STUDIES ON PARAMYOSIN

SENIOR THESIS

submitted in partial fulfillment
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Ch 80

by

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I. INTRODUCTION

The purpose of these experiments was to study the structure of the protein paramyosin. Paramyosin is readily obtainable from the adductor muscle of Venus mercenaria (clams). This protein seems to be restricted to clam muscle and is quite possibly involved in the "catch" mechanism of these muscles, the mechanism which enables the clam to hold its shell tightly shut for long periods of time with little expenditure of energy. Knowing the structure of paramyosin might suggest a model for the catch mechanism. Also, this knowledge might be combined with the expensive studies on collagen to suggest a pattern or evolutionary relationship among the several fibrous proteins (collagen, paramyosin, myosin, tropomyosin, etc.)

At the start of the present investigation a fairly large body of data had been accumulated concerning the physical and chemical properties of paramyosin, but such basic properties as the monomer molecular length and weight (the protein fiber consists of a staggered array of monomer units). Paramyosin extracted under acid conditions according to Hodge's method (see appendix) exhibits a molecular weight of about 110,000. Paramyosin extracted by other investigators

at neutral pH exhibited molecular weights in the vicinity of 220,000. We used the acid extraction procedure from Hodge's PhD. thesis because at neutral pH myosin is co-extracted.

From x-ray diffraction and optical rotatory dispersion, paramyosin is known to be a long rod-shaped molecule composed of two side-by-side α-helices, the helical content being ~90%.[1] The x-ray diffraction pattern of native paramyosin can be interpreted formally by the array reproduced in fig. 1,[2] the principle axial repeat periods are 145 A and 725 A. This pattern may reflect the presence of additional components, however.

Noelkin quotes the following values for some physical properties of paramyosin:

Property	value
shape	rod
molecular weight	225,000
intrinsic viscosity	1.92 dl/gm
radius of gyration	300 A
Length	1330 A
Diameter	19 A
sedimentation coeff.	3.1 S
partial specific vol.	0.733 cc/g
b_o	-600°

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[1] Milton Noelkin, The Denaturation of Paramyosin, PhD. thesis, Washington University, 1962, p. 109.

[2] A.J. Hodge, "Fibrous Proteins of Muscle," Rev. Modern Phys., 31, p.419 (1959).

fig 1

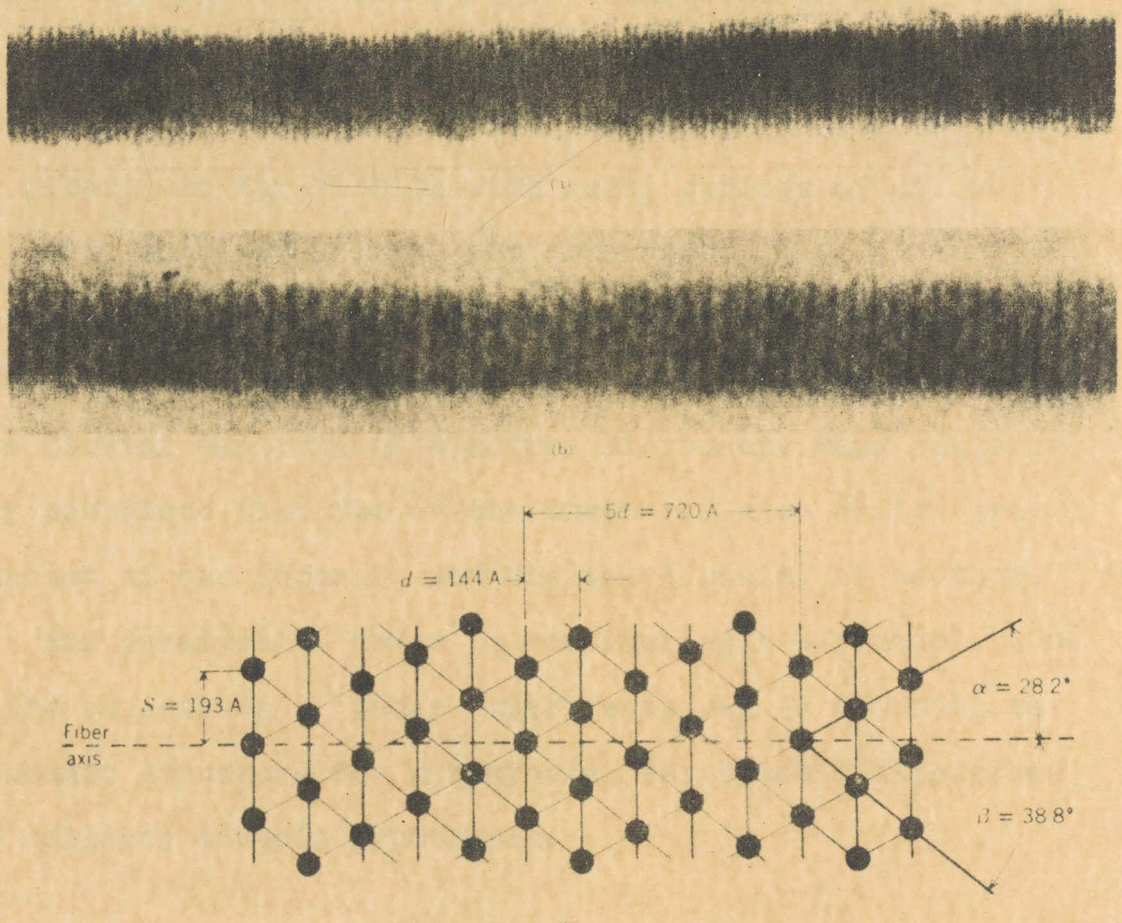

FIG. 13. Native "paramyosin fibrils" isolated from the adductor muscles of *Venus mercenaria* in dilute salt solutions. These usually show (a) a transverse cross striation with an apparent period of 145 A.[37,38] (b) a pattern consisting of the 145 A cross striation and a superposed dot pattern of symmetry such that the true repeat period is 5×145 A $= 7.25$ A (from F. O. Schmitt, R. S. Bear, C. E. Hall, and M. A. Jakus, Ann. N. Y. Acad. Sci. **47**, 799 (1947).[39] (c) shows the node pattern derived from electron micrographs. This formal net structure is in good agreement with the results of low angle x-ray diffraction studies (from C. E. Hall, M. A. Jakus, and F. O. Schmitt, J. Appl. Phys. **16**, 459 (1945)].[40] (a) and (b) × 190,000.

The amino acid analysis of paramyosin reveals no cystines[1] hence we do not expect to find disulfide linkages in paramyosin.

Typical of fibrous proteins, paramyosin can form several crystalline structures distinguishable under the electron microscope. The chains of paramyosin have a suitable distribution of electron-stain binding side chains (mainly lysine and argenine) and stagger themselves in a regular array in native and artificially precipitated fibrils so that a band pattern is observed transverse to the axis of the fiber. So far fibrils with axial periods of ca. 70, 145, 725, and 1400 A have been observed[2].

Hodge and his co-workers have recently proposed a subunit structure for the collagen molecule. Proceeding from fourier analysis of electron microscope band patterns, they postulate that two of the three collagen chains are composed of one subunit, and the third chain, of another.

For paramyosin, Hodge is considering a 4-5 model. One of the objectives of these experiments was to demonstrate a subunit structure for paramyosin, which would strengthen the collagen subunit hypothesis.

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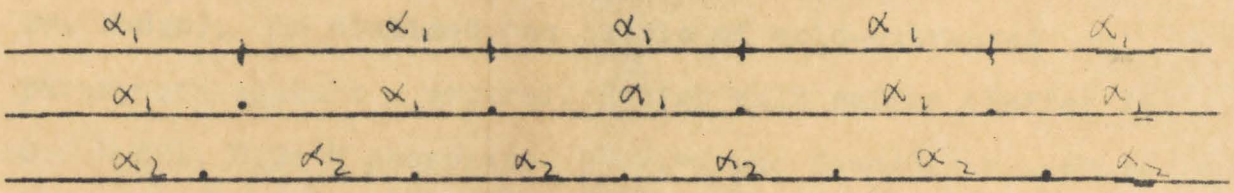
[1] Kominz et. al. Conference on the Chemistry of Muscular Contraction 1957, p. 69.

[2] Hodge, op.cit., p. 418.

COLLAGEN MODEL

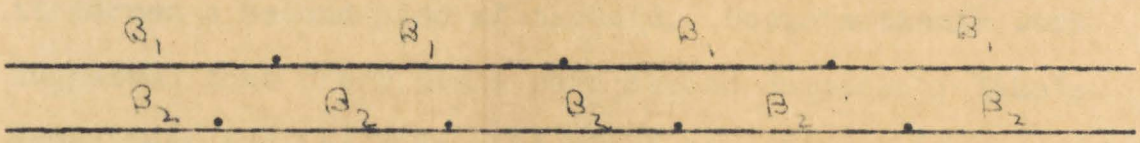


three chains:

two composed of α_1 subunits of legth L/5

one composed of α_2 subunits of legth L/6

PARAMYOSIN MODEL



Two chains:

one composed of β_1 subunits of length L/4

one composed of β_2 subunits of length L/5

II. Experimental

Our first experiments investigated an interesting result reported by Albert Kahn (a former co-worker with Dr. Hodge). He dialized one sample of acid-extracted paramyosin against a neutral pH high salt buffer (Buffer I: 0.6 M KCl, 0.06 M phosphate, pH 7.4) and found that approximately 20% of the protein precipitated. He discarded the precipitate and dialyzed the remainder against 0.35 M ammonium acetate, pH 7.25. All of the protein precipitated in the form of thin ribbons (tactoids) with a characteristic band pattern in the electron microscope. He then dialyzed another acid-extracted against 0.1 M KAc brought to pH 3.7 with 60ml/l acetic acid (Buffer II). When this sample was then dialyzed against the 0.35 M ammonium acetate buffer (Buffer III), it formed a second kind of tactoid. Hodge's thesis work suggested that there might be a second component; Kahn's experiment implied that this might be a dialyzable component. We redissolved each tactoid against its original buffer and measured its molecular weight using sedimentation equilibrium. In both cases the molecular weight was nearly the same as exhibited by untreated paramyosin under the same conditions of pH and ionic strength (F-232, 237, 340,341 ultracentrifuge runs). This does not eliminate the possibility of a second component; however, we decided not to pursue this attack any further.

We used the technique of sedimentation equilibrium exclusively to measure molecular weights of paramyosin because it has proven more reliable than light scattering for large protein molecules. The molecular weight is calculated by measuring the concentration as a function of radial distance in the analytical ultracentrifuge, using Rayleigh interference optics. We used Hodge's Zeiss optical interferometer to measure the initial protein concentration. The molecular weight is given by[1]

$$M = \frac{2RT}{1-\bar{v}\rho} \frac{d(\log c)}{d(r^2)}$$

M = molecular weight
v = partial specific vol.
ρ = solvent density

A plot of concentration versus r^2 should give a straight line for a weight-homogenous solute. Unless otherwise noted, all of our samples analyzed met this critereon for homogeneity, except for a small amount of heavy material at the bottom of the cell. The above formula is readily derived by equating the chemical potential to the gravitational potential in the centrifugal field; however, it neglects any contribution from an electric field due to the charge on the sedimenting molecules. The electrostatic force tends to oppose the concentration gradient of the solute and lead to a reduced value of molecular weight. Since paramyosin is a highly charged molecule, this error can be quite large, as we observed (see run P-288). Although theoretical corrections can be applied, the most satisfactory

[1] Schachman, Ultracentrifugation in Biochemistry, p. 202.

procedure is to run the solute in the presence of a supporting electrolyte. A concentration of 0.05-0.1 M NaCl was found to be sufficient to depress the charge effect.

Paramyosin has an isoelectric point at pH 6.2. Centrifugation was done on both sides of the isoelectric point under various ionic conditions. The results confirm our belief that the molecular weight of acid-extracted paramyosin is in the vicinity of 110,000.

Riddiford extracted paramyosin at neutral pH and then lyophilized her samples. She has observed molecular weights of 220,000 and 330,000 for these samples. We tried the Riddiford extration procedure, without lyophilizing, we saw a molecular weight of 210,000 (F-348). Upon heating to 35°C. in the ultracentrifuge this material showed a molecular weight of 105,000. We interpret her 330,000 MW species as a trimer, probably an artifact of lyophilization. Lyophilizing is known to produce multimers in the case of ribonuclease.

Concurrently, we studied our preparations under the electron microscope. Sequential dialysis of acid-extracted paramyosin against various combinations of buffers I, II, and III produced a variety of crystalline forms, all previously reported. Riddiford's prep behaved like acid-extracted paramyosin.

The collagen investigators derived valuable information from the "SLS" crystals, a side-by-side array of monomers with their ends in register. This form is precipitated by adding ATP. We attempted to produce a paramyosin analog of the SLS form. Starting with acid-extracted material, 1mg/ml, we added 0.2% ATP and looked without success for the presence of SLS-like particles in the electron microscope (shadow-casting the grids). We also tried the citrate-extracted material that Hodge used to precipitate PLS fibrils with. The Davies model for muscle contraction suggested that a divalent cation might help our cause; we tried 10^{-4}M Ca^{++}. Even though none of these tricks brought success, we feel that this approach deserves further pursuit. ADP and AMP might be tried in place of ATP, for instance. The addition of ATP did cause the protein samples to gel; perhaps under different ionic or pH conditions SLS-types could be found.

We also attempted to obtain the molecular length of the paramyosin molecule directly by electron microscopy. The molecules are barely visible under negative staining (uranyl formate worked the best of the stains tried), but the molecules tend to clump, making measurements exceedingly difficult.

The other line of approach we took was to look for subunits by degrading the molecule. Firstly, we tried to separate the two chains of paramyosin. Guanidine hydrochloride and thiocyanate are known to denature paramyosin, as revealed by a decreased helix content.[1] They are believed to act by

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[1] Noelkin, op. cit.

disrupting hydrogen bonds. Therefore, we would expect the two chains of paramyosin to be separated in these reagents if the chains are only held together by hydrogen bonding. We find that the MW of paramyosin is about 120,000 (we assume the $\bar{v}$ is still 0.733) in both of these reagents (F-342, 343), suggesting that the chains are crosslinked and not separated 6M G-HCl or 5M G-SCN.

We also tried adding 1M NH_2OH·HCl, pH 7.3, to the G-SCN buffer, and heating the sample to 50°C for 2 hours. When brought to equilibrium at low speed, the pattern indicated a two-component system of molecular weights 25,000 and 35,000. These are very close to the size fragments expected from Hodge's 4-5 subunit model. However, when the centrifuge was run up to higher speed to achieve the Yphantis condition, the pattern indicated a single species of molecular weight ca. 110,000. We would like to interpret this result as meaning that paramyosin was degraded into its two subunits, which subsequently renatured, either thermally or by concentration at the higher centrifuge speed. We then tried to separate subunits from freshly denatured paramyosin by acrilomide gel electrophoresis, but the samples consistently failed to penetrate the gels we used. A sample heated in the presence of G-HCl gave exactly the same behavior in the ultracentrifuge as the NH_2OH·HCl sample.

Schmitt reported[1] that trypsin did not change the sedimentation velocity of paramyosin, but did increase its diffusion rate markedly. If the effect of the trypsin were to divide the molecule into to rods of half the original length, we would not expect to see a significant change in the sedimentation velocity, because this property is a very insensitive measure of the length of a long, thin rod.

We looked at the molecular weight of trypsin-treated paramyosin in the ultracentrifuge and found that it was indeed homogenous, of molecular weight one-half of the value obtained for untreated paramyosin (F-340, 351).

We know that trypsin cleaves at the lysines and argenines in the polypeptide chain. If we assume that each subunit has trypsins located at its ends and middle (as electron microscopy suggests is the case), then we would expect trypsin to cleave paramyosin in half, according to the Hodge model, because only in the middle is there a co-incidence of labile loci. Even though there would be a nick at every lys/arg site in the molecule, the cross-linking of the chains would hold the molecule together everywhere except at its center. Note that this is a feature of any n-m model, where n is even and m is odd, not just of the 4-5 model.

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[1] Locker and Schmitt, J. Biophys Biochem Cytol, 3, 895 (1957)

A persistent problem in chemical denaturation of paramyosin is that the protein tends to flocculate when the denaturant is added. Sometimes the problem could be solved by dialyzing the sample against the denaturant. Usually the precipitate would dissolve slowly if the sample were allowed to stand at room temperature; after 4-5 days there was sufficient protein in solution to do analytical ultracentrifugation.

The paramyosin problem is still far from under control. Most of our data can be rationalized by the Hodge model. The complete results of our analytical data appear, complete with some inconsistencies. At this point it would be foolhardy to try to summarize this data or elaborate on the Hodge model: more experimental evidence is needed.

I wish to thank Prof. Alan J. Hodge for his patient assistance and helpful advice throughout this project. Also, I would like to acknowledge the contributions of Albert Kahn, who helped me get started, and the late Alex Lielausis, who taught me electron microscopy and helped me in innumerable ways.

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These investigations are being continued by Don Robberson, a graduate student in Dr. Hodge's group.

Ultracentrifuge Runs

summary of analytical data

log no	pH	buffer	protein conc.	Mol. Wt.	sample treatment
F-232	6.95	0.6 NaCl 0.1 phosphate	4.6 mg/ml	144,000	
F-238	3.4	0.1 NaCl	5.2	80,000	
F-288	3.4	-----	4.3	16,000	
F-289	3.4	0.15 NaCl	4.8	100,000	
F-296	3.4	0.05 NaCl	4.7	101,000	
F-340	7.2	0.6 NaCl 0.06 phosphate (Buffer I)	3.4	151,000	dialyzed against buffers I→III→I
F-341	3.3	0.1 K Acetate (Buffer II)	5.3	90,000	dialyzed against buffers II--III--II
F-342	7.4	6 M G-HCl 0.1 phosphate		25,000 and 35,000 @ 15,220 rpm 120,000 @ 21,740	
F-343	7.4	5 M G-SCN	6.4	120,000	
F-348	7.1	Buffer I	0.5	105,000 and 210,000 @ 18°C 105,000 @ 35°C	
		sample extracted by Riddiford's method (see appendi			
F-350	7.4	5M G-SCN 1M $NH_2OH \cdot HCl$	1.6	23,000 and 32,000 @ 10,509 rpm 112,000 @ 15,220 rpm	
F-351	7.3	Buffer I	3.0	73,000	digested with .05% trypsin - 2 hr. @ 35°

All runs made on Hodge's Beckman Model E Analytical Ultracentrifuge "Felicity"

Temperature 20° C unless otherwise noted.

Equilibrium established by taking two pictures at 12 hour intervals, during which time the pattern does not change.

Where the molecular weight is quoted for two centrifuge speeds, the higher speed represents the Yphantis condition (protein concentration → 0 at the meniscus)

 Hodge's Extraction Procedure

II. PREPARATIVE METHODS AND ELECTRON MICROSCOPIC TECHNIQUE

A. Purification of Paramyosin Fibrils

The adductor muscles of the clam, Venus mercenaria, were used as the source of material throughout the present investigation, firstly because previous work has been mainly concerned with paramyosin from this source, and secondly because Venus is available in quantity throughout the year.

Fresh clams were cracked open and the adductors excised, care being taken to remove as much adhering mantle and other material as possible. Since the white part of the adductor is considerably richer in paramyosin than the yellow, it alone was usually employed in the preparation of suspensions of paramyosin fibrils. The separation of the two parts is quite simple since they are clearly demarcated. The white parts of the adductors were then placed in ice-cold 0.3 M KCl solution to await further treatment. The most effective way of liberating the paramyosin fibrils from the intact muscle structure was homogenization in a Waring blendor for a short time. In a typical procedure, 15 to 20 grams of the white material was blended in 200 ml. of .3 M KCl for about one minute. The resulting homogenenate was then centrifuged in the cold in the International refrigerated centrifuge at about 4000 rpm for ten minutes. This was sufficient to pack the paramyosin fibrils, together with larger aggregates and cell membranes, etc., at the bottom of the tube, leaving a somewhat turbid

supernatant, which was discarded (except in a few instances when it was retained for experimentation). The sediment was easily separated at this stage into a lighter and heavier fraction since the larger aggregates, etc., tended to pack very firmly at the extreme bottom of the tube, the lighter material being more easily dispersed on the addition of further KCl solution. Both fractions were redispersed in KCl solution by stirring and use of a syringe. The suspensions were then centrifuged under identical conditions, and again the separation into light and heavy fractions carried out in each case. This procedure of centrifugation and resuspension was carried out five or six times, or until the supernate appeared to be free of dissolved protein. By this differential method it was possible to obtain two suspensions, one containing only relatively fine paramyosin fibrils (as judged by EM examination, Fig. 3), the other, coarser fibrils and partially broken muscle fibers, etc. The suspensions were then stored in the cold. It was found that although the use of low temperatures was desirable in the above procedure in order to minimize the possibility of denaturation, it was not really necessary, since no differences were observed in the properties of suspensions prepared at room temperature.

B. Preparation of Paramyosin Solutions

The thick white suspensions prepared by the above method could be brought into solution merely by the addition of acid, usually acetic acid, since this has some buffering capacity in

Appendix-- Rudiford's Extraction prodedure

Preparation of the Protein --The preparative method of Johnson et al. (1959) was used with a few changes (Szent-Gyorgyi, 1959). The umbo portion of the shell adjacent to the dorsal margin was cracked, and pieces of the shell chipped off, proceeding both anteriorly and posteriorly. The white portion of the two adductor muscles was separated from the pink-tinted portion. The excised muscle was placed in a beaker surrounded by ice water, and 10 volumes of cold 0.1M KCl were added. The following steps were done at 4° C. except where noted. All centrifugation was done in a refrigerated Servall centrifuge at 0° C. at 3000 rpm except where noted. After blending for one minute in the Waring blendor, the suspension was centrifuged for two hours. The precipitate was washed three times with 20 volumes of cold 0.1M KCl and centrifuged for 10 minutes after each washing. All supernatants were discarded. After extraction for 15 minutes with gentle stirring in three volumes of 0.6M KCl, 0.01M pH 7.5 Tris buffer, the extract was separated by filtration through cheesecloth[2] and the residue discarded. Three volumes of 95% ethanol were added to the extract at room temperature (25° C.) to denature the actomyosin. After two hours at room temperature, the precipitate was separated by a 30 minute centrifugation, resuspended in 0.6M KCl, 0.01M pH 7.0 phosphate buffer and dialysed for 24 hours against 6 volumes of the same solvent. Centrifugation (after dialysis) for 10 minutes at 10,000 rpm sufficed to remove the last traces of precipitated denatured actomyosin. The supernatant was then dialysed against three volumes 0.01M pH 6.0 phosphate buffer for 24 hours. The paramyosin crystals were recovered by 10 minute centrifugation at 10,000 rpm and subsequently redissolved in 0.6M KCl, 0.01M pH 7.0 phosphate buffer. To recrystallise the protein, the pH 7.0 solution was dialysed against 6 volumes 0.01M pH 6.0 phosphate buffer for 30 hours. The crystals were treated as before except that, after they were redissolved, the solution was lyophilised. This procedure yielded a stable form of the protein which was stored in the lyophilised state at 4° C.