Physicochemical Studies on Bovine Cardiac Tropomyosin*

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ABSTRACT: Bovine cardiac tropomyosin has been isolated in homogeneous form using the Bailey procedure with trace metal-free $(NH_4)_2SO_4$ and dithiothreitol in all preparative solutions. The resulting preparation has been subjected to a detailed physicochemical investigation using such techniques as sedimentation velocity, viscometry, Archibald ultracentrifugation, osmometry, and optical rotatory dispersion. The molecular weight of the monomeric protein is about 70,000 from osmometry, Archibald ultracentrifugation, and sedimentation diffusion, and the hydrodynamic data are compatible with the representation of the cardiac tropomyosin molecule as a rod 496 A long and about 20 A in diameter. Optical rotatory dispersion measurements indicate that the molecule is essentially a complete α helix in solution. This observation and the mass and diameter per unit length found suggest a molecular structure for cardiac tropomyosin of a double-stranded α-helical coiled coil. Osmometric measurements in 6.67 M urea–0.1 M β-mercaptoethanol indicate that the two polypeptide chains may be dissociated from one another, and that the molecular weight of the subunits is 37,000, about one-half the value deduced for monomeric tropomyosin. Since amino acid analyses on performic acid oxidized and carboxymethylated cardiac tropomyosins reveal that the native protein contains 5–7 free SH groups/10^5 g and no disulfide bridges, it seems that the two chains are not covalently linked by disulfide bands. In view of the essential parallel physicochemical behavior of cardiac and skeletal tropomyosins in high ionic strength neutral solutions and denaturing media, it is concluded that they are virtually identical in terms of molecular size, shape, secondary and tertiary structures, and substructure.

Although the tropomyosin of skeletal muscle has been extensively studied and characterized, much less is known about the corresponding protein from cardiac muscle. A comparative physicochemical study by Katz and Converse (1964) suggested that the two proteins possessed identical sedimentation constants and intrinsic viscosities, and from these parameters a molecular weight of 54,800 was calculated for both. Since this study, the molecular weight of rabbit skeletal tropomyosin has been redetermined as 64,000–74,000 using the more elaborate techniques of sedimentation equilibrium and light scattering (Woods, 1965; Holtzer *et al.* 1965). Woods (1965, 1966) has also demonstrated that the native skeletal tropomyosin molecule can be dissociated in 8 M urea containing 0.1 M mercaptoethanol into subunits of molecular weight between 30,000 and 35,000. In addition, important improvements in the tropomyosin preparative procedure of Bailey (1948) have been made by Mueller (1966), who suggested the use of highly purified ammonium sulfate, free of trace contamination with heavy metals, as well as the incorporation of a sulfhydryl protective reagent, 0.5 mM dithiothreitol (DTT),[1] in all preparative solutions. The studies of both Woods and Mueller suggested that in the absence of these precautions, the SH groups of tropomyosin are easily autoxidizable and that during preparation higher molecular weight species may result due to the formation of disulfide bridges.

In view of the recent improvements in preparative techniques as well as methods of macromolecular characterization (in particular, osmometry), it seemed opportune to undertake a detailed physicochemical study of the molecular state of cardiac tropomyosin in solution. We wish to present here a complete summary of sedimentation, viscosity, and osmometry studies on bovine cardiac tropomyosin in neutral high ionic strength salt solutions as well as in a strongly denaturing medium (6.67 M urea containing 0.1 M mercaptoethanol). In addition, the cardiac tropomyosin molecule has been characterized by optical rotatory dispersion (ORD) studies, and the number of free SH groups/10^5 protein has been ascertained.

Experimental Section

Reagents. Distilled water was deionized before use. Guanidine·HCl (Eastman) was recrystallized from methanol and urea (Mann) was the highest purity available. Ammonium sulfate was Fisher Certified

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[1] Abbreviation used: DTT, dithiothreitol.

Reagent and heavy metal free. All other reagents were reagent grade.

Protein Preparation. Tropomyosin was prepared from beef cardiac muscle by the method of Bailey (1948) using ultrapure ammonium sulfate for the salting-out cycles, and by a modification of this procedure (Mueller, 1966), incorporating the SH-protecting reagent dithiothreitol (DTT) at a concentration of 5×10^{-4} M in all preparative solutions. Three isoelectric precipitations and three cycles of salting-out with $(NH_4)_2SO_4$ were employed. Prior to lyophilization the solutions of tropomyosin were chromatographed on DEAE-cellulose columns to remove nucleic acid contaminants. The ratio of the 277:260-mμ extinction after column passage was 2.0:2.1. The protein was lyophilized from a salt-free water solution and stored in a desiccator at 0°.

Concentration. Concentrations were measured routinely by ultraviolet absorption at 277 mμ, using an $E^{1\%}_{1\,cm}$ value of 3.45, determined independently in this study in the usual fashion. The determination of protein concentration in solutions containing large amounts of urea was carried out in the following fashion. Lyophilized tropomyosin was dissolved in 6.67 M urea–0.067 M potassium phosphate buffer (pH 7.4)–0.1 M β-mercaptoethanol. The resulting solution was then dialyzed against its respective solvent. A 1-ml sample of the dialysate and a 1-ml sample of the protein solution were separately introduced into preweighed beakers, and the beakers and their contents were dried to constant weight in a vacuum oven at 110°. By subtraction, the amount of protein in the complex solvent was thus readily determined. Accurate dilutions were then made on this stock solution, This method presumes that preferential binding of urea by cardiac tropomyosin is negligible, a conclusion reinforced by the study of Kay (1960), which suggested that the partial specific volume of skeletal tropomyosin decreases by less than 1% (from 0.732 to 0.728 ml/g) on transferring the native protein to 8 M urea. The proximity of the two values renders the error due to urea–protein interactions probably small. The extinction coefficient of cardiac tropomyosin in the urea denaturant was determined by measuring the optical densities of solutions made by quantitatively diluting protein solutions of known concentration with 6.67 M urea–buffer solvent. At the wavelength maximum of 277 mμ, the $E^{1\%}_{1\,cm}$ value was found to be 3.12. Alternatively, protein concentrations containing definite amounts of urea were prepared by the method suggested by Holtzer *et al.* (1965), wherein known amounts of stock protein solution and solid denaturant were introduced into common volumetric flasks and the contents dissolved and diluted to the mark with dialysate.

Osmometry. All measurements of osmotic pressure were performed in a Mechrolab Model 503 high-speed membrane osmometer. These studies were carried out at 5° using membranes of type B19. When the concentration of the solution under study had to be changed, usually to higher values, it could be shown that readings following flushing with the more concentrated solution coincided with measurements obtained after flushing with solvent, checking solvent level, and then flushing with the more concentrated solution. The concentration-dependent reciprocal number-average molecular weight, π/RTc, where π is the osmotic pressure, was calculated as $(\rho h/RTc)$ where ρ (g cm^{-3}) is the density of solvent in the osmometer elevator, h (centimeters) is the height of the solvent column, and c (g l.$^{-1}$) is the solute concentration. For the solvent system 1 M KCl–0.067 M phosphate buffer (pH 7.4), RT/ρ was evaluated as 2.247×10^4 l. cm mole^{-1}, and for 6.67 M urea–0.067 M phosphate buffer (pH 7.4)–0.1 M β-mercaptoethanol as 2.129×10^4 l. cm mole^{-1}. It was assumed that the apparent reciprocal number-average molecular weight, $(1/\bar{M}_{n(c)})$, was given by

$$\frac{1}{\bar{M}_{n(c)}} = \frac{\pi}{RTc} = \frac{1}{\bar{M}_{n(o)}} + Bc \qquad (1)$$

The number-average molecular weight $\bar{M}_{n(o)}$ (g mole^{-1}), as well as the second virial coefficient B could thus be established. Of interest in this connection is the recent successful determination of $\bar{M}_n$ for myosin using the same apparatus and approaches (Tonomura *et al.*, 1966).

Viscosity measurements were made with Ostwald–Fenske viscometers at 20 ± 0.02°. Flow times for solvents were in the range of 100–130 sec. No kinetic energy corrections were made. In these measurements, as in osmometry and optical rotatory dispersion, the solvents were rendered dust free by passing them through Millipore filters of pore size 0.45 μ. The protein solutions were passed through 5-μ Millipore filters for clarification purposes.

Ultracentrifugation. A Spinco Model E analytical ultracentrifuge equipped with an electronic speed control was used throughout. Sedimentation velocity runs were carried out at 60,000 rpm for aqueous solutions, and in the case of urea solutions at 68,000 rpm using a titanium rotor (type H) and sapphire windows in the cell. Most runs were carried out in standard Kel f cells, but in some cases experiments were performed in a double-sector cell, with the respective solvent in one sector. Sedimentation coefficients were determined at or near 20°, and the usual corrections were made for solvent viscosity and density. The latter quantities were obtained from Timmermans' (1960) tables or in the case of urea from the data of Kawahara and Tanford (1966).

The partial specific volume of beef cardiac tropomyosin in 1 M KCl–0.067 M potassium phosphate buffer (pH 7.4) was assumed to be identical with that of rabbit skeletal tropomyosin in the same solvent, and was taken as 0.733 ml/g at 20° after Kay (1960).

Apparent diffusion constants were evaluted from sedimentation velocity measurements using a method described by Pickels (1942). Normally at the concentrations required for the schlieren optical system and at the high speeds employed during the run, a sedimenting boundary often shows a marked self-sharpening, and, before a meaningful diffusion coefficient can be

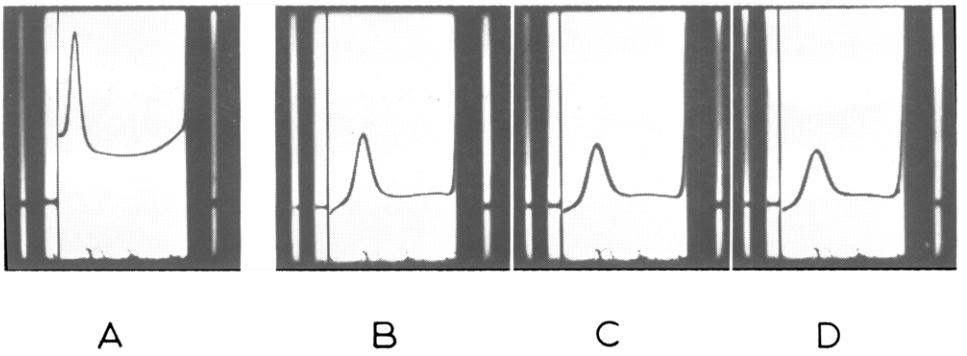

FIGURE 1: Representative sedimentation velocity patterns of bovine cardiac tropomyosin in 1 M KCl 0.067 M potassium phosphate buffer (pH 7.4) during the two phases of the Pickels-type run. Pattern A corresponds to the high-speed phase of the run (60,000 rpm) while patterns B, C, and D were obtained at 0, 32, and 64 min after deceleration and speed maintenance at 8000 rpm.

derived from the boundary spreading, a large correction based on a knowledge of the dependence of sedimentation upon concentration has to be made (Fujita, 1956). Schachman (1951), in a discussion of boundary spreading experiments with tobacco mosaic virus, drew attention to an earlier paper by Pickels (1942), in which it was suggested that the effects of self-sharpening could be circumvented by studying boundary spreading under low-speed ultracentrifugation, after the boundary had been brought into view at a higher speed. With present day improvements in ultracentrifugation and in particular the problem of rotor precession at the low speeds required for these studies eliminated through the use of the electronic speed-control system, it seemed feasible to invoke the method of Pickels in our studies for these reasons, and also because of its relative simplicity. A similar approach using the light absorption method has been used by Möller (1964) for evaluating the diffusion coefficients of mono- and paucidisperse systems containing ribonucleic acids. The method has also been applied successfully for the evaluation of the diffusion constants of wheat germ tRNA (Kay and Oikawa, 1966) and Mengo virus (Scraba *et al.*, 1967).

In the present work, the diffusion experiments were performed in two phases of the same operation: a relatively short, high-speed ultracentrifugation at 60,000 rpm in order to bring the tropomyosin into view, followed by a 2–3-hr, low-speed centrifugation at 8000 rpm, suitable for analysis of the boundary spreading by the maximum-ordinate area method as suggested by Fujita (1962). The speed of 8000 rpm was sufficiently low to permit an analysis of the diffusion process uninfluenced by back diffusion, and the system showed no evidence of self-sharpening (see Figure 1). The method was initially applied to two reference proteins in our laboratory, bovine fibrinogen and bovine fetuin, for which apparent corrected diffusion coefficients at 20° of 1.88×10^{-7} and 5.51×10^{-7} cm^2/sec, respectively, were calculated, in good agreement with values obtained from free diffusion experiments (Shulman, 1953; Green, 1963).[2] After standardizing the normal sedimentation constants and diffusion coefficients to water at 20°, molecular weights were calculated from the Svedberg equation (Svedberg and Pedersen, 1940).

As a check on this procedure, molecular weight determinations by the Archibald technique were carried out as outlined by Schachman (1957), with the cell-top pictures only. The initial concentration was obtained from a separate ultracentrifuge run with a standard 12-mm Kel f cell. The speed of the ultracentrifuge was 12,000–14,000 rpm. Measurements of the concentration gradients were carried out as described by Kay *et al.* (1961). No appreciable salt distribution was detected at any of the speeds employed.

Optical Rotatory Dispersion. A Cary Model 60 recording spectropolarimeter was used for these measurements in accordance with methodology described by Kay and Oikawa (1966). A 1-cm path-length cell was employed in the visible region of the spectrum, and 1-cm and 0.1-cm cells were used in the ultraviolet. The ORD data were analyzed in terms of the Moffitt equation (Moffitt and Yang, 1956), the protein conformational Cotton trough and peak at 233 and 198 mμ, respectively (Davidson *et al.*, 1966), and the equations given by Shechter and Blout (1964).

Amino acid analyses were performed on a Beckman–Spinco Model 120B amino acid analyzer according to Spackman *et al.* (1958). Hydrolysis of samples of native, performic acid oxidized and carboxymethylated tropomyosin was carried out in 6 N HCl at 110°. Times of hydrolysis ranged from 10 to 40 hr. Per-

[2] While it is recognized that considerable uncertainty exists in the resultant apparent D values due to the presence of the high concentration of salt, as well as the considerable asymmetry in the macromolecule, the same approach has been applied successfully to other asymmetric protein systems such as myosin in 5 M guanidine·HCl (Kielley and Harrington, 1960) and *Escherichia coli* ribosomal proteins in 8 M urea, with a frictional ratio of 3.05 (Möller and Chrambach, 1967).

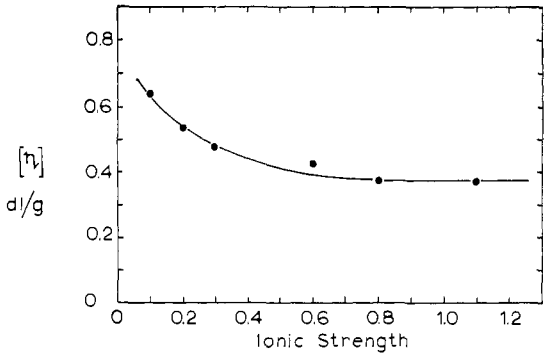

FIGURE 2: Plot of the weight intrinsic viscosity, $[\eta]$, of bovine cardiac tropomyosin as a function of ionic strength.

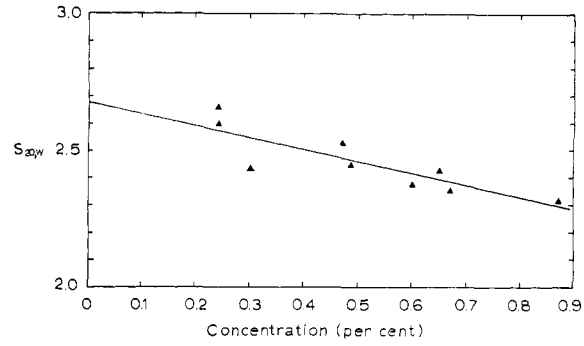

FIGURE 4: Sedimentation coefficients of bovine cardiac tropomyosin in 1 M KCl–0.067 M potassium phosphate buffer (pH 7.4).

formic acid oxidation was effected in 80% formic acid at $-5°$ for 4 hr in order to obtain as cysteic acid all the cystine and cysteine present in the protein. Carboxymethylation was achieved by treating the protein in 8 M urea with a large excess of iodoacetamide. The excess iodoacetamide was removed with a thiol (DTT), and the solution was dialyzed against water followed by lyophilization.

Results

Viscosity. Because of the tendency of skeletal tropomyosin to undergo aggregation with decreasing ionic strength, it was of importance to establish if the same phenomenon occurred with the cardiac homolog, as well as to select an optimum solvent system in which to study the molecular kinetic properties of monomeric tropomyosin. Viscosity was the technique of preference in view of its significance in molecular characterization and its sensitivity to the presence of aggregates. The results of viscosity measurements as a function of ionic strength at pH 7.4 are displayed in Figure 2. It is to be noted that the weight intrinsic viscosity, $[\eta]$ at pH 7.4, decreases with increasing ionic strength, becoming constant after 0.6 M. That the variation in viscosity is due to aggregation and not to a conformational change is attested by the constancy of the trough of the protein conformational Cotton effect at 233 $m\mu$; $[m']_{233}$ is $-15{,}000°$, and remains invariant over the entire ionic strength range examined. A minimum $[\eta]$ of 0.37 dl/g is recorded in solutions of ionic strength in excess of 0.6 M, and since the sedimentation patterns in the same media suggest a single peak, it is concluded that this limiting viscosity value corresponds to the monomeric state. A representative plot of the reduced specific viscosity as a function of concentration for cardiac tropomyosin in 1 M KCl–0.067 M potassium phosphate buffer is shown in Figure 3. The data, linear over the entire concentration range, were found to fit the Huggin's (1942) relationship, from which an intrinsic viscosity of 0.37 dl/g and a Huggin's constant, k', of 2.56 were calculated. It is to be noted that the Huggin's constant is considerably in excess of 1 in the solvent system showing the minimum viscosity. This is reminiscent of a similar finding with skeletal tropomyosin (Holtzer *et al.*, 1965), and is not indicative of aggregation since molecular weight measurements in this solvent system are completely self-consistent and show no anomalous concentration dependence (see below). In order to ensure that all physicochemical

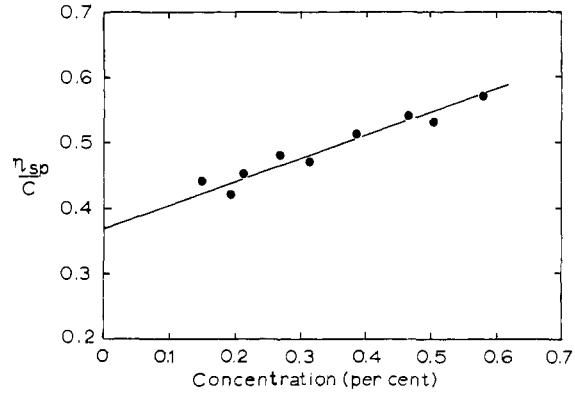

FIGURE 3: Plot of the reduced specific viscosity of bovine cardiac tropomyosin in the monomeric solvent as a function of concentration.

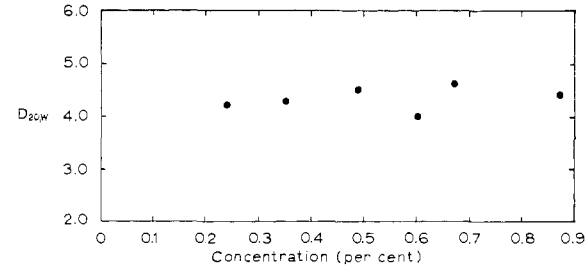

FIGURE 5: The apparent diffusion coefficients of monomeric bovine cardiac tropomyosin as a function of concentration.

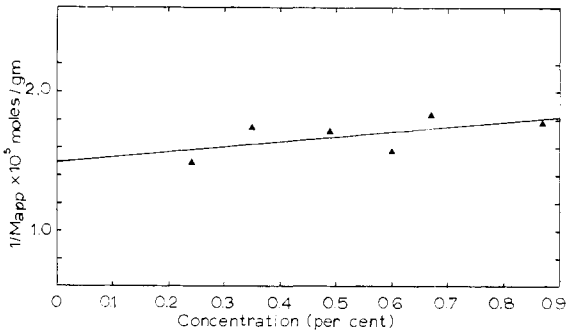

FIGURE 6: Concentration dependence of the reciprocal of the apparent molecular weight (obtained by the Svedberg equation) of cardiac tropomyosin in 1 M KCl–0.067 phosphate buffer (pH 7.4).

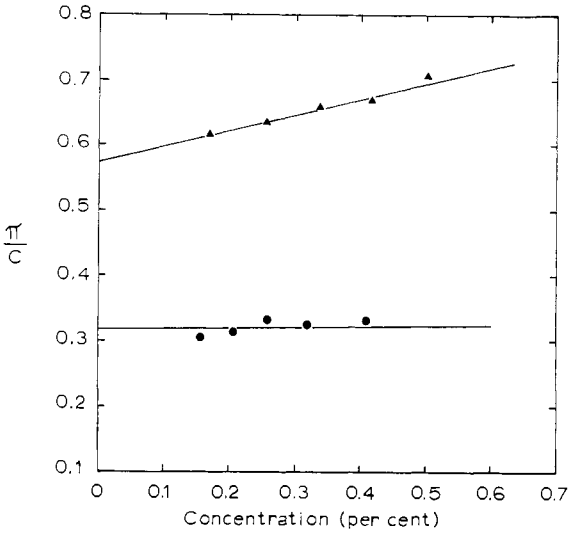

FIGURE 7: Reduced osmotic pressure of cardiac tropomyosin as a function of concentration in: (a) 1 M KCl–0.067 M phosphate buffer (pH 7.4) (●——●), and (b) 6.67 M urea–0.067 M phosphate buffer (pH 7.4)–0.1 M β-mercaptoethanol (▲——▲).

measurements on native cardiac tropomyosin pertain to the monomeric state, they were confined exclusively to studies of the protein in a solvent medium of 1 M KCl–0.067 M potassium phosphate buffer (pH 7.4), hereafter referred to as the monomeric solvent.

Sedimentation Velocity. All the cardiac tropomyosin samples used in this study satisfied the criterion of sedimentation as a single peak in the ultracentrifuge when observed in neutral high ionic strength solutions (see Figure 1a). Results of some of the sedimentation velocity measurements are presented in Figure 4. For the native monomeric protein the intrinsic sedimentation constant, $s^0_{20,w}$, is 2.68 S and the equation for the regression line is

$$s_{20,w} = 2.68\ (\pm 0.06) - 0.45\ (\pm 0.1)c \qquad (2)$$

where c is the protein concentration in g/100 ml.

Diffusion. Measurements of diffusion constants of cardiac tropomyosin at differing concentrations in 1 M KCl–0.067 M potassium phosphate (pH 7.4) are summarized in Figure 5. The apparent diffusion coefficients at the various protein concentrations and the calculated $s_{20,w}$ values at the same concentrations were inserted into the Svedberg equation in order to evaluate apparent molecular weights. Figure 6 illustrates the concentration dependence of the reciprocal of the apparent molecular weight of cardiac tropomyosin in the monomeric solvent. Least-mean-squares treatment of the data results in a calculated molecular weight average of 66,700 ± 5400.

Osmometric Measurements. Figure 7 illustrates that the concentration dependence of osmotic pressure for cardiac tropomyosin fits the linearized form of eq 1. Least-squares fits to the data resulted in a number-average molecular weight, $\bar{M}_n$, for cardiac tropomyosin in the monomeric solvent of 70,600 ± 4000 amu and a second virial coefficient of 4.08×10^{-4} mole cc/g^2.

Figure 7 also reveals the results of osmometry of cardiac tropomyosin in a solvent consisting of 6.67 M urea–0.1 M β-mercaptoethanol–0.067 M phosphate buffer (pH 7.4). The molecular weight in this medium, evaluated from the limiting ordinate intercept, is 37,000 ± 1000 amu, essentially the same value obtained by Woods (1966), using sedimentation equilibrium, for skeletal tropomyosin in a similar solvent system.

Archibald Measurements. The molecular weight data for native, monomeric tropomyosin, obtained by the Archibald method, are summarized in Figure 8. Least-mean-squares treatment of the data shows M = 68,000 ± 4500 and the second virial coefficient is essentially zero.

It should also be noted that if beef cardiac tropomyosin is prepared by the classical Bailey (1948) method, without the incorporation of a SH-blocking reagent such as DTT, the resulting preparation is invariably aggregated. Figure 9 summarizes the concentration dependence of the reciprocal of the apparent molecular weight (obtained by the Archibald method) for beef cardiac tropomyosin (prepared in the absence of DTT) in 1 M KCl–0.067 M potassium phosphate buffer (pH 7.4). The resulting weight-average molecular weight is 100,000 and the intrinsic sedimentation constant (3.08 S) is also higher than that of SH-protected tropomyosin (Figure 10).

ORD measurements on native monomeric cardiac tropomyosin suggest that almost all of the molecule is in the α-helical configuration. A summary of the relevant parameters, presented in Table I, indicates that calculations of apparent α-helix content from Moffitt treatment, the ultraviolet conformational Cotton effects at 233 and 198 mμ, and Shechter–Blout analysis are all self-consistent and result in a helical content of about 92%. In the denaturing solvent, 6.67 M urea–0.1 M β-mercaptoethanol, the resulting subunits are essentially

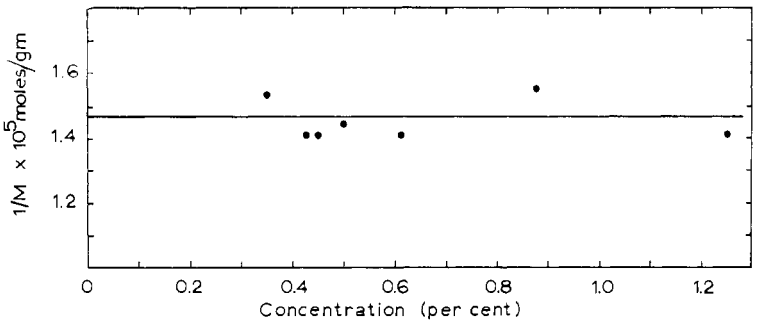

FIGURE 8: Reciprocal of the apparent weight-average molecular weight of cardiac tropomyosin as a function of concentration by the Archibald method.

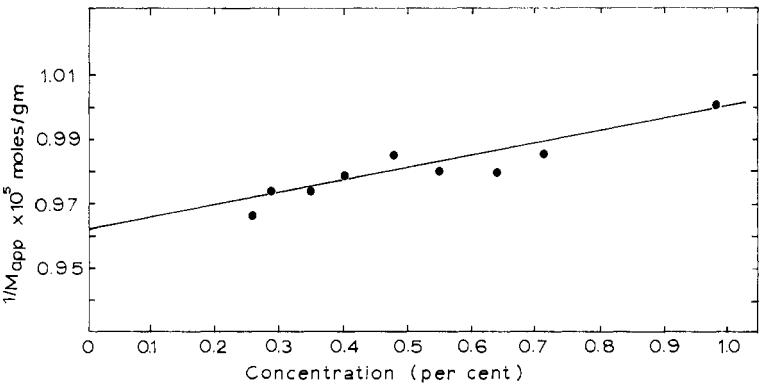

FIGURE 9: Reciprocal of the apparent molecular weight (Archibald method) for bovine cardiac tropomyosin, prepared in the absence of dithiothreitol. Solvent system is 1 M KCl–0.067 M phosphate buffer (pH 7.4).

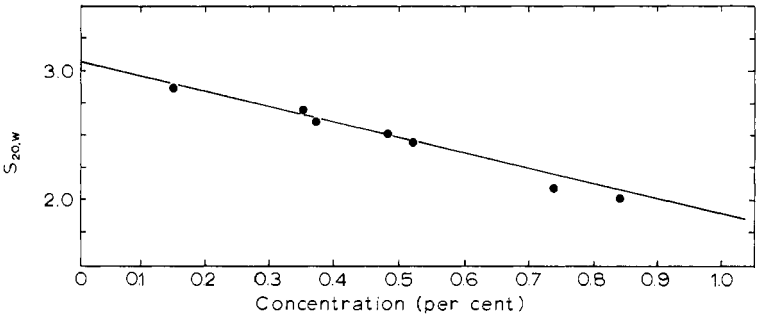

FIGURE 10: Plot of the corrected sedimentation coefficient as a function of concentration for cardiac tropomyosin prepared in the absence of dithiothreitol.

in the random coil form, as shown by a dispersion constant (λ_c) of 219 mμ and a corresponding calculated helical content of about 5%.

Amino Acid Analysis. An amino acid analysis of cardiac tropomyosin after performic acid oxidation suggested that there are 5–7 moles of cysteic acid produced/100,000 g of protein. Amino acid analysis of tropomyosin, after treatment with iodoacetamide in 8 M urea, indicated that there were 5–6 moles of carboxymethylated cysteine/100,000 g of protein. It would thus appear that native cardiac tropomyosin possesses some 5–7 free SH groups/10^5 g of protein and no disulfide bridges.

Urea-Reversibility Experiments. The dissociation of cardiac tropomyosin into its subunits was found to be a reversible process. This was demonstrated by exposing the protein for 6 hr at room temperature to the solvent: 6.67 M urea–0.1 M β-mercaptoethanol, followed by exhaustive dialysis for 36 hr *vs.* 1 M KCl–0.067 M phosphate buffer (pH 7.4) plus 5×10^{-4}

TABLE I: Optical Rotatory Dispersion Parameters of Beef Cardiac Tropomyosin in 1 M KCl-0.067 M Potassium Phosphate Buffer (pH 7.4).

Parameter	Value
a_0 (deg)	+36
b_0 (deg)	−601
(% helix)$_{b_0}$	94
A_{225} (deg)	−1,960
A_{193} (deg)	2,807
H_{225} (%)	95
H_{193} (%)	97
$[m']_{233}$ (deg)	−14,870
(% helix)$_{233}$	92
$[m']_{198}$ (deg)	68,700
(% helix)$_{198}$	92

M DTT. The ORD Moffitt parameters of this preparation were established as: $a_0 = -28°$ and $b_0 = -540°$, corresponding to about 85% α-helix, essentially the same value as recorded for the native molecule. Archibald ultracentrifugation of this material at a concentration of 0.55% resulted in an apparent molecular weight of 74,000, which fell on the $(1/M)$ *vs.* c plot shown in Figure 8 for the protein not exposed to the dissociating solvent. If the urea-treated protein was dialyzed against the monomeric solvent in the absence of a SH-blocking reagent, a higher molecular weight product (about 90,000) resulted, suggesting that some random oxidation of SH groups to disulfide bridges had occurred.

Discussion

The molecular weights found in this study for bovine cardiac tropomyosin dissolved in 1 M KCl–0.067 M potassium phosphate buffer (pH 7.4) by the techniques of osmotic pressure, sedimentation diffusion, and Archibald ultracentrifugation are quite self-consistent, yielding an average value of about 70,000. The agreement between the results from the various techniques is certainly within the ±10% that is regarded as satisfactory in macromolecular physical chemistry. The value reported here is considerably larger than the 54,800 value calculated by Katz and Converse (1964) from sedimentation viscosity data and using the β function of Scheraga and Mandelkern (1953). The lack of agreement is not surprising if one considers that β is derived for a particular hydrodynamic model, an idealization which may not be applicable to the real protein molecule. When accurate values are desired, the only recourse is the use of absolute methods of molecular weight determination such as were used herein.

It is to be noted that while there is very little difference in the extrapolated molecular weights deduced by osmometry and Archibald ultracentrifugation, the second virial coefficient, B, from osmometry is appreciably larger than that determined from the Archibald measurements. Whether this is an inherent artifact or expresses a variable interparticle interaction differing from preparation to preparation is difficult to say.[3] A similar variability in the slope term derived from osmometric and Archibald molecular weight data has also been noted for skeletal myosin (Tonomura *et al.*, 1966).

Since the cardiac tropomyosin molecule is almost completely α-helical, a rodlike model may be employed to represent its solution properties. The measured viscosity corresponds to an axial ratio of 24.6, using the equation of Kirkwood and Auer (1951) for the intrinsic viscosity of elongated particles. The measured sedimentation coefficient, used with the axial ratio from viscosity in the sedimentation equation of Kirkwood and Riseman (1950), gives a diameter of 20.2 A. The length of the tropomyosin particle calculated from its axial ratio and diameter is 496 A. The partial specific volume, used in its equation along with the measured molecular weight and length (Holtzer and Lowey, 1959), gives a value for the diameter of 18.2 A, in fair agreement with the result deduced from the sedimentation viscosity relationships. The success of these calculations suggests that the hydrodynamic data are internally consistent insofar as the rod model is concerned and, further, the diameter so deduced (18–20 A) for cardiac tropomyosin is very similar to that previously calculated for skeletal tropomyosin (Holtzer *et al.*, 1965), *Venus mercenaria* paramyosin (Lowey *et al.*, 1963), and light meromyosin fraction I (Lowey and Cohen, 1962). It is noteworthy that the absolute value of the diameter in the latter cases is consistent with a side-to-side packing of two α-helices, and, in fact, two-chain molecules have been suggested for the substructure of these proteins.

The number of α-helical chains in cardiac tropomyosin may also be calculated. Taking the molecular weight as 70,000 and assuming an average amino acid residue weight of 115, the number of amino acid residues per molecule is calculated to be 609. Since the α-helical content is close to 100% and using an axial translation for the α-helix of 1.5 A, the length of α-helix in one molecule is $1.5 \times 609 = 914$ A. Dividing by the molecular length calculated from the rod model, 496 A, we obtain 1.85 α-helical chains/molecule, or an average of two chains within experimental error. Cardiac tropomyosin thus joins the other proteins noted above in that the molecule consists of two α-helical chains, side to side, and twisted about one another.

The osmotic pressure studies on cardiac tropomyosin

[3] The possibility also exists that the second virial coefficient estimated from Archibald experiments may be in error in the sense that apparent molecular weights were plotted *vs.* initial sample concentrations rather than corresponding meniscus concentrations. However, extrapolation to infinite dilution should lead to the correct molecular weight regardless of whether the apparent molecular weights are plotted against sample or meniscus concentrations.

TABLE II: Comparison of Physicochemical and Chemical Properties of Cardiac and Skeletal Tropomyosins.

Property	Cardiac Tropomyosin (this study)	Skeletal Tropomyosin	References
$s^0_{20,w}$ (S)	2.68	2.59	Holtzer *et al.* (1965)
$[\eta]$ (dl/g)	0.37	0.34	Holtzer *et al.* (1965)
$M_{\text{osomotic pressure}}$	70,600 ± 4,000		
$M_{\text{Archibald}}$	68,000 ± 4,500	72,000 ± 2,000	Holtzer *et al.* (1965)
$M_{S,D}$	66,700 ± 5,400		
$M_{\text{light scattering}}$		77,000 ± 10,000	Holzter *et al.* (1965)
$M_{\text{sed-equil}}$[a]		64,000	Woods (1966)
% helix	92	94	Cohen and Szent-Györgyi (1957)
$a:b$[b]	24.6	24.5	Holtzer *et al.* (1965)
Length of rod model (A)	496[c]	490[d]	Holtzer *et al.* (1965)
Mol wt in urea–mercaptoethanol	37,000 ± 1,000	30,000–35,000	Woods (1966)
No. of SH groups/10^5 g of protein	5–6	5–7	Mueller (1966); Drabikowski and Nowak (1965)

[a] Sedimentation equilibrium. [b] Axial ratio, $a:b$, from Kirkwood–Auer (1951) equations and $[\eta]$. [c] Length deduced from axial ratio and diameter calculated from Kirkwood–Riseman (1950) equation. [d] Length deduced from radius of gyration.

in 6.7 M urea–0.1 M β-mercaptoethanol suggest that the two polypeptide chains may be dissociated from one another, and that the molecular weight of the subunits is 37,000, about one-half the value (within experimental error) deduced for monomeric tropomyosin. A similar finding was noted by Woods (1966), using sedimentation equilibrium for skeletal tropomyosin. The dissociation is reversible on the basis of ORD and Archibald ultracentrifuge measurements. Since amino acid analyses on performic acid oxidized and carboxymethylated cardiac tropomyosins suggest that the native protein contains some 5–7 free SH groups/10^5 g and no disulfide bridges, it would appear that the two chains are not covalently linked by SS bonds. The incorporation of the reducing agent in the dissociating medium is to prevent recombination of the dissociated chains by random oxidation of the SH groups to form nonnative disulfide bridges. The two chains are no doubt stabilized in the native molecule largely by strong side-chain interactions of the hydrophobic and electrostatic type, which are destroyed in a strongly denaturing solvent such as urea. Further support for this view comes from preliminary molecular weight studies on cardiac tropomyosin in high ionic strength alkaline solutions (1 M KCl–0.1 N KOH, pH 13), which suggest that the subunit (34,000 ± 3000) is also produced in this medium.

Table II summarizes the physicochemical properties of cardiac tropomyosin deduced in this study with those reported by other investigators for the skeletal homolog. In view of the essential parallel behavior of the two proteins in high ionic strength neutral solutions and denaturing media, it is concluded that they are virtually identical in terms of molecular size, and secondary and tertiary structures, as well as substructure.

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Isolation and Partial Characterization of a New Amino Acid from Reduced Elastin*

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ABSTRACT: This paper describes the isolation of an amino acid, $C_{18}H_{34}N_4O_6$, which appears in the hydrolysis products of elastin from young animals when the protein has been treated with alkali followed by borohydride reduction. Like desmosine and isodesmosine the new amino acid incorporates the label from [^{14}C]lysine in tissue culture experiments. Evidence from the mass spectrum of the tetra-*N*-trifluoroacetyl tri-*n*-butyl ester (mass 954) indicates that the compound can be regarded as derived from three molecules of lysine with the loss of two nitrogen atoms. From its empirical formula and relationship to lysine a structure is proposed for the new amino acid and preliminary chemical and spectral evidence is given in support. To mark its structural relationship to the isomeric desmosines we propose to name the new compound merodesmosine.

Feeding experiments with labeled amino acids carried out by a number of authors have shown that the turnover rate of elastin becomes extremely slow as the animal reaches maturity and growth ceases. Walford *et al.* (1964) extended their observations with rats up to 930 days after the injection of [^{14}C]lysine and found that after an initial decline due to the growth of the rat during the first 120 days the radioactivity remained constant throughout life. These experiments suggest that apart from the repair of lesions, the elastic structures of the large blood vessels are retained unchanged once growth has ceased. However, it is equally clear that during the early life of mammals there is both growth and remodeling of elastin in structures such as the arterial walls (Gillman and Hathorn, 1958), and that in young animals the elastic tissue is in a state of active metabolism.

The problem of the biogenesis of elastin is complicated by the circumstance that in the mature protein the peptide chains are cross-linked by covalent bonds to form a continuous network (Partridge, 1962). It has become clear that elastin fibres are formed from a soluble precursor protein by a process of cross-linking which is dependent upon the oxidation of certain lysine side chains in the precursor molecule. The cross-links so formed are stable structures which retain their configuration after enzymic or acid hydrolysis of purified elastin (Partridge *et al.*, 1963). Two unusual amino acids, containing cyclized structures forming the link, have been

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