 0.01 M EDTA, and pH variation (from 7.0 to 9.5) was investigated. However, no measurable effect on enzymatic activity or on the sedimentation pattern of Fig. 5B was detected.

Present evidence indicates that myosin contains approximately 45 sulphydryl groups per mole (3). Therefore, the possibility of intra- or intermolecular disulfide bond formation during the refolding process was also considered. In earlier studies, the —SH groups of myosin were blocked by reaction with N-ethylmaleimide before dissociation of the three subunit polypeptide chains to prevent —S—S-bridge formation (1). However, in the present investigation it was of interest to follow the refolding process, and therefore no blocking agent was used. Under these conditions, it was observed that slow aggregation of myosin in 5 M guanidine HCl occurred over a period of 24 hours at room temperature.

1 Sulfhydryl group titration of this material indicated a loss of 90% of the titratable —SH groups of native myosin, and ultracentrifugal analysis showed the presence of high molecular weight aggregates. On the other hand, if the subunit chains are formed from the parent molecule and dialysis to remove guanidine- HCl is begun immediately, refolding occurs, and in this instance 55% of the —SH groups are now titratable, regardless of the presence or absence of β-mercaptoethanol (0.2 M) during removal of guanidine- HCl.

The possibility exists that failure to recover the full complement of —SH groups of native myosin is caused by masking of 1 The development of aggregates through the mechanism of disulfide bridge formation occurs to only a minor extent under the conditions and time intervals summarized in Fig. 2.
Fig. 5. Sedimentation patterns of guanidine-HCl-myosin under various conditions. All runs at 50,780 r.p.m. A, guanidine-HCl-myosin, 6.0 mg per ml, in 0.5 M KCl. Bar angle of 80°, 32 minutes, temperature at 5.4°; B, same solution as in A after 144 minutes, bar angle of 55°; C, guanidine-HCl-myosin, 4 mg per ml in 5 M guanidine-HCl, 352 minutes, bar angle of 80°, temperature at 20.9°; D, guanidine-HCl-myosin, 4 mg per ml in 5 M guanidine-HCl, 0.01 M β-mercaptoethanol, 448 minutes, bar angle of 60°, temperature at 20.0°.

these groups during refolding. The following experiment indicates, however, that oxidation to the disulfide has occurred during the time required to remove guanidine-HCl by dialysis: guanidine-HCl-myosin was made 5 M with respect to guanidine-HCl, whereupon two relatively sharp boundaries were observed in the ultracentrifuge (Fig. 5C). The slower boundary possessed the sedimentation properties of the myosin subunit. The faster boundary, however, appeared to be an aggregate formed through disulfide bond formation. When β-mercaptoethanol (0.01 M) was added to this solution, the more rapidly sedimenting boundary disappeared together with a concomitant increase in area of the myosin subunit peak. These experiments indicate that the more rapidly sedimenting component is transformed into the more slowly sedimenting component as a result of reduction of intermolecular disulfide linkages. When dialysis to remove guanidine-HCl was performed in the presence of a continuous stream of nitrogen bubbling through the dialysate, the relative concentration of the more slowly sedimenting component of Fig. 5B was appreciably increased.

Table 1 presents a comparison of some of the physical parameters of native myosin and guanidine-HCl-myosin in 0.5 M KCl and the myosin subunit in 5 M guanidine-HCl.

DISCUSSION

In solutions of guanidine-HCl, the myosin molecule undergoes a complex set of reactions involving both unfolding and dissociation of the constituent polypeptide chains. These processes are further complicated in the intermediate range of guanidine-HCl.
concentrations by a marked tendency for particle-particle interactions leading to the formation of high molecular weight aggregates as judged by their sedimentation behavior. The sequence of these events may be appreciated by considering, in parallel, the optical rotatory, viscosity, and sedimentation properties which have been described above.

The initial event would appear to be an unfolding reaction since there is an immediate elevation in the optical levorotation which senses, primarily, the configurational pattern of the polypeptide chain backbone. In view of the probable occurrence of the a-helical pattern in myosin (13), it seems likely that the unfolding reaction involves the specific destruction of elements of this hydrogen-bonded peptide chain configuration. This transition leads to a partial collapse of the molecule, which is reflected in an abrupt depression in the reduced viscosity of the system.

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We turn now to a consideration of the reversal of these reactions, i.e. the reformation of a myosin-like complex through association of the subunit chains. The reassociation of protein subunits to give a specific and unique quaternary structure has received considerable attention during the past few years. We have evidence from several sources that protein subunits, which have been dissociated from the native protein complex, can be recombined under appropriate conditions into a structure with similar physical, chemical, and biological properties to that of the parent molecule. In the case of globular proteins, the work on various normal and abnormal hemoglobins is a well known example. The hemoglobin molecule undergoes a reversible dissociation in acid solution yielding dimers of the a and b chains (15). The dimers may be recombined on raising the pH and the work of Itano and Singer (16, 17) has shown that it is possible to construct hybrid hemoglobin molecules through mixing of the a and b dimers of aberrant hemoglobins.

A similar type of phenomenon has been reported for the subunit protein of tobacco mosaic virus. In mildly alkaline solution, pH 10 to 10.5, or in the presence of concentrated acetic acid, dissociation of the tobacco mosaic virus rod can be controlled to give native protein subunits with a molecular weight of 17,800 (18, 19). On adjustment of the pH of such solutions freed of RNA, to values between pH 3 and 6, the subunits aggregate spontaneously into rodlike particles with indefinite lengths. X-ray diffraction and electron microscopy studies on the repolymerized tobacco mosaic virus protein show that the protein subunits are arranged in a helix of the same pitch and with the same number of subunits per turn as the intact virus (20, 21). Moreover, the configuration of the polypeptide chains of the subunits appears to be unchanged. It is difficult to escape the conclusion that the primary structure of the protein subunit alone contains the information required to construct the highly specific quaternary structure of the tobacco mosaic virus rod.

The specific reassociation of protein subunits in fibrous protein systems has received attention only in the case of collagen.
Present evidence favors the view that collagen is a three-stranded structure in which each polypeptide chain is coiled into a helix with a 3-fold left-handed screw axis, the three helices coiling gradually about one another to form a three-stranded rope with an over-all right-handed twist (22, 23). Soluble collagen on heating undergoes a striking transformation over a narrow temperature range which involves the "melting" of the rigid macromolecule to yield single chain, essentially randomly coiled molecules of gelatin. On cooling such solutions many of the properties of native collagen return. X-ray diffraction, optical rotatory, and infrared studies on both hot- and cold-cast gelatin gels indicate that the configuration of the molecules in the cold-cast state resembles that found in native collagen, while the hot-cast specimens show essentially complete loss of ordered structure (24-26). Moreover, recent electron microscope examination of solutions of calf skin collagen before and after heating indicate that if proper cooling schedules are employed, the reappearance of rod-shaped particles resembling native "tropocollagen" can be demonstrated (27, 28). It should be emphasized that in contrast to the studies on the reassociation of the subunits of globular proteins, the polypeptide chains of collagen appear to be completely unfolded during the heating step, and the reversal involves both refolding of the backbone into the specific left-handed poly-L-proline II-type helix as well as the specific association of individual chains. The reassociation of the separated subunit chains of collagen at low temperatures gives a well organized complex than that of the native collagen macromolecule (29). A part of this decrease in order may be related to the recently observed difference in the composition of the individual polypeptide chains (30), since the specific packing of such chains would be expected to require a higher level of discrimination than would be the case with identical subunits. This latter consideration is of importance in re-naturation studies on myosin since we have been unable to detect any physical or chemical difference between the individual chains of the myosin complex.

It is evident from the optical rotatory evidence presented above that removal of the denaturing agent allows the dissociated chains of myosin to refold into a molecular configuration closely similar to that found in the native molecule. Moreover, this occurs within a minute after alteration of the solvent environment, in contrast to the reformation of the collagen fold which requires a period of several hours at low temperature. The sedimentation studies favor the view that a significant fraction of the renatured complex has closely similar hydrodynamic properties to those of the native macromolecule and suggest that the specific reformation of structure may be largely a matter of finding the appropriate environmental conditions for the reversion process. For example, Stracher (31) has shown that at myosin concentrations considerably higher than those employed in the present study, removal of urea from urea-myosin solutions does not result in reversibility of either the viscosity or optical rotation.

Recent investigations of ribonuclease suggest that the "information" determining the secondary and tertiary interactions of the native protein resides in the primary amino acid sequence (32). Should this mechanism for synthesis of the native molecular configuration from the extended polypeptide chain apply to multistranded proteins such as myosin, the information required by the cell for protein synthesis would necessarily be greatly reduced. Such an hypothesis suggests that if the sulphydryl groups of myosin could be rendered unreactive toward oxidation and if guanidine-HCl removal could be carried out in sufficiently dilute solution to minimize unfavorable interactions such as aggregation of the chains, reformation of the native configuration, through cooperative hydrogen bonding, hydrophobic bonding, and electrostatic interactions, might be accomplished. Experiments are now in progress to examine these possibilities.

SUMMARY

The dissociation of myosin into its constituent subunit polypeptide chains by guanidine-HCl has been studied by optical rotation, sedimentation, and viscosity.

Myosin in guanidine-HCl undergoes an initial rapid, time-independent transformation characterized by a fall in intrinsic viscosity and an increase in levorotation, indicating progressive destruction of the α-helical structure and collapse of the molecular backbone. These changes are followed by time-dependent aggregation of the partially collapsed molecules, a process which is inhibited by high concentrations of guanidine-HCl. At guanidine-HCl concentrations between 3 and 4 M, myosin behaves as a collapsed structure from which the individual subunit chains have not dissociated. Finally, between 4 and 5 M guanidine-HCl, the parent molecule dissociates to yield the subunit chains.

The reversal of these transformations has also been studied. The optical rotatory and spectral changes are completely reversible and a significant fraction of the molecules after guanidine-HCl removal possess sedimentation properties closely similar to those of native myosin. Although enzymatic activity and the full complement of sulphydryl groups are not recovered, these results suggest the possibility that, given proper environmental conditions, the recombination of subunits of multistranded proteins might be accomplished in vitro.

REFERENCES

The Dissociation and Reassociation of the Subunit Polypeptide Chains of Myosin
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