Wade, R. S., and Castro, C. E. (1973a), *J. Amer. Chem. Soc. 95,* 231.
Wade, R. S., and Castro, C. E. (1973b), *J. Amer. Chem. Soc. 95,* 226.
Watson, H. C. (1969), *Progr. Stereochem. 4,* 299.
Yandell, J. Y., Fay, D. P., and Sutin, N. (1973), *J. Amer. Chem. Soc. 95,* 1131.
Yonetani, T. (1960), *J. Biol. Chem. 235,* 845.

Proteolysis of Paramyosin from *Mercenaria mercenaria* and Properties of Its Most Stable Segment†

Robert W. Cowgill

ABSTRACT: The helical muscle protein paramyosin appears to consist of three segments of approximately equal size that differ in stability to guanidine hydrochloride and heat. The N-terminal segment is most stable and the C-terminal segment is least stable. These differences in stability serve as a basis for design of proteolytic digestions to specifically remove segments of low and intermediate stability. Thus, at room temperature only the C-terminal region was susceptible to digestion by pepsin or trypsin. Proteolytic removal of the latter region resulted in the accumulation of the remaining ⅔ of the paramyosin molecule as a segment (PPC-1) of 140,000 daltons that was still in a stable helical conformation. Proceeding to more rigorous conditions, papain digestion of either paramyosin or PPC-1 in 4 M guanidine · HCl that would be expected to destabilize all but the N-terminal segment did result in cleavage of all except that region. The N-terminal region accumulated as a helical segment of 74,000 daltons (PPC-2) if digestion was limited to 1.5 hr or a smaller segment of 58,000 daltons (PPC-3) if digestion continued for 24 hr. Stability of the three PPC segments to guanidine · HCl and heat was measured by change in fluorescence of tyrosyl residues upon loss of the helical conformation. The stability of the segments corresponded well with the stability of those regions in the paramyosin molecule from which the segments were believed to have come. Amino acid composition of the PPC segments and of paramyosin were all very similar, and prediction of relative stability of these helical proteins from inspection of gross amino acid composition does not appear promising.

Paramyosin is the protein that constitutes the core of thick filaments in adductor muscle of the clam (Kahn and Johnson, 1960,; Szent-Györgyi *et al.*, ,1971). It is a large molecule of 200,000 daltons that is rod shaped, 1255 Å long by 20 Å diameter, and consists of two intertwined α-helical chains (Lowey *et al.*, 1963; Cohen *et al.*, 1971). About 90% of the polypeptide chain is in the α-helical conformation on the basis of optical rotatory dispersion measurements by Cohen and Szent-Györgyi (1957). Subsequent optical measurements indicated that the α-helical conformation could be disrupted in distinct stages by elevation of temperature or addition of Gdn · HCl[1] (Riddiford, 1966; Olander, 1971; Cowgill, 1972, 1974; Halsey and Harrington, 1973). The stepwise loss of helical conformation is ascribed to differences in stability of regions or segments of the molecule, and the most explicit description of these regions is given in Figure 1.

Location of the regions of different stability in paramyosin was greatly aided by the ability to partially digest the molecule with proteolytic enzymes and to isolate proteolysis-resistant segments. Cowgill (1972) exposed paramyosin to attack by five proteolytic enzymes and in each case obtained a single high molecular weight segment. Two of these, the pepsin-resistant core (PPC-1) and the trypsin-resistant core (PTC-1) of 140,000 daltons, were studied extensively and appear to be identical except for minor differences at the C-terminal region. Halsey and Harrington (1973) digested paramyosin with trypsin and obtained a segment termed light paramyosin. From a comparison of digestion procedures and reported properties of the product, PTC-1 and light paramyosin are the same segment. This segment is believed to arise from the N-terminal two-thirds of paramyosin on the basis that cysteine is N-terminal in PPC-1 and PTC-1 as well as paramyosin (Cowgill, 1972). If Figure 1 is correct and the middle segment of paramyosin is less stable than the N-terminal region, it should be possible to continue this digestive process under conditions that are believed to destabilize all except the N-terminal segment and to observe the proteolysis of all of the paramyosin or PPC-1 except for this most stable segment. This prediction has proved to be true and the digestive procedures and some properties of the smaller segments designated PPC-2 and PPC-3 are described in this paper.

Materials and Methods

Materials. The enzymes mentioned in Table I were all of the highest purity available from Sigma Chemical Co. or Worthington Biochemical Corp. Gdn · HCl was of the Ultra Pure grade supplied by Schwarz/Mann Research Lab. and was found free of any fluorescent impurities in the region of interest (270–350 nm).

Preparation of Paramyosin, PPC-1, and PTC-1. Par-

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[1] Abbreviations used are: Gdn · HCl, guanidine hydrochloride; PPC-1, paramyosin pepsin-resistant core; PTC-1, paramyosin trypsin-resistant core; PPC-2, the paramyosin papain-resistant segment of 74,000 daltons; PPC-3, the paramyosin papain-resistant segment of 58,000 daltons; Mops, morpholinopropanesulfonic acid.

Table I: Enzymatic Digestions of Paramyosin and Its PPC Segments.

No.	Digestion Conditions	Gel Electrophoresis, pH 2.3
		Origin / PPC-1 / PPC-2 / PPC-3
1	Paramyosin + 2% papain + 4 M Gdn·HCl	↓ ↓ ↓ ↓
	1.5 hr at 20°	|
	24 hr at 20°	|
2	Paramyosin + 2% papain, 24 hr at 20°	| | |
3	PPC-1 + 2% papain + 4 M Gdn·HCl	
	1.5 hr at 20°	|
	24 hr at 20°	|
4	PTC-1 + 2% papain + 4 M Gdn·HCl	
	1.5 hr at 20°	|
	24 hr at 20°	|
5	PPC-1 + 1% trypsin, 4 hr at 20°	|
6	PPC-1 + 2% trypsin, 16 hr at 50°	| |
7	PTC-1 + 1% pepsin, 6 hr at 20°	|
8	PPC-1 + 2% pepsin, 16 hr at 50°	(loss of all nondialyzable segments)
9	PPC-1 + 2% chymotrypsin, 16 hr at 20°	| | |
10	PPC-2[a] + 1% pepsin, 4 hr at 20°	|
11	PPC-2 + 2% papain, 24 hr at 20°	| | |
12	PPC-2 + 1% trypsin, 24 hr at 20°	| |

[a] In this digestion with pepsin, the same result was obtained whether the PPC-2 was formed from paramyosin, PPC-1, or PTC-1 by the standard 1.5-hr digestion with papain in 4 M Gdn·HCl.

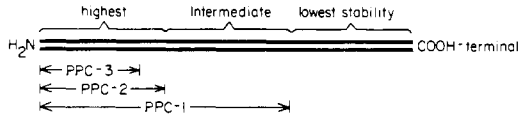

FIGURE 1: Proposed location of proteolysis-resistant segments and their relationship to regions of different stability. Solid lines represent the two polypeptide chains of the paramyosin molecule.

amyosin of *Mercenaria mercenaria* varies slightly in molecular size, depending upon the isolation procedure (Stafford and Yphantis, 1972). As isolated in this laboratory from whole adductor muscle by the classic method of Johnson *et al.* (1959) the paramyosin is of the β form of 200,000 daltons. Procedures for formation of PPC-1 and PTC-1 were described earlier by Cowgill (1972).

Preparation of PPC-2. The paramyosin papain-resistant core could be prepared from paramyosin and also from PPC-1 in a completely analogous fashion. Paramyosin that had been purified by repeated crystallization was dissolved at a concentration of 5 mg/ml in a solution of the following composition at pH 7.0: 4 M in Gdn · HCl, 1 mM in ethylenediaminetetraacetic acid, 0.02 M in Mops buffer (Sigma Chem. Co.), 0.01 M in cysteine, and 0.01 M in dithiothreitol. To this solution of paramyosin at 20° was added 2% by weight of papain relative to the paramyosin. The papain was freshly activated as specified in the Worthington enzyme manual. (Papain was selected for these digestions because of its high stability according to Sluyterman (1967).)

After an optimal digestion time of 1.5 hr at 20°, the proteolytic action was stopped by the addition of HCl to a concentration of 0.04 N. The solution was dialyzed at 0° against three changes of 0.02 N HCl to remove salts and dialyzable peptide fragments. Dialysis was continued against 0.25 M NaCl–0.05 M sodium acetate at pH 5.0 at 0° to precipitate all nondialyzable protein. The precipitated PPC-2 was centrifuged down and redissolved in 0.02 N HCl for storage at 0°. The average yield of protein was 29.4 mg/100 mg of paramyosin digested or 88% of theoretical yield for PPC-2 of 74,000 daltons. These preparations of PPC-2 were homogeneous by the criterion of gel electrophoresis without further purification.

Preparation of PPC-3. Paramyosin or PPC-1 was digested by papain as described above except that the digestion period was 24 hr rather than 1.5 hr for PPC-2. After dialysis against 0.02 N HCl, the solution was dialyzed against 0.25 M NaCl–0.05 M sodium acetate at pH 4.6 for optimal precipitation of PPC-3. For both PPC-2 and PPC-3 the presence of salt was essential at this step for completeness of precipitation. PPC-3 was dissolved in 0.02 N HCl for storage at 0°. The average yield of protein was 17.0 mg/100 mg of paramyosin digested or 65% of theoretical yield for PPC-3 of 58,000 daltons. (Precipitation at pH 4.6, although optimal, was not complete and some PPC-3 was lost in the supernatant solution at this step.) These preparations of PPC-3 also were electrophoretically homogeneous without additional purification.

Preparation of Oxidized and Reduced Forms of PPC-2 and PPC-3. The Cys residues in PPC-2 and PPC-3 were in the reduced cysteinyl state as these segments were prepared above. The pair of Cys residues could be oxidized to the cystinyl state by O_2 with Cu^{2+} ions as catalyst as described previously for paramyosin and PPC-1 (Cowgill, 1972).

Other Proteolytic Digestions Summarized in Table I. Digestion of paramyosin and PPC-1 with 2% papain in the absence of Gdn · HCl was done as described above except that Gdn · HCl was omitted from the digestion mixture. General procedures for digestions with chymotrypsin, pepsin, and trypsin were described earlier (Cowgill, 1972). In all cases, proteolysis was stopped by change of pH (acidification to pH 2–3 for all enzymes except pepsin which was destroyed by adjustment to pH 8 for 15 min). Products were dialyzed against 0.02 N HCl at 0° to remove small peptide frag-

ments. The nondialyzable products were characterized by gel electrophoresis at pH 2.3.

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 (Weber and Osborn, 1969) was altered only by the replacement of mercaptoethanol by dithiothreitol; the electrophoresis conditions were 6 hr at 150 mA in 2.5% acrylamide gels.

Determination of total cysteinyl residues was by a method with 5,5′-dithiobis(2-nitrobenzoic acid) as the colorimetric reagent (Cowgill, 1974). Amino acid analyses were done in this school on a Bendix Model 2500 gas chromatographic amino acid analyzer. Circular dichroism measurements were made in the laboratory of Dr. Gerald D. Fasman at Brandeis University. The instrument was a Cary 60 ORD instrument with the 6001 circular dichroism attachment, and the experimental conditions are given in Table IV.

Fluorescence of tyrosyl residues is expressed in arbitrary values as relative emission and also in specific values (R_{Tyr}) relative to the fluorescence of tyrosine as a standard. Refer to an earlier paper from this laboratory (Cowgill, 1968) for additional remarks about the R_{Tyr} term and for details of fluorescence measurements with an Aminco spectrophotofluorometer. The thermal transition studies were done in a fluorometer constructed in this laboratory and described in the earlier paper.

Results

Preparation and Properties of PPC-2 and PPC-3. Digestion of paramyosin with papain rapidly led to extensive proteolysis and a single nondialyzable fragment or segment PPC-2 accumulated. Upon continued digestion, PPC-2 was slowly converted to a second nondialyzable segment PPC-3. The electrophoretic characteristics of these products at various times of digestion are shown in Figure 2a–c and the band pattern of a mixture of these segments plus paramyosin and PPC-1 is represented in Figure 2d. From the band positions in these photographs, the migration distances relative to PPC-3 are as follows: paramyosin (0.37); PPC-1 (0.71); PPC-2 (0.93); and PPC-3 (1.00). A plot of these values *vs.* log of molecular weight is linear for the three PPC segments but paramyosin moves too slowly relative to the others. This suggests that the charge distribution per unit length of the protein rod at pH 2.3 is uniform for the PPC segments. A similar relationship was observed by Biro *et al.* (1972) for helical segments of myosin. The fact that paramyosin did not conform could be due to nonuniformity of charge distribution or to its greater tendency to self-aggregate. Amino acid analyses to be presented below show that charge distribution is uniform; hence, self-aggregation probably accounts for the slow rate of electrophoresis.

Nondialyzable products of proteolytic attack on paramyosin and preformed PPC segments accumulated when digestions were done under a variety of conditions and with different enzymes. The general conditions for the digestions are described in the Methods section, and the electrophoretic properties of these products are summarized in Table I. The significance of these results will be discussed later.

Some properties of PPC-2 and PPC-3 are summarized in Table II and compared with values for paramyosin and PPC-1. Molecular weights by sodium dodecyl sulfate disc gel electrophoresis were calculated on the assumption that two polypeptide chains, each of the molecular size determined by the electrophoresis, made up the original segment or molecule. The fact that only a single size of polypeptide chain was observed in each case signified that the preparation was homogeneous, both chains were of the same length, and that no breaks in the polypeptide chains had occurred during proteolysis (see Figure 2e–g). The latter point was also established by reversibility of denaturation of both PPC-2 and PPC-3 in 7 M Gdn · HCl at pH 2 or pH 8 as shown below. The cysteinyl analysis in Table II is significant because earlier studies showed that of the 4 cysteinyl residues of paramyosin, two are in the readily digested C-terminal region and two are at the N-terminus. These latter two cysteinyl residues should be retained if proteolysis is as depicted in Figure 1. This is demonstrated for all except PPC-3; for PPC-3 the cysteinyl content varied from one preparation to another and 0.8 SH/molecule is an average value. It would appear that some proteolysis at the N-terminal region occurred during this most extensive digestion, but this implied heterogeneity in the PPC-3 fraction was too slight to detect by gel electrophoresis.

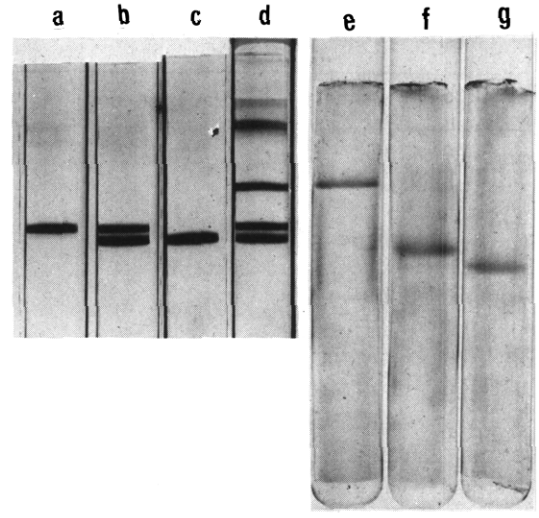

FIGURE 2: Photographs of disc gels. Photographs a–d were of gels run at pH 2.3 and stained with Buffalo Black, these were of a papain digest of paramyosin after 1.5 hr (a); 6 hr (b); and 24 hr (c) of reaction at 20°. Disc (d) represents a separate electrophoresis in a separate experiment of an equal mixture by weight of the following proteins listed in order from the fastest band at the bottom to the slowest at the top: PPC-3; PPC-2; PPC-1; and paramyosin (the faint band above paramyosin is observed with the pure protein and is ascribed to self-aggregation). Photographs e–g were of molecular weight determinations by the sodium dodecyl sulfate electrophoresis procedure; the samples were PPC-1 (e), PPC-2 (f), and PPC-3 (g).

Paramyosin has been tested by a sensitive fluorescence method and found free of tryptophan (Cowgill, 1968). Therefore, the absorbance at 276 nm is attributable to tyrosyl residues. In the earlier paper from this laboratory the high fluorescence of tyrosyl residues in helical sections was noted. The values of R_{Tyr} in Table II would suggest that all the PPC segments are helical for the R_{Tyr} values at pH 2.3 are high, especially for PPC-3. The somewhat lower values for the other segments may reflect the location of some tyrosyl residues in helical regions of transient stability or the existence of some other fluorescence quenching effects. The lower R_{Tyr} values at neutral pH were ascribed in the earlier paper to partial quenching of tyrosyl residues in helical regions by hydrogen-bonding to neighboring aspartyl and glutamyl residues.

Circular dichroism measurements of PPC-2 and PPC-3 are summarized in Table III and these indicate that both

Table II: Comparison of Properties of Paramyosin and Its Proteolysis-Resistant Segments.

Property	Paramyosin	PTC-1	PPC-1	PPC-2	PPC-3
Molecular weight by gel electrophoresis	200,000	140,000	140,000	74,000	58,000
Total cysteinyl SH groups per molecule (based on the above molecular weights)	4.1	2.0	2.0	2.1	0.8
$A_{278\,nm}$ at 1.0 mg/ml	0.31	0.27	0.27	0.34	0.32
Fluorescence efficiency (R_{Tyr})[a]					
At pH 2.3	0.65	0.70	0.70	0.70	0.85
At pH 7.0	0.45	0.45	0.45	0.45	0.55

[a] As defined in the Materials and Methods section.

Table III: Circular Dichroism Spectral Values.[a]

Segment	Wavelength	Obsd $[\theta]$	Calcd % α Helix[b]
In 0.01 M Tris buffer of pH 7.0 at 23°			
PPC-2	222	−39,083; −38,425	
	209	−30,083; −34,409	97
	191	+64,000; +78,898	
PPC-3	222	−41,900; −40,160	
	209	−32,900; −36,480	106
	191	+79,000; +80,160	
In 0.02 N HCl at 23°			
PPC-2	222	−35,217	
	209	−32,464	98
	192	+70,580	
PPC-3	222	−36,829	
	209	−34,797	106
	192	+74,146	

[a] Mean residue ellipticity $[\theta]$ in deg cm²/dmol was measured for the compounds under the conditions as indicated. [b] % α helix = $([\theta]_{209nm} - 4000)/(33,000 - 4000)$ (Greenfield and Fasman, 1969).

segments are completely helical.

The amino acid content of paramyosin and the various segments is given in Table IV. For this comparison, values of Halsey and Harrington (1973) are included for paramyosin and their light paramyosin. Values comparable to these for paramyosin have also been published by Riddiford (1966).

Stability of PPC-2 and PPC-3. Loss of the helical conformation of the PPC segments can be followed by fluorescence because the fluorescence yield for tyrosyl residues is high in helical regions and much lower for residues in regions of random coil.[2] The former, classified as type I, have values of R_{Tyr} about 0.8–1.0; the latter, classified as type II, have values of R_{Tyr} about 0.25–0.35 (Cowgill, 1968). The course of denaturation of PPC-2 and PPC-3 is compared in Figure 3 with the earlier observations for paramyosin and PPC-1. (The original data for paramyosin have been changed in the region 0–3 M Gdn · HCl. We now are able to observe an initial drop in fluorescence at 1.5 M Gdn · HCl without interference by light scatter that obscured earlier measurements in this region.) In 6–8 M Gdn · HCl, optical rotatory dispersion measurements indicate that paramyosin has completely lost the helical conformation (Riddiford, 1966; Olander, 1971). Fluorescence measurements would indicate that this was also true for the PPC segments. The decreases in relative emission in Figure 3 are to 47–55% of the values in the absence of Gdn · HCl. These values correspond to R_{Tyr} values for type II tyrosyl residues in regions of random coil. In substantiation of this conclusion, elevation of the temperature in 7 M Gdn · HCl did not produce any evidence of further uncoiling on the basis of fluorescence measurements (Cowgill, 1972). The denaturation was reversible for all PPC segments on the basis that upon removal of Gdn · HCl by dialysis the proteins had the original electrophoretic properties shown in Figure 2d and the same R_{Tyr} values as in Table II.

Both PPC-2 and PPC-3 appear in Figure 2 to be unchanged in conformation when in 5 M Gdn · HCl at 20°. If the 5 M Gdn · HCl solution was heated, the conversion to the random coil was accomplished with transition temperatures of 40–46° as shown in Figure 4. These transition curves were reversible upon decrease of temperature. Because the earlier studies had shown that the N-terminal pair of Cys residues could contribute to stability of the structure when they shared a disulfide bond in the case of both paramyosin and PPC-1, this point was examined for PPC-2. The oxidized form of PPC-2 (O-PPC-2) with a disulfide bond was more stable than the reduced form (R-PPC-2). This is shown by the shift of the midpoint of the transition from 40° for R-PPC-2 to 46° for O-PPC-2.

Values of R_{Tyr} for PPC-2 and PPC-3 in 5 M Gdn · HCl at 55° but corrected for thermal effects on fluorescence could be estimated on the basis of an extrapolation that was described more fully in the earlier paper (Cowgill, 1972). This extrapolation is shown by the light dotted line in Figure 4. The relative emission extrapolated to 20° is 42% of the value for the unheated protein. The R_{Tyr} value that the heat-denatured PPC-2 would have at 20° can be calculated by multiplication of the extrapolated 42% times the R_{Tyr} of

[2] It should be emphasized that conclusions about loss of helical conformation must be 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 molecule, that is, every six turns of helix. Therefore, some small regions may not be monitored by the fluorescence technique.

Table IV: Amino Acid Composition of Paramyosin and Its Proteolysis-Resistant Segments.

	Residues/100 Residues						
	Analyses in This Laboratory					Analyses by Halsey and Harrington (1973)	
Amino Acid[a]	Paramyosin	PTC-1	PPC-1	PPC-2	PPC-3	Paramyosin	PTC-1[b]
Alanine	12.0	15.2	15.4	15.0	18.0	12.2	14.1
Arginine	10.7	8.0	7.9	9.5	9.3	10.1	8.7
Aspartic acid	13.3	14.6	15.6	15.0	17.0	13.1	14.5
Glutamic acid	21.4	22.8	21.1	21.4	18.9	21.1	20.6
Glycine	1.8	0.4	0.9	0.8	0.4	2.2	1.8
Histidine	1.0	1.0	0.5	0.5	0.3	0.6	0.4
Isoleucine	3.3	2.7	2.2	3.8	3.6	3.4	3.3
Leucine	12.4	14.2	14.3	13.6	14.0	12.1	13.5
Lysine	7.0	5.9	5.8	6.9	7.0	7.4	6.9
Methionine	1.2	0.8	0.7	0.4	0.1	1.5	1.0
Phenylalanine	0.5	0.4	0.5	0.1	0.0	0.8	0.5
Proline				Not Detected			
Serine	4.8	3.7	4.5	3.0	2.8	5.0	4.3
Threonine	4.1	4.8	4.6	4.2	3.6	4.2	4.7
Tyrosine	2.3	1.9	1.9	2.3	2.2	2.1	1.7
Valine	4.0	3.7	4.1	3.5	2.8	4.3	4.1

[a] Cysteine content of these proteins is given in Table II; tryptophan was not detected by a sensitive fluorescence technique described by Cowgill (1968). [b] Values are for the segment designated by these authors as "light paramyosin" which is presumed to be the same as PTC-1 as isolated in this laboratory.

0.70 in Table II, or R_{Tyr} = 0.30. The latter value is in the middle of the predicted range of R_{Tyr} = 0.25–0.35 for type II tyrosyl residues in a randomly coiled polypeptide.

Discussion

The greater susceptibility of one-third of the paramyosin molecule to proteolytic digestion has been attributed to the greater instability of the segment (Cowgill, 1972; Halsey and Harrington, 1973). It is assumed that the polypeptide chain is resistant to attack by the proteolytic enzyme while in the α-helical conformation (Mihalyi and Harrington, 1959). This could account for the accumulation of the helical segment PPC-1 as in Figure 5. The transition on the left that is denoted by the double-headed arrow is probably represented in an oversimplified fashion. For example, this C-terminal segment may have only a small region that is nonhelical at the outset of digestion, or portions and possibly all of the segment may undergo transient helix ↔ random coil conversions. If the above supposition about the C-terminal region is correct then it should be possible to carry this process further. That is, if the segment of intermediate stability in the middle of paramyosin (or the C-terminal half of PPC-1) were subjected to proteolytic attack under conditions that destabilize this region then further digestion should occur and the most stable N-terminal segment of the molecule should accumulate. On the basis of the above mentioned denaturation experiments, it appeared that two-thirds of paramyosin (or one-half of PPC-1) would be nonhelical in 4–5 M Gdn · HCl. (See Figure 3 for evidence of this kind at pH 2.3 or the data of Riddiford (1966) at pH 7.3.) Papain digestion in 4 M Gdn · HCl did result in the accumulation of a stable helical segment PPC-2 of 74,000 daltons, and the presence of cysteine in the segment shows that it came from the N-terminal region of the paramyosin. During prolonged digestion this initial product was slowly converted to a smaller helical segment PPC-3 of 58,000 daltons. A stepwise digestion of the double helical portion of myosin as described by Biro *et al.* (1972) to yield three helical segments of decreasing size appears to be strikingly analogous to the present observations.

Digestions of paramyosin and PPC segments under a variety of conditions that are summarized in Table I show that PPC-1, PPC-2, and PPC-3 consistently accumulate as stable segments. Several of these results confirm the conclu-

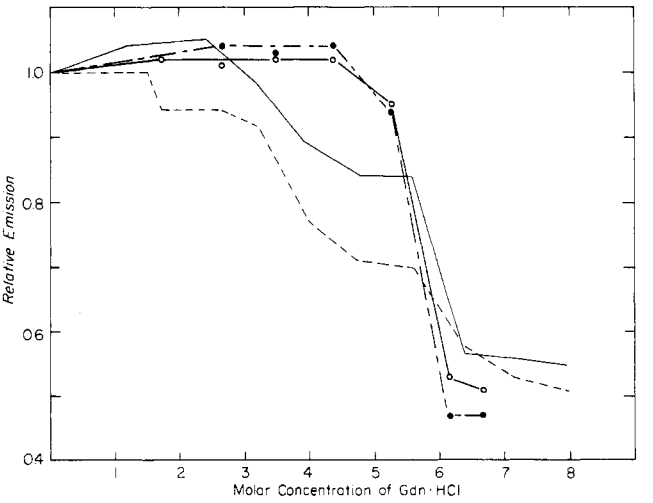

FIGURE 3: Denaturation of paramyosin (- - -); PPC-1 (—); PPC-2 (○); and PPC-3 (●) by Gdn · HCl in 0.02 N HCl at 20°. Relative emission of fluorescence of tyrosyl residues was measured at 305 nm following activation at 280 nm. The data have been corrected for a small enhancement of fluorescence for phenolic compounds in solutions at high Gdn · HCl concentration (Cowgill, 1972).

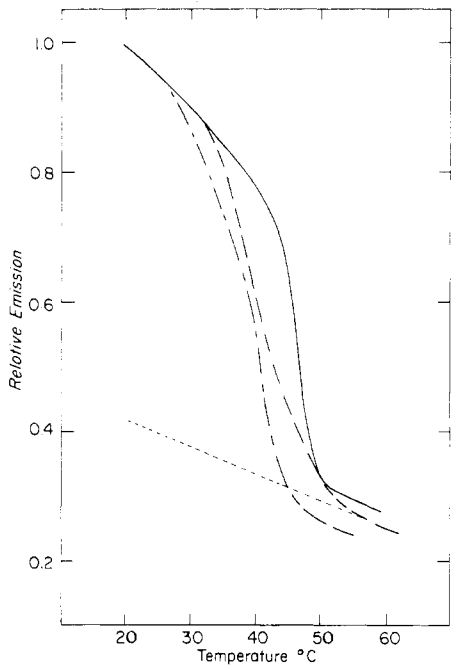

FIGURE 4: Recorder tracings of the course of denaturation of reduced PPC-2 (— —); oxidized PPC-2 (—); and PPC-3 (- — -) in 5 M Gdn · HCl, 0.02 N HCl upon increase of temperature. Relative emission of fluorescence was measured as in Figure 3. (- - -) An extrapolation as explained in the text.

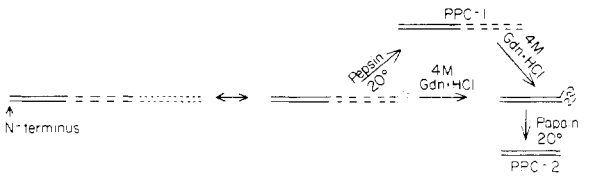

FIGURE 5: The relationship of the stability of regions of paramyosin to their susceptibility to proteolysis. Straight lines represent the helical conformation of the two polypeptide chains of the molecule. The regions of different stability are represented by solid lines (most stable); dashed lines (intermediate), and dotted lines (least stable). The structure in 4 M Gdn · HCl is intended to show that only the N-terminal region remains helical regardless of whether the random coil region represents the remainder of either paramyosin or PPC-1.

sion by Cowgill (1972,1974) that PPC-1 and PTC-1 come from the same region of the paramyosin molecule and retain their original stability. (Compare the similar results from digests 1, 3, and 4; contrast digests 5 and 7; and note the similarity of results in digest 10.) Other results in Table I are consistent with the supposition that attack was initially at the most labile C-terminal region and that progressive digestion to PPC-1, then PPC-2, and finally PPC-3 occurred in that order. This was shown by the occurrence of these PPC segments and only these segments in digestions 2, 6, 9, and 10. This was shown also by digestions 11 and 12 but in each of these cases one other product appeared. These other products moved faster than PPC-3 on electrophoresis and presumably were of smaller size.

Stability of PPC-2 and PPC-3 was compared to stability of regions of paramyosin and PPC-1. These results appear consistent with the origins of the PPC segments as in Figure 1 and the conclusion that the PPC segments retained the stability they had possessed in the original paramyosin molecule. Both PPC-2 and PPC-3 were stable in acidic Gdn · HCl at room temperature until the concentration exceeded 5 M Gdn · HCl. Beyond that concentration, denaturation occurred as an apparently single-stage transition at 5.6 M Gdn · HCl as in Figure 3. This transition occurred in the same concentration region of Gdn · HCl required to denature the last one-third segment of paramyosin or one-half segment of PPC-1; therefore, PPC-2 appears not only to come from this region but also to have retained the stability it possessed in the original molecule. Denaturation of PPC-2 and PPC-3 in acidic 5 M Gdn · HCl could be effected also by elevation of the temperature as in Figure 4. Similar thermal transition curves have been observed for denaturation of the most stable portions of paramyosin and PPC-1 (Cowgill, 1972), and the same transition temperature of 46° was observed for the oxidized forms of paramyosin, PPC-1 and PPC-2. It is interesting that this constancy of the transition temperature would suggest that the amount of randomly coiled polypeptide chain at the C-terminal end did not influence the stability of the N-terminal segment.

The amino acid composition of paramyosin from *Mercenaria mercenaria* as analyzed in this laboratory (Table IV) is in good agreement with values reported by others and mentioned in the Results section. For paramyosin and all the PPC segments the amino acid composition is very similar except for an increase in alanine and aspartic acid in the N-terminal region (PPC-3) and concomitant decreases in glutamic acid and some of the amino acids present in minor amounts. Prediction of stability of these helical proteins from inspection of gross amino acid composition does not appear promising. Halsey and Harrington (1973) calculated a helical potential parameter for paramyosin and light paramyosin on the basis of a rating of α-helical forming tendency of various residues by Robson and Pain (1971). Similarly, calculations can be made with the more recent P_α rating of Chou and Fasman (1974) of residues as helix formers. These P_α values range from 0.53 for Gly (strongly α-helix disruptive) to 1.53 for Glu (strongest helix former), and a value of 1.00 denotes a weak tendency to favor the helical conformation. The average of these values for residues in proteins of Table IV can be calculated as the sum of P_α × mole %/100 for all the amino acids. This value for the most unstable C-terminal region (paramyosin minus PPC-1) was lower (1.11) than the value of 1.16 for paramyosin. However PPC-1, PPC-2, and PPC-3 all gave the same value of 1.19 even though PPC-2 and PPC-3 are more stable to Gdn · HCl than a portion of PPC-1 according to data in Figure 3. Employment of such parameters based on frequency of occurrence of specific amino acids in the helical regions of globular proteins probably is not a valid measure of stability of fibrous proteins because no consideration is given to the importance of hydrophobic stabilization forces between the intertwined α-helical polypeptide chains. The greater stability of some segments in the paramyosin may arise from a more favorable primary sequence that permits stronger hydrophobic forces in those regions. Another stabilizing feature that might account for the ability to isolate these distinct PPC segments may be the occurrence of small clusters of residues contributing high stability in the regions at the transition from one segment to another. An example (although an atypical one) of such stabilizing effects is that of the disulfide bond on stability of the N-terminal region.

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References

Biro, N. A., Szilagyi, L., and Balint, M. (1972), *Cold Spring Harbor Symp. Quant. Biol. 37,* 55.
Chou, P. Y., and Fasman, G. D. (1974), *Biochemistry 13,* 211.
Cohen, C., and Szent-Györgyi, A. G. (1957), *J. Amer. Chem. Soc. 79,* 248.
Cohen, C., Szent-Györgyi, A. G., and Kendrick-Jones, J. (1971), *J. Mol. Biol. 56,* 223.
Cowgill, R. W. (1967), *Biochim. Biophys. Acta 133,* 6.
Cowgill, R. W. (1968), *Biochim. Biophys. Acta 168,* 417.
Cowgill, R. W. (1972), *Biochemistry 11,* 4532.
Cowgill, R. W. (1974), *Biochemistry 13,* 2467.
Davis, B. J. (1965), *Ann. N. Y. Acad. Sci. 121,* 404.
Greenfield, N., and Fasman, G. D. (1969), *Biochemistry 8,* 4108.
Halsey, J. F., and Harrington, W. F., (1973), *Biochemistry 12,* 693.
Johnson, W. H., Kahn, J. S., and Szent-Györgyi, A. G. (1959), *Science 130,* 160.
Kahn, J. S., and Johnson, W. H. (1960), *Arch. Biochem. Biophys. 86,* 138.
Lowey, S., Kucera, J., and Holtzer, A. (1963), *J. Mol. Biol. 7,* 234.
Mihalyi, E., and Harrington, W. F. (1959), *Biochim. Biophys. Acta 36,* 447.
Mitchell, W. M. (1967), *Biochim. Biophys. Acta 147,* 171.
Olander, J. (1971), *Biochemistry 10,* 601.
Riddiford, L. M. (1966), *J. Biol. Chem. 241,* 2792.
Robson, B., and Pain, R. H. (1971), *J. Mol. Biol. 58,* 237.
Stafford, W. F., and Yphantis, D. A. (1972), *Biochem. Biophys. Res. Commun. 49,* 848.
Sluyterman, L. A. (1967), *Biochim. Biophys. Acta 139,* 418.
Szent-Györgyi, A. G., Cohen, C., and Kendrick-Jones, J. (1971), *J. Mol. Biol. 56,* 239.
Weber, K., and Osborn, M. (1969), *J. Biol. Chem. 244,* 4406.

Functional Differences in Protein Synthesis Between Rat Liver tRNA and tRNA from Novikoff Hepatoma†

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ABSTRACT: Synthesis of ovalbumin in fragmented oviduct magnum explants of immature, estrogen-stimulated chicks has been studied in the presence of exogenous tRNA. tRNA from Novikoff hepatoma specifically inhibited ovalbumin synthesis, determined by precipitation with antisera. In addition, the major protein(s) synthesized in the presence of hepatoma tRNA had higher electrophoretic mobility than ovalbumin, as shown by sodium dodecyl sulfate polyacrylamide gel electrophoresis. tRNAs from rat liver, rooster liver, and hen oviduct did not affect ovalbumin synthesis, although oviduct tRNA is stimulatory during the earlier stages of estrogen stimulation (Sharma *et al.* (1973), *J. Biol. Chem. 248,* 7622).

A comparison of elution profiles of aminoacyl-tRNAs from rat liver and Novikoff hepatoma, on methylated albumin Kieselguhr columns, has revealed the presence of new species of histidine tRNA, tyrosine tRNA, and asparagine tRNA among tRNAs extracted from hepatoma (Baliga *et al.,* 1969). Recently tyrosine tRNA and histidine tRNA have been shown to differ quantitatively in methylated ribonucleosides (Nau, 1974). The enzymes which introduce methyl groups at macromolecular level, tRNA methyltransferases are also aberrant in the hepatoma (Tsutsui *et al.,* 1966; Sharma, 1973). In a variety of other neoplasms tested, different isoacceptor species of tRNAs have been found (Borek and Kerr, 1972), and it has been suggested that chromatographically distinct atypical tumor tRNAs might play a regulatory role in protein synthesis. Morris hepatoma 5123D contains increased amounts of chargeable tRNA (Ouellette and Taylor, 1973). However, attempts to show functional differences in the behavior of tumor tRNAs compared to thier normal counterparts in protein synthesis have been unsuccessful either with synthetic messenger in an *Escherichia coli* cell-free system (Gonano *et al.,* 1971), or in hemoglobin synthesis on rabbit reticulocyte ribosomes (Mushinski *et al.,* 1970).

To study functional differences in tRNAs from rat liver and Novikoff hepatoma, we have used explanted oviduct magnum fragments from estrogen-stimulated immature chicks (Palmiter *et al.,* 1971). The oviduct magnum fragments in culture synthesize proteins identical with those made in the intact oviduct of chicks. In such a system, therefore, the control mechanisms within the cell remain intact and the probability of artifactual effects is minimized.

We have shown earlier that exogenous oviduct tRNA potentiated ovalbumin synthesis during the lag phase (Sharma *et al.,* 1973), in fragmented oviduct magnum explants of primary and secondary estrogen stimulated immature chicks. As an extension of these observations, tRNA from Novikoff hepatoma was added to estrogen-stimulated ovi-

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