Studies on the Proteins of Fish Skeletal Muscle

7. DENATURATION AND AGGREGATION OF COD MYOSIN*

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Earlier papers in this series (Connell, 1958*a, b*) showed that, compared with rabbit myosin, in the ease with which it aggregates cod myosin appears to be markedly unstable. Since myosin is the principal myofibrillar protein it seems likely that this instability is related to the general experience that the structural organization of cod muscle is much more easily damaged by, for example, dehydration or freezing than is mammalian muscle. Freezing and storing myosin or actomyosin in the frozen state produce complex aggregation and disaggregation reactions (Nord, 1936; Snow, 1950; Connell, 1959) that are little understood.

It was therefore decided to examine in detail the processes of denaturation and aggregation in solutions of myosin and to study the effects on these processes of varying conditions such as protein concentration, ionic strength and pH which are likely to alter markedly during freezing and drying.

An attempt has been made to measure both the intramolecular changes and the intermolecular interactions which occur during the storage of unfrozen solutions of cod myosin at a temperature, in the main, of 0°. While this work was nearing its completion Lowey & Holtzer (1959) published the results of an investigation of the aggregation of rabbit myosin which, though differing in scope from the present work, complements it in some particulars.

EXPERIMENTAL

Preparation of cod myosin

Some of this work has been carried out on cod myosin prepared by dissociating cod actomyosin and separating the liberated myosin by means of preparative ultracentrifuging (Connell, 1958*b*). The maximum concentration of pure myosin which can be prepared by this method is about 0.5%, though concentrations up to 0.8% can be prepared at the sacrifice of some contamination with actomyosin. As it was desirable to investigate more concentrated solutions than this, another method of preparing cod myosin has been mostly used. In this, the coarsely minced skeletal muscle of cod (*Gadus callarias* L.) is washed three or four times in the cold with phosphate buffer, pH 7.5, 1.0-0.05 (Connell, 1958*b*) to remove the sarcoplasmic proteins, and the residue is then extracted at 0° by slowly stirring for

1 hr. with 3-4 vol. of 0.45M-KCl-3.38 mM-KH₂PO₄-15.5 mM-Na₂HPO₄-10 mM-tetrasodium pyrophosphate-5 mM-MgCl₂, pH 7.5. Such extracts contain 1.5-1.8% of protein and generally show a single boundary in the ultracentrifuge (Fig. 1*a*) sedimenting with a velocity characteristic of myosin. Occasionally a much smaller and faster component is observed in the ultracentrifuge, presumably actomyosin or actin. Presumably such extracts also contain the 'extra-protein' fraction of muscle (Szent-Györgyi, Mazia & Szent-Györgyi, 1955). The amount of myosin in the extracts, judged by the area under the ultracentrifuge peak, is about 75-80% of the total protein content. The electrophoresis diagram of such a preparation showed a single, sharp main peak and a smaller (5-10% of the total area) faster peak. The values for some of the properties of the preparation, e.g. optical rotation, intrinsic viscosity, differ slightly from those of pure cod myosin (Connell, 1958*b*), providing an additional indication of the presence of impurities. However, no attempts were made to purify further this type of preparation since for the present investigation it was considered to be sufficiently pure. The usual dilution method of purifying myosin cannot be used with cod (Connell, 1958*a, b*); as will be shown below, fractionation with ammonium sulphate is impracticable because salting out renders the protein insoluble.

All the protein preparations have been made from muscle (sometimes frozen) which was excised before the onset of *rigor mortis*. The myosin concentrations referred to in this paper are calculated from the total protein nitrogen of the solution.

Methods

The methods used for measuring diffusion, sedimentation, optical rotation and viscosity have been described previously (Connell, 1958*b*), with the exception that in most of the sedimentation runs a phase plate was used in place of the diagonal wire or bar. All sedimentation runs were at between 0° and 5°, and viscosity and diffusion were measured at 0.5°.

The areas under the peaks of sedimentation pictures have been measured, after subtraction of appropriate base lines, either directly from the photographic plate with a two-way travelling microscope and trapezoidal integration, or, when comparison of areas in arbitrary units only was required, from photographic enlargements of the image followed by planimetry or by cutting out and weighing.

Adenosine triphosphatase activity was determined at 25° essentially by Perry's (1955) method. With the present myosin preparations the amount of hydrolysis of adenosine triphosphate (ATP; Sigma Chemical Co.) was linear with time up to 10 min. incubation and therefore usually only the amount of hydrolysis at 10 min. was measured.

* Part 6: Connell & Howgate (1959).

Digestion of myosin by trypsin (L. Light and Co. Ltd.) was followed in 0.45M-KCl-3.38 mM-KH₂PO₄-15.5 mM-Na₂HPO₄, pH 7.5, by measuring the liberation of ninhydrin-reacting compounds, Yemm & Cocking's (1955) reagent being used after precipitation of protein with trichloroacetic acid.

Sulphydryl groups were determined at 0° by titration with mm-*N*-ethylmaleimide or mm-*p*-chloromercuribenzoate, the nitroprusside pink colour being used as indicator.

Ultraviolet-absorption spectra were measured in a Unicam SP. 500 spectrophotometer with matched 1 cm. cells.

RESULTS

General course of cod-myosin aggregation

The process of aggregation of rabbit myosin has been well described by Lowey & Holtzer (1959) and the stepwise side-by-side nature of the reaction demonstrated mainly by the light-scattering method. Fig. 1 shows the changes in the sedimentation picture which occur when cod myosin is stored at 0°; similar changes occur within the temperature range 0–23°. In dilute solutions of greatly aggregated myosin sometimes as many as five discrete peaks are visible. That the changes are a reflexion of a real increase in molecular weight (*M*) and not, for example, an unlikely but possible stepwise alteration of shape at constant *M* has been shown in the present case by measurements of the diffusion coefficient, *D* (Fig. 2) and of weight-average *M* (Fig. 3). The latter values were obtained by the Archibald procedure (Connell, 1958*b*). For aggregated samples, *M* falls progressively during the run owing to the removal of heavier components from the meniscus, and in order to find *M* of the

uncentrifuged solutions the values obtained during any particular run were extrapolated to zero time. The decrease in *D* [as reflected in progressively smaller values of $(1/t) \cdot (1/h^2)$] coupled with the increase in *S* during storage can only mean that *M* is increasing, a conclusion substantiated by the results of the Archibald experiments. The form of the curve shown in Fig. 3 is different from that of the curves of increase of weight-average molecular weight as measured by light-scattering [Lowey & Holtzer's (1959), figs. 5, 6], which show that the rate of increase falls off gradually. This disparity might be accounted for by the very rapid removal of large aggregates from the meniscus in the Archibald experiments, which would tend to make the results lower than they should be, especially in the earlier stages of aggregation.

Fig. 2. Diffusion of a cod-myosin solution at 0.5°, pH 7.5, *I* 0.5. Protein concentration, 0.87%. *h*, Maximum ordinate (in arbitrary units) of the diffusing peak.

Fig. 1. Ultracentrifuge diagrams of a cod-myosin solution stored at 0°, pH 7.5, *I* 0.5 for (a) 0, (b) 1, (c) 2, (d) 3, (e) 6 and (f) 10 days. Sedimentation from right to left. (a) 87 min. and (b)–(f) 69 min. after reaching full speed (56 100 rev./min.). Temperature of runs, 3.3°. Protein concentration, 0.43%.

Fig. 3. Increase of weight-average molecular weight (*M*) during storage of cod myosin at 0°, pH 7.5, *I* 0.5, as measured by the Archibald procedure. Protein concentration, 0.79%.

After the stage when the monomeric myosin peak has disappeared from the ultracentrifuge diagram, the aggregates continue to grow in size as indicated by a progressive increase in average S . The total area under the peaks of the ultracentrifuge diagram does not change much even when the monomer peak has disappeared, which suggests that the formation of 'precipitable' aggregates is slow. However, cod myosin does precipitate if kept long enough and it has been found, for example, that if a 1.5% solution of the protein is kept overnight at 23° and then for about a week at 0° it is converted into a rigid gel.

Kinetics of the aggregation

The detailed course of the aggregation has been followed by measuring the rate of disappearance of the monomeric myosin. The accuracy of this procedure is limited by a number of factors. The first is the shape of the myosin 'peak', which tends to be very sharp and difficult to define at the top. Secondly, owing to the sector-dilution effect, uncorrected areas at the photographic plate are not true measures of protein concentrations in the uncentrifuged solution. No attempt has been made to correct areas for this effect, which has been minimized by performing runs under as similar conditions of protein concentration, ionic strength, temperature, centrifugal field and time as possible, so that the peak is always measured at very nearly the same level in the cell. Areas of the monomer peak in aggregated solutions were then expressed as a fraction of the area of the monomer peak in the initial, unaggregated solution of myosin run under identical conditions. (In this respect Fig. 1*a* is exceptional in that the duration of the run is longer than the others in the series.) As the amount of monomer becomes less, under a given set of conditions its boundary will travel to a position slightly further down the cell (because of the inverse dependence of S for myosin on concentration), and the correction necessary for dilution will become larger. Neglect of this last effect is estimated to be at most 5% at the lowest concentrations measurable. Thirdly, the correction necessary because of the effect described by Johnston & Ogston (1946) will become greater as the ratio of monomer to aggregates becomes smaller (i.e. as the amount of monomer diminishes). Neglecting sector-dilution and assuming that the mixture contains only monomer and dimer, it was calculated from a typical set of results at a total protein concentration of 0.5% that the factor necessary to correct the observed to the actual concentration of monomer was 0.95, 0.89 and 0.67, when the amounts of monomer fell to 43, 28 and 15% respectively of their original amount. Above 50% of monomer remaining the factor is

negligible whereas below this stage there will be a tendency for the Johnston & Ogston correction to be balanced by the dilution correction. However, the uncertainty involved in these corrections means that even corrected data below 20% of monomer remaining are likely to be considerably in

Fig. 4. First-order rate of disappearance of cod-myosin monomer during storage at 0°, pH 7.5, I 0.5. □, Mean values of four experiments run at protein concentrations of 1.2-1.5%; ○, mean values of six experiments run at protein concentrations of 0.3-0.5%. x , Fraction of the initial amount of monomer remaining. Vertical bars represent the standard deviations of the results; only two results were obtained at 10 days.

Fig. 5. Second-order rate of disappearance of cod-myosin monomer during storage at 0°, pH 7.5, I 0.5. Symbols are the same as in Fig. 4.

error, and, in fact, measurements have not been carried beyond this stage.

If the data from experiments such as that shown in Fig. 1 are analysed by means of the integrated forms of the first- or second-order-rate equations, the plots shown in Figs. 4 and 5 result. For more concentrated solutions (1.2–1.5%) the data fit the first-order plot down to 30–40% of monomer remaining; below this stage the order always becomes higher than unity and this is true even when the full Johnston & Ogston correction is applied. For dilute solutions (0.3–0.5%) there is a tendency for the data during the initial stage of the reaction to fit the second-order plot slightly better than the first-order plot; at 40% of monomer remaining the order is greater than unity.

For a given set of conditions the rate of disappearance of monomer varies erratically, though not very widely, from one preparation to another. A similar phenomenon was noted by Lowey & Holtzer (1959). This behaviour has necessitated making as many comparisons of different conditions as possible on the same preparation.

For any one preparation of myosin it has been found repeatedly that the first-order constant of disappearance is concentration-dependent over a wide range of concentrations, the rate increasing markedly at higher concentrations (Fig. 4). Extraneous factors that might influence the rate of aggregation in these experiments have been minimized by diluting the same preparation of myosin to different degrees, dialysing all the diluted solutions in the same buffer at 0° and then analysing them within a few hours of each other after adjusting the protein concentration to that of the most dilute solution.

This finding is in contrast with the observation of Lowey & Holtzer (1959) that the rate of aggregation of rabbit myosin as measured by light-scattering was (for two low concentrations of 0.02 and 0.04%) independent of the initial concentration. It is possible that the reconciliation of these results lies in the fact that the absolute concentrations studied are so different. The present results suggest that the rate of aggregation is relatively constant at the lower concentrations examined and it is possible that at the very low concentrations studied by Lowey & Holtzer (1959) the rates as measured by light-scattering, which is particularly sensitive to large aggregates, would appear the same.

For the initial portion of the first-order-rate curve the reaction constants at 0° from the mean results of several experiments varied between $0.14 \times 10^{-5} \text{ sec.}^{-1}$ for a 0.3–0.5% solution and $0.45 \times 10^{-5} \text{ sec.}^{-1}$ for a 1.2–1.5% solution in 0.45M-KCl-phosphate, pH 7.5, I 0.05. Although an exact comparison is not possible because the conditions are so different, Lowey & Holtzer (1959) calcu-

late a first-order-rate constant of $1.8 \times 10^{-5} \text{ sec.}^{-1}$ from the rate of increase of the average M determined by light-scattering during the aggregation of rabbit myosin at 0.02 and 0.04% and at 25° (0.6M-KCl, pH 6.3). At 4° it is stated (Lowey & Holtzer, 1959) that the rate is about one-tenth of that at 25°.

In order to compare reaction rates under different conditions, the protein concentration has been kept constant in any one experiment (usually at about 0.3–0.4%) and the rates quoted are for convenience those calculated from the initial portions of the first-order-rate curve, i.e. before the stage when about 30% of the monomer remains.

Effect of temperature, ionic strength and pH on the aggregation

Temperature. At 0°, 3.5° and 8°, one preparation (concentration 0.48%) aggregated at first-order rates of 0.12, 0.21 and $0.75 \times 10^{-5} \text{ sec.}^{-1}$ respectively, corresponding to an activation energy of approx. 41 kcal.mole⁻¹. Almost no monomer was observed in the ultracentrifuge diagram of a 1.5% solution which had stood 18 hr. at 23°.

Ionic strength. The effect of ionic strength is shown in Fig. 6. In order to avoid high local concentrations of salt, portions of the stock myosin solution were diluted with an equal volume of phosphate buffer, pH 7.5, I 0.05, containing sufficient KCl to bring the final ionic strength to 0.3, 0.5, 1.0, 2.0 and 3.0, and then the diluted solutions were dialysed against KCl-phosphate buffer of total ionic strength 0.3, 0.5, 1.0, 2.0 and 3.0 respectively. With the solution diluted to I 0.3 this procedure resulted in the precipitation of about 10% of the protein.

Fig. 6. Effect of ionic strength on aggregation of cod myosin at 0°, pH 7.5. Protein concentration, 0.47%. k , First-order-rate constant calculated as described in the text.

The increase of rate of aggregation at higher ionic strength is in line with Lowey & Holtzer's (1959) observations that the rate is greater in 1.5 than 0.6M-KCl. Exposure of cod myosin to 1.0 or above produces small amounts of a polydisperse ultracentrifugal component of low S . More of this material is produced at higher ionic strengths though the amount never exceeded about 10% of the total protein present. As described below, this component, or one like it, often appears when cod myosin is subjected to extreme conditions. It is possibly a dissociation product of low M like that observed by Kominz, Carroll, Smith & Mitchell, (1959) in alkali-treated rabbit myosin.

Precipitation of cod myosin once, by diluting with water, results in its irreversible aggregation (Connell, 1958*a, b*). The protein becomes progressively more difficult to dissolve when repeatedly precipitated in this way and that which does dissolve always shows considerable amounts of aggregated material in the ultracentrifuge. In addition, salting-out by half-saturation with ammonium sulphate renders cod myosin insoluble.

pH. As shown in Fig. 7, aggregation is minimal between pH 7.0 and 8.0 and increases on both sides of this range. Below pH 5.5–5.7, of course, the protein precipitates in the presence of salt. Below pH 6.2 and above pH 9.2 at I 0.5 progressively more of the polydisperse component of low S accompanies the increased tendency to aggregate.

Cod myosin which has been precipitated by dilution and then washed with water can be dissolved in acetate buffer (I 0.05, pH 4). In this

solvent the protein (concentration 0.41%) shows a single asymmetric ultracentrifuge component of $S_{20,w} = 4.1 \times 10^{-13}$ sec. Presumably under these conditions the protein is considerably denatured because it has $[\alpha]_D^{25} - 78^\circ$ [compared with $[\alpha]_D^{25} - 29^\circ$ and -104° for native and urea-denatured cod myosin respectively at I 0.5, pH 7.5 (Connell, 1958*b*)], and on returning it to neutrality or adding salt it precipitates.

Effects of additives on the aggregation

Sulphydryl reagents. Lowey & Holtzer (1959) reported that oxidation with sulphite or treatment with mM-thioglycollate did not affect the rate of aggregation of rabbit myosin. Connell (1959) noted that the aggregation of cod myosin is not inhibited by 10 mM-monothioglycol. As shown in Fig. 8, 0.1M-monothioglycol accelerates aggregation whereas 0.1M-ethylene glycol is without effect. It has also been found that oxidation by iodosobenzoic acid or alkylation by *N*-ethylmaleimide or *p*-chloromercuribenzoate increases the rate of aggregation of cod myosin. Aggregation does not therefore seem to occur by reactions involving the 'reactive' sulphydryl groups. Since the sulphydryl reagents described react with only about 50% of the total sulphydryl groups of cod myosin (J. J. Connell, unpublished work) it is conceivable that the aggregation may occur through a sulphydryl-disulphide-exchange reaction involving the remaining sulphydryl groups.

The accelerating effects of the sulphydryl reagents suggests that the sulphydryl and disulphide groups are necessary for the stability of the protein.

Urea and sodium dodecyl sulphate. In experiments with these reagents care was taken that the protein was not exposed to concentration greater than the final one aimed at.

Urea concentrations from 0.3 to 1.0M accelerate aggregation (Fig. 9). Polydisperse material of low S is evident even at 0.3M and the amount of this increases with the urea concentration. This fact, together with the pronounced curvature of the base line in ultracentrifuge runs at high urea concentrations, makes the interpretation of effects above 1.0M uncertain. However, as judged from the amounts of aggregates formed after equivalent periods of storage there are indications that aggregation is retarded or, at least, not accelerated, in 2M-urea and above (Fig. 9).

Sodium dodecyl sulphate at a concentration of 0.04% does not precipitate the protein but produces large amounts of an ultracentrifuge component of $S_{20,w} 14 \times 10^{-13}$ sec., presumably an aggregate different in type from that produced during normal storage. Sodium dodecyl sulphate at this concentration also causes the formation of polydisperse material of low S .

Fig. 7. Effect of pH on the aggregation of cod myosin at 0° , I 0.5. Protein concentration, 0.3%. k has the same significance as in Fig. 6.

Actin. Fig. 10 demonstrates that keeping myosin in the form of actomyosin has a considerable protective effect.

Changes in viscosity, optical rotation, sulphhydryl groups and ultraviolet-absorption spectrum

Viscosity. Lowey & Holtzer's (1959) light-scattering and sedimentation results on rabbit myosin and sedimentation results on cod myosin (Connell, 1958a) show that the main aggregation mechanism is a side-by-side process. However, viscometry, which ought to be a sensitive indicator of this type of aggregation in such an asymmetric

molecule as myosin, suggests that the process cannot be simply an exclusive side-by-side one. For example, the intrinsic viscosity of cod myosin does not change during its extensive aggregation (Fig. 11) and Lowey & Holtzer (1959) found that during the aggregation of rabbit myosin the intrinsic viscosity actually increased. It would therefore appear that a full description of aggregation must await further investigation.

Optical rotation. No change in this property of cod myosin could be detected during storage of up to 10 days at 0° but small increases in laevorotation accompany storage for 20 hr. at 23°, e.g. $[\alpha]_D^{23}$ changed in one experiment from -34.2° to -36.5° .

Fig. 8. Ultracentrifuge diagrams showing the effect of (a) 0.1 M-ethylene glycol and (b) 0.1 M-monothiolglycol on the aggregation of cod myosin for 2 days at 0°, pH 7.5, I 0.5; (c) is the untreated solution. Protein concentration, 0.57%. Sedimentation from right to left; 69 min. after reaching full speed (56 100 rev./min.). Temperature of runs: (a) 1.4°; (b) 1.5°; (c) 1.1°.

Fig. 9. Ultracentrifuge diagrams showing the effect of urea on the aggregation of cod myosin for 3 days at 0°, pH 7.5, I 0.5. (a) Untreated; (b) 0.3 M-urea-treated; (c) untreated; (d), (e) and (f) M-, 2M- and 4M-urea-treated respectively. (a) and (b) are the same preparation (protein concentration, 0.63%). (c), (d), (e) and (f) are the same preparation (protein concentration, 0.44%). Sedimentation from right to left; 69 min. after reaching full speed (56 100 rev./min.). Temperature of runs: (a) 4.6°; (b) 2.0°; (c) 3.9°; (d) 5.0°; (e) 1.3°; (f) 0.9°.

Fig. 10. Ultracentrifuge diagrams of (a) cod actomyosin + mM-pyrophosphate, pH 7.5, I 0.5; (b), (a) after 3 days at 0°; (c) cod actomyosin used for (a) kept 3 days at 0° and then treated with mM-pyrophosphate. Protein concentration, 0.59%. Sedimentation from right to left; 69 min. after reaching full speed (56 100 rev./min.). Temperature of runs: (a) 1.5°; (b) 3.6°; (c) 1.7°.

Fig. 11. Intrinsic viscosity of a 1.44% cod-myosin solution during storage at 0°, pH 7.5, I 0.5. Time of storage (days): ○, 0; □, 1; △, 2; ×, 3; ▽, 6. η_{sp} is the specific viscosity.

Sulphydryl groups. No changes in either the sulphydryl groups of cod myosin that react freely with *p*-chloromercuribenzoate or the total sulphydryl groups (determined after denaturation in 8*M*-urea) have been found during aggregation for 6 days at 0°.

Ultraviolet-absorption spectrum. Laskowski, Widom, McFadden & Scheraga (1956) and Scheraga (1957) respectively have proposed that, in insulin and ribonuclease, formation of hydrogen bonds involving tyrosyl groups is revealed by shifts in the ultraviolet-absorption spectra of the proteins. The shifts are observed by differential spectrophotometry, the maximum difference in absorption occurring at 287 m μ . Spectral changes occurring during the polymerization of fibrin also suggests that tyrosyl hydrogen bonds are formed (Scheraga & Laskowski, 1957).

An attempt was made to observe similar shifts during the aggregation of cod myosin by reading aggregated samples against the monodisperse protein adjusted to the same nitrogen content. However, apart from very small differences in absorption at 276 m μ , probably attributable to slight differences in protein concentration, the absorptions of the aggregated and monodisperse samples were identical between 265 and 295 m μ .

Changes in adenosine triphosphatase activity during aggregation

Aggregation at 0° is paralleled by a loss in enzyme activity. The rate of loss of activity is first-order with respect to time up to 3 days' storage but becomes of higher order later. Yasui, Fukazawa,

Hashimoto, Kitagawa & Sasaki (1958) found the same behaviour with rabbit myosin or actomyosin. The initial inactivation rate, like that of aggregation, is somewhat erratic; the mean of several experiments in which the myosin was stored at a concentration of 1.2–1.5% gave an inactivation constant [$k_0 = (1/t)/\ln (A_0/A_t)$, where A_0 and A_t are the initial activity and activity after time t respectively] of 0.6×10^{-5} at 0°. The comparable figure of Yasui *et al.* (1958) for rabbit myosin is $< 0.01 \times 10^{-5}$ at 1°.

Although aggregation is accelerated at higher protein concentrations, one experiment (Table 1) indicated that the reverse is true for the inactivation of the enzyme. In other experiments enzyme activity was not lost when cod myosin was aggregated by precipitating and redissolving. Actomyosin retains its activity better on storage than myosin (Table 2).

Tryptic digestion of native and aggregated myosins

Although earlier experiments (Haurowitz, Tunca, Schwerin & Göksu, 1945) had shown that heat coagulation of rabbit myosin had no effect on the rate of its digestion by trypsin, a recent report that an estimate of the amount of 'crystalline' or 'folded' region in a fibrous protein like myosin could be arrived at by measuring its rate of tryptic digestion prompted a similar investigation of cod myosin. Harrington, Hippel & Mihalyi (1959) suggest that digestion can be accounted for by two parallel first-order reactions, the slower involving the 'crystalline' regions and the faster the 'amorphous' regions of the molecule. During the proteolysis of myosin at 6–25° approximately 20% of the bonds are split by the fast reaction whereas at 41° the proportion

Table 1. *Inactivation of cod-myosin adenosine triphosphatase during storage at 0°, pH 7.5, I 0.5*

Time of storage (days)	Concn. of protein (%)		
	1.20	0.40	0.20
	Percentage of original activity remaining		
1	85	83	63
2	72	50	45
3	54	41	32

Table 2. *Inactivation of adenosine triphosphatase of cod myosin and actomyosin compared at the same protein concentration (0.23 %)*

Time represents period of storage at 0°, pH 7.5, I 0.5.

Time (days)	Percentage of original activity remaining	
	Myosin	Actomyosin
1	66	85
2	47	80
3	47	71
6	34	64

Fig. 12. Tryptic digestion of cod myosin at 17° and 40°. ○, Native myosin; □, myosin denatured by heating at 40° for 30 min. $\beta = 10 (E_{\infty} - E_t)$, where E_{∞} and E_t are the extinctions of the ninhydrin-treated digests after 24 hr. and t min. of digestion respectively.

is 50%; the conclusion drawn is that the molecule is becoming unfolded ('denatured') at the higher temperature. It was therefore of obvious interest to see if a similar change in the kinetics of tryptic digestion of cod myosin occurred during its aggregation.

The kinetic observations of Harrington *et al.* (1959) have been broadly substantiated, but as shown in Fig. 12 the rate of digestion is the same at any one temperature for aggregated as for monomeric myosin. Aggregation was produced by keeping the myosin at 40° for 30 min. and ultracentrifugal analysis revealed the complete absence of monomer. These results imply that if any such configurational alteration as postulated by Harrington *et al.* (1959) does occur on raising the temperature from 17° to 40°, then it is completely reversible. It is therefore clear that any change in the cod-myosin molecule that accompanies its irreversible aggregation is not revealed by differences in rates of tryptic digestion.

DISCUSSION

The kinetic results just described can be accounted for most simply and at least qualitatively by assuming that the aggregation reaction involves two steps: a first-order step which does not affect the sedimentation coefficient followed by a second- (or higher-) order step. Such a scheme is indicated by the facts that the reaction is concentration-dependent, is apparently first-order at high protein concentrations and of indeterminate order at low concentrations. At the concentrations studied the second step must be more rapid but not enormously more rapid than the first. If the second step were very much more rapid than the first the kinetics would be first-order throughout. If the rates of the two steps were similar deviations from first-order kinetics would appear in the early stages of the reaction.

The two steps of this kinetic scheme can be rationalized in terms of a small configurational alteration in the myosin monomer (M) preceding the step-wise aggregation of the altered molecule (M_D):

It is not, however, possible to exclude reactions such as

and

A similar two-step scheme has been proposed for rabbit myosin by Lowey & Holtzer (1959), though on very different grounds.

One consequence of this kinetic scheme is that if the measured ultracentrifuge component were all monomer (M) then its rate of disappearance should remain first-order under all conditions. The fact that this is not observed experimentally could be accounted for by assuming that part of the component is due to the presence of the product of step (1), i.e. M_D . In other words the sedimentation behaviour of M_D is similar to that of M. Among the relatively few ultracentrifugal investigations of the denaturation and aggregation of proteins by heat there are two precedents for this suggestion. Heat denaturation of both thyroglobulin (Lundgren & Williams, 1939) and bovine-serum albumin (Warner & Levy, 1958) produces species that have the same sedimentation velocity as the native molecule. Yasui *et al.* (1958) remark on the fact that the monomer peak in the ultracentrifuge picture becomes broader during the aggregation of rabbit myosin. The same phenomenon has been noted with cod, but although it might be due to an alteration in the molecule it could also be explained on the grounds that self-sharpening of the boundary will be less and diffusion greater at the lower myosin concentration.

The first-order reaction (1) is presumably a form of denaturation because the activation energy is high and because extremes of pH or ionic strength and reagents such as urea accelerate aggregation. However, only small increases in laevorotation accompany aggregation, indicating that any configurational disorders are much less than those accompanying urea-denaturation, which produces a fourfold increase in laevorotation. The fact that the numbers of 'free' sulphhydryl groups do not change during aggregation is another indication that the degree of unfolding is small.

The rates of aggregation during storage are similar to the rates of enzymic inactivation. For cod myosin, the rate constants for the aggregation and enzymic inactivation of a 1.2–1.5% solution at 0° are 0.45 and $0.6 \times 10^{-5} \text{ sec.}^{-1}$ respectively; values for rabbit myosin at 25° of $0.18 \times 10^{-4} \text{ sec.}^{-1}$ for aggregation (Lowey & Holtzer, 1959) and of $0.12 \times 10^{-4} \text{ sec.}^{-1}$ (interpolated from the data of Yasui *et al.* 1958) and $0.13 \times 10^{-4} \text{ sec.}^{-1}$ (Ouellet, Laidler & Morales, 1952) for enzymic inactivation have been reported. However, the relation between the two processes is not clear since protein concentration has different effects and since precipitated cod myosin retains its enzymic activity.

The present results emphasize that on keeping at low temperatures cod myosin denatures much more readily than the rabbit protein. At 0° inactivation of cod-myosin adenosine triphosphatase proceeds at least 60 times as quickly as that of rabbit myosin. It has also been observed that the ultracentrifuge diagram of rabbit myosin was not

changed by storage for 7 days under the conditions of Fig. 1. The ease with which apparently low-molecular-weight fragments are split off cod myosin, e.g. by urea concentrations as low as 0.3 M, may point to a relatively loose internal cohesion of the molecule.

The results of Lowey & Holtzer (1959) and the present ones have given little help in discovering the nature of the bonds holding myosin aggregates together. There is no evidence for sulphhydryl interactions being involved. The slight inhibition or reversal of aggregation in concentrated urea solutions suggests that hydrogen bonds might be concerned. The negative results given by the ultraviolet-difference spectra of aggregated versus monomeric cod myosin suggest that tyrosyl groups do not participate in any hydrogen bonding which may occur.

SUMMARY

1. The aggregation of cod-myosin monomer at concentrations of 0.2–1.5% has been studied by following changes in weight-average molecular weight, sedimentation, diffusion, viscosity and optical rotation.

2. The kinetics of the disappearance of monomer have been analysed and can be plausibly accounted for by assuming that the reaction involves two steps: a denaturation stage followed by a relatively rapid stepwise side-to-side aggregation.

3. Additional evidence for a denaturation stage has come from a study of the reaction at different temperatures, conditions of pH and ionic strength, from the effects of added substances and from an examination of the concurrent enzymic inactivation.

4. Aggregation, which under extreme conditions is often accompanied by a dissociation of the molecule, cannot be explained on the basis of sulphhydryl–disulphide interactions.

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Intracellular Distribution of Carbonic Anhydrase

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The presence of carbonic anhydrase in various mammalian tissues has been recognized for many years. The only known function of the enzyme is catalysis of the reaction

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In certain organs, such as stomach, kidney and pancreas, it appears that carbonic anhydrase is involved in adjusting the rate of a steady state of production of carbon dioxide or carbonic acid to specific functional demands. The necessity for an increased rate of the above reaction is most clearly understood for the example of elimination of