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Purification and Properties of Tuna Myosin*

C.-S. Chung,† E. G. Richards, and H. S. Olcott

ABSTRACT: Myosin was extracted from the white muscle of skipjack tuna (*Euthynnus pelamis*) and purified by the chromatographic method of Richards *et al.* (Richards, E. G., Chung, C.-S., Menzel, D. B., and Olcott, H. S. (1967), *Biochemistry 6*, 528). The product showed a hypersharp boundary in the ultracentrifuge, $s^0_{20,w} = 6.22$ S, and possessed adenosine triphosphatase (ATPase) activity and amino acid composition similar to that of rabbit myosin. Molecular weight by high-speed sedimentation equilibrium was approximately 530,000, compared to 510,000 obtained for rabbit myosin under the same conditions. The pH–activity curve showed two maxima similar to that of the unpurified preparation.

The purified tuna myosin tended to aggregate during storage; this was not prevented by dimethyl sulfoxide, 1,3-butanediol, *N*-ethylmaleimide, or 2-mercaptoethanol.

Disagreements in the reported molecular weight of myosin and in details of its subunit structure are thought to be due to the different methods of extraction and purification used (Tonomura *et al.*, 1966; Dreizen *et al.*, 1966). Among these may be mentioned the conventional precipitation–resolution method (Szent-Györgyi, 1943) and the ammonium sulfate fractionation procedure (Szent-Györgyi, 1951; Tsao, 1953; Kielley and Bradley, 1956; Small *et al.*, 1961). Column chromatographic methods have been applied to the purification of myosin (Brahms, 1959; Perry, 1960; Asai, 1963; Smoller and Fineberg, 1964; Baril *et al.*, 1964, 1966). Recently we described a modified chromatographic procedure with DEAE-Sephadex A-50 and KCl gradient in pyrophosphate or phosphate buffer, which has provided preparations of highly purified myosin from several sources (Richards *et al.*, 1967).

The present investigation describes (1) the preparation and purification of skipjack tuna myosin by the modified chromatographic procedure, (2) determination of its molecular weight by the high-speed sedimentation equilibrium method of Yphantis (1964), (3) study of some of its enzymatic properties, and (4) its amino acid composition. These properties are compared with those of highly purified rabbit myosin.

Experimental Procedure

Materials and Methods. Methods and reagents used for the extraction of myosin, the chromatographic procedures, and methods for the analysis for protein and ATPase[1] activity and identification of RNA were those previously described (Richards *et al.*, 1967). DMSO (Stepan Chemical Co., Chicago) and 1,3-butanediol (Eastman) were used as obtained.

Live skipjack tuna (*Euthynnus pelamis*), about 45 cm in length (obtained at the Research Laboratory of the Fish and Wildlife Service in Honolulu, Hawaii), were denervated by cutting the spinal cord and brought to the Biomedical Research Center of the University of Hawaii for further handling. The time between denervation and extraction of myosin was about 1 hr.

The preliminary processing of the myosin, including the first high-speed centrifugation, was carried out in Hawaii. At this stage an equal volume of glycerol was added to the myosin solutions and the resulting solutions were held at −20°. When needed, the myosin was precipitated from the 50% glycerol solution by the addition of nine volumes of water; the precipitate

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[1] Abbreviations used: ATPase, adenosine triphosphatase; DMSO, dimethyl sulfoxide; NEM, *N*-ethylmaleimide.

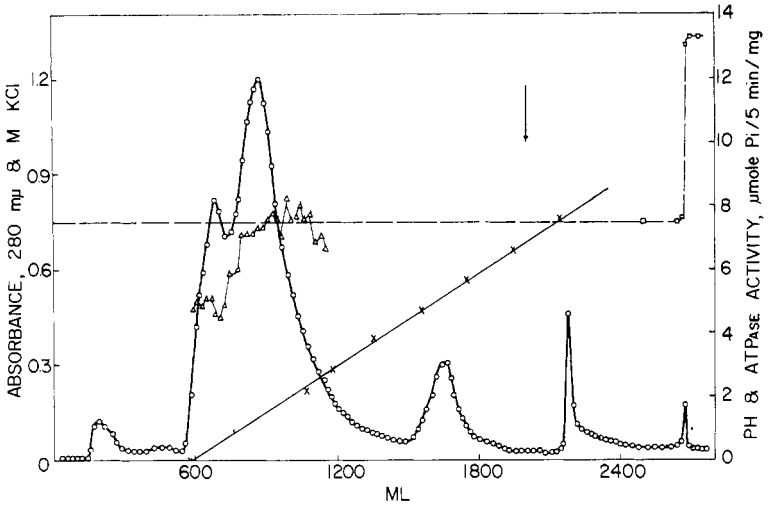

FIGURE 1: Chromatography of skipjack tuna myosin prepared by the method of Asai (1963) on DEAE-Sephadex A-50. Protein solution (90 ml of 1.1%) was applied to a 2.5 × 94 cm column and eluted with a linear gradient of KCl (1 l. each of 0.04 M pyrophosphate and 0.04 M pyrophosphate-1 M KCl, pH 7.5) at 20 ml/hr; 20-ml fractions were collected. KOH (0.5 M) was applied (at arrow) until the pH of the eluate was 13. (O) absorbance at 280 mμ, (X) KCl concentration in eluate, (Δ) ATPase activity, and (□) pH.

was then collected and dissolved in the appropriate buffer.

Amino Acid Analysis. The proteins were hydrolyzed according to the procedure of Moore and Stein (1963) in 6 N HCl at 110° for 24, 48, and 72 hr, and the hydrolysates were chromatographed on a Spinco Model 120 amino acid analyzer. Values for serine, threonine, ammonia, tyrosine, and methionine were extrapolated to zero time to correct for losses during hydrolysis. Recoveries of nitrogen were 92–98%. Half-cystine was determined by oxidation to cysteic acid according to the method of Moore (1963). Tryptophan was determined spectrophotometrically (Beavan and Holiday, 1952).

Sedimentation Experiments. The Spinco Model E ultracentrifuge used for all sedimentation experiments was equipped with a phase plate, Rayleigh and schlieren optics, and a temperature control and measuring unit. The optical systems were aligned according to the procedure of E. G. Richards, R. H. Haschemeyer, D. C. Teller, and H. K. Schachman (in preparation).

Sedimentation velocity studies were usually conducted at 59,780 rpm at 4°. Schlieren patterns, photographed through a Wratten 77A filter on Eastman Kodak metallographic plates, were read with a Nikon Shadowgraph Model 6C equipped with a 20 power objective lens.

For sedimentation equilibrium studies the high-speed method of Yphantis (1964) was employed. Observations were made by Rayleigh interference optics, with 0.44-mm symmetrical slits as the upper aperture and a Baird Atomic 546-mμ interference filter. The pictures were taken on Eastman Kodak type II-G spectroscopic plates and read in a Nikon Shadowgraph with a 50 power objective lens.

An aliquot of purified myosin solution was dialyzed overnight against 0.6 M KCl–0.025 M histidine (pH 6.8). Concentrations of about 0.75, 0.50, and 0.25 mg/ml were obtained by dilution with the dialysate. A rectangular three-channel Yphantis centerpiece was employed. The solution of 0.75 mg/ml was placed in the centripetal channel, that at 0.50 mg/ml in the central channel, and the 0.25 mg/ml solution, in the centrifugal channel. The equilibrium speed was calculated so that the cell containing the 0.25-mg/ml solution would have resolvable fringes at the bottom (Teller, 1965).

Equilibrium distribution was attained more rapidly by starting the centrifuge at a higher velocity, and then lowering it to the calculated speed (Richards, 1960). Equilibrium patterns were analyzed by reading radial positions at 0.25, 0.375, 0.50, 0.625, 0.75, 1, 1.5, 2, 2.5, 3, 4, 5, etc. fringes. Concentrations (in units of fringes) were corrected for base-line distortion using patterns obtained at the beginning of the run and also at the end of run after redistribution of the cell contents (Richards, E. G., Teller, D. C., and Schachman, H. K., in preparation). The log of the concentration was then plotted against the radius squared, and the molecular weight was calculated from the slope according to the usual equation (Yphantis, 1964). The value of 0.720 was used for the partial specific volume (Woods *et al.*, 1963), and the density was corrected for the buffer which contained 0.6 M KCl–0.025 M histidine (pH 6.8).

Results

Chromatography. The chromatographic pattern of the preparation of skipjack tuna myosin obtained

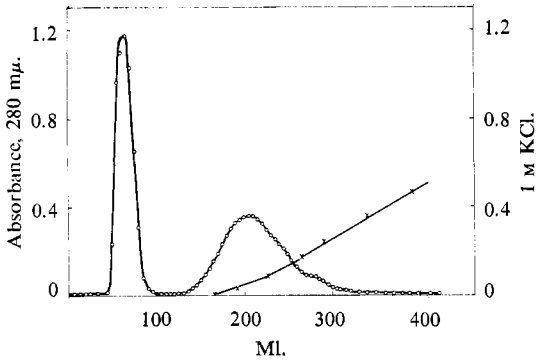

FIGURE 2: Chromatography of aggregated skipjack tuna myosin. The trailing part of myosin peak in Figure 1 was dialyzed against 0.04 M pyrophosphate (pH 7.5) at room temperature overnight and applied to a 2 × 27 cm column of DEAE-Sephadex A-50. A linear KCl gradient (200 ml each of the buffers given in Figure 1) was applied at 15 ml/hr. Symbols are the same as in Figure 1.

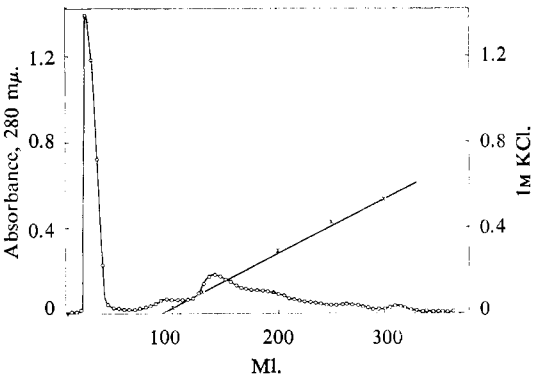

FIGURE 3: Chromatography of skipjack tuna myosin prepared by the method of Connell (1960). Protein (13.5 ml of 0.74%) was applied to a 1.5 × 26 cm column. Elution was accomplished as in Figure 2. Symbols are the same as in Figure 1.

by a modified method of Asai (Richards *et al.*, 1967) resembled those obtained with rabbit and yellowfin tuna preparations (Richards *et al.*, 1967), except that in this case the myosin was eluted in two peaks (Figure 1). The first began to emerge at the start of the KCl gradient, and the main peak appeared at 0.13 M KCl. Sedimentation velocity experiments showed that the two peaks were probably due to the overlapping of aggregates and myosin monomer. A fraction from the leading edge contained two components by schlieren optics: a fast-moving boundary, presumably a dimer of myosin; and the characteristic hypersharp peak of myosin. In contrast, a fraction from the trailing edge showed no heterogeneity, and was highly pure as judged by its enzymatic activity.

The positions occupied by aggregated myosins on the chromatogram were determined as follows. Purified skipjack tuna myosin (30 ml of 3 mg/ml) was dialyzed against 0.04 M pyrophosphate (pH 7.5) overnight at room temperature (about 22°). By sedimentation velocity the resultant solution contained at least three components, presumably myosin monomer, dimer, and trimer. The aggregated myosin was chromatographed on a 2 × 27 cm DEAE-Sephadex A-50 column, according to the standard procedure (Figure 2). Compared to the untreated preparation the rapidly moving peak was much larger, and the myosin peak was smaller. A fraction from the first peak showed at least three boundaries in the ultracentrifuge. This fraction had an ATPase activity of 2.1 units, less than one-half that of the nonaggregated myosin. The leading edge of the myosin peak itself showed slight heterogeneity in the ultracentrifuge but the trailing edge exhibited no heterogeneity. The ATPase values were 1.8 and 3.1 units, respectively, for the front and tail of the peak.

These results indicate that small aggregates elute at very low concentrations of KCl. When the amount of dimer was large, its peak overlapped with that of monomeric myosin, but when the amount was small it could be separated. A similar observation was made in the rechromatography of rabbit myosin (Richards *et al.*, 1967).

The higher aggregates, presumably trimer and higher polymers, were not retained by DEAE-Sephadex A-50 and were eluted together at the void volume of the column. It is noteworthy that the aggregates retained some enzymatic activity, but the level was only one-third that of the original myosin solution.

When myosin was prepared from fresh skipjack tuna muscle by a modified method of Connell (1960) and chromatographed on a 1.5 × 30 cm column of DEAE-Sephadex A-50, the preparation was aggregated since most of the protein appeared at the void volume (Figure 3).

The ultraviolet spectra of myosin and its aggregates (not shown) were very similar so that no conclusion could be reached with respect to the affect of aggregation on the aromatic amino acid groups. The solutions containing aggregates were usually turbid.

Enzymatic Properties. The preparations of skipjack tuna myosin had high enzymatic activity: 5.4 units for the crude sample and 7.0–8.0 units for the chromatographed protein. This activity was somewhat higher than that of the best rabbit myosin (Table I). Preparations obtained from yellowfin tuna (Richards *et al.*, 1967) and from bluefin tuna, black fish, and striped-bass myosins (Chung, 1967) were considerably less active.

The effect of pH on ATPase activity was measured with both crude and purified skipjack tuna myosins (Figure 4). The purified protein was the more active within the pH range studied. The profiles of the two curves were similar, with maxima at pH 6.5 and 9.5, and minima at pH 7.9. The ATPase of purified myosin appeared to fall at pH 10.2 while that of the crude enzyme remained high.

Effect of Chemical Agents on Aggregation. Chroma-

TABLE I: ATPase Activities of Chromatographed Tuna and Rabbit Myosins.

Myosin Source	Method of Prepn	ATPase Act. (μmoles of P_i/5 min per mg)			
			Chromatographed[a]		
		Crude	1	2	3
Skipjack tuna	Connell (1960)	2.4	0.7	6.4	3.5
	Asai (1963)	3.9	4.1	5.0	7.0
	Asai (1963)	5.6	4.8	8.4	6.7
	Same, stored[b]	5.7	3.8	8.0	4.5
Rabbit	Asai (1963)	3.9		6.7–7.9	
	Tonomura *et al.* (1966)	5.4	5.2	6.7	6.4
	Leading fraction[c]		3.9	5.8	4.4
	Trailing fraction[c]		5.8	8.0	6.8

[a] ATPase activities under 1–3 are values from leading edge of myosin peak, maximum activity, and from trailing edge of myosin peak, respectively. [b] Experiment performed after 5 months of storage in 50% glycerol at −20°. [c] Values obtained after rechromatography of leading and trailing edges of preceding sample (data from Figures 1 and 4, Richards *et al.*, 1967).

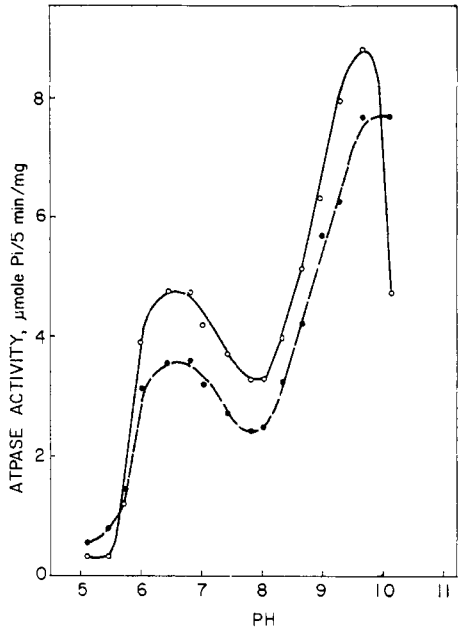

FIGURE 4: Effect of pH on ATPase activity of skipjack tuna myosin. (O) Chromatographed myosin and (●) unchromatographed myosin.

tographed skipjack myosin was incubated in a water bath at 25° for 18 hr with 5, 10, and 20% DMSO or with similar concentrations of 1,3-butanediol in order to determine if these agents might prevent aggregation of myosin. In all cases the $s_{20,w}$ increased to 7.2 and 9.0, indicating some degree of aggregation. The broadness of the peaks also suggested that the material was polydisperse. The slightly different $s_{20,w}$ values observed (not shown) were probably due to the solvent present in the solution, since no correction for the viscosity of these reagents was made. The effect of sulfhydryl agents, 2-mercaptoethanol and NEM, on myosin was also studied; neither prevented aggregation.

The amino acid compositions of the purified myosins from tuna and rabbit are shown in Table II which also includes for comparison results from other authors on rabbit and cod preparations. The values for the present study and that of Kominz *et al.* (1954) with rabbit myosin were based on the value of 16.7% nitrogen (Bailey, 1948), while the data of Connell and Howgate (1959) for cod myosin and those of Lowey and Cohen (1962) for rabbit myosin were based on 16.4 and 16.1% N, respectively. However, when the percentage of nitrogen in myosin was calculated from our amino acid compositions, the values obtained were 17.3% for tuna and 17.1% for rabbit. Although the analyses of all the myosins are very similar the potential errors are large enough to preclude any final conclusion concerning the question as to whether their amino acid compositions are identical.

Sedimentation Properties and Molecular Weight. There were no differences in the sedimentation patterns of tuna and rabbit myosins before and after chromatography except that a small amount of aggregate was present in the crude tuna preparation.

Plots of $1/s_{20,w}$ against concentration for skipjack tuna and rabbit myosins are presented in Figure 5. Several fractions from the chromatographic peak were used to measure the sedimentation coefficient for tuna myosin. All values fell on the same line, indicating that the material from the top and the trailing part of the myosin peak had the same sedimentation properties. The tuna myosin data fitted the equation $1/s_{20,w} = 0.1608 + 0.0162c$, $s^0_{20,w} = 6.22$ S; that for rabbit myosin fitted the equation $1/s_{20,w} = 0.1626 + 0.0151c$, $s^0_{20,w} = 6.15$ S, where c is concentration in milligrams

TABLE II: Comparison of Amino Acid Composition of Myosins from Several Sources (number of amino acids for 10^5 g of protein).

	Tuna[a]	Rabbit[a]	Cod[b]	Rabbit[c]	Rabbit[d]
Asp	84	80	85	85	85
Thr	41	42	36	41	44
Ser	38	40	45	41	39
Glu	155	157	145	155	157
Pro	18	19	25	22	22
Gly	41	35	44	39	40
Ala	73	73	71	78	78
Val	43	39	44	42	43
Met	24	23	20	22	23
Ile	37	38	35	42	42
Leu	79	77	77	79	81
Tyr	20	22	15	18	20
Phe	26	27	23	27	29
Lys	86	88	72	85	92
His	18	16	13	15	16
Arg	40	40	38	41	43
Trp	4	3	4	4	
Cys (half)	8	7	7	9	9
Amide NH_2	98	78	99	86	92
% N[e]	17.3	17.1	17.3	17.0	17.2
Net Weight[e]	95,400	94,500	89,900	96,000	98,200

[a] Present data. [b] Connell and Howgate (1959). [c] Kominz (1954). [d] Lowey and Cohen (1962). [e] Calculated on basis of stated composition.

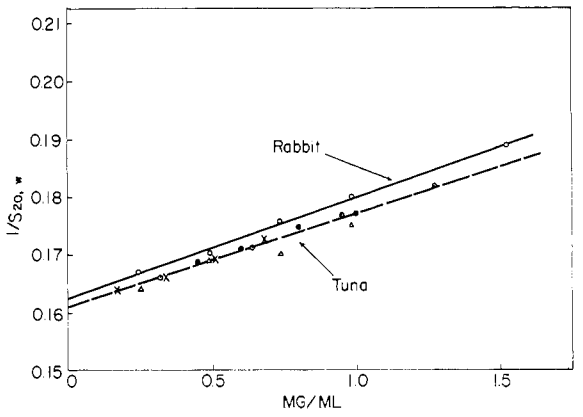

FIGURE 5: Concentration dependence of sedimentation coefficients of myosins. Solid line: Rabbit myosin; dashed line; skipjack tuna myosin. Different symbols denote different fractions; solvent, 0.6 M KCl–0.025 M histidine (pH 6.8).

per milliliter.

The molecular weights of rabbit and tuna myosins as determined by the high-speed sedimentation equilibrium method of Yphantis (1964) are given in Table III. The average values, not corrected for effects of nonideality, were 510,000 for rabbit and 530,000 for tuna. The precision in these experiments was approximately ±5%. The solutions had been oversped at 16,200 rpm for 4–8 hr, and then at 8766 rpm to achieve equilibrium. Experiment *c* was performed shortly after myosin was eluted from the column, and *d* was carried out after the myosin had stood for 1 week at 0°.

The solutions reached equilibrium after about 45 hr of centrifugation as determined by the constancy of fringe position. Even after 5 days of centrifugation, the apparent molecular weight of rabbit myosin remained the same without any upward curvature in the plot of the concentration *vs.* the radius squared. The sample that had been stored for 1 week at 0° showed no aggregation. The data from fresh tuna myosin also gave a straight line up to 182 hr of centrifugation (Figure 6A). However, with the tuna myosin that had been held for 1 week at 0°, the presence of aggregates was indicated by the upward curvature of the plot at the bottom of the cell (Figure 6B). Nevertheless, if only the points near the meniscus were used for the slope, the same molecular weights were obtained as those of the other myosins in the absence of aggregates. The high speed used in this method accomplished the fractionation of molecular species, as suggested by Yphantis (1964).

TABLE III: Molecular Weights of Myosins by Sedimentation Equilibrium.[a]

Sample	Hr	Mol Wt × 10^{-3} for Cell[b] 1	2	3
Rabbit[c]	54	518	534	531
	73	514	530	505
	95	523	508	506
	118	523	491	494
Rabbit[d]	51	496	486	509
	74	480	493	520
	94	515	520	523
Average (rabbit)				510
Tuna[c]	47	541	535	502
	55	530	514	506
	71	535	538	510
	82	533	544	534
	97	561	574	528
Tuna[d]	70	554	524	560[e]
	93	539	506	598[e]
	99	522	514	577[e]
	99	531	531	591[e]
Average (tuna)[e]				530

[a] Oversped at 16,200 rpm for 4–8 hr, then down to 8766 rpm. [b] The concentration of protein in cells 1–3 were *ca.* 0.75, 0.50, and 0.25 mg/ml, respectively, in 0.6 M KCl–0.025 M histidine (pH 6.8). [c] Run shortly after elution from column. [d] Same solution as *c*, but ran after 1-week storage at 0°. [e] Omitted from average for tuna molecular weight because of aggregation.

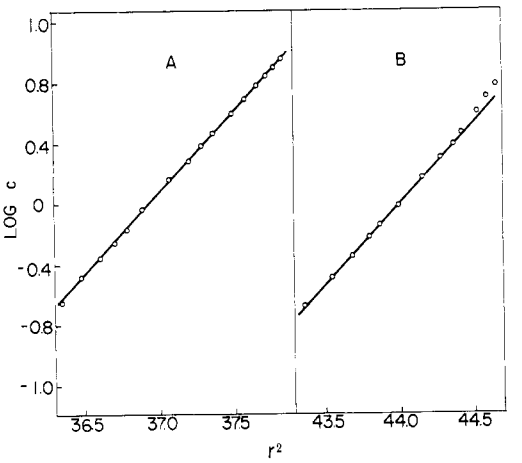

FIGURE 6: Log *c vs.* r^2 plot for skipjack tuna myosin. (A) Fresh myosin, cell 1. (B) Same myosin, but stored at 0° for 1 week, cell 2.

Discussion

The column chromatographic procedure of Richards *et al.* (1967) is applicable to fish myosins as well as to those of rabbit and chicken (Morey *et al.*, 1967). The present experiments showed that although the front edge of the myosin peak may contain some dimer and trimer, fractions obtained from the trailing edge were highly purified myosin, as judged from high ATPase activity, sedimentation patterns, and absence of nucleic acids. Although the enzymatic activity was lower at the trailing part of the peak as compared to the top fraction, sedimentation coefficients, amino acid composition, and molecular weights revealed no significant differences.

In contrast to rabbit myosin, the main peak for skipjack tuna myosin was heterogeneous. This may be related to the state of fish before death or the instability of the fish myosin itself. The particular tuna used in this work had not been feeding for several days—a problem with many tuna in captivity. The loss in muscle mass, the amount of which was not known, may have led to changes in some of the myosin molecules.

Myosin is a relatively unstable protein. Its instability is reflected in changes in aggregation leading to precipitation and loss of ATPase activity. Connell (1959, 1960, 1963) and Mackie and Connell (1964) have described the instability of cod myosin. The tendency of rabbit myosin to aggregate has been studied by Lowey and Holtzer (1959) and Johnson and Rowe (1961). In the present study it was found that tuna myosin is more stable than cod but less than rabbit myosin. When yellowfin tuna myosin was rechromatographed, the ATPase activity was reduced below that of the starting sample (Chung, 1967). Precipitation of skipjack tuna protein by dialysis against dilute buffer also introduced aggregates in contrast to the behavior of rabbit and chicken myosin under the same conditions. The implication from these observations is that the large amount of dimers seen in the chromatograms of skipjack tuna myosin may have arisen during the course of preparation and handling.

However, the nature of the interactions involved in the aggregation of myosin is not yet known. The results of Lowey and Holtzer (1959) and the results obtained in this study suggest that sulfhydryl groups do not participate in the reaction. Connell (1960) reported that the number of free SH groups did not change during aggregation of cod myosin and suggested that it was not likely that hydrogen bonding by tyrosyl groups was involved.

Tonomura *et al.* (1961) showed that organic solvents such as ethanol, chloroethanol, and dioxane promote aggregation. The ability of DMSO to preserve organs under frozen conditions has been reported (Block, 1963; Smith *et al.*, 1963), but in the present investigation, it was found not to prevent the aggregation of myosin. 1,3-Butanediol, a solvent which has a density approximately the same as water and characteristics similar to glycerol and ethylene glycol, was also ineffective.

It has been thought that the pH-dependence curve for myosin ATPase (Engelhardt and Ljubimova, 1939;

Mommaerts and Green, 1954) might disappear after extensive purification of the enzyme. However, the chromatographed myosin still possessed the two pH optima of the crude protein. A possible explanation is that the activity of myosin is determined simultaneously by the ionization of the enzyme as well as that of the substrates. The summation of these two then results in the irregular shape of the curve. In this connection, it is interesting that the participation of histidine and sulfhydryl groups in the active site of myosin has been postulated (Stracher, 1965). These two groups have ionization constants near 6.0 and 10.7, respectively.

With purified myosin, the enzyme activity appeared to decline above pH 10, while the crude protein remained active. This observation is being repeated but it may be mentioned that the pH at which ATPase activity appeared to fall corresponds to the pH at which small subunits dissociate from the myosin molecule (Gershman *et al.*, 1966).

The importance of the difference in amino acid composition in the kinds of myosins is difficult to assess because of their large molecular weights. When only the values obtained in the present study were compared, the composition of tuna myosin was similar to that of rabbit myosin, except that asparagine, glycine, valine, and histidine were higher in tuna protein. The values for rabbit myosin in the present study were generally lower than those found by Kominz *et al.* (1954) and Lowey and Cohen (1962). This was possibly due to the lower recovery of nitrogen in the present determination. Nevertheless, it should be mentioned that the rabbit protein in this study was obtained only from the back muscle of the animal rather than from mixed back and leg muscle, hence the composition may represent a better figure for a homogeneous protein.

The high-speed sedimentation equilibrium method of Yphantis (1964) proved to be very useful for the determination of molecular weights. The protein that eluted from the column did not have to be concentrated, and the time to attain equilibrium was moderate, thus reducing the possibility of aggregation of native myosin. The values for myosin found in the present study were slightly higher than those obtained by Tonomura *et al.* (1966), but were within the range reported by many workers (Mueller, 1964; Connell and Mackie, 1964; Mackie and Connell, 1964; Lowey and Cohen, 1962; Richards *et al.*, 1965, 1967). The molecular weight for skipjack tuna myosin (530,000) was approximately 4% higher than that of rabbit (510,000) when fresh preparations were compared, possibly due to the presence of a small amount of aggregates which appeared during centrifugation. Assuming that all the aggregates present were dimer, and that the value for rabbit myosin was the correct one, the amount of aggregates in skipjack protein was approximately 4%.

The present investigation revealed that other than instability, the properties of skipjack tuna myosin are very similar to those of rabbit protein. The magnitude of enzymatic activity was comparable, and the pH–activity curves coincided. Except for differences of doubtful significance, the amino acid compositions were similar. The sedimentation coefficients and molecular weights also agreed within experimental error.

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