Segments of Paramyosin Formed by Cleavage at Sites of Cysteine Residues†

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ABSTRACT: The helical muscle protein β-paramyosin of 200,000 daltons was treated by the general method of G. R. Jacobson et al. ((1973), *J. Biol. Chem. 248,* 6583) for cleavage of the polypeptide chain at the site of Cys residues. The protein cleaved into two segments: CCF-1 of 140,000 daltons and CCF-2 of 60,000 daltons. The two segments were separated and some properties were compared. Circular dichroism measurements indicated that CCF-1 was completely helical and that CCF-2 was 85% in the α-helical form. The molecular size, resistance to pepsin digestion, stability to heat and urea, and solubility of CCF-1 were all similar to corresponding properties of a pepsin-resistant segment PPC-1 described earlier (Cowgill, R. W. (1972), *Biochemistry 11,* 4532). By contrast, the properties of CCF-2 were distinctly different. It was concluded that the CCF-1 segment, like the PPC-1 segment, arose from the N-terminal two-thirds of the paramyosin molecule. The CCF-2 segment from the C-terminal one-third of paramyosin had limited solubility at neutral pH that matched the low solubility of paramyosin. It was concluded that the CCF-2 region is responsible for the self-aggregating tendency of paramyosin at neutral pH and low ionic strength.

The double helical muscle protein paramyosin from the clam *Mercenaria mercenaria* contains four Cys residues. Two of these are at the N-terminus and the other two occur as a pair in the C-terminal third of the molecule. In the course of establishing the location of the latter pair the polypeptide chains were cleaved at the site of the Cys residues by the specific chemical method of Jacobson et al. (1973). Two segments were observed as products of the reaction and the molecular weights of these segments served to establish the position of the pair of Cys residues in the C-terminal region (Cowgill, 1974).

The present paper describes the separation of these two segments CCF-1 and CCF-2, and compares properties of these segments to those of paramyosin and a segment (PPC-1) formed by proteolysis.[1] (The relationship of these various segments and their sites of origin in the paramyosin molecule are diagramed in Figure 1.) The most significant of the observed properties are the low stability of the C-terminal third of the paramyosin molecule and the self-aggregating tendency which also is attributed to this same region.

Materials and Methods

Materials. The Ultra Pure grade of urea was obtained from Schwarz/Mann Research Labs. and was free of any fluorescent impurities in the 270–350-nm region of interest. All other materials were described in an earlier paper (Cowgill, 1974).

General Procedures. Analytical procedures, fluorescence measurements, and the isolation of reduced β-paramyosin as the 200,000-dalton form (Stafford and Yphantis, 1972) were described earlier (Cowgill, 1974). Circular dichroism (CD) 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 I.

Formation and Separation of Segments CCF-1 and CCF-2. Reduced β-paramyosin was subjected to the procedure of Jacobson et al. (1973) for chemical cleavage of polypeptide chains at the site of Cys residues. This method is based on the quantitative conversion of Cys residues to *S*-cyanocysteinyl residues and subsequent cleavage of the aminopeptide bond of the *S*-cyanocysteinyl residue. Detailed description of the application of this method to paramyosin was described earlier (Cowgill, 1974). The products were recovered in 90% yield and consisted of approximately 18% paramyosin, 44% CCF-1, and 38% CCF-2 as reported in the earlier paper. This mixture was treated as follows in order to separate the products.

The mixture was dialyzed extensively against 0.5 *M* NaCl, 0.05 *M* $NaPO_4$ (pH 7.2), and then against 0.01 *M* $NaPO_4$ (pH 7.2) at 0°. The mixture was centrifuged and a precipitate of paramyosin plus a small amount of CCF-2 was discarded. Dialysis of the clear supernatant solution was continued against 0.01 *M* $NaPO_4$ (pH 6.0) at 0°. The turbid solution was centrifuged and the supernatant solution which contains the CCF-1 was saved for further treatment as described below. The pellet of CCF-2 was washed briefly with cold 0.1 *M* $NaPO_4$ (pH 7.2) to remove any CCF-1 and paramyosin. The washed pellet of CCF-2 was dissolved in 0.02 *N* HCl. Yield was usually about 20% of the amount of protein taken for the cysteinyl cleavage. The product contained at most a trace of paramyosin or CCF-1 by disc gel electrophoresis. The CCF-2 could be stored at 0 or −20° in 0.02 *N* HCl or after lyophilization from 0.02 *N* HCl solution.

The supernate that contained impure CCF-1 was adjusted at room temperature to pH 5 by addition of dilute acetic acid. The precipitated CCF-1 was centrifuged and the supernatant solution was discarded. The pellet was extracted overnight with 0.05 *M* $NaPO_4$ (pH 6.5) and centrifuged to remove a small residual amount of insoluble paramyosin and CCF-2. The supernatant solution at room temperature

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[1] Abbreviations used are: CCF-1, cysteine cleavage fraction of 140,000 daltons; CCF-2, cysteine cleavage fraction of 60,000 daltons; PPC-1, paramyosin pepsin resistant core (Cowgill, 1972).

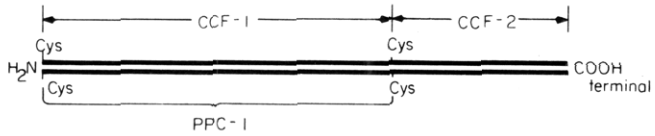

FIGURE 1: Proposed location of segments from paramyosin. Solid lines represent the two polypeptide chains of the paramyosin molecule and the two pair of Cys residues are represented.

Table I: Properties of Cysteinyl Cleavage Segments CCF-1 and CCF-2.

	CCF-1	CCF-2
Molecular weight[a]	140,000	60,000
Gel electrophoresis at pH 2.3[b]	0.88	1.26
Gel electrophoresis at pH 9.5[b]	0.84	*c*
Fluorescence at pH 2.5[d]	0.70	0.70
Fluorescence at pH 7.5[d]	0.40	0.40
Percent α helical[e]	100	85

[a] See text. [b] Values refer to migration relative to PPC-1 taken as unity. [c] No distinct bands appeared, probably because of insolubility of CCF-2 at low ionic strength in alkaline solution. [d] Values are relative to the fluorescence of tyrosine at pH 7.0 (R_{Tyr}); see Cowgill (1972). [e] Based on circular dichroism measurements in 0.02 *N* HCl at 20°. Percent α helix was calculated by the method of Greenfield and Fasman (1969).

was adjusted to pH 5.9 with dilute acetic acid and centrifuged to remove a precipitated mixture of paramyosin, CCF-1, and CCF-2. The supernate was adjusted as above to pH 4.9 and the insoluble CCF-1 was centrifuged. The supernate was discarded and the pellet of CCF-1 was dissolved in 0.02 *N* HCl. Yield was about 35% of the amount of protein taken for the cysteinyl cleavage. The CCF-1 was homogeneous by disc gel electrophoresis and could be stored as described above for CCF-2.

Solubility Measurements. The solubility determinations depicted in Figure 4 were done as follows. Stocks of the appropriate protein in solutions of 0.2 *M* KCl or 1.0 *M* KCl and 0.05 M KPO_4 at pH 7.0 were adjusted to 2 mg of protein/ml. Aliquots of 5.0 ml were placed in small dialysis bags and weighed. The bags were equilibrated overnight against 300 ml of 0.05 *M* KPO_4 buffer of the appropriate KCl concentration and pH in a rocking dialyzer at 20°. At the end of this period, the pH was measured to assure that it was unchanged. The dialysis bag was blotted to remove surface solution and reweighed to correct for any volume change. The contents of the dialysis bag was transferred to a centrifuge tube and centrifuged at 20°. Absorbance at 276 nm of the supernatant solution permitted calculation of the amount of protein still in solution.

Results and Discussion

Cleavage of β-paramyosin gave only two large (nondialyzable) segments whose total mass was found within experimental error to be equivalent to the mass of the paramyosin molecule of 200,000 daltons. Despite many similarities in the properties of those two segments, it was possible to separate them on the basis of differences in solubility upon suitable variation of pH, ionic strength, and temperature. These procedures are described in detail in the experimental section. Some properties of the separated CCF-1 and CCF-2 segments are presented in Table I. Molecular weights by sodium dodecyl sulfate disc gel electrophoresis

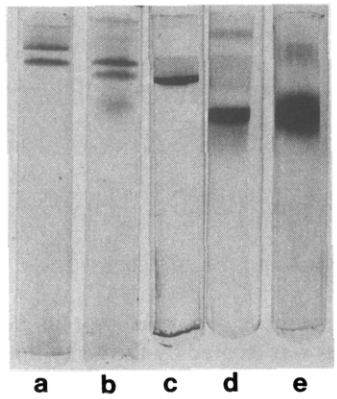

FIGURE 2: Disc gel electrophoresis at conditions described earlier by Cowgill (1972). Discs a and b were in the presence of sodium dodecyl sulfate and dithiothreitol for determination of molecular weight; in (a) the upper band is CCF-1 and the lower band is ovalbumin and in (b) the upper band is ovalbumin and the second band is CCF-2. Electrophoresis run at pH 2.3 is shown in c and d; disc c contained CCF-1 and disc d contained CCF-2. Electrophoresis of CCF-1 at pH 9.5 is shown in disc e and a broad band is typically observed.

were calculated on the assumption made for all double helical proteins that two polypeptide chains each of the molecular size determined by the electrophoresis made up the segment. The fact that only a single size of polypeptide chain was observed in each case signified that the preparation was homogeneous and that both chains were of the same size. Homogeneity was indicated also by the occurrence of a single band in each case upon electrophoresis at pH 2.3 (see Figure 2). The fact that the chemically cleaved segment CCF-1 moved at only 88% of the rate of the enzymatically cleaved segment PPC-1 upon electrophoresis at pH 2.3 as mentioned in Table I indicates that both cleavages were not at the same site. The slower migration rate for CCF-1 is consistent with a larger molecular size for CCF-1 of 140,000 daltons by dodecyl sulfate gel electrophoresis as compared to 132,000 daltons for PPC-1 by the same method (Cowgill, 1972). It has been observed that the migration rate on gel electrophoresis is proportional to the logarithm of molecular weight for several helical segments both from paramyosin (Cowgill, 1974) and myosin (Biró et al., 1972). The high fluorescence yield of both CCF-1 and CCF-2 as noted in Table I would suggest that both segments had preserved the α-helical conformation and this was confirmed by the CD measurements.

Earlier studies in this laboratory (Cowgill, 1972) as well as by Halsey and Harrington (1973) had indicated that the N-terminal ⅔ of the paramyosin molecule was more stable than the C-terminal third; for example, the former portion accumulated as a helical segment (PPC-1) of 140,000 daltons after proteolytic digestion with pepsin, whereas the C-terminal portion was degraded to small peptides. If the CCF-1 and CCF-2 segments arose by the cleavage of paramyosin as diagramed in Figure 1, then stability of CCF-1 should be similar to that of PPC-1, and CCF-2 should resemble the less stable C-terminal third of paramyosin. The following observations are consistent with this proposal. (1) Pepsin digestion of the separated CCF-1 or CCF-2 for 4 hr at 20° and subsequent analysis of products by gel electrophoresis showed retention of the CCF-1 band as in Figure 2 and loss of the CCF-2 band. (2) Heat lability tests of the protein and segments in 0.5 *M* NaCl–0.05 *M* $NaPO_4$ (pH 7.5) revealed that both paramyosin and CCF-2 were irreversibly denatured as denoted by strong turbidity after 5

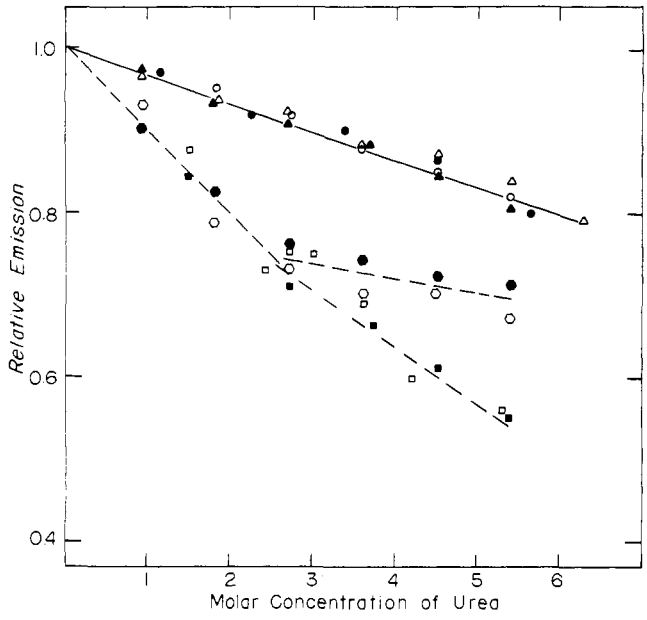

FIGURE 3: Effect of urea on fluorescence from Tyr residues in 0.02 *N* HCl at 20° for paramyosin with disulfide bonds oxidized (●) or reduced (○); PPC-1 with the disulfide bond oxidized (▲) or reduced (△); CCF-1 (two sets of data, ○ and ●); CCF-2 (two sets of data, □ and ■). Fluorescence excitation was at 280 nm and emission was measured at 305 nm.

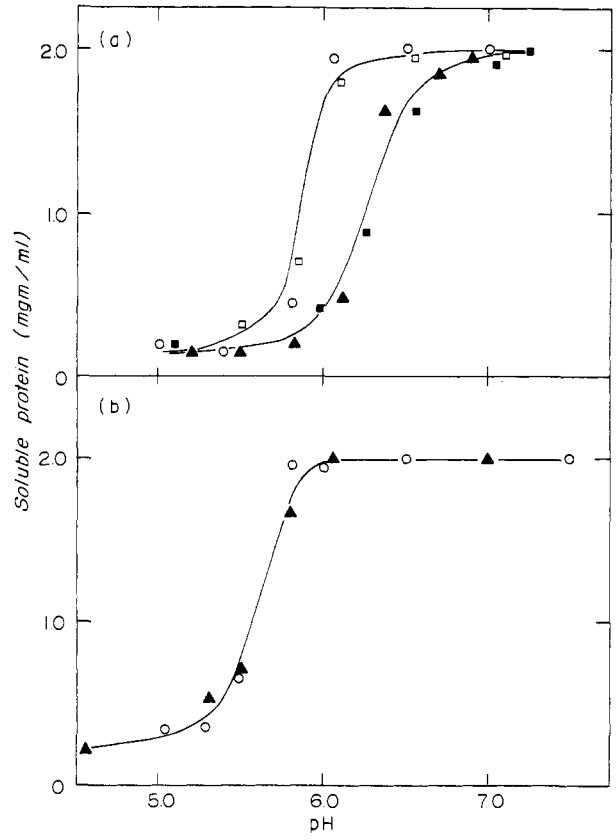

FIGURE 4: Solubility comparison at 20°. In (a) the proteins are paramyosin (○, ▲) and CCF-2 (□, ■); open symbols were for 1.0 *M* KCl–0.05 *M* KPO_4 and solid symbols were for 0.2 *M* KCl–0.05 *M* KPO_4. In (b) the protein segments are PPC-1 (○) and CCF-1 (▲) and for both segments the same curve was obtained either at 0.2 or 1.0 *M* KCl.

min at 45°. By contrast solutions of PPC-1 and CCF-1 remained clear even after 5 min at 65°. (3) Susceptibility to denaturation by urea is compared in Figure 3. Decrease in fluorescence from Tyr residues was employed to follow change in conformation as described in an earlier paper (Cowgill, 1972). Decreases in fluorescence were the same for CCF-1 and PPC-1 and suggest that a slight loss of helical conformation may have occurred. The decrease was much greater for CCF-2 and paramyosin at low concentrations of urea and then diverged at about 3 *M* urea. These observations indicated more extensive conformational changes in CCF-2 and in the C-terminal region of paramyosin. The decrease was less marked for paramyosin at higher concentration of urea, and this would be consistent with the proposal that only ⅓ of its length was of low stability.

The self-aggregating properties of paramyosin as denoted by its insolubility at low ionic strength and neutral pH and its deposition in crystals by lateral alignment are of significance in terms of the function of paramyosin as the core of thick filaments in the muscle fiber. Solubility comparisons in Figure 4 are quite interesting in this regard. These comparisons show that the segments CCF-1 and PPC-1 from the N-terminal portion of paramyosin are soluble above pH 5.5 and their solubility is independent of KCl concentration at least in the range 0.2–1.0 *M*. By contrast paramyosin and the CCF-2 segment are less soluble in the region of pH 5.5–6.5 and their solubility is dependent upon the ionic strength. Similar solubility curves for paramyosin that also demonstrate the dependence upon ionic strength have been presented by Johnson et al. (1959). The present comparison strongly suggests that the C-terminal third of paramyosin not only has a less stable double helical conformation but also that this is the region involved in self-aggregation of the molecule. In an apparently analogous fashion the self-aggregating properties of myosin seem to depend upon the L-meromyosin region (Szent-Györgyi, 1953; Biró et al., 1972).

Acknowledgments

The author thanks Dr. Gerald D. Fasman for doing the CD measurements in his laboratory at Brandeis University. The expert technical assistance of Miss Gail Melson is deeply appreciated.

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