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Secondary structure of synthetic peptides derived from the repeating unit of a giant secretory protein from *Chironomus tentans*

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The secretory proteins of *Chironomus tentans* larvae, which are used to construct underwater feeding and pupation tubes, assemble into complexes in vitro. Members of a family of 1000 kDa proteins, the spls, appear to form the fibrous backbone of the assembled complexes. The spls consist of a core of tandemly repeating units of 60 to 90 amino acids that can be subdivided into two regions: the subrepeat region, made up of short internal repeats, and the constant region, which lacks simple subrepeats. We have synthesized peptides representative of the constant and subrepeat regions of one of the spls, and have examined their secondary structure using Fourier transform IR and CD spectroscopy. The IR spectrum of the constant peptide indicates that this peptide has α -helical regions and β -turns. The CD spectrum confirms this. The IR spectrum of the subrepeat peptide is similar to that of the poly(Gly)II helix, and also may indicate the presence of β -turns. The CD spectrum is consistent with this helical structure. Extrapolation of these results to intact spls is in agreement with secondary structure prediction and modeling studies. Our results indicate that the α -helices and poly(Gly)II-like helices are not arranged as coiled-coils, which are often found in fibrous proteins. We suggest that these structural elements may be in an unusual arrangement in the spls, organized as alternating α -helices and poly(Gly)II or collagen-like helices, interspersed with β -turns.

Introduction

Salivary glands of the aquatic larvae of *Chironomus tentans* contain a family of secretory proteins (SPs). SPs are secreted as insoluble threads that larvae spin underwater to construct feeding and pupation tubes [1]. We are interested in the structure and unusual properties of these proteins, and in determining the molecular architectural plan that dictates these properties. The largest of these proteins, members of the spl family (spl_a, spl_b, spl_c and spl_d), are estimated to be

from 750 to 1000 kDa in size [2–4]. These four different proteins have similar primary structure, including several features that are characteristic of fibrous proteins: periodicities in the amino acid sequence and a large percentage of charged residues (for reviews see Refs. 5–7). They consist mainly of a core sequence of 60 to 90 amino acids, tandemly repeated more than 100 times. Each core repeat has two regions, a constant (C) and a subrepeat (SR) region (Fig. 1A). The SR region is rich in proline and charged amino acids; within this region are internally repeated segments which tend to have the tripartite motif (basic residue – proline – acidic residue). The basic residues are typically arginine or lysine, and the acidic residues are glutamate, or phosphorylated serine or threonine. The C region, in contrast, has no internally repeating motif and lacks proline. There are 6 invariant amino acids in the C region: 4 cysteines, 1 methionine and 1 phenylalanine.

Hamodrakas and Kafatos [8] have proposed roles for some of these features in the packing and cross-linking of the spls; they predicted that the C regions of

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Abbreviations: SPs, secretory proteins; spl family, a family of approx. 1000-kDa SPs; C region, constant region of an spl; SR region, subrepeat region of an spl.

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spls have regions of α -helix, and suggested that these regions could pack into higher-order structures that could be stabilized by intermolecular electrostatic interactions and disulfide bonds between cysteine residues. They suggested that the charged residues would ensure hydration, a necessary property for these proteins because they function at the bottom of freshwater lakes. They pointed out that intermolecular disulfide bonds would render the protein complex insoluble, and that the size of the proteins would ensure that this insolubility occurred as soon as cross-links began to form. Due to the short precise subrepeats within the SR region, its secondary structure could not be predicted.

We have begun studies to determine the mechanisms of assembly of SPs and the interactions that may occur between them. We have previously shown that the SPs can be isolated as complexes and that the spls are the fibrous backbone of these complexes [9]. Because the spls alone are capable of forming complexes, we have focused our studies on them. We measured the CD spectra of spls and confirmed that spls had regions of α -helix and β -turn [9], as predicted [8]. The CD spectra of spls were, in addition, consistent with the presence of β -sheet structures. β -Sheet structure was not predicted by Hamodrakas and Kafatos in the C region, and although they could not predict secondary structure of the SR region, they suggested, from modeling studies, that it might exist as an extended collagen-like left-handed helix.

Several attempts to determine the secondary structure of the proteins within isolated larval feeding and pupation tubes, using various methodologies such as X-ray diffraction, laser-Raman spectroscopy and IR

spectroscopy, have failed, mainly because of the high percentage of contaminants, especially of cellulose. Larvae spin threads of secretory proteins underwater and construct their tubes by attaching these threads to plant debris, which contains cellulose fibers. Therefore, in order to determine the secondary structure elements contributed by regions of the repeat unit, we have synthesized peptides from the C and SR regions of a core repeat of an spl, and have investigated their secondary structure using Fourier transform IR and CD spectroscopy. We used the sequence of spla to design our peptides; however, since the four spls are considered equivalent structurally (for review, see Ref. 7) and functionally [10], and are predicted to form similar secondary structure [8], the results obtained for these spla peptides may be applicable to all four spls.

Materials and Methods

Peptides. The amino acid sequences of C and SR peptides (Fig. 1B and C) were deduced from nucleotide sequences of cDNA clones [11] and genomic clones [12] that code for the protein spla of *C. tentans* (see Refs. 2 and 13). All or nearly all serines and threonines are phosphorylated in three of the four native spls, including spla [14,4]. Co-translational phosphorylation of these serines and threonines apparently neutralizes the net charge resulting from the numerous lysines and arginines [15]. The fourth spl has no serines and threonines; instead there are glutamic acid residues at corresponding positions. Three-dimensional molecular modeling studies of spl core repeats indicated that glutamic acid is a structural analogue of phosphoserine or phosphothreonine in spls [8]. There-

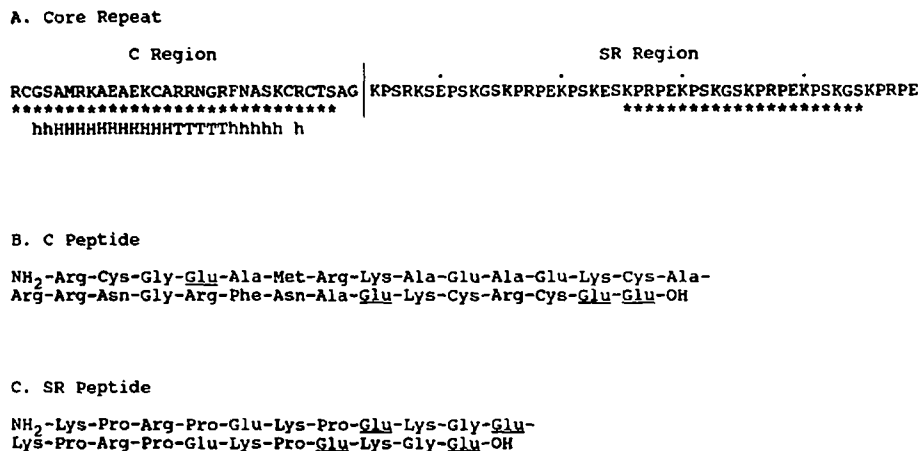


Fig. 1. (A) Primary structure of a core repeat. One repeat unit of the protein spla from *C. tentans* is shown. The amino acid sequence was deduced from cDNA and genomic DNA sequences [11,12]. Subrepeating elements (in some cases inexact repeats) within the subrepeat region are marked at their boundaries by dots. The sequences corresponding to the peptides are indicated by the asterisks. The letters H and T indicate regions where α -helix and β -turn, respectively, were predicted to occur; upper-case letters indicate that the predictions were particularly strong [8]. (B, C) Sequences of synthetic peptides. (B) C peptide. The Glu residues that are underlined were substituted for Ser residues and a Thr residue in spla. (C) SR peptide. The Glu residues that are underlined were substituted for Ser residues in spla. These substitutions were made because Ser and Thr residues in spla are phosphorylated in vivo [14,4].

fore, we chose glutamic acid as a substitute for serine and threonine. Peptides were synthesized on an Applied Biosystems Model 430 automated peptide synthesizer using standard cycles for t-BOC chemistry. Peptides were cleaved from the resin with HF as described [16]. C peptide was dissolved in 8 M urea, 0.2 M dithiothreitol and 0.1 M Tris-HCl (pH 8.0) and dialyzed as described [17] to remove scavengers. Both peptides were chromatographed through Sephadex G-50 in 10 mM triethylammonium bicarbonate (pH 8) and were purified using reverse-phase high-pressure liquid chromatography on a C_{18} column. C peptide was dissolved in 6 M guanidine hydrochloride, 200 mM dithiothreitol, 10 mM Tris-HCl, 0.25 mM EDTA for injection into the column. SR peptide was dissolved in 0.1% trifluoroacetic acid and injected into the column heated to 50°C. Amino acid analysis showed that both peptides contained the predicted ratios of amino acids.

IR spectroscopy. Samples for IR spectroscopy were in the form of solid KBr pellets. Peptides were lyophilized from aqueous solutions, thoroughly ground in a vibrating mill and mixed with KBr to a final concentration of approx. 2% (w/w). IR spectra were recorded on a Fourier transform Bruker 113v vacuum spectrometer. Each spectrum is the result of signal averaging of 100 scans at a resolution of 2 cm^{-1} .

CD spectroscopy. Peptides for CD spectroscopy were dissolved in 10 mM phosphate buffer (pH 7.0) containing 90 mM NaF, or, where noted, in trifluoroethanol. Concentrations (over a 10- to 20-fold range) were chosen that gave suitable absorption at the path length to be used [32]. Peptide samples were spun in a microcentrifuge for 15 min before they were used in measurements. CD measurements were made at room temperature or at 5°C on a Jasco J-500 spectropolarimeter in a cell with 0.1 cm or 1 cm pathlength. The fractions of secondary structures were estimated using the secondary structure estimation programs from Jasco, which are based on the Chang-Wu-Yang method [18]. The spectra of seven standard proteins are provided by Jasco for calculation of the basis spectra. A subset that gave the best fit to the data was used.

Results

IR spectrum of C peptide. Table I summarizes the diagnostic IR bands of characteristic protein secondary structures and lists the corresponding locations for bands observed in the IR spectrum of the C peptide. The IR spectrum of C peptide is shown in Fig. 2. There are bands in the amide I region at 1682 cm^{-1} and at 1653 cm^{-1} ; in the amide II region bands appear at 1542 and 1514 cm^{-1} . The band at 1682 cm^{-1} is diagnostic of β -turns [19], and the other three bands indicate the presence of α -helices [20]. No bands characteristic of β -sheet were observed.

CD spectrum of C peptide. The CD spectrum of C peptide at a concentration of 120 $\mu\text{g}/\text{ml}$ in phosphate buffer shows a large negative band at 206 nm, a smaller negative band centered at 222 nm, and a positive band at 193 nm (Fig. 3). A crossover from positive to negative occurs at 197 nm. This spectrum is similar to that of lysozyme, an $\alpha + \beta$ protein [21]. The fractions of secondary structures found in the peptide were estimated by fitting the spectrum to reference spectra. The reference spectra were those of myoglobin, lysozyme, papain, cytochrome *c* and hemoglobin. The results of this analysis were 26% α -helix, 13% β -sheet, 28% β -turn and 32% other structure. In 100% trifluoroethanol, the fraction of β -sheet structure was reduced to about 5% and the fraction of other conformation was increased to 39% (data not shown). The fraction of α -helix was unchanged.

Qualitative differences in the CD spectra of C peptide were observed at different concentrations; at a concentration of 9 $\mu\text{g}/\text{ml}$, C peptide gave a spectrum with bands of decreased intensity. However, there were no changes in secondary structure estimated from this spectrum. Several methods of detecting aggregation of the peptide (measurement of turbidity or removal of complexes with high-speed centrifugation; data not shown) indicated that large complexes were not formed at either of the concentrations used for measurement of CD spectra. We cannot eliminate the possibility that C peptide forms concentration-dependent small

TABLE I

Summary of diagnostic IR amide bands (cm^{-1}) and their occurrence in the C and SR peptides

	α -Helix ^a	β -Sheet ^a	poly(Gly)II ^b	β -Turn ^c	C peptide	SR peptide
Amide I	1650-1652	1633	1639, 1644	1663, 1670, 1683 1688, 1694	1682 1653	1670 1643
Amide II	1540-1546 1516	1530	1551, 1554		1542 1514	1552

^a Parker, 1971.

^b Suzuki et al., 1966.

^c Byler and Susi, 1986.

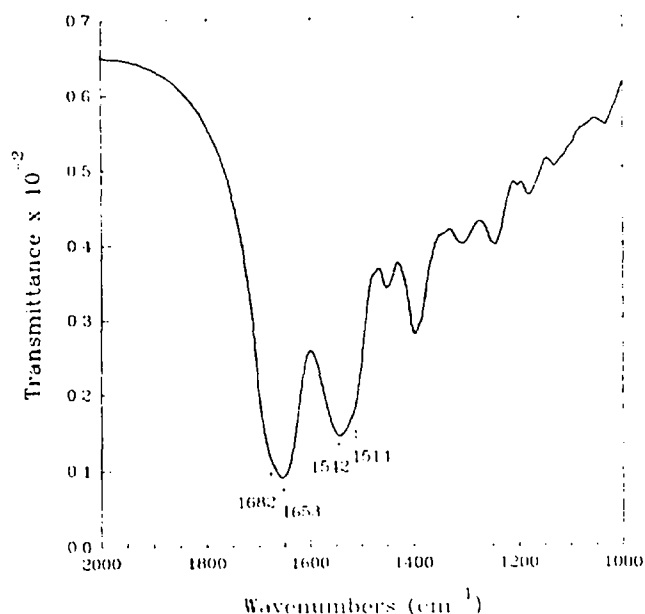


Fig. 2. IR spectrum of C peptide. C peptide was pressed into a solid disk with KBr at a final concentration of about 2% (w/w).

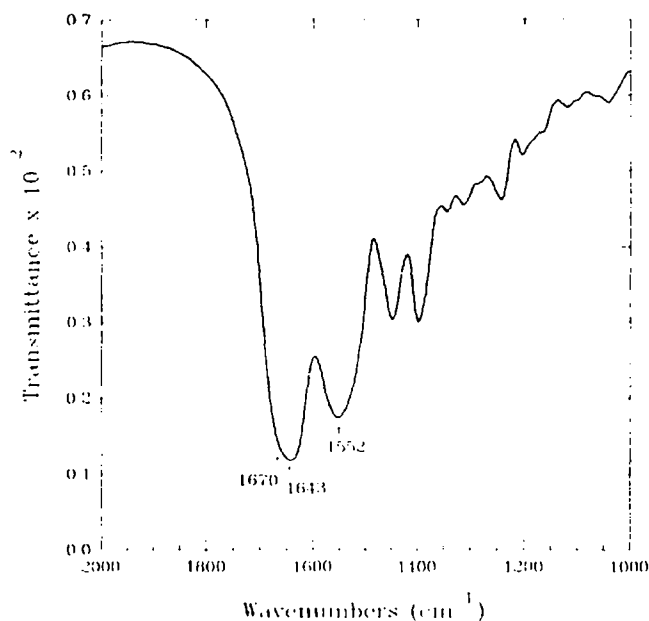


Fig. 4. IR spectrum of SR peptide. SR peptide was pressed into a solid disk with KBr at a final concentration of about 2% (w/w).

oligomeric complexes, but if such complexes are formed, their formation does not induce changes in secondary structure.

IR spectrum of SR peptide. The amide I and amide II bands of SR peptide were at 1670 cm^{-1} , 1643 cm^{-1} and 1552 cm^{-1} (Fig. 4). Bands near 1643 cm^{-1} and 1552 cm^{-1} occur in the spectrum of the poly(Gly)II helix [22], and the band at 1670 cm^{-1} is diagnostic of

β -turns [19]. Neither α -helix nor β -sheet appears to be present in this peptide (Table I).

CD spectrum of SR peptide. CD spectra of SR peptide are shown in Fig. 5. This peptide has a large negative peak at 203 nm and a crossover from positive to negative at 193 nm. The spectra resemble CD spectra calculated for peptides with collagen-like structure, especially (Gly-Ala-Pro)₂ and (Gly-Pro-Ala)₂ [23]. Because of the lack of reference spectra for collagen-like

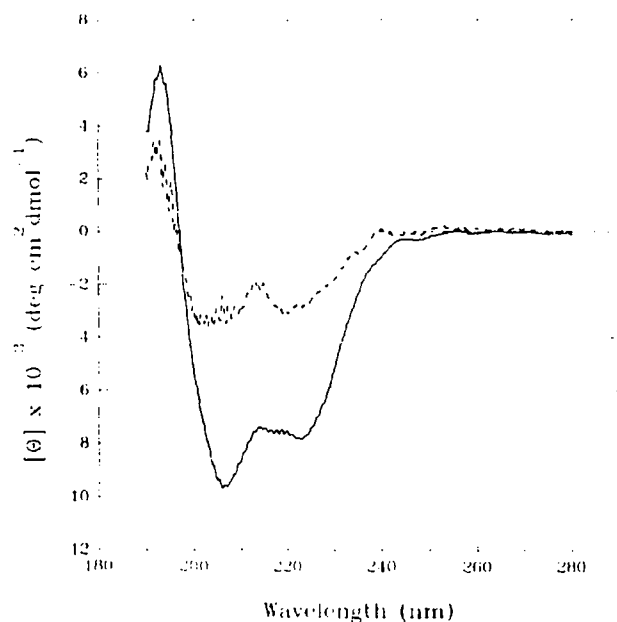


Fig. 3. CD spectrum of C peptide. C peptide was dissolved in 90 mM NaF, 10 mM phosphate buffer (pH 7.0) at a concentration of 120 $\mu\text{g/ml}$ (solid line), or at a concentration of 9 $\mu\text{g/ml}$ (dashed line). The spectra were measured at 5°C. $[\theta]$ was calculated using a mean residue weight of 116.80 for C peptide.

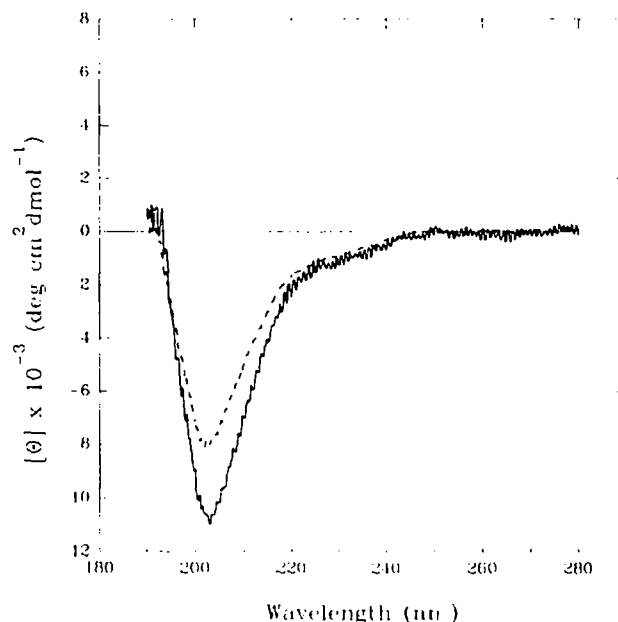


Fig. 5. CD spectrum of SR peptide. SR peptide was dissolved in 90 mM NaF, 10 mM phosphate buffer (pH 7.0) at a concentration of 280 $\mu\text{g/ml}$ (solid line) or at a concentration of 22 $\mu\text{g/ml}$ (dashed line). $[\theta]$ was calculated using a mean residue weight of 116.86 for SR peptide.

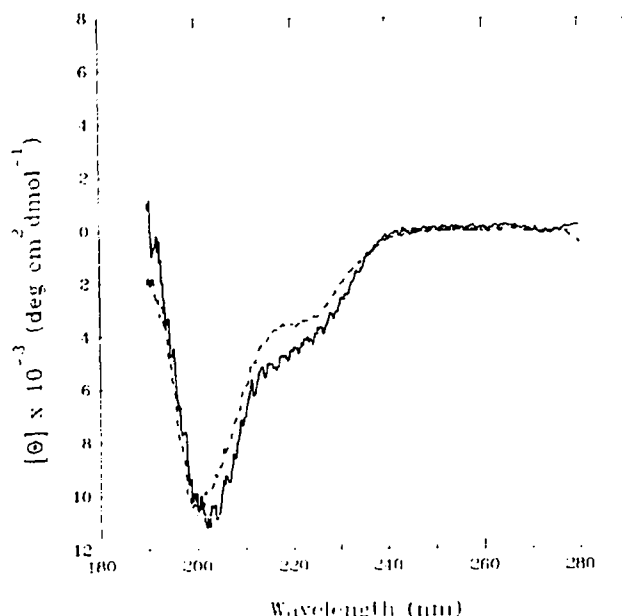


Fig. 6. CD spectrum of a mixture of C and SR peptides. Peptides were dissolved in 90 mM NaF, 10 mM phosphate buffer (pH 7.0). For the measured spectrum, the molar ratio of C to SR was 1.4 to 1 (peptide), or 1.9 to 1 (residue). The final concentration was 69 $\mu\text{g/ml}$. $[\theta]$ was calculated using a mean residue weight of 116.82. For the 'calculated' spectrum, $[\theta]$ of C alone was multiplied by the factor 0.66 and $[\theta]$ of SR alone was multiplied by the factor 0.34, the factors corresponding to their relative fractions of residues in the mixture. Solid line, measured spectrum; dashed line, 'calculated' spectrum.

peptides and proteins, we were unable to perform quantitative analysis of the CD spectrum of SR peptide. The CD spectra of SR peptide at concentrations of 280 $\mu\text{g/ml}$ and 22 $\mu\text{g/ml}$ were nearly identical.

CD spectrum of mixture of C and SR peptides. In order to determine if the secondary structure of C and SR peptides would be altered if they were mixed together, which would be an indication of interactions between them, the peptides were mixed together in approx. a 1:1 ratio (mole peptide) and the CD spectrum of the mixture was measured. In Fig. 6 is shown the measured spectrum (converted to $[\theta]$) of the peptide mixture compared to a 'calculated' spectrum: the appropriate fractions of the spectra of the individual peptides (as $[\theta]$) were added together to obtain the 'calculated' spectrum (see legend to Fig. 6). The measured and 'calculated' spectra were nearly identical.

Discussion

Structure of C peptide. The IR spectrum of C peptide suggests the presence of β -turns and α -helical structure. This is consistent with the analysis of Hamodrakas and Kafatos [8], who, using six different predictive methods, suggested that α -helical structure and β -turns occur in the corresponding C regions of spI core repeats (see Fig. 1). No β -sheet was predicted,

nor was there evidence of β -sheet in the IR spectrum. The CD spectrum in aqueous solution confirms the presence of α -helix and β -turn. Analysis of the CD spectrum also suggests that β -sheet structure may be present, but the IR measurements, which do not show β -sheet, are more likely to be correct, as agreement between values observed from X-ray data and values computed from CD data is sometimes poor for β -structures [24,18,25]. For example, Manavalan and Johnson [25] calculated correlation coefficients between values determined with the two methods of from -0.27 to 0.76 for β -sheet and for β -turn, from 0.18 to 0.69. In contrast, correlation coefficients for α -helix were in the range 0.95-0.98. Because prediction of β structures from CD data is not as reliable, we give greater weight to the IR data.

It is possible that the α -helices in C peptide are distorted, and result in an aberrant CD spectrum. Parrish and Blout [26] concluded that the peptide poly-L-alanine in hexafluoroisopropanol formed a distorted α -helix; the CD of this peptide resembles that of C peptide, with a positive band at about 190 nm, a negative band at 206 nm, a negative shoulder at 219 nm and the crossover from positive to negative at 198-199 nm.

It is also possible that the structure of the peptide is different in solution (as determined from the CD spectrum) than as a solid (as determined from the IR spectrum), with β -sheet structures present only in solution. This is an interesting possibility, because the proteins themselves are in solution in the salivary glands, but become insoluble when they are secreted into the aqueous environment. Perhaps the interactions that render the proteins insoluble involve a conformational change. Another possibility is that at the high concentrations of peptide necessary for IR measurements, interactions between peptides result in differences in secondary structure that do not occur at the concentrations that must be used for CD measurements. In light of the absence of predicted β -sheet in modeling studies, we consider these possibilities to be less likely.

We conclude that C peptide contains α -helix and β -turns; structural predictions [8], the IR data and the CD data are consistent with this conclusion. We think that β -sheet does not exist in this peptide in solution; structural studies and the IR data support this conclusion.

Structure of SR peptide. SR is likely to form an extended, collagen-like structure because of the tripeptide motif and the proline content. The IR spectrum of SR peptide indicates that it has regions in a conformation similar to poly(Gly)II, a collagen-like left-handed helix, and β -turns. The CD spectrum of SR cannot be compared to that of poly(Gly)II itself; poly(Gly)II is optically inactive, as it can form both left- and right-

handed helices [27]. However, other peptides have this helical structure, and the CD spectrum of SR peptide can be compared to their spectra. Caldwell and Applequist [23] calculated CD spectra for a number of tripeptides that form collagen-like helices. These repeating peptides contained glycine, alanine and proline. In their calculations they used four different backbone structures proposed for collagen-like triple-helices. They calculated spectra for a single chain or for a complex of three chains. They observed in all cases a negative peak near 200 nm. Interactions between chains shifted this peak toward longer wavelength. The calculated spectra were sensitive to amino acid composition. These calculated spectra were similar to observed spectra for some peptides and for collagen, and several of the spectra, especially those of (Gly-Pro-Ala)₂ and (Gly-Ala-Pro)₂, were similar to that of SR. The spectrum of SR peptide and these calculated spectra have two features that are different from observed spectra of collagen and other collagen-like peptides; they lack a weak positive peak at about 220 nm, and show a weak positive peak at 185–187 nm. We don't know the reasons for these deviations, but the presence of β -turns would be expected to alter the spectrum. Differences in amino acid content may also have an effect (see above). The higher-order structure also influences the CD spectrum [23], and SR may not form the triple-helix found in collagen and many collagen-like peptides. Poly(Gly)II forms a hexagonal array of chains rather than a triple-helix, and some collagen-like peptides form sheet-like aggregates of helices [28,29]; The collagen-like helices of SR may interact to form one of these structures.

Comparison of the structures of C and SR peptides and spls. Core repeats in tandem arrays account for at least 90% of the sequence of each spl (reviewed in Ref. 7). We reasoned that analysis of peptides based on the constituents of a core repeat, the C and SR regions, might provide structural information that could be extrapolated to the entire protein. Some of our CD results for the peptides are in surprisingly good agreement with our data for purified spls. The fraction of α -helix estimated by quantitative analysis of the CD spectrum of C is reasonably consistent with that estimated for purified spls [9]. Our analysis of spls indicated that the α -helical content of spls was about 15%. We find no α -helix in SR peptide, so we would expect that all of the α -helical structure would be contributed by C peptide; in this case, C peptide should be 30–35% α -helical. The fraction of α -helix that we determined was 26%. A straightforward explanation for this small discrepancy is that the α -helix in a C region of an spl is stabilized by the contiguous SR regions. Also, if α -helices are stabilized by interactions with other helices from different C regions, then the SR regions might align the C regions so that these specific interactions

can occur. We estimated that the fraction of β -turn in C peptide was about 28%. This would correspond to about two β -turns in C peptide. For the spls themselves, we estimated that the fraction of β -turn was about 28%. To account for this fraction, β -turns must be present in the SR regions of spls, which we would predict from the IR data for the SR peptide.

We suggest that the C and SR peptides have similar secondary structures in the corresponding regions of the spl proteins. The SR region probably has extended, collagen-like helices. This region may also contain turns. The C region probably has an α -helix in the N-terminal portion and at least 1 β -turn in the C-terminal portion. The large, fibrous spls thus appear to consist of 100 or more alternating domains of α -helix and collagen-like helix, punctuated by β -turns. The alternation of these helical domains would impart to these proteins regions of contrasting physical properties such as degrees of flexibility, tensile strength and elasticity.

A wide range of interactions between these secondary structure elements could result in assembly of spls into threads. The α -helices in the C region could form bundles of several helices, and the collagen-like helices in the SR region could associate as sheets or bundles. Long coiled coils, of either α -helices or of triple-stranded collagen-like helices, would be precluded by periodic β -turns. While this particular organization of secondary structure elements has not been described in other fibrous proteins, arrangements other than coils of α - or collagen-like helices exist; for example, microtubules (see Ref. 30) and actin filaments (see Ref. 31) are formed by the association of globular subunits. Furthermore, it is easy to envision the β -turns contributing to the branching that we observe in electron micrographs [9].

While the lack of association between peptides obtained from proteins known to associate in vitro and in vivo may seem surprising, each peptide represents only half of the [C + SR] core repeat unit. Association may, in fact, require complete core repeats. We wish to test this notion; however, synthesis of the complete core repeat unit is beyond our capacity for peptide synthesis. Further clues on the assembly and structure of spls may be obtained with the expression of a gene that codes for a peptide with both C and SR regions.

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