Substructure of the Paramyosin Molecule*

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ABSTRACT: Paramyosin is shown to consist of two covalent molecular subunits, each having molecular weight of about 100,000. Light-scattering molecular weight measurements and calculations of molecular weight using sedimentation–viscosity and viscosity–molecular weight relationships demonstrate that appropriate modification (reduction of disulfide bonds with β-mercaptoethanol and carboxymethylation to block the resulting sulfhydryl groups) and denaturation (with guanidine hydrochloride at 25°) separates the paramyosin molecule into the apparently monodisperse molecular units. Disulfide bonds, if present in the native molecule, are not required for maintenance of the native conformation, since light-scattering and ultracentrifugation measurements show that modified paramyosin has, in benign solvents, a molecular weight of 217,000 amu as does unmodified paramyosin. The intrinisic viscosity (215 cm^3/g), intrinsic sedimentation coefficient (3.0 S), and b_0 (−675°) of the modified and native paramyosin are also the same in benign media. Optical rotatory dispersion studies indicate that the disulfide bonding, if present in the native paramyosin, does not significantly alter the dependence on denaturant concentration of the guanidine hydrochloride induced denaturation of the protein at room temperature. Both the unmodified and the modified proteins exhibit a plateau at approximately 30% helix content between 3.5 and 5 M guanidine hydrochloride. Thus the existence of an extrastable portion of the paramyosin structure is confirmed and it is shown that disulfide bonding is not responsible for this structural stability.

In benign media[1] molecules of the protein paramyosin consist of two α helices arranged side by side and slightly twisted about one another (Lowey *et al.*, 1963; Cohen and Holmes, 1963) and this unit has a molecular weight of about 200,000 amu (Lowey *et al.*, 1963; Olander, 1969). Optical rotatory dispersion studies have shown that a portion of the paramyosin molecule possesses unusual conformational stability since appreciable helix content is found, at 25°, up to a guanidine hydrochloride concentration of 6.0 M (Cohen and Szent-Györgyi, 1957; Noelken, 1962; Riddiford, 1966; Olander, 1969). The presence of cysteine or cystine in paramyosin has not been unequivocally established; Riddiford and Scheraga (1962a) report about 8 sulfhydryl groups and possibly 1 disulfide bond per molecule while Szent-Györgyi *et al.* (1959) found at most one sulfhydryl group per molecule and no evidence for a disulfide bond.

This paper presents evidence bearing on the following questions. (1) Is the paramyosin molecule one, long, α-helical, polypeptide chain, or is it two or more separate helical chains arranged to form the side-by-side helices of the native molecule? (2) Are there any covalent cross-linkages such as disulfide bonds (cystine) between the parallel segments? (3) Are such cross-linkages, if any, required to maintain the native conformation? (4) Do such cross-linkages, if any, have influence on the extrastable portion of paramyosin's structure?

Experimental Methods

Reagents. During most of this study, guanidine hydrochloride (Gdn·HCl) was prepared from ethanol-recrystallized guanidine carbonate (Eastman Highest Purity, and, later, Matheson Coleman and Bell's Superior Grade, which was of better quality) by the method of Anson (1941). If a 5 M solution of the product had a pH no less than 5 and an optical density of less than 0.1 at 277 nm in a 1-cm cell, further purification was deemed unnecessary since these specifications ensured that the concentration of impurity was so low it did not effect the spectrophotometric measurements of protein concentrations, the only quantity found to be adversely effected by the impurity in Gdn·HCl. More recently, Mann Research Laboratories has produced an ultrapure grade of Gdn·HCl which is far superior.

Matheson Chromatoquality reagent β-mercaptoethanol, Eastman Organic Chemicals' iodoacetic acid, and Baker and Adamson Biological grade KCl were used. All other chemicals used were reagent grade or the equivalent.

Distilled water was passed through an activated charcoal column, then an Amberlite MB-1 mixed-bed ion-exchange column before use.

Protein Preparation. 1. NATIVE PARAMYOSIN. Paramyosin was prepared from whole adductor muscles of *Venus mercenaria* according to the procedure described by Johnson *et al.* (1959) and by Noelken (1962). The protein was usually lyophilized from $(KCl)_{0.6}(K[PO_4])_{0.06}(7.0)$[2] and stored at −20°. If the intrinsic viscosity in KCl solvents of the para-

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[1] Benign media is a term used to designate solutions, such as 1.0 M KCl–0.1 M $K[PO_4]$, pH 7.4, or 0.6 M KCl–0.06 M $[PO_4]$, pH 7.4, in which the protein has its native conformation, *i.e.*, media similar to physiological solutions. Gdn·HCl is used as an abbreviation for guanidine hydrochloride.

[2] $K[PO_4]$ is used to signify a mixture of potassium phosphates. The frequent necessity of referring to complex aqueous solvent media makes shorthand notation desirable. Such a solvent will be designated by writing the chemical formula (or name) of each component (omitting water) with its molarity as a subscript, followed by parenthetical specification of pH (Holtzer *et al.*, 1965).

myosin preparation was within experimental error of 192 cm^3/g (Lowey *et al.*, 1963) and/or if its schlieren sedimentation peak was symmetrical at concentrations ranging from 0.15 to 0.60%,[3] the preparation was considered satisfactory.

2. "MODIFIED" PARAMYOSIN (REDUCTION AND CARBOXYMETHYLATION). The reduction of disulfide bonds (cystine) was accomplished by treatment with β-mercaptoethanol and the carboxymethylation of free sulfhydryl groups (cysteine) with iodoacetate according to a procedure based on methods which had been developed for other proteins (Cecil, 1963; Anfinsen and Haber, 1961; White, 1961; Thompson and O'Donnel, 1961; Gundlach *et al.*, 1959; Sela *et al.*, 1959). No attempt was made to quantitatively determine whether any free sulfhydryl group remained after the carboxymethylation through the conditions used for the reaction, as described below, are sufficiently extreme that all the free sulfhydryls are expected to be blocked.

Paramyosin was dissolved in $(Gdn\cdot HCl)_{7.5}(K[PO_4])_{0.1}$-(6.0–6.4) (plus small amounts of KCl sometimes) at a concentration of 0.6–1.0%. One of two procedures was then followed. Either: (a) this solution was made 0.2–1.0 M in β-mercaptoethanol, by pipetting in the required amount of the concentrated reagent, and the reduction reaction was allowed to proceed 12 hr at room temperature; or, (b) the solution was dialyzed against the same solvent as above which had, however, been deoxygenated by flushing with N_2 and which contained β-mercaptoethanol so that the final solution was 0.3 M in the reducing agent. The dialysis was continued overnight at room temperature. Procedure b was used to make sure that the addition of the liquid β-mercaptoethanol directly to a protein solution did not denature the paramyosin and to make sure that the oxygen present in the protein solution (one cannot bubble N_2 through paramyosin solutions without denaturing the protein) did not decrease the effectiveness of the reducing procedure. No difference between the protein reduced by procedures a and b was detected.

The reduction was stopped and all free sulfhydryl groups carboxymethylated in the following manner. A solution containing a number of moles of iodoacetate equal to twice the number of moles of β-mercaptoethanol in the protein solution was prepared by dissolving the required amount of iodoacetate acid in 45% KOH plus just enough water for dissolution and bringing the pH to about 8.0 with 45% KOH. The iodoacetate and protein solutions were mixed under a stream of nitrogen to prevent any reoxidation of the protein, and the pH was adjusted to 8.0–8.1 with about 4 M KOH (delivered from a buret with an immersed tip, in order to provide rapid mixing of the concentrated KOH). The pH adjustment and reaction time were carefully monitored in order to minimize carboxymethylation of amino groups on the protein.

After exactly 15 min, the carboxymethylation was stopped by adding β-mercaptoethanol, providing a twofold molar excess over the iodoacetate. The pH was adjusted to about 6.2 and the mixture was allowed to stir under N_2 for 0.5 hr at room temperature, in order for the β-mercaptoethanol to react with all the excess iodoacetate.

The reduced and carboxymethylated (modified) protein was then exhaustively dialyzed *vs.* $(KCl)_{1.0}(K[PO_4])_{0.1}$(7.4 or 8.5) to remove the excess modifying reagents, or it was annealed (see below). It was then ready for use, and was either used directly or lyophilized.

[3] The designation of concentration in per cent means g of isoionic protein per 100 ml of solution.

There were two annealing procedures used. (a) The protein solution was dialyzed against fresh and successively more dilute solutions of Gdn·HCl down to 3 M, allowing 1–3-days dialysis at each concentration; *i.e.*, the incremental dilutions varied from ~0.5 M decreases of Gdn·HCl with 1–2-days dialysis against a single solution at each concentration to ~1.0 M decreases of Gdn·HCl with 3–4-days dialysis at each concentration. Thus, the Gdn·HCl and the β-mercaptoethanol concentration were lowered gradually. (b) The protein solution was dialyzed against a 5 M Gdn·HCl solution which was gradually diluted in roughly the same kind of steps as in (a) with $(KCl)_{1.0}(K[PO_4])_{0.1}$ (7.4) again allowing 1–3-days dialysis at each Gdn·HCl concentration. The major difference between the two methods is that in b, the β-mercaptoethanol concentration is always much higher than in a. The β-mercaptoethanol is essentially undetectable in the final 3 M Gdn·HCl dialysate of annealing procedure a, but its odor can quite easily be detected (in fact, the β-mercaptoethanol concentration was at least 0.1 M) in the final dialyzate of procedure c. The important difference between the procedures is that in b the β-mercaptoethanol is diluted and not removed as it is in a. The final step in both cases is, as in the ordinary procedure, exhaustive dialysis against the KCl solvent.

Protein Concentration Determination. In KCl solvents, protein concentrations were determined by the micro-Kjeldahl method, using 18.2% as the nitrogen content (Kominz *et al.*, 1957; Bailey and Ruegg, 1960) or from the optical density at 277 nm using extinction coefficients based on micro-Kjeldahl analyses. The extinction coefficient (ϵ_{277}) was found to be 3.24 dl g^{-1} cm^{-1} for native paramyosin in $(KCl)_{1.0}$-$(K[PO_4])_{0.1}$(7.4) or $(KCl)_{0.6}(K[PO_4])_{0.06}$(7.4). The extinction coefficient for modified paramyosin varied from one modification preparation to another over a ± 2% range around 3.39 dl g^{-1} cm^{-1} in the same solvents.

Analysis of the protein content of Gdn·HCl solutions was made by measurement of optical density. The extinction coefficients in 7 and 8 M Gdn·HCl were determined as described by Holtzer *et al.* (1965) while the procedure for measuring those at lower Gdn·HCl concentrations was altered in that pipetted amounts of an 8 M Gdn·HCl stock solution were used. The extinction coefficients of unmodified and modified paramyosin differ by about 5% and both were found to decrease linearly with increasing concentration of Gdn·HCl. For the unmodified protein ϵ_{277} follows the equation $\epsilon_{277} = 0.035$ (M Gdn·HCl) + 3.24 while for the modified samples the equation becomes $\epsilon_{277} = 0.34$ (M Gdn·HCl) + 3.39.

Viscosity. Viscosities of protein solutions in benign media were measured using Ostwald-Fenske viscometers at 20.0 ± 0.02°, while those of paramyosin in Gdn·HCl solvents were measured using either Ostwald-Fenske or Ubbelohde semimicro dilution viscometers at 25.0 ± 0.02°. The viscometer capillary size was chosen so that solvent flow times were always greater than 100 sec and solution flow times were at least 50 sec greater than solvent flow times. Solvents and paramyosin solutions in high concentrations of Gdn·HCl were cleaned of particulate matter for viscometry by filtration through Corning medium-porosity, sintered-glass filters. Solutions of the protein in benign media and those containing low concentrations of Gdn·HCl were cleaned by centrifugation for 2 hr at 40,000*g* and removed from the tubes by careful pipetting.

Optical Rotatory Dispersion. The Rudolph automatic recording spectropolarimeter (260/655/850/810–614), equipped

with a high-pressure, high-intensity xenon–mercury lamp, was used to measure the optical rotatory dispersion of precentrifuged paramyosin solutions (concentrations of 0.2–0.8%). All measurements were done at room temperature using a 0.50-dm cell with quartz windows. Readings were taken at wavelengths corresponding to the mercury emission lines in the range 302–578 nm, which have relatively high intensities, thus improving spectral purity. The instrument was repeatedly checked against the standard quartz plates with which it is equipped, and the monochromator was readjusted when necessary. On one occasion a Cary 60 spectropolarimeter was used in a parallel optical rotatory dispersion experiment to check the Rudolph instrument; satisfactory agreement was obtained.

The parameter $-b_0$ was calculated from the data using Moffit's equation (Moffit, 1956), taking λ_0 equal to 213 nm and M, the mean residue weight, equal to 115 (Bailey, 1948; Hodge, 1959). The required values of refractive indices of the Gdn·HCl solvents were obtained from measurements reported by Kielley and Harrington (1960). No dispersion corrections were made, nor was any adjustment used to account for the added KCl and phosphate buffer since such corrections do not alter the b_0 results significantly. A linear least-squares fit of the optical rotatory dispersion data to the Moffit equation was used to determine $-b_0$. This parameter has been taken as proportional to the helix content (Noelken, 1962; Nagasawa and Holtzer, 1964) with a value of about $-600°$ indicating 100% helix while values close to 0° indicate no helix content.

Ultracentrifugation. 1. INTRINSIC SEDIMENTATION COEFFICIENTS. The Beckman Spinco Model E analytical ultracentrifuge was operated, with schlieren optics, either at 59,780 or 60,000 rpm and at 20°. Cells used were 4°, 12-mm standard and wedge quartz windowed, single-sector type, though occasionally double-sector cells and/or sapphire windows were used. The usual corrections for solvent density and viscosity were made in calculations of the intrinsic sedimentation coefficient. The apparent partial specific volume, $\bar{v}$, is taken to be 0.730 ml/g at 20° for paramyosin in KCl and Gdn·HCl solvents (Lowey *et al.*, 1963; Kielley and Harrington, 1960; Holtzer *et al.*, 1965).

2. ARCHIBALD DETERMINATION OF MOLECULAR WEIGHTS. Two procedures were used. One employing only schlieren optics has been described elsewhere (Lowey and Holtzer, 1959; Lowey and Cohen, 1962; Lowey *et al.*, 1963). To obtain the molecular weight, $1/M_{app}$ was plotted against the meniscus concentration and extrapolated to zero concentration. Again $\bar{v} = 0.730$ ml/g was used.

The second procedure, which involves a combination of schlieren and interference optics, has been suggested and its use demonstrated by Richards and Schachman (1959) and Chervenka (1966). The technique is described in detail in Olander (1969).

3. HIGH-SPEED SEDIMENTATION EQUILIBRIUM DETERMINATION OF MOLECULAR WEIGHTS. The meniscus depletion method was used exactly as described by Yphantis (1964). Details concerning the experimental procedure and the methods of reading the plates and making the calculations appear in Olander (1969).

Light Scattering. The instrumentation and techniques are essentially those employed previously (Holtzer and Lowey, 1959; Holtzer *et al.*, 1962; Schuster, 1963; Holtzer *et al.*, 1965). Light-scattering erlenmeyer cells with no back-reflection correction were used for measurements of paramyosin in KCl solvents; cells with slight back-reflection [*i.e.*, cells having a dissymmetry measured in a semioctagonal cell, which has no back-reflection (Tomimatsu and Palmer, 1963; Holtzer *et al.*, 1965)] were occasionally used with 6.0 M Gdn·HCl solvent where the dissymmetry of the protein solutions is so small that this slight back-reflection could not matter. This was done since, at the time of these experiments, only one cell with no back-reflection was available.

Dust-free water was obtained from an all-glass still which has been described by Schuster (1963). Because all distilled, deionized water we could obtain routinely contained some kind of oily organic matter which apparently dispersed as small particulates in the distillate, the feed water had to be treated before introduction into the light-scattering still. The treatment is detailed in Olander (1969).

The solvents and paramyosin solutions in 6 M Gdn·HCl were cleaned by filtration through Corning ultrafine fritted filters (nominal pore size 0.9–1.4 μ) under a pressure of about 12 psi of N_2 (see description in Hawkins, 1967). The ultracentrifugation method of cleaning was used for paramyosin in KCl (Schuster, 1963).

The light-scattering data were treated exactly as described by Holtzer *et al.* (1965).

The refractive index increment in KCl for the native paramyosin is 0.179 ± 0.001 ml/g (Lowey *et al.*, 1963) and the same value was used for the modified protein. For the 6 M Gdn·HCl medium, the refractive index increment was estimated since the light-scattering experiment was done only to determine if, in this medium, the paramyosin molecule would separate into constituent chains and, if so, the number of such chains per paramyosin molecule; it was felt that the error incurred in such an estimation would not be so great as to obscure the information desired. The calculation of the refractive index increment is based upon the Gladstone–Dale equation and data available for tropomyosin; the details are presented in Olander (1969). The estimated value used was 0.129 ml/g.

Results

Unmodified Paramyosin in Guanidine Hydrochloride Medium at 25°. Optical rotatory dispersion measurements performed with solutions of unmodified paramyosin containing Gdn·HCl at concentrations greater than or equal to 6.0 M gave b_0 of $0 \pm 20°$ indicating zero helix content.

The intrinsic viscosities of the unmodified protein in $(Gdn \cdot HCl)_{7.5}(KCl)_{0.2}(K[PO_4])_{0.03}(7.4)$ are extremely irreproducible, covering a range of values from 78 to 153 cm^3 per g; the values obtained depend markedly on the duration of dialysis used in preparing the solution, on the age of the protein sample, and on the particular paramyosin preparation. The probable cause of this irreproducibility is aggregation caused by disulfide-bond formation and/or interchange which can take place above pH 6 and is favored in denaturing media (Cecil, 1963; Thompson and O'Donnel, 1961). The viscosity experiments described below support this explanation.

Because of the irreproducibility of the intrinsic viscosities, no attempt was made to measure a molecular weight of the unmodified paramyosin in high Gdn·HCl concentrations.

Modified Paramyosin in Guanidine Hydrochloride Medium at 25°. As is detailed in the Experimental Methods section, modified paramyosin has all of its disulfide linkages reduced to sulfhydryl and then all the sulfhydryls blocked with carboxymethyl groups.

Again b_0 is found to be $0 \pm 20°$ at Gdn·HCl concentrations equal to or greater than 6.0 M.

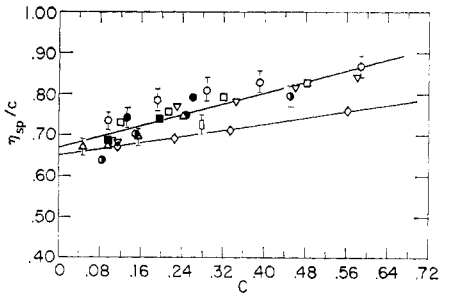

FIGURE 1: Viscosity data for modified paramyosin in $(Gdn\cdot HCl)_{7.5}(KCl)_{0.2}(K[PO_4])_{0.03}$(7.4) at 25°. ◊ designates unmodified paramyosin dissolved in $(Gdn\cdot HCl)_{7.5}(KCl)_{0.2}(K[PO_4])_{0.03}$($\beta$-mercaptoethanol)$_{0.1}$(7.4). All the other symbols designate different preparations of modified paramyosin: 5 modifications were made on paramyosin preparation V and 3 on preparation VI; c is in per cent.

The viscosity of unmodified paramyosin in $(Gdn\cdot HCl)_{7.5}(KCl)_{0.2}(K[PO_4]_{0.03}$($\beta$-mercaptoethanol)$_{0.1}$(7.4) solution is displayed in Figure 1. The intrinsic value of 65 cm³/g is seen to be much lower than any value obtained in the denaturing solvent in the absence of the disulfide-bond reducing agent, β-mercaptoethanol. This clearly shows that disulfide bonding is present in such paramyosin solutions. Figure 1 also presents the viscosity data for eight different preparations of modified paramyosin in $(Gdn\cdot HCl)_{7.5}(KCl)_{0.2}(K[PO_4])_{0.03}$-(7.4). In contrast to results for the unmodified protein, these data are reproducible, giving an intrinsic viscosity of 68 cm³/g, a value in close agreement to that obtained above for denatured paramyosin in the presence of β-mercaptoethanol. Intrinsic viscosities of modified paramyosin were also measured at intermediate Gdn·HCl concentrations; these data are presented in Table I.

In the 7.5 M Gdn·HCl medium, the intrinsic sedimentation coefficient is 2.6 S. In $(Gdn\cdot HCl)_{3.0}(KCl)_{1.0}(K[PO_4])_{0.1}$(7.4), $s^0_{20,w}$ is 2.8 S and careful examination of the schlieren patterns, obtained using double-sector ultracentrifuge cells, shows single symmetrical peaks, *i.e.*, there is no evidence that two components are present (the marked concentration dependence exhibited by all sedimentation data of paramyosin could mask such evidence).

The number of amino acid residues (n) is obtained from the viscosity of the completely denatured modified paramyosin in 6 M Gdn·HCl (65 cm³/g) using Tanford's (Tanford *et al.*, 1967a) determination of the dependence of viscosity on chain length. Then, taking 870 as the number of residues/10^5 g of paramyosin (Kominz *et al.*, 1957; Bailey and Ruegg, 1960) a molecular weight of 106,000 is calculated. A molecular weight of 92,000 is obtained from the Scheraga–Mandelkern (1953) relationship taking $[\eta]$ = 65 cm³/g, $s^0_{20,w}$ = 2.6 S, and $\beta = 2.5 \times 10^6$ (for a coiled polymer).

Light scattering was used to obtain an estimate of the molecular weight of modified paramyosin in $(Gdn\cdot HCl)_{6.0}(KCl)_{0.5}(K[PO_4])_{0.01}$(7.4), by direct measurement, which was sufficiently accurate to distinguish between molecular units the size of the native molecule (200,000), one-half this size (100,000), and one-third the native size (67,000). For this purpose a calculated estimate of the $\Delta n/dc$ (see Experimental Methods section) was employed to obtain the data displayed in Figure 2. An analysis of the data (as described in Holtzer *et al.*, 1965) shows that for zero angle the curve is represented by $Kc/R_0 = 0.89 \times 10^{-5} + 2(5.82 \times 10^{-4})c + 3(8.67 \times 10^{-3})c^2$ which gives a molecular weight of 102,300. Any reasonable curve through the data will give a weight close to this value and any error in the $\Delta n/dc$ estimate is not expected to change the final result sufficiently either; *i.e.*, a weight of 67,000 could not be obtained. Furthermore, this light-scattering molecular weight is in close agreement to the indirectly determined molecular weights reported above. It is apparent, then, that the molecular unit of the modified paramyosin in 6 and 7.5 M Gdn·HCl is one-half as massive as the native molecule.[4]

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TABLE I: Intrinsic Viscosities of Modified Paramyosin.

Solvent	$[\eta]$ (cm³/g)
$(KCl)_{1.0}(K[PO_4])_{0.1}$(7.4) and $(KCl)_{0.6}(K[PO_4])_{0.06}$(7.4)	213[a]
$(Gdn\cdot HCl)_{2.0}(KCl)_{0.5}(K[PO_4])_{0.1}$(7.4)	158[b]
$(Gdn\cdot HCl)_{2.0}$ + ?[c]	118[b]
$(Gdn\cdot HCl)_{3.0}(KCl)_{1.0}(K[PO_4])_{0.1}$(7.4)	95
$(Gdn\cdot HCl)_{4.0}$ + ?[c]	83
$(Gdn\cdot HCl)_{4.0}(KCl)_{1.0}[K(PO_4])_{0.1}$(7.4)	77
$(Gdn\cdot HCl)_{5.0}(KCl)_{0.5}(K[PO_4])_{0.1}$(7.4)	83
$(Gdn\cdot HCl)_{5.5}(KCl)_{0.5}(K[PO_4])_{0.1}$(7.4)	66
$(Gdn\cdot HCl)_{6.0}(KCl)_{0.5}(K[PO_4])_{0.1}$(7.4)	70[d,e]
$(Gdn\cdot HCl)_{6.0}(KCl)_{0.4}(K[PO_4])_{0.1}$(7.4)	58[d]
$(Gdn\cdot HCl)_{7.5}(KCl)_{0.2}(K[PO_4])_{0.03}$(7.4)	68
$(Gdn\cdot HCl)_{7.5}(KCl)_{0.2}(K[PO_4])_{0.03}$-($\beta$-mercaptoethanol)$_{0.1}$(7.4)	65

[a] The viscosities in KCl media were measured at 20° while those in Gdn·HCl solutions were measured at 25°. [b] The discrepancy in the intrinsic viscosities measured in 2 M Gdn·HCl solutions may be the result of turbidity which sometimes occurs with paramyosin solutions of Gdn·HCl $\leq$ 3.0 M and which may not have been noticed. No explanation for this aggregation has been verified. [c] Measurement of these viscosities was made by students of M. F. Emerson at Webster College, St. Louis, Mo. The solvents were prepared by weighing Gdn·HCl into a graduated cylinder and diluting to volume with $(KCl)_{1.0}(K[PO_4])_{0.1}$(7.4) dialysate. Protein solutions were dialyzed against these solvents at least 3 days. [d] The discrepancy in the intrinsic viscosities measured in 6 M Gdn·HCl solutions is not understood though it is suspected that the 58 cm³/g value was obtained with a solution which was for some reason defective. [e] One of the protein samples used to obtain this intrinsic viscosity was modified and annealed by method a (see Experimental Methods section). The annealing had no noticeable effect on the measured viscosities. Also, three solutions were cleaned for light scattering and their viscosities were not affected.

[4] Direct measurement of the Archibald molecular weight of modified paramyosin in 7.5 M Gdn·HCl medium was unsuccessful because the extreme concentration dependence of the apparent molecular weight did not allow meaningful extrapolation to zero meniscus concentration. Furthermore, the high-speed sedimentation equilibrium (meniscus depletion) method of molecular weight measurement (Yphantis, 1964), which is ideally suited to highly nonideal systems because it allows the use of very low initial protein concentrations and thus minimizes the extrapolation errors, also failed. The method proved too sensitive to the small amounts of low molecular weight material which are inevitably present in paramyosin samples and to which the other physicochemical measurements here employed are insensitive.

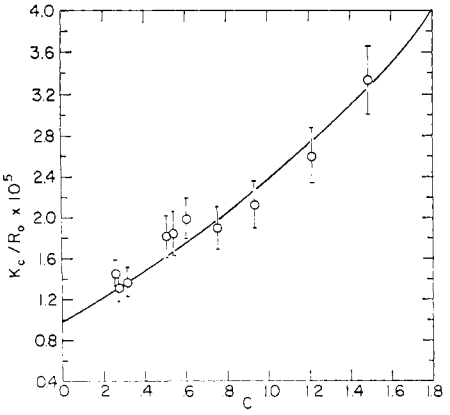

FIGURE 2: Concentration dependence of the zero-angle scattering of modified paramyosin in $(Gdn \cdot HCl)_{6.0}(KCl)_{0.5}(K[PO_4])_{0.01}(7.4)$. Each point represents a single solution; c is in per cent.

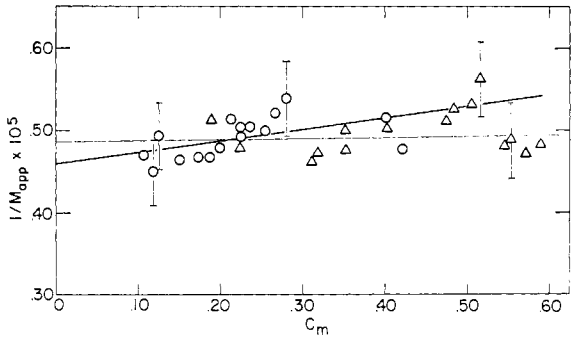

FIGURE 3: Archibald data for the modified and unmodified paramyosin in benign media at 20°. Opened circles designate data for native paramyosin in $(KCl)_{0.6}(K[PO_4])_{0.06}(7.4)$ obtained using schlieren optics and containing data from 5 runs done at varying speeds. Opened triangles designate data for modified paramyosin in $(KCl)_{1.0}$-$(K[PO_4])_{0.1}(7.4)$ obtained using the combined schlieren interference optics method with all 15 runs done at the same speed; c_m is in per cent.

lability, to make certain that the filtration technique used to clean the solutions for light scattering (see Experimental Methods section) does not degrade or aggregate the protein. This was checked in two ways. The viscosity of solutions cleaned for light scattering is indistinguishable from data obtained with solutions cleaned more gently (see Experimental Methods section for cleaning of solutions for viscometry). Furthermore, one experiment was performed where only ultracentrifugation was used to clean the solution for light scattering (c = 1.493% and Kc/R_0 = 3.340 in Figure 2) and it is seen to fall right on the curve with the rest of the data.

Modified and Unmodified Paramyosin in Benign Media. An intrinsic viscosity of 213 cm^3/g is obtained at 20° with 5 separate preparations of modified paramyosin dissolved in KCl–K[PO_4] pH 7.4 media. This value is the same at both the 1.27 and 0.76 ionic strengths used. This result is in very close agreement with the intrinsic viscosity of native paramyosin, 217 cm^3/g, measured under identical conditions and, in one case, obtained with a freshly prepared, unlyophilized protein sample.

Both forms of the protein have an intrinsic sedimentation coefficient of 3.0 S in $(KCl)_{1.0}(K[PO_4])_{0.1}(7.4)$.

If a b_0 of −675° is taken to represent 100% right-handed, α-helix content, then the optical rotatory dispersion data for unmodified paramyosin in benign media, indicate, within experimental error, 100% helix content in all the preparations used.[5]

The optical rotatory dispersion experiments performed with modified paramyosin in benign medium gave somewhat complicated results. It appears that full helix content is not regained after modification followed by direct dialysis of the modified protein from the reaction medium into benign media; such paramyosin samples have a b_0 of only about −518°. This finding led to the annealing procedures, described in the Experimental Methods section, on the supposition that if the chains were given more time amidst favorable surroundings, they might reassociate properly. After annealing the reduced and blocked protein by method a (dialysis into solvents of successively lower Gdn·HCl concentration) the b_0 for the protein in benign medium rose to an intermediate value of −604°, while method b (successive dilution of a 5 M Gdn·HCl solvent) brought the b_0 to a value of −659° which is in the range of b_0's found for the native paramyosin. Thus annealing does provide a way to regain the full helix content.

The implication of these annealing experiments, if they are taken at face value, appears to be that the process does increase the helix content, but that a relatively high concentration of β-mercaptoethanol (~0.1 M) is necessary for success, even though all the sulfhydryl groups on the protein have been blocked. This is a surprising result and it is likely that some factor other than the presence of β-mercaptoethanol is responsible for the greater success of annealing by procedure b over procedure a.

The molecular weights of modified and native paramyosin in benign media, as obtained by the Archibald method, are shown in Figure 3. The combined optics technique gives 205,000 amu as the molecular mass of the modified material with a second virial coefficient (B) of 0.07×10^{-4} mole-cm^3/g^2; this mass agrees very well with the 216,000-amu value obtained for the native protein using only schlieren optics, though B is 0.68×10^{-4} mole-cm^3/g^2 in the latter. A careful examination of Figure 3 reveals that the two sets of data appear, in fact, indistinguishable. The experimental error is such, that were the range of concentrations (0.1–0.4%) of the native protein solutions extended, the least-squares line of the resulting data might well coincide with the data for modified paramyosin which had been obtained over a wider range of protein concentrations (0.2–0.6%). Thus the second virial coefficient is probably closer to the 0.07×10^{-4} mole-cm^3/g^2 value for both forms of the protein.

The light-scattering molecular weight of modified paramyosin in $(KCl)_{1.0}(K[PO_4])_{0.1}(7.4$ or $8.5)$, independent of whether or not the protein had been annealed by method a, was found to be 228,000 ± 1700 and the light-scattering length of the molecule is 1100 Å (approximately 10 solutions were measured). This weight is somewhat higher than the 203,000 ± 2200 (L = 1200 Å) obtained with native, mostly unlyophilized paramyosin in $(KCl)_{1.0}(K[PO_4])_{0.1}(7.4)$ (approximately 20 solutions were measured). There is apparently some aggregation of the modified paramyosin in benign media. The reason for this has not been determined though it appears that if concentrations of β-mercaptoethanol less

[5] There was, however, a slight but consistent rise in b_0 values for each new preparation of protein; *i.e.*, earlier preparations have b_0 = −641° while the most recent one has a b_0 = −711°. The Rudolph polarimeter and lyophilization of the protein were both ruled out as causal factors of this drift which leaves the possibilities that in each successive preparation a little more of some impurity was removed or added, or that the mastery of the investigator increased.

TABLE II:[a] Molecular Parameters of Modified and Unmodified Paramyosin.

Medium[b]	$[\eta]^u$	$[\eta]^m$	$s^0_{20,w}{}^u$	$s^0_{20,w}{}^m$	M^u	M^m	% Helix^u	% Helix^m
Benign	217[c,d]	213[c,d]	3.0[c]	3.0[c]	216,000[d,e]	205,000[c,e]	100[c]	75–100[c,g]
					203,000[c,f]	228,000[c,f]		
(Gdn·HCl)	78[h]	68[h]		2.6[h]		102,000[i,f]	0[h,i]	0[h,i]
≥6.0 M	153	70[i]				106,000[i,j]		
						92,900[h,k]		

[a] Columns whose headings contain superscript m(u) refer to the property obtained when the modified (unmodified) protein is dissolved in the medium given. Intrinsic viscosities, $[\eta]$, are in cm³/g; intrinsic sedimentation coefficients, $s^0_{20,w}$, are in Svedbergs; % helix is based on the assumption that $b_0 = -675°$ corresponds to 100% helix content and $b_0 = 0°$ to zero helix content. [b] The detailed composition of the benign or Gdn·HCl solvent is given in a footnote to each piece of data. [c] $(KCl)_{1.0}(K[PO_4])_{0.1}(7.4)$. [d] $(KCl)_{0.6}(K[PO_4])_{0.06}(7.4)$. [e] Measured by the Archibald method. [f] Measured by light scattering. [g] The % helix depends on the method of modifying the paramyosin, *i.e.*, whether or not annealing was a part of the procedure. [h] $(Gdn\cdot HCl)_{7.5}(KCl)_{0.2}(K[PO_4])_{0.03}(7.4)$. [i] $(Gdn\cdot HCl)_{6.0}(KCl)_{0.5}(K[PO_4])_{0.01}(7.4)$. [j] Calculated from Tanford *et al.*'s (1967a) curve: $[n] = 0.716\ n^{0.66}$. [k] Calculated from the Sheraga–Mandelkern relation with $\beta = 2.5 \times 10^6$.

than 0.1 or greater than 1.0 M are used in the modification procedure or if modified material is carried through the entire modification procedure again, the aggregation becomes more severe.

Effect of the Modification on Paramyosin's Conformational Stability. Denaturation curves at 25° for both the unmodified and modified paramyosin are displayed in Figure 4. Both curves possess a plateau region between about 3.5 and 5 M Gdn·HCl. This region falls at approximately 30% helix ($b_0 = -200°$) for the unmodified and at 22% helix ($b_0 = -159°$) for the modified molecule. In view of the reproducibility of the data (including experiments performed with entirely different preparations) and of the expected error in b_0 measurements, the plateau must be real. The separation between the two curves as they are drawn appears to be just outside of experimental error; however, the b_0 values for the modified paramyosin consistently fall below those for unmodified paramyosin. It should be noted that no b_0's were measured for method b—annealed modified paramyosin at low Gdn·HCl concentrations and such b_0 values may fall closer to those of unmodified protein.

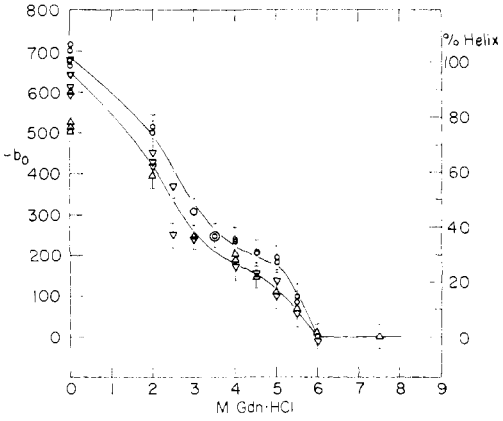

FIGURE 4: Denaturation profile of modified and unmodified paramyosin at room temperature. ○ designates unmodified paramyosin; △ designates modified paramyosin; and ▽ designates annealed modified paramyosin. Some averaging of the b_0 values in KCl and in 6 M Gdn·HCl was done in order to make the plot less cluttered. The greater scatter of some of the data in solutions ≤3.0 M Gdn·HCl may be the result of turbidity which sometimes occurs in such solutions with both forms of the protein. Solutions 0–4 M in Gdn·HCl also contained $(KCl)_{1.0}(K[PO_4])_{0.1}(7.4)$; solutions 4.5 and 5 M in Gdn·HCl contained $(KCl)_{0.5}(K[PO_4])_{0.1}(7.4)$; 5.5 M Gdn·HCl solutions had $(KCl)_{0.4}(K[PO_4])_{0.1}(7.4)$; 6.0 M Gdn·HCl solutions had either $(KCl)_{0.2}(K[PO_4])_{0.1}(7.4)$ or $(KCl)_{0.5}(K[PO_4])_{0.01}(7.4)$; and solutions 7.5 M in Gdn·HCl contained $(KCl)_{0.2}(K[PO_4])_{0.03}(7.4)$.

The molecular parameters deduced from several different kinds of measurements for both forms of paramyosin in benign and denaturing media are summarized in Table II.

Discussion

Characterization of Modified and Unmodified Paramyosin Dissolved in Guanidine Hydrochloride Media at Room Temperature. The presence of a small number of sulfhydryls and/or disulfide bonds in a molecule as large as paramyosin is difficult to detect from amino acid analysis, and the literature is understandably inconclusive on this point. The data of Riddiford and Scheraga (1962) indicate the presence of about 8 sulfhydryl residues per protein molecule (if one uses 200,000 as the molecular weight rather than their 330,000 value), and possibly one disulfide bond. Szent-Györgyi *et al.* (1959), on the other hand, find at most one sulfhydryl per molecule and no evidence for a disulfide bond. It should, however, be possible to establish the presence, if not the number, of interchain disulfide bonds by physical methods. With this purpose in mind, paramyosin dissolved in Gdn·HCl denaturing medium was modified (its disulfide bonds were reduced with β-mercaptoethanol and its sulfhydryls carboxymethylated with iodoacetate to prevent reoxidation); the intrinsic viscosity, sedimentation velocity, and light-scattering molecular weight of the resulting material were determined.

The intrinsic viscosity, 68 cm³/g, is considerably lower than the corresponding values for unmodified paramyosin in the same Gdn·HCl medium and, as detailed in the Results section, the intrinsic viscosity of this modified paramyosin

now becomes a reproducible quantity. Thus, intrinsic viscosity alone is sufficient to tell us that disulfide bonding must be present in paramyosin under the conditions of these experiments.

This change in intrinsic viscosity must reflect an underlying molecular change. The expected intrinsic viscosity of the completely denatured molecule, if it were a single polypeptide chain of the native molecular weight (200,000), would be 99 cm^3/g, using the Mark–Houwink coefficient determined by Tanford *et al.* (1967a). If the molecular unit, in this denatured state, is a chain half as large, one expects $[\eta] = 62$ cm^3/g. It appears, therefore, that the molecule splits into two chains when it is reduced in denaturing media.

Subsequent light-scattering, viscosity, and sedimentation experiments clearly show that at concentrations of Gdn·HCl greater than or equal to 6 M, where optical rotatory dispersion experiments indicate the protein contains no α helix, the molecular weight of modified paramyosin is indeed 100,000—one-half that of the native molecule (see Table II). This result has been confirmed recently by McCubbin and Kay (1969) who have reported the molecular weights of paramyosin in denaturing, reducing media determined with a membrane osmometer. They find values of 109,000 in $(urea)_{8.0}(KCl)_{0.1}$-$(K[PO_4])_{0.01}(dithiothreitol)_{5\times10^{-4}}(7.4)$ and 116,000 in $(Gdn\cdot HCl)_{6.0}(KCl)_{0.2}(K[PO_4])_{0.01}(dithiothreitol)_{5\times10^{-4}}(7.4)$. It is important to remember that 5×10^{-4} M dithiothreitol in the medium should *reduce* any existing cystines and prevent the oxidation of any cysteines (Cleland, 1964).

Optical rotatory dispersion measurements gave a b_0 of 0°, at room temperature, for both modified and unmodified paramyosin in 6 M Gdn·HCl. However, Noelken (1962) obtained a $b_0 = -117°$ at this Gdn·HCl concentration while Riddiford's data (1966) indicate that the protein is not completely denatured until a Gdn·HCl concentration of 7 M is reached. The discrepancy is not understood but it may arise from the difference in the methods of preparing the protein solutions. Noelken's method involved the dialysis of protein solutions against $(Gdn\cdot HCl)_{6.0}(K[PO_4])_{0.01}(7.4)$ for about 6 hr before measurement of b_0. Riddiford added paramyosin in $(KCl)_{0.6}(K[PO_4])_{0.01}(7.3)$ to weighted quantities of Gdn·HCl in a volumetric flask and then diluted the solutions quantitatively with the 0.6 M KCl dialysate;[6] measurement was made after a few minutes to a few hours of solution preparation. In the experiments reported here, a modification of Noelken's method was used: dialysis against 6 M Gdn·HCl solvent (which contained at least 0.4 M KCl and 0.01 M $K[PO_4]$) was continued for a minimum of 3 days before the optical rotatory dispersion measurements were made. It was found that considerable time, on the order of 36 hr or more, is required to reach dialysis equilibrium in concentrated Gdn·HCl solutions. It might be that to obtain complete denaturation in 6 M Gdn·HCl, some time or a considerable amount of added KCl is required.

In summary, both the unmodified and the modified proteins are completely denatured at room temperature in solutions which are 6 M or more in Gdn·HCl, and the molecular weight of the modified paramyosin is one-half that of the native molecule in these media.

Characterization of Modified and Unmodified Paramyosin in Benign Media at Room Temperature. The agreement between the Archibald molecular weight of the native paramyosin (216,000) and the light-scattering value (203,000) is very good. Furthermore, this agreement extends to the literature values as well; Lowey *et al.* (1963) obtained 211,000 and 250,000, by the same methods of measurement, while McCubbin and Kay (1968) got 212,000 ± 10,000 by osmometry.

TABLE III: Comparison of b_0 Values of Paramyosin in Benign Media.

$-b_0$	λ_0 (nm)	Reference
675	213	This work
540	213	Noelken (1962)
600	212	Riddiford (1966)
570	212	Riddiford and Scheraga (1962)
600	210	Cohen and Szent-Györgyi (1957)
650	210	Kay and Bailey (1959)
680	210	Lowey (1965)

The value found for the intrinsic viscosity (217 cm^3/g) and for the intrinsic sedimentation coefficient (3.0 S) of the native material are close to those (192 cm^3/g and 3.1 S) found by Lowey *et al.* The intrinsic viscosity (213 cm^3/g) and sedimentation coefficient (3.0 S) of the modified paramyosin in benign medium are the same as those of the native protein. This is also true of the molecular weight of the modified protein for which 205,000 is obtained by the Archibald method and 228,000 is gotten by light-scattering measurements (see Table II). The light-scattering length of the unmodified protein was found to be 1200 Å which is ~8% less than the 1300 Å reported by Lowey *et al.* (1963). The light-scattering length of the modified paramyosin is 1100 Å.

A comparison of the average b_0 value of −675° reported here for native paramyosin with values from the literature is presented in Table III. The variations in b_0 are greater than would result from the different λ_0's used. Though no causes for the variations have been examined, it seems possible that differences in the purity of the paramyosin preparations could lead to such changes.

Although molecular weight, length, and intrinsic viscosity assume the native values when paramyosin is modified and placed in benign medium, total helicity ($b_0 = -675°$) does not unless the modified protein is annealed. The need for a fairly high concentration of β-mercaptoethanol (~0.1 M) in the annealing medium is not understood, nor has it been firmly established that β-mercaptoethanol is uniquely necessary. Alternative annealing methods were not studied. The effect of annealing is manifested only in the optical rotatory dispersion measurements; the molecular weight and length of the modified protein in KCl (measured by light scattering) is unchanged by annealing. Thus, when placed in benign medium, the modified paramyosin essentially regains its native conformation which means that the disulfide bonding, if it is present in the native state, is not required to maintain this conformation.

The possibility remains, as pointed out earlier (Olander *et al.*, 1967), that the two chains may be invariably parallel, or invariably anti-parallel, in every native molecule, whereas the modified chains might recombine in both ways, *i.e.*, indiscriminately under the experimental conditions used. Such a subtle difference would not be apparent from the

[6] In Riddiford's method of solution preparation, the KCl concentration varied from solution to solution though it is estimated, from experience gained by working with concentrated Gdn·HCl solutions, that her 6 M Gdn·HCl solutions contained somewhat less than 0.4 M KCl.

physicochemical experiments described here. However, if Crick's (1953) suggestion that a coiled coil owes its stability to knobs-into-holes packing is correct, then it is unlikely that any side-to-side packing other than that characteristic of the native protein would be very stable.

Comparison of the Guanidine Hydrochloride Denaturation at Room Temperature of Modified and Unmodified Paramyosin. The presence of the plateau region in the b_0 *vs.* Gdn·HCl curve for unmodified paramyosin confirms the existence of an extrastable, α-helical structure in this molecule, first noted by Cohen and Szent-Györgyi (1957) and Noelken and Holtzer (1964). The plateau shown here falls at about 30% helix and between 3.5 and 5 M Gdn·HCl, while the helix content of the protein drops to zero at 6 M Gdn·HCl. The plateau which Noelken and Holtzer (1964; Noelken, 1962) use as a basis for the postulation of an extrastable α helix falls at 23% helix and between 3.5 and 6 M Gdn·HCl at room temperature; a 7 M Gdn·HCl (rather than 6 M) concentration is required to obtain a b_0 equal to zero. Riddiford (1966) reported finding two plateaus; one located between 1.5 and 2.5 M Gdn·HCl and the other occurring at 3.5–4.5 M Gdn·HCl. The denaturation curve did not attain its final value until a 7 M Gdn·HCl concentration was reached. The differences between the denaturation curve reported here and those just described may be the result of differences in the preparation of the protein solutions (discussed earlier in this section) and/or differences among the protein preparations themselves. For example, the first plateau reported by Riddiford was not observed either by Noelken or by the author because, at Gdn·HCl concentrations less than 3 M, precipitation took place before the b_0 measurement could be made.

There is a plateau in the denaturation profile of modified paramyosin which falls at the same place as that for unmodified protein (3.5–5 M Gdn·HCl), but which has a slightly lower b_0 value (−150°). The entire denaturation curve for modified paramyosin falls slightly below and closely parallel to that of the unmodified protein. Though this difference appears to be real experimentally, it need not reflect a correspondingly lower helix content of the protein. While it might be argued that breakage of a disulfide bond and carboxymethylation of reactive groups could decrease the stability of the helix, it seems peculiar that, if the molecule did become less stable, its denaturation profile should parallel that of the unmodified paramyosin so closely. One should also recall that Noelken found the plateau in the denaturation profile of unmodified paramyosin to center around 23% helix; *i.e.*, the same helix content as is found here for the stable region of the modified protein. This alone should make one suspicious of interpretations, in terms of helix content changes, of such small differences in b_0 as are involved here. The decrease in b_0 upon modification of paramyosin cannot be ascribed simply to changes in residue rotations caused by the breakage of disulfide bonds in the molecule. Tanford *et al.* (1967b) and Coleman and Blout (1968) have shown that the reduction of a disulfide bond leads to a more positive specific rotation at any wavelength in the 250–589-nm range; the specific rotation becomes more negative when paramyosin is modified.[7]

[7] Tropomyosin's b_0 denaturation profile also shows a drop in the b_0 values upon modification of the protein. M. F. Emerson (personal communication) calculated that the difference in rotation at 436 nm of this protein on reduction is *much* larger and in the opposite direction to that expected for the breakage of one disulfide bond per molecule.

It is, thus, shown clearly that disulfide bonding is not responsible for the unusually stable portion of the α-helical structure in paramyosin, since the plateau is found with or without such bonds being present. Additional support for this conclusion would be confirmation of the preliminary evidence which indicates the presence of plateaus between 4 and 5 M Gdn·HCl in the intrinsic viscosity denaturation profiles of both forms of the protein.

Naturally one would next like to answer questions such as these. (1) At what Gdn·HCl concentration do the chains of modified paramyosin separate? (2) How "cooperative" is the process? (3) Does the extrastable portion of the molecule's α helix remain after the chains have separated? Some viscosity and sedimentation data are reported here for the modified paramyosin at intermediate Gdn·HCl concentrations, but these are insufficient to answer any of the above questions. Ambiguities inherent in interpreting the viscosity and sedimentation results in terms of single- or double-chain molecules make it clear that to get a proper answer to such questions will require extensive absolute molecular weight measurements at a variety of Gdn·HCl concentrations.

In summary, the existence of the extrastable portion of the α helix has been confirmed for native paramyosin and found in modified paramyosin.

Conclusions

Paramyosin is composed of two α-helical polypeptide chains and contains sulfhydryl groups capable of interchain bonding in the native state. Such bonding may be present, but is not *required* for maintenance of the native conformation nor does it significantly alter the stability of the protein with respect to Gdn·HCl denaturation. That the paramyosin molecule, both native and modified, has an extraordinarily stable α-helical region is confirmed by the plateaus found in the optical rotatory dispersion denaturation profiles. It has been established here that disulfide bonds are not responsible for this stable region. To regain 100% of the native b_0 after the protein has been modified and separated into two randomly coiled chains, the molecule must be annealed as it reassembles in benign medium.

Whether or not paramyosin's two chains are identical has not been investigated. It would be most interesting to see if they can be separated (perhaps by ion-exchange column chromatography) and, after successful separation, to characterize them. It might be possible to obtain data relevent to this question by isolation of peptides containing the thiol groups in paramyosin and determination of their sequences using the techniques applied to myosin by Weeds and Hartley (1968).

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