von Hofsten, B., van Kley, H., and Eaker, D. (1965), *Biochim. Biophys. Acta 110*, 585.
Waley, S. G., and Watson, J. (1953), *Biochem. J. 55*, 328.
Woods, K. R., and Wang, K. I. (1967), *Biochim. Biophys. Acta 133*, 369.
Yamada, S., and Itano, H. A. (1966), *Biochim. Biophys. Acta 130*, 538.

Susceptibility of Paramyosin to Proteolysis and the Relationship to Regions of Different Stability†

Robert W. Cowgill

ABSTRACT: Similar proteolytic-resistant cores were obtained by digestion of paramyosin from the adductor muscles of the clam, *Mercenaria mercenaria*, with a wide variety of enzymes (chymotrypsin, nagarse, pepsin, Pronase, and trypsin). Detailed studies were made on paramyosin pepsin-resistant core (PPC) and paramyosin trypsin-resistant core (PTC). Both cores were from the N-terminal portion of paramyosin and Cys was the only N-terminal amino acid found. Both cores were completely helical by circular dichroism and fluorescence, and the high intrinsic viscosities of 120 cm^3/g at pH 7.4 and 157 cm^3/g at pH 2.0 were consistent with the helical structure. Reversibility of denaturation (5 M guanidine hydrochloride (Gdn·HCl), pH 2.0, 55°) as well as sodium dodecyl sulfate gel electrophoresis indicated that each core consisted of two intact polypeptide chains. Determination of molecular weight by three methods gave the same average value of 140,000 for both cores. It is concluded that PPC and PTC are identical except for minor differences at their C-terminal ends. Proteins similar to PPC or PTC could not be detected in extracts of adductor muscle of *M. mercenaria*. Regions in the paramyosin molecule differ in their stability to heat and Gdn·HCl. The C-terminal region is least stable and the N-terminal region is most stable. The C-terminal one-third of paramyosin lost its helical structure upon heating at pH 2 (transition temperature 57°) whereas PPC and PTC (from the N-terminal two-thirds of paramyosin) were stable over the region 20–70°. Denaturation by Gdn·HCl at pH 2 and 20° occurred in two major stages at 5 and 7 M Gdn·HCl. From changes in fluorescence, it was estimated that the loss of helical structure in 5 M Gdn·HCl was 64% for paramyosin and 34% for PPC and PTC; again, the C-terminal segment seemed least stable. In 7 M Gdn·HCl both paramyosin and the cores behaved as completely random coils. Conversion from the intermediate stage of denaturation in 5 M Gdn·HCl at 20° to completely random coil could also be effected by heat. The transition temperature of 46° was the same for paramyosin, PPC, and PTC. This is in accord with the other denaturation studies and supports the conclusion that the most stable region of paramyosin is at the N-terminal portion.

Paramyosin is one of the major contractile proteins extractable from adductor muscle of molluscs (Hodge, 1952; Bailey, 1957; Kominz *et al.*, 1957). The contractile units of molluscan muscle consist of thick and thin filaments (Hanson and Lowry, 1959; Philpott *et al.*, 1960), and paramyosin is the major component of the thick filaments (Hanson and Lowry, 1961). More recently, Szent-Györgyi *et al.* (1971) have demonstrated that the paramyosin molecules align to form a bipolar core of the thick filament which is covered by a surface layer of myosin. This paramyosin core seems to modify the interaction of myosin with actin of the thin filament and thereby to have a specific regulatory role in maintenance of muscle tension. The paramyosin molecule appears to be well suited to such a role for it is a large molecule of 220,000 atomic mass units that is rod shaped, 1330 Å long by 20 Å diameter, and consists of two intertwined α-helical chains (Lowey *et al.*, 1963).

Our laboratory is engaged in studies of the detailed structure of the paramyosin molecule both to help clarify the specific role of this molecule in the molluscan thick muscle filament and as a model system for more general studies of the stability of the helical structure and of interaction between helical rodlets. In the course of these studies it was observed that paramyosin from *Mercenaria mercenaria* upon digestion by any one of several proteolytic enzymes yielded a large segment of the original molecule resistant to further digestion by any of these enzymes. Proteolytic digestion of paramyosin from *Pinna nobilis* had been reported (Bailey and DeMilstein, 1964; Bailey *et al.*, 1964) to yield a large segment of 102,000 atomic mass units that was nearly two-thirds of the mass of the original molecule. These results are similar to those observed for paramyosin from *M. mercenaria*. The principal difference was that the segment from *P. nobilis* was largely nonhelical (35% helical) whereas the segment isolated from *M. mercenaria* has been found to be completely helical. Because this large helical segment permitted interesting chemical and physical comparisons with the parent molecule, the size and shape of this segment and its location within the parent molecule were determined. In addition, some of the comparative studies, notably stability to Gdn·HCl[1] and heat, will be reported, and regions of different stability will be

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[1] Abbreviations used are: TNBS, trinitrobenzenesulfonic acid; Gdn·HCl, guanidine hydrochloride; PPC, paramyosin pepsin-resistant core; PTC, paramyosin trypsin-resistant core; T_{tr}, transition temperature.

TABLE I: Comparison of Proteolytic-Resistant Cores Isolated from Digests.[a]

Enzyme for Digestion	% Migration of Protein Bands from the Digest Rel to Total Gel Length		Yield of Isolated Core as % of Paramyosin Digested	Fluorescence R_{Tyr} Values of Isolated Cores	
	At pH 2.3	At pH 9.5		At pH 2	At pH 7
Control	12 (4)[b]	6	0	0.67	0.40
Chymotrypsin	19	10 (8) (65)	66	0.63	0.41
Nagarse	19	11	62	0.69	0.40
Pepsin	19	11	62	0.68	0.41
Pronase	18 (15)	10 (16)	60	0.61	0.41
Trypsin	19	12	65	0.66	0.39

[a] Digestion conditions: 2.5 hr, 20°, 1% enzyme. Paramyosin at 5 mg/ml in 0.5 M KCl–0.05 M Tris (pH 8.0) for all enzymes except pepsin (in 0.01 N HCl). For isolation procedures, see Materials and Methods. [b] Values in parentheses are for minor bands.

identified. The degree of oxidation of the Cys residues in paramyosin has interesting effects on certain physical properties of the molecule and these will be reported later. However, the degree of oxidation had no effect on the proteolysis studies reported in this paper and all of these experiments were done on paramyosin in which all sulfhydryl groups were in the disulfide form.

Materials and Methods

Materials. The enzymes mentioned in Table I as well as carboxypeptidase A (DFP), all of the highest purity available, were obtained either from Sigma Chemical Co. or Worthington Biochem. Corp. except for the broad-spectrum proteases of microbial origin, Pronase β (Calibiochem) and nagarse (Biddle Sawyer Co.). Rabbit muscle tropomyosin was isolated as described earlier (Cowgill, 1968). Gdn·HCl was of the Ultra Pure grade supplied by Mann Research Laboratory and was found free of any fluorescent impurities in the region of interest (275–350 nm). Standard dansylamino acids were obtained in part from Pierce Chemical Co. and in part by synthesis in this laboratory, by the general method of Boulton and Bush (1964).

Paramyosin. Extraction of paramyosin from adductor muscle of the clam, *Mercenaria mercenaria*, was by the procedure of Riddiford and Scheraga (1962a). Whole adductor muscle as well as separated smooth and striated portions both from fresh and frozen muscle were used in various lots. The extracted paramyosin appeared to be identical in all cases although the yield of extracted paramyosin was higher from smooth muscle (17–25 mg/g) than from striated muscle (10–15 mg/g) as was noted also by Rüegg (1961). The paramyosin was recrystallized at pH 6.0 four times for these studies (and it was during these recrystallizations that sulfhydryl groups became oxidized to the disulfide form). After these recrystallizations, the protein was pure by the criteria of disc gel electrophoresis at pH 2.3 and 9.5 as well as the absence of any detectable tryptophan fluorescence (Cowgill, 1968); the latter criterion relies upon the observation that paramyosin lacks tryptophan (Bailey, 1957; Riddiford and Scheraga, 1962a).

Isolation of Paramyosin Pepsin Core (PPC). Paramyosin at a concentration of 5 mg/ml in 0.02 N HCl was adjusted to pH 2.0. At 20°, a freshly prepared solution of pepsin in water was added to a concentration of 0.05 mg/ml (1% pepsin). After 3 hr at 20°, the solution was adjusted to pH 8 to destroy pepsin. It was then dialyzed 24 hr at 0° against a large volume of 0.02 M $NaPO_4$ (pH 6.0) and centrifuged to remove any residual paramyosin (usually negligible). The supernatant solution was adjusted to pH 5.0 with dilute acetic acid to precipitate PPC. After 1–2 hr at 0°, the PPC was centrifuged down and redissolved in 0.02 N HCl. The clear solution was dialyzed at 0° against 0.02 N HCl plus sufficient solid KCl to bring the final solution to 1.0 M KCl. Again, the PPC was centrifuged down and redissolved in water. The stock solution was extensively dialyzed against water to remove KCl and stored at 0°. The yield and purity of PPC will be reported in the Results section.

Isolation of Paramyosin Trypsin Core (PTC). Paramyosin was brought to a concentration of 5 mg/ml in 0.5 M NaCl–0.05 M Tris (pH 8.0). At 20°, a freshly prepared solution of trypsin in the pH 8 buffer was added to a concentration of 0.05 mg/ml (1% trypsin). After 3 hr at 20°, the digestion was stopped by addition of lima bean trypsin inhibitor in an amount equal to the weight of trypsin. The dialysis against pH 6 buffer and all subsequent steps were the same as for preparation of PPC.

Analytical Procedures. Acrylamide disc gel electrophoresis was done by the general method of Davis (1965), but with elimination of the stacking gel as suggested by Mitchell (1967). The sodium dodecyl sulfate gel electrophoresis procedure for determination of molecular weight (Shapiro *et al.*, 1967; Weber and Osborn, 1969) was altered only by the replacement of mercaptoethanol by dithiothreitol. In addition, molecular weights were confirmed at lower concentrations of protein by the fluorescent modification of Talbot and Yphantis (1971). Protein assays were by the method of Lowry *et al.* (1951), with a protein standard of bovine serum albumin prepared by Schwarz Mann Research Laboratories. Peptide formation during proteolysis experiments was followed by TNBS assay of the digestion mixture for amino-terminal groups reactive with trinitrobenzenesulfonic acid (Habeeb, 1966). Absorbance was measured with a Beckman Model DB spectrophotometer standardized with a holmium oxide glass. The pH was measured on a Radiometer instrument.

Terminal Amino Acid Determinations. The N-terminal amino acids were determined by the method of Gros and Labouesse (1969) and their procedures were followed for dansylation of the protein, hydrolysis, and separation of the dansylamino

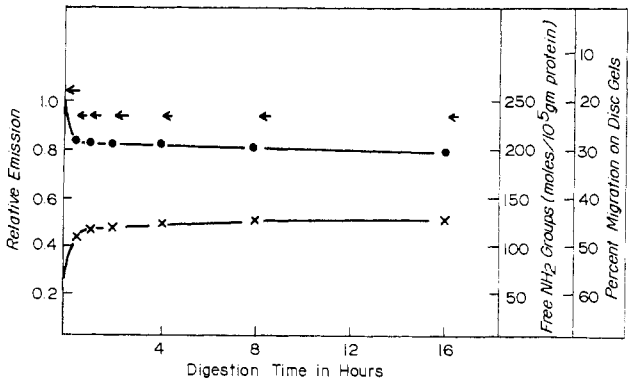

FIGURE 1: Digestion of paramyosin by trypsin (1%) at pH 8.0, 20°. Course of digestion was followed by relative fluorescence emission of Tyr residues at pH 2, 280/305 nm (●); increase in NH_2 groups by the TNBS assay (×); and gel electrophoresis at pH 2.3 (←).

acids by thin-layer chromatography. The C-terminal amino acids were released by the action of carboxypeptidase A with method 2 of Ambler (1967) that employs $(NH_4)_2CO_3$ as the volatile buffer. The buffer was removed by evaporation to dryness three times in a flask evaporator. The residue was dissolved in 0.2 ml of H_2O and the free amino acids were identified by thin-layer chromatography (Brenner and Niederwieser, 1967).

Viscosity. Viscosities were measured at 25 ± 0.02° in Cannon-Ubbelohde semimicro dilution viscometers. Flow times for solvents were in the range of 400–500 sec and flow times for protein samples were higher by at least 60 sec so that kinetic energy corrections were unnecessary. All solutions routinely were centrifuged 1 hr at 35,000*g* prior to measurements. In some measurements, paramyosin solutions were filtered through a Millipore GS filter of 0.22-μ mean pore size. The measured viscosities were the same as for centrifuged samples without the additional filtration step.

Circular Dichroism. These measurements were made in the laboratory of Dr. Gerald D. Fasman at Brandeis University. The experimental conditions are given in Figure 2.

Fluorescence. Fluorescence of tyrosyl residues is expressed in arbitrary values as relative emission and also in specific values relative to the fluorescence of tyrosine as a standard. For the latter, the term R_{Tyr} represents the ratio of fluorescence output of the protein relative to that of tyrosine in 0.05 M phosphate (pH 7.0). Experimentally, it was calculated as follows

$$R_{Tyr} = \frac{(a)_1}{(a)_2}\frac{(A)_2}{(A)_1}$$

where a_1 and a_2 refer to the areas under the emission spectra for the protein and for tyrosine, respectively; A_1 and A_2 refer to the absorbance at the wavelength of excitation for the protein and tyrosine, respectively. Refer to an earlier paper from this laboratory (Cowgill, 1968) for additional remarks about R_{Tyr} and for details of fluorescence measurements with an Aminco spectrophotofluorometer. The thermal transition studies were done in a fluorometer constructed in this laboratory. Light from a 150-W xenon lamp was passed through a Bausch and Lomb (33-86-01) monochromator. The selected 280-nm light was split by a quartz plate into a beam directed to a photocell to monitor lamp intensity and a second beam that passed through the sample cuvet. The cuvet was 4 × 4 cm but only a 1 × 1 cm segment in one corner was used for fluorescence measurements at 90° relative to the excitation beam. The fluorescent light passed through a Corning (CS 75-54) filter to eliminate scattered 280-nm light and pass only the fluorescent light (300–330 nm). The fluorescent light was directed to a second photocell. Signals from the two photocells were amplified by Aminco photomultiplier microphotometers and the double-ended outputs of the two photometers had their negative outputs connected together. This gave one differentiated output proportional to the difference between the two photometer currents and thus corrected for any variance in lamp intensity. This differential output went to the *Y* axis of an *X*–*Y* recorder. By means of continuously adjustable slits, the two light beams were adjusted to yield the same initial reading on both photometers, and the recorder was adjusted to match the scale of the photometer for the photocell that monitored fluorescence. The 25-ml sample for fluorescent study was centrifuged 1 hr at 35,000*g* before addition to the cuvet in order to remove particulate material that might create scatter. The cuvet rested in an aluminum block and the temperature was varied by circulation of streams of hot or cold water through the block. The sample was magnetically stirred for rapid temperature equilibration and temperature was measured by a thermocouple positioned in the center of the cuvet. The output of the thermocouple was passed through a telethermometer of Yellow Springs Instrument Co. and to the *X* axis of the recorder. Calibration of the *X* axis was done with the thermocouple in solutions at two different temperatures. In the experiments continuous traces of net fluorescence *vs.* temperature were obtained at heating rates which could be varied but were usually 5–7°/min.

Results

Action of Proteolytic Enzymes on Paramyosin. An earlier paper from this laboratory (Cowgill, 1968) briefly mentioned that a segment of the paramyosin molecule was resistant to digestion by trypsin. The experiment summarized in Figure 1 demonstrates that digestion by trypsin quickly led to the loss of the paramyosin band on disc gel electrophoresis and to its replacement by a band that moved more rapidly than paramyosin both at pH 2.3 and 9.5 (see the earlier paper of Cowgill (1968) for photographs of gels). The proteolytic change was accompanied by a decrease in relative emission of the Tyr residues and an increase in free amino groups. These results are consistent with the gel electrophoresis in suggesting a rapid digestion of paramyosin to leave a segment that was resistant to further attack. Upon long digestion this segment also was subject to slow hydrolysis as denoted by the small but significant changes in fluorescence and free amino groups in the period 2–18 hr.

A similar segment of paramyosin was resistant to proteolysis by a variety of other enzymes. These studies are summarized in Table I. For all enzymes tested, a segment of the paramyosin molecule accumulated that behaved the same as the trypsin core upon disc gel electrophoresis. Chymotrypsin and Pronase led to somewhat greater heterogeneity of products, in fact, digestion at a higher level of pronase (5%) had been used earlier (Cowgill, 1968) for complete disruption of helical order of paramyosin. The cores from digestion by nagarse, pepsin, and trypsin were so similar that their bands could not be resolved from one another upon coelectrophoresis at either pH 2.3 or 9.5. The marked differences in solubility of paramyosin and the proteolytic-resistant segment or

core were the basis for isolation of the cores for these studies. In all cases, the cores were soluble in distilled water at neutral pH, whereas paramyosin is quite insoluble at low ionic strength (Johnson *et al.*, 1959). The yield of isolated core was similar for each enzyme. The isolated and electrophoretically homogeneous cores all had the same absorbance from Tyr residues, $E^{1\%}_{1\ cm}$ (276 nm) = 2.6–2.7, which is lower than for paramyosin, $E^{1\%}_{1\ cm}$ (276 nm) = 3.1. Also, the fluorescence yields from isolated cores in Table I were similar, and the magnitude of these R_{Tyr} values would indicate that the cores were still helical (Cowgill, 1968). The comparison in Table I would suggest that the same segment of paramyosin was resistant to attack by all of these enzymes so that cores of very similar properties accumulated in all cases. To further test this conclusion, the trypsin-resistant core was isolated and found resistant to 4 hr attack by chymotrypsin, pepsin, or Pronase as judged by disc gel electrophoresis. Similarly, the pepsin-resistant core was isolated and found resistant to attack by trypsin. Because of the similarity of all these cores the experiments that follow were restricted to paramyosin pepsin core (PPC) and paramyosin trypsin core (PTC). The selection of these two cores was made on the basis of maximal differences in paramyosin at the time of hydrolysis. That is, the pepsin attack was at pH 2 and low ionic strength whereas the trypsin (and all other enzymes in Table I) acted at pH 8 and high ionic strength.

Site of Initial Proteolytic Attack on Paramyosin. The values in Table I for recovery of cores indicate that proteolytic attack left a core of approximately two-thirds the size of the original paramyosin molecule. This could arise from a random attack of multiple sites in the other third of paramyosin or from a specific attack at a highly susceptible site one-third the distance down the double-helical rodlet. Electrophoretic characterization of the products of short-term proteolysis might be expected to reveal which type of attack had occurred. On the one hand, random attack at multiple sites should yield initially a multiplicity of segments that should be converted finally to a single core resistant to further attack. Disc gel electrophoresis of samples at digestion times of 1, 2, 4, 8 min, etc., should then show a multiplicity of bands leading to a single band of the resistant core as incubation continued (route A). On the other hand, specific attack at a highly susceptible site one-third the length of the molecule should yield disc gels (route B) showing early replacement of the paramyosin band by the final core band. In fact, both types of attack were observed. Trypsin digestion clearly conformed to route A. That is, during the first 10-min digestion the paramyosin band was lost, numerous transient bands appeared, but the band corresponding to PTC was minor; between 10 and 60 min the transient bands were lost and the PTC band increased. Pepsin digestion conformed to route B. That is, the band corresponding to PPC appeared within 2-min digestion and increased in intensity as the paramyosin band decreased. Transient bands were minor at all stages in the pepsin digestion. After 60-min digestion no large peptide fragment other than PPC or PTC could be found under a variety of conditions that included digestions at 0° and the use of resin-bound trypsin.

Location of PPC and PTC Segments in Paramyosin. The N-terminal and C-terminal end-group determinations were made on paramyosin and the purified PPC and PTC. Cysteine was the only N-terminal residue found for paramyosin, PPC, and PTC. This determination by the dansylation method of Gros and Labouesse (1969) was based on the following observations of the tlc of dansylamino acids after hydrolysis.

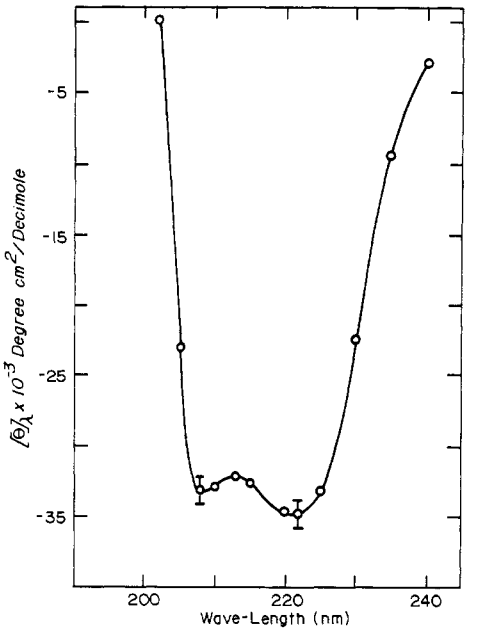

FIGURE 2: Circular dichroism of PTC. The PTC at 5 mg/ml in 0.01 N HCl was measured in 0.5-mm cells at 23°. Accuracy of ±1000 (deg cm^2)/dmole is indicated for 208 and 222 nm.

(1) Monodansylcystine was the only dansylated amino acid in the initial acetone·HCl extract except, of course, *O*-dansyltyrosine and ε-dansyllysine. (2) Ether extraction at pH 3.5 showed the solvent distribution expected for monodansylcystine, that is, the material stayed in the aqueous phase. (3) Treatment of the extract at pH 7 with dithiothreitol followed by iodoacetic acid gave a loss of the spot on tlc corresponding to monodansylcystine. The C-terminal residues were determined by hydrolysis with carboxypeptidase and tlc of the liberated amino acids. The tlc method was not reliable enough for unequivocal identification of C-terminal amino acids, but the two-dimensional tlc pattern of liberated amino acids was distinctly different for paramyosin, PPC, and PTC. On the basis of these studies it was concluded that both PPC and PTC came from the N-terminal segment of paramyosin.

Size and Shape of PPC and PTC. In preliminary purification by chromatography on G-200 Sephadex, the fact that both PPC and PTC eluted at the void volume indicated that these cores were large and asymmetric. Circular dichroism of PTC in 0.01 N HCl is shown in Figure 2. From these data a helical content of 100% for PTC was calculated by the formula of Greenfield and Fasman (1969) in which $[\theta]_{208}$ is the mean residue ellipticity observed at 208 nm.

$$\alpha \text{ helix} = \frac{[\theta]_{208} - 4000}{33{,}000 - 4000} = \frac{33{,}037 - 4000}{33{,}000 - 4000} = 100\%$$

High intrinsic viscosity also is indicative of an asymmetric molecule. The viscosity data in Figure 3 for the PPC and PTC at pH 7.4 gave an intrinsic viscosity of 120 cm^3/g for both PPC and PTC. This value, which is intermediate between that for paramyosin of 195 cm^3/g and that for rabbit muscle tropomyosin of 42 cm^3/g, is reasonable for a helical segment of paramyosin but inconsistent with a nonhelical segment. Figure 4 shows similar viscosity data measured in HCl solution at pH 2.

The molecule weights of PPC and PTC were determined in several ways. The yield of core recovered after digestion

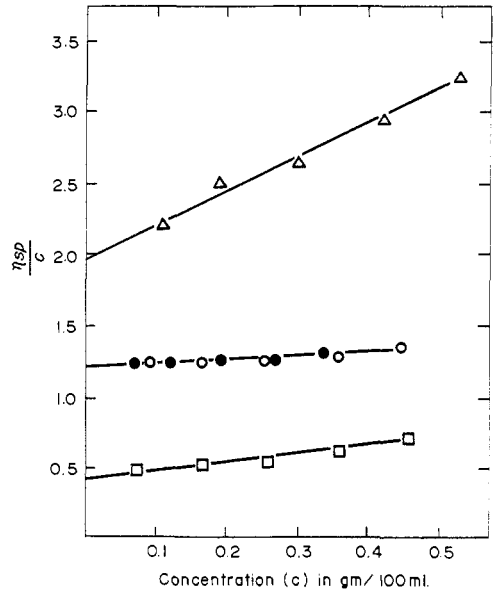

FIGURE 3: Viscosity in 0.6 M KCl–0.06 M KPO_4 (pH 7.4) at 25° of paramyosin (Δ), PPC (○), PTC (●), and tropomyosin (□).

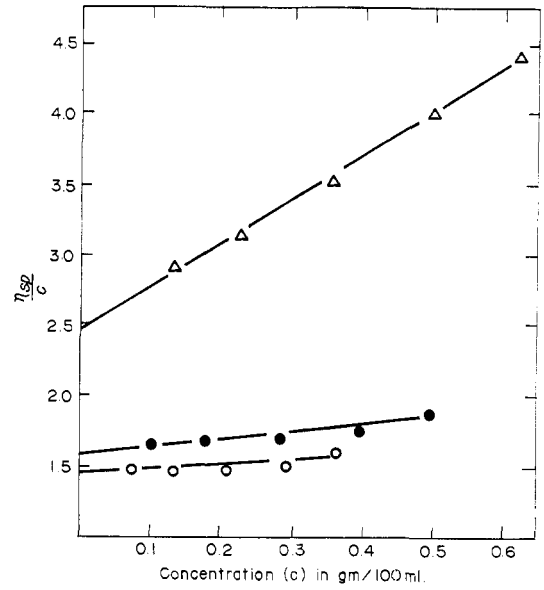

FIGURE 4: Viscosity in 0.02 N HCl (pH 2.0) at 25° of paramyosin (Δ), PPC (○), and PTC (●).

of a known amount of paramyosin of an assumed molecular weight of 220,000 (Lowey *et al.*, 1963) gave an estimate of 136,000–142,000. A determination by sodium dodecyl sulfate gel electrophoresis has been demonstrated to be reliable for helical as well as globular proteins (Weber and Osborne, 1969). By the regular method of Weber and Osborne (1969) and by the modification of Talbot and Yphantis (1971), the single chains from both PPC and PTC migrated at the same rate. From this rate, it was possible to calculate a molecular weight of 132,000 for both PPC and PTC. A third calculation of molecular weight was based on the viscosity data in Figure 3. For rigid rods of the same diameter, *i.e.*, double α helices in this instance,[2] the empirical Mark–Houwick equation

[2] The possibility that the two polypeptide chains of the core were significantly different in length so that the core was in part double helical and in part a single helix did not seem probable because only a single size polypeptide chain was observed on sodium dodecyl sulfate gel electrophoresis.

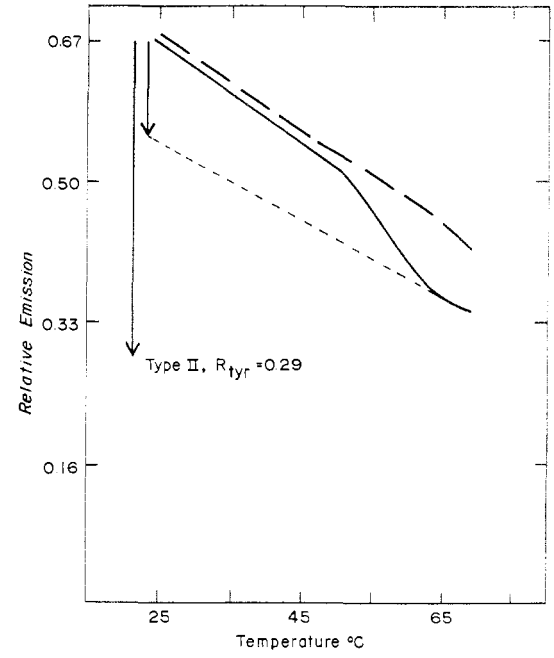

FIGURE 5: Thermal stability of paramyosin (———) and PPC (— — —) each at 1 mg/ml in 0.02 N HCl (pH 2.0). See Materials and Methods for experimental details and Discussion for the significance of the dotted line.

$[\eta] = K(M)^{1.8}$ should apply, where M is molecular weight, $[\eta]$ is intrinsic viscosity, and K is a constant for a particular solute–solvent system and temperature (Bradbury, 1970). For evaluation of K, data for paramyosin of M = 220,000 (Lowey *et al.*, 1963) and $[\eta]$ = 195 cm³/g (Figure 3) yielded $K = 4.8 \times 10^{-8}$; and data for tropomyosin of M = 74,000 (Holtzer *et al.*, 1965) and $[\eta]$ = 42 cm³/g (Figure 3) yielded $K = 7.2 \times 10^{-8}$. For a third value, data for light meromyosin obtained at similar conditions of pH and ionic strength were selected (Seifter and Gallop, 1966). These values of M = 146,000 and $[\eta]$ = 108 cm³/g yielded $K = 5.5 \times 10^{-8}$. With an average of 5.8×10^{-8} from these three values of K and with $[\eta]$ = 120 cm³/g for both PPC and PTC, the calculated molecular weight was 149,000.

Stability of PPC and PTC. Change from the ordered helical conformation to the random chain can be followed by fluorescence because fluorescence yield is high for Tyr residues in helical regions (classified as type I) and low for Tyr residues of type II in regions of random chain (Cowgill, 1968). Paramyosin has been reported more stable in acid solution than at neutral pH (Riddiford and Scheraga, 1962b) and both PPC and PTC appear stable at pH 2 as judged by their retention of helical, asymmetric structure on the basis of R_{Tyr} in Table I, circular dichroism in Figure 2, and viscosity in Figure 4. If these helical proteins pass through a helix-to-random chain transition upon heating, the transition temperature (T_{tr}) should be marked by an abrupt drop in fluorescence. Figure 5 compares the stability of paramyosin and PPC to elevated temperature at pH 2. The recorder trace for PPC had a regular decrease of fluorescence of −0.9%/deg which was due to thermal quenching customarily seen with phenolic compounds. Lack of any discontinuity would indicate that PPC was stable

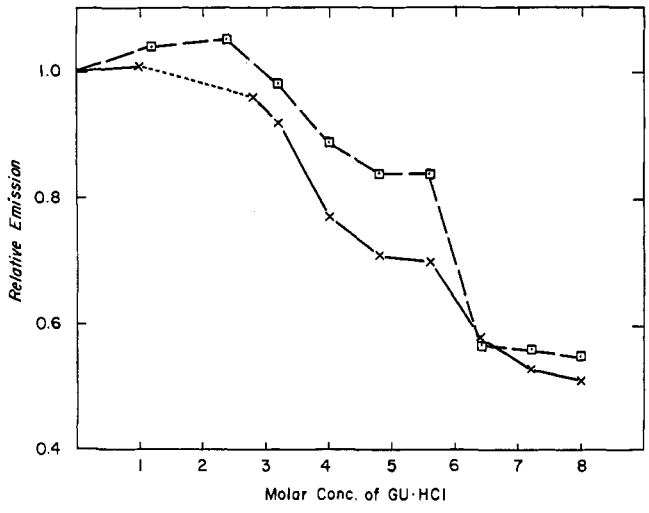

FIGURE 6: Effect of Gdn·HCl on fluorescence (280/305 nm) of Tyr residues of paramyosin (×) and PTC (□) each at 0.1 mg of protein/ml in 0.02 N HCl (pH 2.0). Values were corrected for a minor elevation of fluorescence by the Gdn·HCl (see text). The dotted line covers a region of scatter interference described in the text.

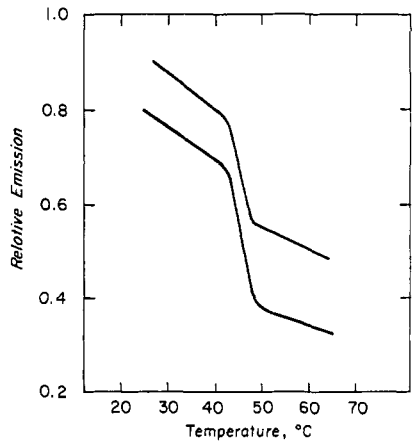

FIGURE 7: Thermal stability of paramyosin (upper curve) and PPC (lower curve) at 1 mg of protein/ml in 5 M Gdn·HCl (pH 2.0). For clarity of presentation, the paramyosin curve was raised ten units on the relative emission scale.

over this temperature region.[3] By contrast paramyosin did show a discontinuity with $T_{tr} = 57°$.

Effect of Gdn·HCl at pH 2 and 20° on stability is shown in Figure 6. The data were corrected for an enhancement of fluorescence by Gdn·HCl that is common to phenolic compounds. This correction was minor; for example, fluorescence was increased by 10% in 6 M Gdn·HCl. Data similar to that shown for PTC was obtained with PPC. There appears to be two major stages of denaturation at 5 and 7 M Gdn·HCl. With the Gdn·HCl at 5 M, it was possible to complete the denaturation by heat and the transitions are shown in Figure 7. A curve identical with that shown for PPC was obtained for PTC. The T_{tr} was 46° for both paramyosin and PPC and the greater fall in fluorescence for PPC from 25–40 to 50–60° regions is consistent with differences in fluorescence levels of denaturation stages at 5 and 7 M Gdn·HCl in Figure 6.

The denaturation curves for both paramyosin and PPC in Figure 7 were reversible. The reversibility of the denaturation was confirmed by heating solutions of paramyosin, PPC, or PTC in 5 M Gdn·HCl (pH 2), to 55° for 5 min. Upon cooling and removing guanidine by dialysis, bands of all three samples on disc gel electrophoresis were identical with untreated samples of paramyosin, PPC, or PTC.

Search for Paramyosin Cores in Muscle Extracts. The possibility that a scission of the paramyosin molecule might occur *in vivo* was investigated. For this purpose adductor muscles from clams under a variety of environmental conditions were extracted by the procedure for isolation of paramyosin. All extract fractions that might contain a core of solubility properties similar to PPC and PTC were examined by disc gel electrophoresis. No such core was observed and it was concluded that a segment of paramyosin similar to PPC or PTC does not occur *in vivo*.

Discussion

Nature of the Proteolytic Attack on Paramyosin. Paramyosin from *M. mercenaria* was rapidly attacked by a variety of proteolytic enzymes; however, a fully helical segment of the molecule that was resistant to further attack soon accumulated. Essentially the same segment of paramyosin appeared to be resistant to hydrolysis by all the enzymes. The basis for this conclusion is that the proteolytic resistant cores all behaved the same on disc gel electrophoresis at both pH 2.3 and 9.5; a core formed by hydrolysis of paramyosin with any one of the enzymes was resistant to attack by other enzymes; and the detailed studies of PPC and PTC established that they were similar in shape, size, and origin from the N-terminal portion of paramyosin. In fact, the PPC and PTC segments appear to be identical except for minor differences at the C-terminal end. These results would indicate that paramyosin has a region in the C-terminal one-third of the molecule that is susceptible to general proteolytic attack whereas the N-terminal two-thirds of the molecule is highly resistant to attack.[4] Incorporation of potentially susceptible peptide bonds into a stable helical conformation may account for the resistance to proteolysis. Arguments in favor of this view have been summarized by Mihalyi and Harrington (1959) for the similar case of myosin which also can be attacked by a variety of proteolytic enzymes to release the helical light meromyosin segment. Because paramyosin is about 90% helical (Cohen and Szent-Györgyi, 1957) at least two possibilities exist. A small nonhelical region may exist one-third distant from the C-terminal end or the entire C-terminal helical region may be so unstable that multiple sites for hydrolysis become exposed. The nature of the products from brief periods of digestion suggest that both possibilities occur at the widely different conditions of pH and ionic strength for the pepsin and trypsin digestions. The fact that complete denaturation of PPC and PTC by Gdn·HCl at 55° was reversible indicates that the two polypeptide chains in these cores were intact and that neither digestion by pepsin nor

[3] It must be emphasized that conclusions of this sort are actually restricted to helical regions bearing Tyr residues. Even if Tyr residues of paramyosin are equally spaced, they would occur only every 33 Å along the rodlet, that is, every six turns of helix. Therefore, some regions may not be monitored by this fluorescence technique.

[4] Paramyosin of *Pinna nobilis* also has been reported to yield a core of two-thirds the original molecular weight upon digestion by trypsin (Bailey *et al.*, 1964). However, the latter core was found to be only 38% helical and to have a globular shape. Also, paramyosin from *P. nobilis* did not yield a segment resistant to either chymotrypsin or subtilisin (Kay and Smillie, 1964).

trypsin had broken any peptide bonds in the N-terminal two-thirds of the paramyosin molecule. The sodium dodecyl sulfate gel electrophoresis data for molecular weight determination of PPC and PTC also are in accord with this conclusion.

Other data in this paper seem consistent with this concept of the degree of susceptibility of the paramyosin molecule to proteolysis. Consider the data in Figure 1. The rather small drop in fluorescence to 80% of the initial level can be assessed in terms of R_{Tyr} values. For native paramyosin, $R_{Tyr} = 0.67$ (Table I) and complete digestion to small segments of random coil should reduce the value to $R_{Tyr} = 0.29$.[5] Therefore, it is possible to calculate that $(0.67 - 0.8 \times 0.67)/(0.67 - 0.29) \times 100 = 36\%$ of Tyr residues were converted from helical to random-chain regions. This would be expected if Tyr residues were distributed uniformly over the molecule and energy-transfer studies (Cowgill, 1968) indicate that this is the case. That is, if the C-terminal one-third of the molecule were digested, about 36% of the Tyr residues should behave as in regions of random chain. In a similar fashion, the free amino group assays in Figure 1 are consistent. There are 65.7 Lys and 82.6 Arg or a total of 148 basic residues/10^5 g of paramyosin (Riddiford, 1966) that might be susceptible to trypsin. The initial value of the TNBS assay was 65 NH_2 groups/10^5 g of protein which agrees very well with ϵ NH_2 groups of Lys. Liberation of amino groups, which was rapid at first, continued slowly, but even at 18 hr the total was only 125 NH_2 groups/10^5 g of protein. Hence, the percent of total Arg and Lys attacked could be calculated as $(125 - 65)/148 \times 100 = 40\%$. This again is consistent with other data as discussed above. The helical nature of the digestion products PPC and PTC was indicated also by the high fluorescence yields given in Table I. Values at pH 2 were very nearly the same for the cores as for paramyosin which is essentially a complete helix (Cohen and Szent-Györgyi, 1957). Lower R_{Tyr} values at pH 7 have been attributed to quenching by ionized Asp and Glu residues contiguous to Tyr residues (Cowgill, 1968) and the similarity of these values for paramyosin and the cores also indicate that the conformation of the cores was the same as for the paramyosin from which they came.

Stability of Paramyosin, PPC, and PTC to Denaturants. The denaturation studies in Figures 5–7 demonstrate that the region of greatest stability is in the N-terminal segment. (That this region actually does extend out from the N-terminal end of the molecule will be demonstrated in a later paper on the effects of disulfide linkages on stability.) In Figure 5, the PPC was inferred to be stable over the 25–65° range because of the absence of a discontinuity. The discontinuity in the data for paramyosin of $T_{tr} = 57°$ indicated some loss of helical structure at this temperature. How extensive this loss was could be estimated within the limitation of this method.[3] For this purpose, an extrapolation was made that would resemble the case if the extent of uncoiling at 65° had been irreversible upon return to 25°. This extrapolation is the dotted line in Figure 5 and runs from the data at 65° to the value at 25° calculated on the basis of a reversal of the normal thermal effect of −0.9%/deg. $(RE_{25} - RE_{65})/RE_{25} = (0.9\%/\text{deg}) \times (65° - 25°)$, and with relative emission (RE_{65}) at 65° observed to be 0.35 it was possible to calculate the relative emission (RE_{25}) at 25° to be 0.55. For clarity the relative emission scale for this figure had been adjusted so that the value 0.67 corresponds to fluorescence of paramyosin at 25°. The reason for this is that $R_{Tyr} = 0.67$ for paramyosin under these conditions; hence fluorescence to be expected for Tyr residues in nonhelical regions (type II) could be compared on the same scale. A value of $R_{Tyr} = 0.29$ is a reasonable one[5] for Tyr of type II. With this value placed on the scale of Figure 5, it may be calculated that the extrapolated loss of fluorescence (short arrow) was 33% of the total loss (long arrow) that would be expected if all Tyr residues went into regions of random chain. This estimated value of 33% loss of helical structure at 65° must be accepted with strong reservation, but this value and the observed stability of PPC would suggest that the loss of helical structure of paramyosin in the region 50–60° represented all of the C-terminal one-third of the molecule.

In Figure 6 three plateaus or levels are apparent. The region 0–3 M Gdn·HCl appears to be a region in which paramyosin and the cores are stable. The slight rise for PTC and omission of data for paramyosin in the region 1–3 M Gdn·HCl are due to interference by scatter in this region, and turbidity of paramyosin in solutions of Gdn·HCl $\leq$ 3.0 M has been noted also by Olander (1971). At higher Gdn·HCl concentrations, a fall in fluorescence did occur to a second plateau at 5 M Gdn·HCl. This plateau was lower for paramyosin than for PTC as might be expected if much of the C-terminal portion of paramyosin and only a small portion of the segment encompassed by PTC had uncoiled. Again, within the limitations of this method,[3] a calculation can be made of the extent of uncoiling in each case. If the level of 0.53 is taken as relative emission of completely random coil, the 5 M Gdn·HCl plateau for paramyosin corresponds to 64% random coil and for PTC as 34% random coil.[6] The difference of 30% is in reasonable agreement with the 36% of paramyosin as the C-terminal portion not in PTC on the basis of the molecular weight determinations. Finally, the fall to the third plateau at 7 M Gdn·HCl is attributed to complete conversion to random chain on the basis of failure to observe a further fluorescence decrease on heating the 7 M Gdn·HCl solutions and on the basis of optical rotatory dispersion values reported in the literature (Riddiford and Scheraga, 1962b; Noelken and Holtzer, 1964).

Additional evidence that the most stable portion of the paramyosin molecule is the N-terminal segment that constitutes PPC and PTC is in Figure 7. Loss of the residual helical structure, estimated from the values calculated above as 36% of paramyosin and 66% of PPC, occurred during heating in 5 M Gdn·HCl. The drop in fluorescence at the transition was greater for PPC because of the greater per cent of its Tyr residues invovled in the uncoiling at that temperature. The transition temperature was identical ($T_{tr} = 46°$) for paramyosin and PPC and is evidence that the same helical segment was involved in both molecules.[6]

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[5] In an earlier paper (Cowgill, 1968) the value for a Tyr residue (type II) in a region of random coil was $R_{Tyr} = 0.29$ for denatured tropomyosin and $R_{Tyr} = 0.27$ for an extensive Pronase digest of paramyosin. $R_{Tyr} = 0.29$ is probably the more representative value.

[6] One indication of the validity of these interpretations of the fluorescence is the fact that Riddiford and Scheraga (1962b) found from ORD measurement that paramyosin was only one-third helical in 5 M Gdn·HCl–1.2 M urea (pH 2) at room temperature and passed to the completely random-coil state with a T_{tr} at 40°.

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References

Ambler, R. P. (1967), *Methods Enzymol. 11*, 155.
Bailey, K. (1957), *Biochim. Biophys. Acta 24*, 612.
Bailey, K., and DeMilstein, C. P. (1964), *Biochim. Biophys. Acta 90*, 492.
Bailey, K., DeMilstein, C. P., Kay, C. M., and Smillie, L. B. (1964), *Biochim. Biophys. Acta 90*, 503.
Boulton, A. A., and Bush, I. E. (1964), *Biochem. J. 92*, 11P.
Bradbury, J. H. (1970), *in* Physical Principles and Techniques of Protein Chemistry, Part B, Leach, S. J., Ed., New York, N. Y., Academic Press, p 123.
Brenner, M., and Niederwieser, A. (1967), *Methods Enzymol. 11*, 39.
Cohen, C., and Szent-Györgyi, A. G. (1957), *J. Amer. Chem. Soc. 79*, 248.
Cowgill, R. W. (1968), *Biochim. Biophys. Acta 168*, 417.
Davis, B. J. (1965), *Ann. N. Y. Acad. Sci. 121*, 404.
Greenfield, N., and Fasman, G. D. (1969), *Biochemistry 8*, 4108.
Gros, C., and Labouesse, B. (1969), *Eur. J. Biochem. 7*, 463.
Habeeb, A. F. S. A. (1966), *Anal. Biochem. 14*, 328.
Hanson, J., and Lowry, J. (1959), *Nature (London) 184*, 286.
Hanson, J., and Lowry, J. (1961), *Proc. Roy. Soc., Ser. B 154*, 173.
Hodge, A. J. (1952), *Proc. Nat. Acad. Sci. U. S. 38*, 850.
Holtzer, A., Clark, R., and Lowey, S. (1965), *Biochemistry 4*, 2401.
Johnson, W. H., Kahn, J. S., and Szent-Györgyi, A. G. (1959), *Science 130*, 160.
Kay, C. M., and Smillie, L. B. (1964), *in* Biochemistry of Muscle Contraction, Gergely, J., Ed., Boston, Mass., Little Brown, p 379.
Kominz, D. R., Saad, F., and Laki, K. (1957), *Nature (London) 179*, 206.
Lowey, S., Kucera, J., and Holtzer, A. (1963), *J. Mol. Biol. 7*, 234.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193*, 265.
Mihalyi, E., and Harrington, W. F. (1959), *Biochim. Biophys. Acta 36*, 447.
Mitchell, W. M. (1967), *Biochim. Biophys. Acta 147*, 171.
Noelken, M., and Holtzer, A. (1964), *in* Biochemistry of Muscle Contraction, Gergely, J., Ed., Boston, Mass., Little Brown, p 374.
Olander, J. (1971), *Biochemistry 10*, 601.
Philpott, D. E., Kahlbrock, M., and Szent-Györgyi, A. G. (1960), *J. Ultrastruct. Res. 3*, 254.
Riddiford, L. M. (1966), *J. Biol. Chem. 241*, 2802.
Riddiford, L. M., and Scheraga, H. A. (1962a), *Biochemistry 1*, 95.
Riddiford, L. M., and Scheraga, H. A. (1962b), *Biochemistry 1*, 108.
Rüegg, J. C. (1961), *Proc. Roy. Soc., Ser. B 154*, 224.
Seifter, S. and Gallop, P. M. (1966), *Proteins 4*, 398.
Shapiro, A. L., Viñuela, E., and Maizel, J. V., Jr. (1967), *Biochem. Biophys. Res. Commun. 28*, 815.
Szent-Györgyi, A. G., Cohen, C., and Kendrick-Jones, J. (1971), *J. Mol. Biol. 56*, 239.
Talbot, D. N., and Yphantis, D. A. (1971), *Anal. Biochem. 44*, 246.
Weber, K., and Osborn, M. (1969), *J. Biol. Chem. 244*, 4406.

Location of Sulfhydryl and Disulfide Groups in Bovine β-Lactoglobulins and Effects of Urea†

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ABSTRACT: As part of a study to understand the role that the single cysteine residue and two cystine residues (per single chain, monomer) play in the unfolding and aggregation reactions of bovine β-lactoglobulins, both in the presence and absence of urea, it is important to locate these residues in the native molecule. It is shown here that alternate positions for the free sulfhydryl group occur in equal proportions in the isolated protein of each β-lactoglobulin variant examined. The cysteine residue is located at position 68 with a disulfide bridge from residues 57 to 70, *or* at position 70 with residue 57 bridged to 68, both forms having a disulfide bridge between residues 123 and 160. These positions have been determined by diagonal peptide mapping, by a method for quantitatively determining the ratio of multiple positions of a free sulfhydryl group if such exist, and by making use of the partial sequence, with corrections indicated by the present work. The significance of the findings is discussed.

It is becoming increasingly evident that one of the keys to the behavior of the ruminant β-lactoglobulins lies in the single sulfhydryl group present in each monomer chain (for reviews, see McKenzie, 1970, 1971). There are also present two disulfide bridges in each chain. The bovine proteins exhibit genetic polymorphism. Near the isoionic point (pH ~

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