Location and Properties of Sulfhydryl Groups on the Muscle Protein Paramyosin from *Mercenaria mercenaria*†

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ABSTRACT: Paramyosin has four Cys residues per molecule. These Cys residues are in the reduced sulfhydryl form as the protein is isolated from adductor muscles of the clam *Mercenaria mercenaria*. The Cys residues are oxidizable in the presence of Cu^{2+} ions as catalyst. Both disulfide bonds formed on oxidation are found to be interchain in this molecule which consists of two intertwined α-helical polypeptide chains. This conclusion is based on disc gel electrophoresis and viscosity measurements of the molecules in both the oxidized and reduced states. The Cys residues occur as two pairs, each pair capable of forming a disulfide bond. One pair (N pair) occurs at the N terminus; the second pair (C pair) is located approximately 400 Å from the C-terminal end of the molecule. Location of the latter pair was based on (1) the preparation of a paramyosin pepsin-resistant segment (PPC-1) which consisted of the N-terminal two-thirds of the paramyosin molecule and which contained only the Cys pair located at the N terminus, and (2) the chemical cleavage of the paramyosin polypeptide chain of 110,000 specifically at the Cys residues and recovery of two fragments of 30,000 and 75,000. Both pairs of Cys residues were equally susceptible to oxidation to the disulfide state with $CuCl_2$ as catalyst. When a Cu^{2+}–phenanthroline complex was the catalyst, the C pair was most susceptible to oxidation. This permitted the preparation of paramyosin with only the C pair oxidized (O/2-paramyosin). The fully oxidized paramyosin could be reduced by dithiothreitol but the C pair disulfide bond was most readily reduced. Therefore, it was possible to prepare half-reduced R/2-paramyosin with disulfide bonding mainly in the N pair. Thermal denaturation studies in acidic solutions of 5 M guanidine-HCl showed differences for the oxidized and reduced forms of paramyosin and PPC-1. These differences indicated that the N pair disulfide bond contributes to the high stability of the N-terminal segment of the molecule. The C pair disulfide bond does not appear to stabilize the C-terminal region.

Paramyosin occurs in the adductor muscles of molluscs. This protein as isolated from the clam *Mercenaria mercenaria* is a large molecule of 220,000 atomic mass units that is rod shaped, 1350 Å long and 20 Å in diameter, and consists of two intertwined α-helical polypeptide chains (Lowey *et al.*, 1963). Published values for the number of Cys residues in paramyosin have varied from four to six based on amino acid analysis by ion-exchange chromatography (Riddiford and Scheraga, 1962a). The value of four Csy/paramyosin molecule as reported in this paper on the basis of a colorimetric method is in good agreement with these earlier values. The cysteine content of paramyosin from other molluscan species is about the same for some species such as *Pinna nobilis* (Bailey, 1957) and as much as twice as high for others such as *Crassostrea commercialis* (Woods, 1969).

Earlier studies from this laboratory have shown paramyosin to be susceptible to proteolysis in such a fashion that a common segment or proteolytic-resistant core remains after attack by any one of a number of enzymes. This is true whether the proteolytic enzyme is pepsin that yields a pepsin core PPC-1[1] or trypsin that yields a trypsin core PTC-1 (Cowgill, 1972). Each of these core segments arises from the N-terminal two-thirds of the paramyosin molecule. They are completely helical and seem to preserve intact the intertwined double α-helical chain structure of the parent molecule. In fact, the PPC-1 and PTC-1 segments seem to be identical except for minor differences at the C-terminal ends. Recently, the formation and general properties of PTC-1 have been verified in another laboratory (Halsey and Harrington, 1973).

The earlier studies that were published from my laboratory were done with paramyosin that had all sulfhydryl groups oxidized to the disulfide form. These results were not affected by the state of oxidation of the paramyosin, for the same PPC-1 and PTC-1 were obtained by proteolysis of paramyosin whether the sulfhydryl groups were oxidized or reduced. However, it did become apparent that some of the physical properties of paramyosin do depend upon the state of oxidation of its sulfhydryl groups. Some of these properties of paramyosin recently have been investigated also by Olander (1971), and he concluded that disulfide bonds do not affect either the molecular size or the stability. The present studies are in agreement that the molecular size is unaffected by oxidation of the sulfhydryl groups. However, my experiments do show an effect of the disulfide bond on stability of the molecule. Because these properties of the Cys residues might affect the physiological role of paramyosin in muscle contraction, the present detailed investigations were made. These studies have been greatly aided by the ability to isolate the segment PPC-1 that consists of the N-terminal two-thirds of the paramyosin molecule. This segment contains only two Cys residues (the N pair) and thus reveals the oxidation–reduction state of that particular pair following any oxidative or reductive treatment of paramyosin. By difference, it then was possible to determine the state of oxidation of the other two Cys residues (the C pair) in the C-terminal region. Sim-

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[1] Abbreviations used are: PPC-1, paramyosin pepsin-resistant core; PTC-1, paramyosin trypsin-resistant core; R-paramyosin (or R-PPC-1) is the form with all Cys reduced; O-paramyosin (or O-PPC-1) is the form with all Cys oxidized to the disulfide state; O/2-paramyosin is the form with only the Cys pair in the C-terminal region oxidized; R/2-paramyosin is the form with only the Cys pair in the C-terminal region reduced; EDTA, ethylenediaminetetracetic acid; Nbs_2, Ellman reagent, 5,5′-dithiobis(2-nitrobenzoic acid); Gdn · HCl, guanidine hydrochloride; MalNEt, *N*-ethylmaleimide.

ilarly, comparisons of the stability of paramyosin and PPC-1 permitted a distinction to be made between stabilizing effects of the disulfide bonds formed between the N pair and C pair of Cys residues.

Materials and Methods

Materials. Guanidine-HCl was of the Ultra Pure grade supplied by Mann Research Labs. The enzymes pepsin and trypsin, proteins for molecular weight standards, and the organic chemicals were of the highest purity available from Sigma Chem. Co. The metal salts were of Analytical grade.

Clams of the *M. mercenaria* species in the various lots were collected at the coast both in Delaware and North Carolina at all seasons of the year. No seasonal or geographic difference could be found.

R-Paramyosin. The paramyosin was isolated by minor modifications (Cowgill, 1972) of the classical procedure of Johnson *et al.* (1959). As isolated, all Cys residues of paramyosin usually were reduced. Four recrystallizations at pH 6 were employed to obtain preparations that were homogeneous by disc gel electrophoresis. However, repeated crystallization at pH 6 and solution at pH 7.5 slowly led to oxidation of the sulfhydryl groups. In fact, the difference in the appearance of the crystals of reduced and oxidized paramyosin was the initial observation that instigated this study. This oxidation was prevented by the presence of 2 mM dithioerythritol plus 1 mM EDTA in the crystallization solutions. A final dialysis against 0.02 N HCl removed the latter agents and R-paramyosin was stored in this acidic solution to prevent oxidation.

O-Paramyosin. Oxidized paramyosin was formed by oxidation of the four cysteinyl residues of R-paramyosin during storage in 0.4 M NaCl–0.05 M $NaPO_4$ solution at pH 7.5. The rate of oxidation was accelerated greatly in the presence of an atmosphere of O_2 and 2×10^{-5} M $CuCl_2$ in the solution. With the latter conditions, oxidation was complete in a few hours.

O-PPC-1 and R-PPC-1. The oxidized and reduced forms of the pepsin-resistant cores of paramyosin could be formed by pepsin digestion of the oxidized and reduced forms of paramyosin. A 3-hr digestion of the paramyosin with 1% by weight of pepsin in 0.02 N HCl at 20° was sufficient for complete conversion to PPC-1. Details of this procedure and subsequent purification procedures were described by Cowgill (1972). The O-PPC-1 also can be prepared by oxidation of R-PPC-1 in the presence of Cu^{2+} ions as catalysis in a fashion analogous to that described above for O-paramyosin formation.

O-PTC-1 and R-PTC-1. The oxidized trypsin-resistant core of paramyosin was formed by 3-hr digestion of O-paramyosin with 1% by weight of trypsin in 0.5 M NaCl–0.05 M Tris (pH 8.0) at 20°. The reaction was stopped by acidification or by addition of trypsin inhibitors. Details of this procedure and subsequent purification procedures were described by Cowgill (1972). R-PTC-1 was prepared in an analogous fashion except for the addition of 10 mM dithioerythritol to the digestion mixture to prevent air oxidation of the sulfhydryl groups on the protein. Conversion of R-PTC-1 to O-PTC-1 was readily accomplished as described above for PPC-1.

Cleavage of the Polypeptide Chain of Paramyosin at the Site of Cys Residues. The specific chemical cleavage reaction of Jacobson *et al.* (1973) was employed. Their method was followed that involves the sequential interaction of the protein with Nbs_2 and then KCN. After the prescribed reaction period, the products were dialyzed *vs.* 0.5 M NaCl–0.05 M $NaPO_4$ (pH 7.5) to remove salts and then analyzed by sodium dodecyl sulfate gel electrophoresis.

General Laboratory Procedures. Acrylamide disc gel electrophoresis was done by the method of Davis (1965) but with elimination of the stacking gel as has been suggested by Mitchell (1967). Sodium dodecyl sulfate gel electrophoresis for determination of molecular weight was by the method of Weber and Osborn (1969). This was routinely done with gels of 5% cross-linkage except for the proteins of high molecular weight in Table III, in which case gels of 2.5% cross-linkage were employed. Also, in this case it was essential to retain disulfide bonds intact and for this reason the dithioerythritol normally present in the preincubation mixture was replaced by 1×10^{-3} M MalNEt. The latter reagent was found to have no direct effect on the electrophoretic behavior of the proteins. Protein assays were by the method of Lowry *et al.* (1951) with a protein standard of bovine serum albumin prepared by Schwarz/Mann Research Labs.

Determination of Sulfhydryl Groups Reactive with Nbs_2. The determination of sulfhydryl groups reactive with Nbs_2 was by a modification of the method of Ellman (1959). The modifications were the inclusion of 0.5% of sodium dodecyl sulfate to enhance exposure and reactivity of SH groups and 1 mM EDTA to bind metal ions and thereby prevent air oxidation of the SH groups during the assay. Based on ϵ_{412} 13,600 for the liberated 2-nitro-5-thiobenzoate anion, the moles of sulfhydryl groups that reacted with Nbs_2 = $(A_{412}/13{,}600)(3\text{ ml}/1000\text{ ml}) = A_{412}2.2 \times 10^{-7}$. Calculation of the number of sulfhydryl groups per molecule of a protein was done as follows: SH/molecule = moles of SH reactive/moles of protein = $A_{412}2.2 \times 10^{-7}$/moles of protein in the 3-ml assay sample.

Determination of Total Cys Residues. The total Cys content of proteins, both oxidized and reduced, was determined by a modification of the method of Butterworth *et al.* (1967). The latter method consists of reaction of Nbs_2 with the protein, removal of excess Nbs_2 and unbound thionitrobenzoic acid, and subsequent measurement of thionitrobenzoic acid that had combined with sulfhydryl groups on the protein. The present modifications for total Cys content involved: (1) denaturation to render all Cys residues reactive and (2) reduction of any disulfide bonds with dithioerythritol prior to reaction with Nbs_2. The detailed procedure for paramyosin is as follows.

Paramyosin at a concentration of 4 mg/ml in 5 M Gdn·HCl–0.02 M $NaPO_4$ (pH 7.5) was placed in a water bath at 40° to denature the protein. Solid dithioerythritol was added to a concentration of 1 mM in the solution. After 0.5 hr at 40°, solid Nbs_2 was added to a concentration of 2 mM in the solution. After 0.5 hr at 40°, the solution was cooled and then mixed with 10 volumes of 0.02 M $NaPO_4$ (pH 6.0) at 0° to precipitate the protein. The insoluble paramyosin was washed well by centrifugation with cold 0.01 M NaOAc (pH 5.0). The pellet was dissolved in a volume of pH 8 buffer (0.5% sodium dodecyl sulfate–1 mM EDTA–0.1 M Tris) to yield a concentration of about 3 mg of paramyosin/ml. The thionitrobenzoic acid bound to the protein was released when a 1.5-ml aliquot of the protein solution was mixed with 1.5 ml of 1 mM dithioerythritol. The released thionitrobenzoic acid was measured at 412 nm and calculations of SH/molecule are as above.

PPC-1 and PTC-1 could be assayed for total Cys in the same fashion except that precipitation of these proteins was done at pH 5 rather than pH 6.

Fluorescence. Instrumentation including procedures for transition temperature studies was described in an earlier paper (Cowgill, 1972).

TABLE I: Number of Reduced Sulfhydryl Groups in Purified Preparations of Paramyosin and Its Proteolytic-Resistant Cores.

Compound	State	Symbol	SH/mole[a]
Paramyosin	Reduced[b]	R-Para	4.04–4.27
Paramyosin	Oxidized[c]	O-Para	0.1–0.2
Pepsin-resistant core	Reduced[d]	R-PPC-1	1.9–2.1
Pepsin-resistant core	Oxidized[e]	O-PPC-1	0.05–0.1
Trypsin-resistant core	Reduced[b,d]	R-PTC-1	1.9–2.1
Trypsin-resistant core	Oxidized[e]	O-PTC-1	0.05–0.1

[a] Determined by the Nbs_2 method for reactive SH groups (see Materials and Methods section). Values represent the range for determination of three or more lots of each protein. Molecular weights were assumed to be 220,000 for paramyosin and 140,000 for PPC-1 and PTC-1. [b] Determination of total cysteine as described in the Materials and Methods section. [c] Formed by oxidation of R-Para with $O_2 + Cu^{2+}$. [d] Formed by enzymatic digestion of R-paramyosin. [e] Formed by enzymatic digestion of O-paramyosin.

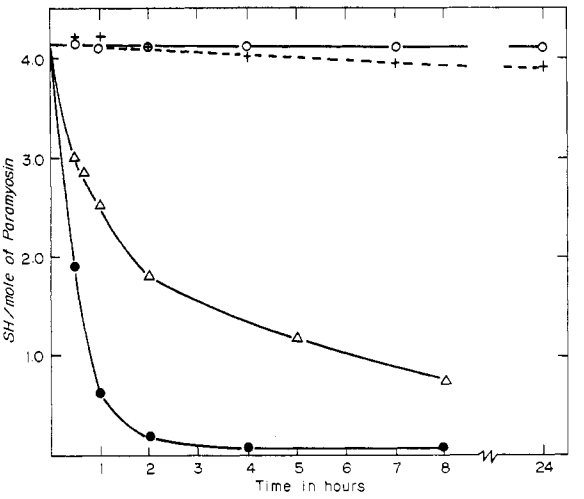

FIGURE 1: Oxidation of R-paramyosin at 5 mg/ml in 0.5 M NaCl–0.05 M $NaPO_4$ (pH 7.3) in an O_2 atmosphere at room temperature. The additives were 5×10^{-3} M EDTA (○); 5×10^{-3} M EDTA plus 5×10^{-5} M $CuCl_2$ (+); 5×10^{-5} $CuCl_2$ (●); or 1×10^{-5} M $CuCl_2$ (△).

Results

Total Cys Content of Paramyosin, PPC-1, and PTC-1. Paramyosin was isolated from both whole adductor muscle and either the striated or smooth portions. These various preparations of paramyosin were found to contain similar amounts of reactive sulfhydryl groups as measured by the Nbs_2 assay. Values ranged from 3.5 to 4.3 SH/molecule of paramyosin at the initial stage of crystallization after extraction of muscle from numerous batches of clams that had been kept in aerated seawater at 5°. Comparable isolations of paramyosin from clams subjected to quite a variety of environmental and contractile conditions gave the same range of reactive SH/mole. These results seem to show that the physiological state of the muscle had little effect on the redox state of the sulfhydryl groups of paramyosin as extracted.

The average value of four SH/mole of paramyosin as isolated was found to correspond to the total Cys residues of paramyosin, that is, all Cys residues were in the reduced state upon isolation. The values for total Cys residues are presented in Table I. These sulfhydryl groups could be completely oxidized, however, as is demonstrated in Figure 1 as well as Table I. When similar experiments were done with PPC-1 and PTC-1 it was found that only two Cys residues remained on these core segments. These Cys residues of R-PPC-1 and R-PTC-1 also could be oxidized under the same conditions, as will be demonstrated later in Figure 3.

Effect of the Oxidation State of the Cys Residues on Crystallization of Paramyosin. In the course of its isolation as R-paramyosin, the protein is exceedingly easy to crystallize and yields sharp needle-shaped crystals. As a result, crystalline suspensions of R-paramyosin upon swirling display a strong birefringence. Crystallization of O-paramyosin was quite different. Crystallization seemed to occur more slowly, no birefringence developed in the solution, and microscopic examination showed clumps of minute crystals. It is interesting that if crystallization was induced in a solution prepared by mixing equal amounts of O-paramyosin and R-paramyosin each form retained its characteristic crystal form.

Absence of Intermolecular Disulfide Bond Formation upon Oxidation. Disulfide bonds in the fully oxidized paramyosin might form between molecules or might be intramolecular. Tests for intermolecular disulfide bonds were done by two techniques that are sensitive to gross changes in molecular size. Disc gel electrophoresis both at pH 2.3 and 9.5 gave a single well-defined band at the same position for the fully oxidized and fully reduced forms of paramyosin. Similarly, electrophoresis of both PPC-1 and PTC-1 gave a single band which was at the same position for oxidized and reduced forms. This indicates that the disulfide bonds were not intermolecular. The absence of intermolecular disulfide linkages also was established by viscosity studies. Viscosity measurements at both pH 2.3 and 7.4 (Cowgill, 1972) yielded intrinsic viscosities for oxidized and reduced forms of both paramyosin and PPC-1 that were identical within the limitation of experimental error. Had intermolecular disulfide bonding given dimers the intrinsic viscosity would have increased markedly.

Location of Cys Residues. The fact that four Cys residues of paramyosin are able to form intramolecular disulfide bonds suggests that they occur as pairs along the polypeptide chains. One pair of Cys residues occur in both PPC-1 and PTC-1 which have been shown to arise from the N-terminal two-thirds of the paramyosin molecule. It was to be expected that some of the Cys residues would be associated with this segment for only Cys had been found as the N-terminal residue of paramyosin, PPC-1, and PTC-1 as in Scheme I, (a) (Cowgill, 1972). The second pair of Cys residues, somewhere in the C-terminal one-third of the paramyosin molecule, was lo-

SCHEME I: Representations of the Possible Distribution of Cys Residues (C) along the Two Polypeptide Chains of Paramyosin and PPC-1 or PTC-1.

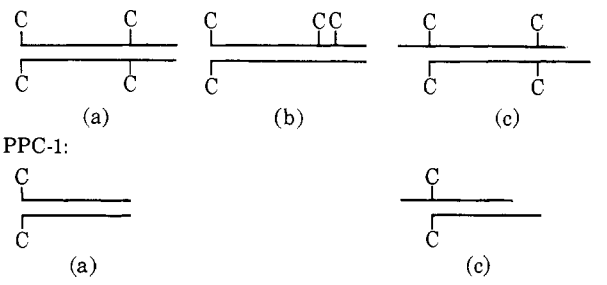

TABLE II: Products from Cleavage of Paramyosin at Sites of Cys Residues.[a]

Conditions for the Cleavage Reaction	% Recovery of the Initial Protein in the Products	Rel Intensity of Bands Corresponding to Products of Mol Wt as Indicated: 110,000 Band (%)	75,000 Band (%)	30,000 Band (%)
Regular procedure	94	18.2	43.0	38.8
Reaction with double the regular amounts of reagent	90	19.3	44.6	36.1
Products of reaction recycled through the reaction a second time	88	19.4	43.6	37.0

[a] Paramyosin in amounts of 50–100 mg was subjected to the method of Jacobson *et al.* (1973) for peptide cleavage specifically at the site N terminal to Cys residues. Consult the Methods section for details of the reaction. Products were subjected to sodium dodecyl sulfate gel electrophoresis and the intensities of the three bands were determined in a densitometer. The relative intensities are expressed as the percentage of the total densitometer band areas that occurred in each band. Molecular weights of products at each of these band positions were determined as described in the Methods section for gel electrophoresis.

cated by the chemical cleavage method of Jacobson *et al.* (1973). This method is based on quantitative conversion of Cys residues to *S*-cyanocysteine residues and subsequent cleavage of the amino peptide bond of the *S*-cyanocysteine residue to yield polypeptide fragments. The size of these fragments in favorable circumstances such as the present case determines the location of the Cys residues in the original molecule. This reaction when carried out with paramyosin gave only three products which constituted 90% of the protein taken for reaction. These products are characterized in Table II. The 110,000 band appears to be a minor amount of the original polypeptide chain of paramyosin. The other two bands are those to be expected if the Cys residue was located 30% of the total molecular length or 400 Å from one end or the other of the original polypeptide chain. That location must be 400 Å from the C-terminal end because the PPC-1

TABLE III: Molecular Weights of Oxidized and Reduced Forms of Paramyosin and PPC-1.[a]

Protein	Molecular weight
R-PPC-1	72,000
O-PPC-1	151,000
R-Paramyosin	113,000
O-Paramyosin	200,000

[a] The molecular weight determinations were done by sodium dodecyl sulfate gel electrophoresis with 2.5% cross-linkage. Oxidized samples were preincubated in the presence of 1×10^{-3} M MalNEt in place of dithioerythritol to prevent disulfide interchange. (See Methods section for details.)

TABLE IV: Comparative Rates of Oxidation of the Cys N Pair and C pair by Oxygen Plus 1×10^{-5} M $CuCl_2$.[a]

Time (hr)	SH/mole by Nbs_2 Assay: In Paramyosin	In PPC-1	% of the Total Reduced SH Present in the N Pair
0.5	3.00	1.38	46
0.67	2.86	1.42	50
1	2.52	1.34	53
2	1.79	0.97	54
5	1.20	0.61	50
8	0.75	0.40	53

[a] R-Paramyosin was oxidized in an oxygen atmosphere at 20° in the presence of 1×10^{-5} M $CuCl_2$–0.5 M NaCl–0.05 M $NaPO_4$ (pH 7.3). Samples were withdrawn at times noted and brought to a level of 1×10^{-3} M EDTA to terminate oxidation. A portion of the paramyosin in each aliquot was converted to PPC-1 by pepsin digestion at pH 2.3.

segment from the N-terminal two-thirds of the molecule contains only the N pair of Cys residues.

Tests for Inter-vs. Intrachain Disulfide Bonds. Distinction between the possibilities of disulfide bonds along the same polypeptide chain or between the two intertwined chains was made in part by molecular weight studies. These are summarized in Table III. It was necessary to retain the disulfide bonds in these experiments and this does introduce a complication.[2] However, the values in Table III show that the reduced form of PPC-1 yields two polypeptide chains, each of 72,000, which is consistent with earlier observations of Cowgill (1972), while the oxidized form of PPC-1 moves as a single polypeptide of 151,000 or twice the mass of the reduced state. This indicates that the N pair of Cys residues form an interchain disulfide bond. If the two chains of paramyosin are held together by the N-terminal disulfide bond the approximately twofold greater size for O-paramyosin relative to R-paramyosin in Table III is to be expected. Therefore, these data do not reveal whether the C pair of Cys residues form an interchain or an intrachain disulfide bond. Further consideration of data in Table II suggests that this bond also is interchain as will be discussed later.

Oxidation of Sulfhydryl Groups. Oxidation of the four cysteinyl residues of R-paramyosin by atmospheric O_2 rapidly occurred at neutral pH when small amounts of cupric ions were present. The data in Figure 1 demonstrate that the rate of oxidation was dependent upon the concentration of cupric ions and did not occur in the present of sufficient EDTA to sequester all of the free metal ions. It was of interest, in light of other experiments to be described below, to determine whether one Cys pair was more susceptible to oxidation than the other. Distinction between the two Cys pairs could be made by study of the N pair separately in PPC-1. The data are summarized in Table IV and show that essentially half of

[2] All disulfide bonds are broken at the time of interaction with sodium dodecyl sulfate under regular conditions for molecular weight determination. The shape and electrophoretic behavior of the detergent–protein complex when disulfide bonds restrict freedom of the polypeptide chain are unknown and probably vary depending upon the number and location of disulfide bonds. These uncertainties should be minimal for PPC-1 with a single disulfide bond at the end of the polypeptide chain.

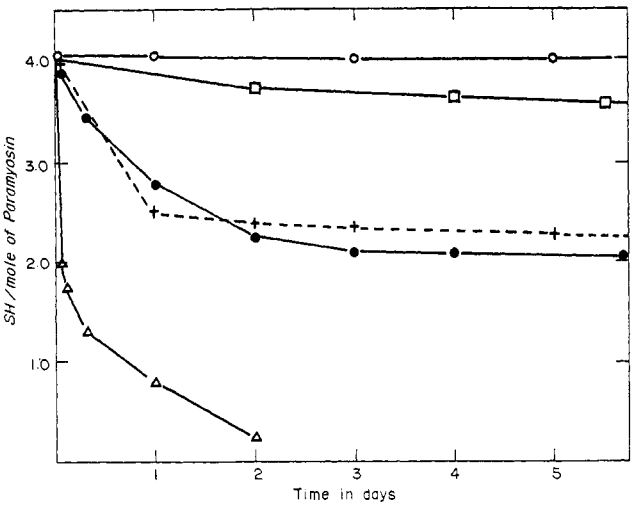

FIGURE 2: Oxidation of R-paramyosin as in Figure 1. The additives were as follows: (□) none; (●) 5×10^{-4} M *o*-phenanthroline; (○) 5×10^{-4} M *o*-phenanthroline + 1×10^{-3} M EDTA; (△) 5×10^{-4} M *o*-phenanthroline + 2×10^{-6} M $CuCl_2$; (+) 5×10^{-4} M *o*-phenanthroline + 1×10^{-5} M $CuCl_2$ + 5×10^{-4} M EDTA.

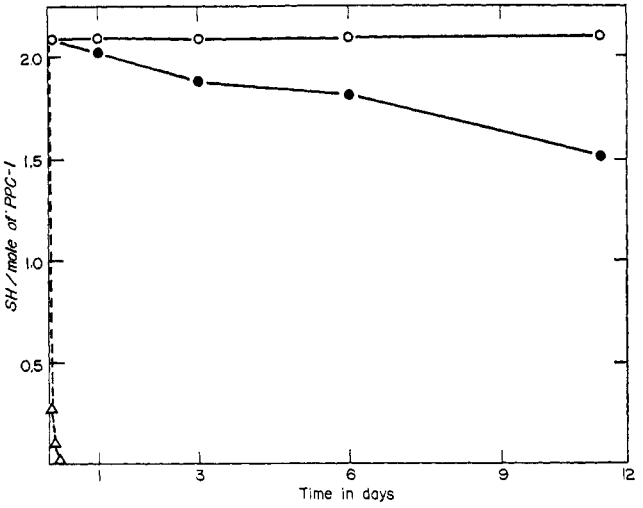

FIGURE 3: Oxidation of R-PPC-1 under conditions as in Figure 1. The additives were 1×10^{-3} M EDTA (○); 5×10^{-4} M *o*-phenanthroline (●); or 5×10^{-5} M $CuCl_2$ (△).

the residual sulfhydryl groups were in the N pair regardless of the degree of oxidation of the paramyosin. Thus, it appears that both Cys pairs were equally susceptible to oxidation when Cu^{2+} was the catalyst. A variety of other metal ions (Mg^{2+}, Mn^{2+}, Ni^{2+}, Sn^{2+}, Zn^{2+}, Fe^{3+}) was tested and failed to catalyze the oxidation.

Kobashi and Horecker (1967) had observed the oxidation of a restricted number of the sulfhydryl groups on aldolase when the catalyst was *o*-phenanthroline. The rate of oxidation increased markedly when the catalyst was a complex of cupric ions plus *o*-phenanthroline. Therefore, the effect of *o*-phenanthroline on the oxidation of R-paramyosin was investigated. Figure 2 shows that oxidation slowly occurred in the absence of an added catalyst even when all reagents were prepared with water that had been both glass distilled and deionized. This rate was increased significantly by the presence of 5×10^{-4} M *o*-phenanthroline. The presence of 2×10^{-6} M $CuCl_2$ in addition to phenanthroline increased the rate further. Virtually all Cu^{2+} ions would be bound to the phenanthroline and the Cu^{2+} phenanthroline complex seems to be the effective catalyst in this latter case as well as for the last set of data (+) in this figure. The fact that oxidation did not occur in the presence of 5×10^{-4} M phenanthroline when 1×10^{-3} M EDTA also was present indicates that the catalyst even when Cu^{2+} was not added probably was a complex of phenanthroline plus some contaminating metal ions.

The most interesting observation in this figure is that the *o*-phenanthroline catalyzed reaction stopped after only half the total Cys was oxidized. This appeared to be true so long as the cupric ion level was low, either because $CuCl_2$ was not added or because EDTA was present as well as $CuCl_2$. In order to determine whether one Cys pair was preferentially oxidized in the presence of *o*-phenanthroline, the experiments described in Table V were done. Oxidation of a small amount (5–10%) of the Cys pair on PPC-1 occurred routinely during the pepsin digestion of R-paramyosin and subsequent isolation of PPC-1. This could account for 46% rather than 50% of the total SH in the PPC-1 at the start of the experiment and the 85% rather than 100% value at days 3–6. However, it is clear that most if not all of the Cys residues oxidized in the presence of *o*-phenanthroline are the C pair. To test this conclusion, the rate of oxidation was measured for R-PPC-1 which bears only the Cys N pair. Figure 3 shows that the Cys N pair was rapidly oxidized in the presence of free cupric ions but extremely slowly in the presence of *o*-phenanthroline.

The oxidation of sulfhydryl groups on aldolase was catalyzed by complexes of Cu^{2+} with *o*-phenanthroline but not by complexes with other divalent cations, such as Zn^{2+} (Kobashi and Horecker, 1967). However, the rate of oxidation of R-paramyosin in the presence of *o*-phenanthroline was increased by high levels of Zn^{2+} (5×10^{-4} M) as well as low levels of Cu^{2+} ions. The rate of oxidation in the presence of the Zn–phenanthroline complex decreased after the level of remaining SH groups had fallen below two SH/mole, just as was the case for the Cu–phenanthroline complex in Figure 2.

Reduction of Disulfide Groups. The disulfide groups of O-paramyosin and O-PPC-1 were reduced by incubation with excess dithioerythritol at neutral pH. The details of these experiments are presented in Table VI and again the conversion of paramyosin to PPC-1 was employed to distinguish between the Cys pairs. These results indicated that reduction was incomplete when the proteins were in their native conformation and reduction occurred mainly with the C pair of cystinyl residues on O-paramyosin. Therefore, reduction was conducted also in 6 M Gdn·HCl. Reduction approached completion for both proteins under the latter conditions which are known to disrupt the helical structure (Cowgill, 1972).

TABLE V: Comparative Rates of Oxidation of Cys N Pair and C Pair by Oxygen in the Presence of *o*-Phenanthroline.[a]

Time (days)	SH/mole by Nbs_2 Assay In Paramyosin	In PPC-1	% of Total Reduced SH Present in the N Pair
0	4.05	1.85	46
0.33	3.40	1.85	54
1	2.75	1.80	66
3	2.10	1.80	86
6	2.05	1.75	85

[a] R-Paramyosin was oxidized in an oxygen atmosphere at 20° in the presence of 5×10^{-4} M *o*-phenanthroline–0.5 M NaCl–0.05 M $NaPO_4$ (pH 7.3). Samples were analyzed as in Table IV.

TABLE VI: Extent of Reduction of Native and Denatured O-Paramyosin and Loci of Affected Groups.[a]

Condition for reduction	SH/mole of the Isolated Paramyosin (N pair + C pair)	SH/mole of the N pair
Helical O-Paramyosin	2.22, 2.44	0.43, 0.35
O-Paramyosin denatured by 6 M Gdn·HCl	3.40, 3.97	1.85, 2.12
Helical O-PPC-1		0.40, 0.60
O-PPC-1 denatured by 6 M Gdn·HCl		1.95, 2.10

[a] O-Paramyosin was reduced by 10 mM dithioerythritol during 24 hr at 20° in 0.5 M NaCl–0.02 M $NaPO_4$–2 mM EDTA (pH 7.0). In the case of denatured paramyosin, conditions were as above except that 6 M Gdn·HCl also was present. In both cases, paramyosin was crystallized by extensive dialysis against 0.05 M $NaPO_4$–2 mM EDTA (pH 6.0) in a N_2 atmosphere. The crystalline paramyosin was dissolved in 0.02 N HCl for assay of SH/mole (values are for two experiments) and for preparation of PPC-1 as a measure of the N pair. O-PPC-1 was reduced as above, and then dialyzed extensively against 0.02 N HCl to remove dithioerythritol and salts.

Preparation of R/2- and O/2-Paramyosin. The experiments described above indicated that it should be possible to preferentially reduce the disulfide bond of the Cys C pair of O-paramyosin to yield R/2-paramyosin (C pair mainly reduced; N pair mainly oxidized). Conversely, it should be possible to preferentially oxidize the Cys C pair of R-paramyosin to yield O/2-paramyosin (C pair mainly oxidized; N pair mainly reduced).

A typical preparation of R/2-paramyosin will be described. O-Paramyosin (50 mg) at a concentration of 5 mg/ml of 0.5 M NaCl–0.05 M $NaPO_4$ (pH 7.5) was employed. Initially the preparation had 0.15 SH/mole by the Nbs_2 assay. Dithioerythritol was added to 10 mM and EDTA to 1 mM concentrations and the preparation was stored under a N_2 atmosphere for 24 hr at room temperature. Then, the paramyosin was crystallized by dropwise addition of 50 ml of 0.01 M NaH_2PO_4, pH unadjusted. The crystalline slurry was centrifuged at 0°. The pellet was dissolved in 10 ml of 0.02 N HCl and dialyzed extensively at 0° *vs.* 0.02 N HCl. The properties of the product are summarized in Table VII.

A preparation of O/2-paramyosin was made in the following fashion. R-Paramyosin (100 mg) at a concentration of 5 mg/ml of 0.5 M NaCl–0.05 M $NaPO_4$ (pH 7.5) was employed. Initially the preparation was completely reduced (4.1 SH/mole). *o*-Phenanthroline was added to a 5×10^{-4} M concentration. The solution was stored at room temperature under an atmosphere of O_2 until an aliquot showed a decrease to 2.15 SH/mole by the Nbs_2 assay. The protein was crystallized in the presence of 1 mM EDTA and dialyzed against 0.02 N HCl as described above for R/2-paramyosin. The properties of the product are summarized in Table VII.

Stability of Paramyosin and PPC-1 at Various Stages of Oxidation of the Sulfhydryl Groups. Earlier work has shown that fluorescence of Tyr residues is an effective way to evaluate the stability of these helical molecules because fluorescence drops dramatically when helical structure is lost (Cowgill, 1968). (These proteins lack Trp residues which otherwise would contribute to the fluorescence.) By means of the fluorescence technique, the stability of O-paramyosin and O-PPC-1 in acidic Gdn·HCl solutions has been investigated by Cowgill (1972). From these studies it was concluded that paramyosin is most stable in the N-terminal region. At a concentration of 5–6 M Gdn·HCl at pH 2 and 25°, it was estimated that only the N-terminal one-third of paramyosin and the N-terminal one-half of PPC-1 were still helical. These conditions now have been employed to compare the stability of the N-terminal regions of these molecules in the presence and absence of the disulfide bond.[3] The residual helical structure in the N-terminal region was disrupted by elevation of the temperature in 5 M Gdn·HCl at pH 2 as shown in Figures 4 and 5. These data show differences between the oxidized and reduced forms of both paramyosin and PPC-1. The initial slow decrease in fluorescence over the region 10–30° is a general thermal quenching phenomenon unrelated to conformational changes. The more marked drop in fluorescence above 30° is attributable to loss of helical structure. The latter change begins at a lower temperature for R-paramyosin and the extent of the transition region is greater than for O-paramyosin in Figure 4. At 50°, denaturation of both forms of paramyosin is complete (Riddiford and Scheraga, 1962b; Cowgill, 1972). Quite similar data in Figure 5 were observed for R-PPC-1 and O-PPC-1 which contain only the Cys N pair. In experiments analogous to those in Figure 4, the transition temperature curve of R/2-paramyosin (N pair oxidized) was identical with the one shown for O-paramyosin and the one for O/2-paramyosin (N pair reduced) was identical with the R-paramyosin curve. All of these studies indicate that the N-terminal disulfide bond stabilizes the helical conformation.

A search for an effect of the Cys C pair disulfide bond on stability was done in 0.02 N HCl. In this acidic solution paramyosin was initially in the helical conformation at room temperature. Upon heating, a transition was noted at 48° as

TABLE VII: Distribution of the Reduced Form of Cysteine in R/2-Paramyosin and O/2-Paramyosin (See text for the preparation of these two forms of paramyosin).

Form of Paramyosin	% Recovery of Protein in the Preparation	Total SH/mole	% in the Reduced State[a]	
			N pair	C pair
R/2-paramyosin	88	2.28	25	90
O/2-paramyosin	85	1.90	85	10

[a] Pepsin digestions of each form and analysis of the SH content of the PPC-1 permitted the calculation of the percent of the reduced Cys in each Cys pair. Percentages do not total 100% because each value is the percent in the SH state for that particular Cys pair.

[3] In the present experiments, consideration had to be given to any effects of Cys residues on fluorescence. The reason for this is that sulfhydryl groups can quench fluorescence weakly and disulfide groups can quench strongly (Cowgill, 1967). The quenching appears to occur, however, only when the Cys and Tyr residues are within van der Waal distances of one another. In the case of both paramyosin and PPC-1 the fluorescence yields were the same for both the oxidized and reduced forms at pH 2 and pH 7.5. Presumably the distance between Cys and Tyr residues is too great for quenching to occur.

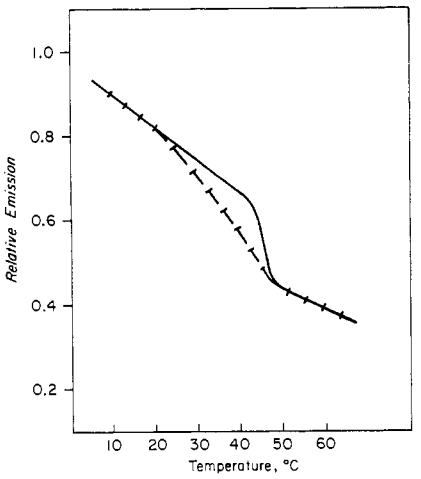

FIGURE 4: Thermal denaturation of paramyosin as followed by loss of fluorescence from Tyr residues. Paramyosin was at 1.0 mg/ml in 5.0 M Gdn·HCl–0.02 N HCl. The recorder traces were for O-paramyosin (—) and R-paramyosin (|——|). Initial and final slopes were −0.9%/deg that characterize thermal effects on general solvent quenching of fluorescence. Transitional slopes were −2.0%/deg for R-paramyosin and −4.0%/deg for O-paramyosin. Denaturation was reversible in both cases.

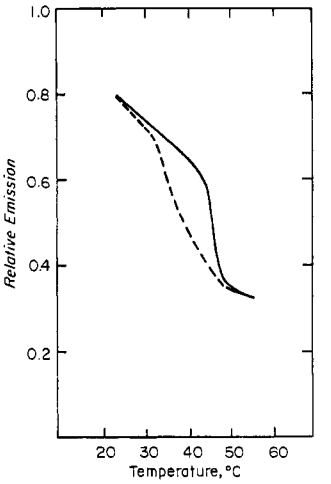

FIGURE 5: Thermal denaturation of O-PPC-1 (—) and R-PPC-1 (- -) under conditions as in Figure 4. Denaturation was reversible in both cases.

shown in Figure 6. This transition appears to relate to changes in the most labile C-terminal portion of the molecule for no comparable transition was noted for PPC-1. The loss of fluorescence was the same for both R- and O-paramyosin. Therefore, stabilization of the C-terminal region does not seem to be dependent upon the oxidation–reduction state of the Cys residues.

Discussion

These results demonstrate that the four cysteinyl residues of paramyosin may be considered as two pairs. Two Cys residues (the N pair) are at the N terminus. One residue is on each of the two polypeptide chains and the residues are so situated that they can form an interchain disulfide bond. This was established by the observation that the molecular weight of O-PPC-1 was twice that of R-PPC-1 in Table III. Only cysteine has been found as the N-terminal amino acid of paramyosin (Cowgill, 1972) but this does not prove that both polypeptide chains terminate in cysteine. However, because the N pair of Cys residues participate in a disulfide bond, the second Cys residue must either be N terminal on the second polypeptide chain or close to the N terminus. (The occurrence of only the two 30,000 and 75,000 fragments in Table II would not be expected if the two polypeptide chains were aligned so that appreciable amount of single chain protruded at each end of the molecule. Contrast (a) *vs.* (c) in Scheme I.)

The second pair (C pair) of Cys residues can be located at about 27–30% of the distance down from the C terminus on the basis of data in Table II. For a rod-like molecule of 1350 Å total length, this would locate the C pair about 950 Å away from the N pair. This Cys pair also is capable of forming an interchain disulfide bond. The basis for this conclusion comes from the data in Table II. Either the two Cys of the C pair are on different polypeptide chains and the disulfide bond is interchain (diagram Ia), or they occur on the same chain and the disulfide bond is intrachain (diagram Ib). Ideally, the results of the Cys cleavage experiment in Table II would yield equal molar amounts of 110,000, 75,000, and 30,000 products if both Cys were on one chain and yield equal molar amounts of only the 75,000 and 30,000 products if one Cys residue were on each chain. The data indicate that the Cys residues are on different chains although the experimental values do not approach the above ideal. Certainly there remains about 19% as 110,000 product (this is on a mass basis and would amount to about 12% on the basis of molecular ratios). However, it would be difficult to account for this low amount of 110,000 product if one of the two polypeptide chains had no Cys residue of the C pair and thus was not subject to cleavage. (The N pair of Cys residues will not of course influence these results.) Conversely, it would be nice to bolster the conclusion that one Cys residue is on each polypeptide chain by being able to account for the minor amount of 110,000 product. It could arise from incompleteness of the cleavage reaction but this does not seem likely for more stringent reaction conditions described in Table II failed to change the amount of the 110,000 product. A recent paper by Degani and Patchornik (1974) provides another explanation that appears more probable. These authors report that the *S*-cyanocysteinyl residue can undergo either the peptide bond cleavage reaction or a β elimination as a side reaction. The β elimination of thiocyanate yields the dehydroalanyl residue and of course the

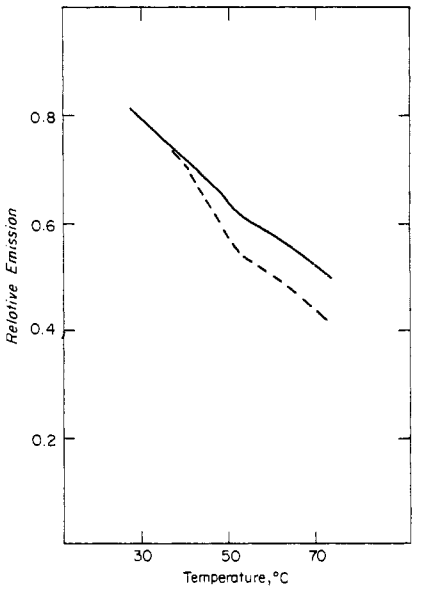

FIGURE 6: Thermal denaturation of proteins at 1 mg/ml in 0.02 N HCl. R-Paramyosin and O-paramyosin both gave the same data (- - -); the other curve is for R-PPC-1 (—).

polypeptide chain is not cleaved. Among the Cys peptides these authors tested, the β elimination reaction accounted for from 5 to 50% of the product, and therefore might account for the amount of 110,000 product in Table II.

All four Cys residues of paramyosin could readily be oxidized when the catalyst was cupric ions and the data in Table IV indicate that both the Cys C pair and N pair were equally susceptible. When the catalyst was the complex of Cu^{2+} plus phenanthroline the rate of oxidation of the Cys N pair was much slower and it was possible to form the O/2-paramyosin with disulfide bonding mainly in the C pair. It is not known whether these differences in oxidation rate with the phenanthroline complex are due to steric hindrance for approach to the Cys N pair or to a more basic difference in the catalytic process for free and complexed cupric ions. Also, there was a difference in the tendency of the two disulfide bonds to be broken by reduction with dithioerythritol. This difference was great enough to permit the isolation of R/2-paramyosin in which 80% of the unreduced disulfide bonds were in the N pair. The ease with which the C pair of Cys residues may be oxidized or reduced suggests that these two residues are situated in positions favorable for formation of the disulfide bond. The resistance of the N pair of Cys residues to both oxidation and reduction may be associated with some energy barriers peculiar to their position at the termination of the polypeptide chains.

The N-pair disulfide bond clearly enhanced the stability of O-paramyosin and O-PPC-1 in acidic solutions of 5 M Gdn·HCl as demonstrated in Figures 4 and 5. The marked similarity of data in the two figures and the fact that PPC-1 contains only the Cys N pair indicate that the stability differences depend upon the N-terminal disulfide bond. These results also establish the location of the remaining helical structure in 5 M Gdn·HCl at room temperature. This helical structure which was estimated to be one-third of the paramyosin molecule and one-half of PPC-1 must be in the N-terminal segment. As the temperature of the 5 M Gdn·HCl solution was increased the N-terminal one-third of paramyosin also lost its helical structure and the molecule separated into two randomly coiled polypeptide chains. The wide temperature range of 28–47° for this transition in the case of R-paramyosin suggests that the process occurred in multiple stages, possibly by an uncoiling initiated at one or both ends of the helical segment. The sharp transition for O-paramyosin indicates that the entire helical segment retained its conformation until the transition temperature of 46° was reached. At that temperature it apparently passed to the random coil in a single stage. (This process was reversible for both oxidized and reduced forms.) Because the only known difference is the presence or absence of the disulfide bond, it seems reasonable to attribute these differences to that bond. That is, the results are consistent with stabilization of the helical segment of O-paramyosin in a temperature region of 28–43° where this segment in R-paramyosin has begun to uncoil from the N terminus. Finally, at 43–47° the stabilizing contribution of the disulfide bond was exceeded and O-paramyosin denatured completely.

Stability of the C-terminal region was assessed in Figure 6. Paramyosin is known to be stable and about 90% helical in acid solution at room temperature (Lowey, 1965) and PPC-1 is completely helical under the same conditions (Cowgill, 1972). As the temperature increased a transition occurred at 48°. This did not occur with PPC-1[4] and therefore appears to involve the loss of helical conformation in the more unstable C-terminal segment. The same thermal denaturation curve was observed with R- and O-paramyosin. Therefore, the presence of the C pair disulfide bond does not appear to affect stability under these conditions.

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References

Bailey, K. (1957), *Biochim. Biophys. Acta 24*, 612.
Butterworth, P. H. W., Baum, H., and Porter, J. W. (1967), *Arch. Biochem. Biophys. 118*, 716.
Cowgill, R. W. (1967), *Biochim. Biophys. Acta 140*, 37.
Cowgill, R. W. (1968), *Biochim. Biophys. Acta 168*, 417.
Cowgill, R. W. (1972), *Biochemistry 11*, 4532.
Davis, B. J. (1965), *Ann. N. Y. Acad. Sci. 121*, 404.
Degani, Y., and Patchornik, A. (1974), *Biochemistry 13*, 1.
Ellman, G. L. (1959), *Arch. Biochem. Biophys. 82*, 70.
Halsey, J. F., and Harrington, W. F. (1973), *Biochemistry 12*, 693.
Jacobson, G. R., Schaffer, M. H., Stark, G. R., and Vanaman, T. C. (1973), *J. Biol. Chem. 248*, 6583.
Johnson, W. H., Kahn, J. S., and Szent-Györgyi, A. G. (1959), *Science 130*, 160.
Kobashi, K., and Horecker, B. L. (1967), *Arch. Biochem. Biophys. 121*, 178.
Lowey, S. (1965), *J. Biol. Chem. 240*, 2421.
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.
Mitchell, W. M. (1967), *Biochim. Biophys. Acta 147*, 171.
Olander, J. (1971), *Biochemistry 10*, 601.
Riddiford, L. M., and Scheraga, H. A. (1962a), *Biochemistry 1*, 95.
Riddiford, L. M., and Scheraga, H. A. (1962b), *Biochemistry 1*, 108.
Weber, K., and Osborn, M. (1969), *J. Biol. Chem. 244*, 4406.
Woods, E. F. (1969), *Biochem. J. 113*, 39.

[4] A slight discontinuity in the curve for PPC-1 may denote a very small amount of uncoiling in the C-terminal end of this molecule at 50°.