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LASER RAMAN STUDIES OF PROTEIN CONFORMATION IN THE SILKMOTH CHORION

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Laser Raman spectroscopy reveals extensive β -pleated sheet structure in a silkmoth chorion. This structure characterizes both developing and mature chorion samples. α -helical structure is much less evident, but β -turns appear to be abundant. In the immature chorion the sulfhydryls are apparently found in diverse environments. The highly localized tyrosine residues appear to be strongly hydrogen-bonded in a hydrophobic environment.

Introduction

The silkmoth chorion has been the subject of numerous biochemical and morphological investigations (reviewed in Ref. 1). Despite considerable complexity of both protein composition and ultrastructure [2,3], certain features of the chorion are relatively simple. The bulk of the chorion consists of multiple proteins belonging to one of two major size classes, A and B. Sequence analysis has shown that the proteins in each class are evolutionarily homologous, and in part of the molecule extremely conservative; moreover, sequence and compositional similarities exist even between classes [4–7]. At the ultrastructural level the bulk of the chorion consists of helicoidally arranged sheets or planes of fibrils [1,8]. Within individual planes, fibrils are oriented parallel to each other and to the surface

of the chorion. Between successive planes the fibril direction rotates progressively, thus giving rise to a helix with its axis perpendicular to the chorion surface. Such helicoidal arrangements are very common in extracellular structures, including plant cell walls, vertebrate tendons and arthropod cuticles, and are analogous to cholesteric liquid crystals [9].

The morphogenesis of the chorion, and of the helicoidal arrangement of fibrils in particular, is the subject of an ongoing investigation [2,3,10]. An essential step in this investigation is elucidation of the preferred secondary structure of the proteins, which must condition their assembly into fibrils and higher-order structures. In a previous paper [11] we have reported computer predictions of secondary structure for chorion proteins of known sequence, which are typical of the predominant A and B chorion protein classes. Here we provide experimental evidence from laser Raman spectroscopic studies, which indicate that chorion proteins are predominantly found in β -sheet and probably also in β -turn conformations, in agreement with the computer predictions. The Raman

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studies also yield information on the state of the abundant cysteine and tyrosine residues of the chorion.

Methods

Raman measurements were obtained by using a microcomputer-interfaced Raman spectrometer described previously [12]. The spectra were obtained in 90° illumination with the beam either at a grazing angle to the chorion surface or radially grazing a cut edge (see Results).

The chorion samples were prepared from immature and ovulated follicles, dissected from developing female *Antheraea polyphemus* pupae. The follicles were cut in half with fine scissors and washed several times in distilled water to remove the yolky oocyte. Swollen epithelial (follicular) cells were peeled off the surface of the underlying chorion. The insoluble chorions were repetitively washed in 95 and 100% ethanol followed by distilled water to remove the vitelline membrane, and were air-dried.

Results

Raman spectra: backbone conformations of chorion proteins

According to extensive theoretical and experimental studies, the frequencies of amide I, II and III bands in Raman spectra are useful indicators of protein and polypeptide secondary structure (reviewed in Refs. 13, 14, 15 and 19). The amide I bands (which occur in the region 1630–1690 cm^{-1}) appear to have contributions from C–O stretching

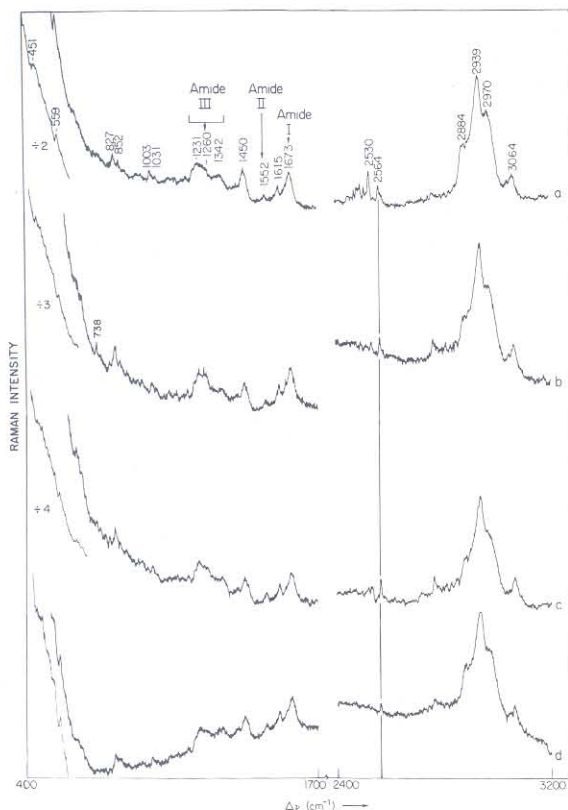
(approx. 70%) and C–N stretching (approx. 16%). The generally weak amide II Raman bands (1510–1570 cm^{-1}) and the amide III bands (1220–1330 cm^{-1}) have significant contributions from N–H in plane bending and C–N stretching. Table I summarizes the diagnostic locations of these bands for α -helical, β -sheet and β -turn structures, and lists the corresponding frequencies observed in laser Raman spectra of chorion samples.

The chorion spectra of four chorion samples are presented in Fig. 1. One chorion (Fig. 1a) was immature, approximately stage III, corresponding to less than 30% of the final protein content [1], and the other three were mature, freshly ovulated. The outer surface was examined tangentially in one ovulated chorion (Fig. 1b), as well as in the immature chorion. The inner structure of another ovulated chorion was also examined tangentially, after scraping off the outer layers (Fig. 1c). The third ovulated chorion was examined in a different orientation: a cut edge was illuminated along the radial dimension (Fig. 1d). The Raman spectra of each of these samples were essentially identical in the 1000 to 1800 cm^{-1} region, and clearly indicate that the β -sheet conformation is predominant in chorion (Tables I and III). Thus, the most intense peaks at 1673 cm^{-1} (amide I region) and 1231 cm^{-1} (amide III region), which are reproducible in location and strength in all spectra, can best be interpreted as resulting from antiparallel β -sheet structures [12]. In addition, relatively prominent peaks at 1552 cm^{-1} (amide II) and 1342 cm^{-1} (amide III) suggest the existence of β -turns [15], while a weak but reproducible shoulder at 1648

TABLE I
SUMMARY OF DIAGNOSTIC AMIDE BANDS AND THEIR OBSERVATION IN CHORION

See Refs. 13, 14, 15 and 19 and Table II.

Band nature	Bands characteristic of			Observed in chorion
	α -helix	β -sheet	β -turn	
Amide I	1650 to 1660	1665 to 1680	1665, 1690	1648 (shoulder) 1673 (strong)
Amide II	1516, 1545	1535, 1560	(I) 1550 to 1555, 1567 (II) 1545, 1555, 1560	1552
Amide III	1260 to 1290	1230 to 1240	1290 to 1330	1231 (strong) 1260, 1342



cm^{-1} (amide I) may indicate the existence of a low proportion of α -helical structure [13,14]. The predominant amide I and III frequencies indicative of β -sheets are compared in Table II to the frequencies observed in other polypeptides and proteins of well-characterized β -sheet structures.

A 738 cm^{-1} peak is observed in the outer surface spectrum from ovulated chorions (Fig. 1b and data not shown), while in the other spectra at most a shoulder is observed; in studies of denatured insulin, which exists in an antiparallel sheet (cross- β) structure, Yu et al. [16] tentatively as-

Fig. 1. Laser Raman spectra of silkmoth chorion. Early developing chorion (a) or ovulated chorion (b-d) samples were used. As described in the text, (a)-(c) were examined tangentially to the surface, and (d) in the radial orientation along a cut edge. In (c), the inner structure was examined tangentially, after scraping off the outer layers of the chorion. Several Raman bands are identified, including those discussed in the text and Table III. A change in scale was necessary for plotting the low end of the spectrum in (a), (b) and (c). Instrumental conditions: excitation wavelength = 5145 \AA ; average slit width = 5.2 cm^{-1} ; integration time = 1 s; laser power at the sample = 100 mW.

TABLE II
COMPARISON OF AMIDE FREQUENCIES OF STRUCTURES WITH β -CONFORMATION

Structure	Amide I (cm^{-1})	Amide III (cm^{-1})	Reference
Silkmoth chorion	1673	1231	This work
Bovine lens	1672	1240	27
Silk fibroin	1667	1229	19
Extended keratin	1672	1237	29
Poly(L-glycine) I	1674	1234	30
Poly(L-valine)	1666	1229	31
Poly(L-serine)	1668	1235	32
Poly(L-lysine) (pH 11.8, 52°C)	1670	1240	33
Denatured insulin	1673	1227	16
Glucagon	1674	1234	34
Ribonuclease A	1667	1239	35
α -chymotrypsin	1669	1246	36
Cobramine B	1672	1235	24
Carbonic anhydrase B	1672	1231	37
	1666	1240	
Basic pancreatic trypsin inhibitor	1664	1243	38
Anthopleurin A	1677	1254	39
BJP Ta (κ)	1672	1243	40
BJP Nag (λ)	1674	1242	
BJP Kob (λ) (solid state)	1670	1246	
BJP Kob (λ) (solution)	1672	1245	
β -lactoglobulin	1665	1242	
Ovalbumin	1665	1245	41

signed a similar (737 cm^{-1}) peak to the β -sheet skeletal bending. However, it is also possible to attribute the 738 cm^{-1} peak to C-S stretching [13]. A 1260 cm^{-1} shoulder in the amide III region may be indicative of either cross β or α -helical conformation, or possibly of disordered structure [13,14,16].

Raman spectra: side-chain environments

Table III tabulates the frequencies and tentative assignments of peaks in the Raman spectra of chorion. Additional minor peaks are resolved but not tabulated, because insufficient data are available for unambiguous assignments.

Tyrosine and cysteine are unusually abundant in silkworm chorion (average of 6.4 molar percent each). Peaks at 827 and 852 cm^{-1} are ascribable to tyrosine, and the intensity ratio of these peaks suggests that, in these dried chorion samples, most tyrosine side chains are strongly hydrogen-bonded in a hydrophobic environment (see Discussion).

A clearly resolved peak at 2564 cm^{-1} present in all chorion samples examined is ascribable to S-H stretching vibration, and thus indicates the ex-

istence of free sulfhydryls. In the immature chorion, several additional peaks are observed at slightly lower frequencies, with the most prominent one at 2530 cm^{-1} , suggesting the existence of additional sulfhydryls in several different environments. As expected, most sulfhydryls, especially those at lower frequencies, disappear in the ovulated samples, presumably due to the formation of disulfide bonds (see Discussion).

Discussion

Laser Raman spectroscopy is a relatively new technique for examining changes in the frequency of emitted radiation due to molecular vibrations (reviewed in Refs. 13, 14 and 19). It is related to, but distinct from, infrared spectroscopy, which examines absorption of radiation due to the same molecular vibrations. Both techniques have been empirically demonstrated to be quite sensitive to protein conformation. The weak emission as opposed to absorption of radiation by water is an important advantage of Raman spectroscopy for studies of biological materials.

The chorion is a relatively favorable structure for Raman spectroscopy studies. It consists almost exclusively of protein [1], and functions as an essentially dry shell; thus it meets the criterion of high protein density which is required for adequate sensitivity in Raman studies. The absence of major admixtures, such as chitin or other carbohydrates, minimizes interference from other than protein vibrations. Furthermore, we have encountered only limited interference from fluorescence in our studies. These features have permitted analysis of the intact chorion structure, as opposed to protein extracts, ensuring that the structural features observed reflect a physiological state.

Our basic conclusion is that β -sheets are a major part of chorion protein secondary structure. The distribution of ϕ and ψ angles in the β -sheets appears to be rather narrow, since the amide I band at 1673 cm^{-1} is sharp [17]: its half-width is approximately 40 cm^{-1} , as compared to the 27 cm^{-1} observed in the very uniform silk fibroin, and to the 76 cm^{-1} observed in the less uniform β -keratin [18,19].

Both mature and early developing chorions show similar evidence for β -pleated sheet structure (cf.

TABLE III
FREQUENCIES AND TENTATIVE ASSIGNMENTS OF PEAKS IN THE RAMAN SPECTRA OF CHORION

Frequency (cm^{-1}) ^a	Tentative assignment
738	Skeletal bending (β -sheet) or C-S stretch
827(+)	Tyr
852	Tyr
1003	Phe or C-C stretch (β -sheet) ^b
1031	Phe ^c
1231(+)	Amide III (β -sheet)
1260(+)	Amide III (α -helix? β -sheet?)
1342	Amide III (β -turn)
1450(+)	Ch ₂ deformation
1552	Amide II (β -turn)
1615	Tyr, Phe, Trp
1648	Amide I (α -helix)
1673 (+)	Amide I (β -sheet)
2564 (and lower)	S-H vibration
2800-3100	C-H stretch

^a (+) indicates a strong peak.

^b See Ref. 13.

^c See Ref. 16.

the 1673 and 1231 cm^{-1} bands in Fig. 1). This is significant in terms of chorion morphogenesis. Early in development a helicoidally twisted chorion framework is set up, which is subsequently modified by expansion and densification [10]. Correlated ultrastructural and biochemical studies suggest that a relatively minor set of chorion proteins (class C) are major components of the early framework, whereas A and B proteins predominate later [10]. The relative invariance (during development) of the bands indicative of β -sheet structure suggests that both the early framework (C proteins?) and the subsequently added A and B proteins probably have extensive β -sheet conformations.

Despite the limited experimental data base for detection of β -turns by Raman spectroscopy, we consider that β -turns are probably abundant in chorion, because of the presence of both 1552 and 1342 cm^{-1} bands, and the computer predictions of β -turns in A and B proteins [11]. It should be noted, however, that the Raman signatures for β -turns are not generally agreed upon; for example, Tu and co-workers [43] place the amide III band for β -turns near 1270 cm^{-1} .

The evidence for α -helical structure is tentative, being based largely on a shoulder observed at 1648 cm^{-1} . Certainly α -helices are considerably less abundant than β -sheets. In the chorion the main amide I peak is at 1231 cm^{-1} , whereas abundant α -helix is characterized by the absence of intensity below 1275 cm^{-1} [13].

The widespread occurrence of helicoidal arrangements in extracellular structures with mechanical strength is intriguing. It would be of considerable interest to know whether or not such helicoidal structures share underlying molecular conformations. Such comparisons are not possible as yet, however. A number of investigators have looked for secondary structure in protein extracts from insect cuticle, but the results depend on the method of preparation and mode of analysis (e.g., Ref. 20). Infrared studies on proteins within intact cuticle are particularly difficult because of the presence of chitin. Neville [21] reported studies on intact *Eutropidacris* tendon but found no evidence for the preferential orientation of proteins. The favorable properties of the chorion may be duplicated in other helicoidal structures consisting

largely of protein, such as the mantid ootheca [22,23]; if so, comparisons may become possible in the future.

In addition to secondary structural features, the Raman spectra have yielded information on two abundant amino acid residues in chorion, tyrosine and cysteine.

Yu et al. [24] have suggested that the intensity ratio of the two tyrosine Raman bands, I_{850}/I_{830} , indicates whether tyrosine side chains are buried in a hydrophobic environment or exposed to an aqueous environment. Siamwiza et al. [25] suggest that the tyrosine doublet results from Fermi resonance between the ring breathing fundamental and the overtone of an out-of-plane ring-bending vibration, and conclude that the I_{850}/I_{830} ratio mainly depends on the nature and extent of hydrogen bonding of the phenolic hydroxyl or its ionization, rather than on the environment of the phenyl ring and the backbone conformation. According to the latter authors, if the phenolic oxygen acts as proton donor for the formation of a strong hydrogen bond in a hydrophobic environment, the I_{850}/I_{830} ratio has low values, ranging between 0.3 and 0.5, whereas if the tyrosine is exposed and acts as a much weaker donor or as an acceptor to an acidic proton, the ratio values range between 0.9 and 1.4. In the chorion spectra, the intensity ratio values are approximately 0.3 ± 0.1 , suggesting that the tyrosines are buried in a hydrophobic environment and strongly hydrogen-bonded. This is significant, since the tyrosine residues are highly localized within both A and B protein sequences [7], [11].

Silkmoth chorion has a high content of cysteine or cystine, depending on the stage of maturation. The mature chorion cannot be dissolved by denaturing agents unless disulfide bonds are reduced [1], although reduction is unnecessary for dissolution of early chorion [26]. These solubility studies and direct chemical estimations of sulfhydryl content as a function of developmental stage (J.C. Regier, unpublished observations) indicate that most cross-linking of chorion proteins via disulfide bonds occurs at or after ovulation. Within the 2500 to 2800 cm^{-1} region, the exact frequency of the S-H stretching vibration differs for different materials, apparently in a conformation-dependent manner [27]. Thus, in the intact lens and in frac-

tionated lens proteins, the S-H line occurs at 2582 cm^{-1} with shoulders at lower frequencies of approx. 2530 to 2560 cm^{-1} (see Fig. 7 in Ref. 27); in oxygenated mammalian hemoglobins it is observed at 2551 to 2564 cm^{-1} , while in oxygen-free hemoglobins it occurs at 2558 cm^{-1} [44]. Therefore, we consider the 2564 cm^{-1} peak of chorion spectra as unambiguous evidence of sulfhydryl content, and tentatively also assign the even stronger, multiple nearby peaks (most prominent at 2530 cm^{-1}) to the S-H mode. Although the latter peaks are distributed over an uncommonly low range of frequencies, their assignment to the S-H mode is strongly supported by their evident suppression and even disappearance in the ovulated chorion samples. If our assignment of several peaks to sulfhydryls is correct, it would suggest that in the chorion the cysteines are found in a variety of different environments; the multiple, low-frequency peaks may be associated with the predominant cysteines which are located near the amino and carboxyl termini in both A and B proteins [4,7,28], in regions which appear to have relatively little secondary structure [11]. The few, internal cysteines located in the midst of β -pleated sheet structure [11] may possibly correspond to the 2564 cm^{-1} peak. In any case, it is notable that the 2564 cm^{-1} peak does not decrease as extensively (or as rapidly) after ovulation as do the lower frequency peaks; this disparity may ultimately provide important insights into the process of crosslinking in the chorion.

Surprisingly, no clear evidence was obtained in the 500–550 cm^{-1} spectral region (which is typically associated with the S-S stretching mode) for the formation of disulfide bonds in parallel with the disappearance of sulfhydryls. A minor but reproducible peak at 559 cm^{-1} may possibly indicate S-S stretching, at a higher than normal frequency [13,14], or alternatively may reflect skeletal bending [16]; we favor the latter alternative, since the 559 cm^{-1} peak is equally prominent in early chorion, where disulfide content is minimal (J.C. Regier, unpublished observations). As for the sulfhydryls in the young chorion, disulfides of the ovulated chorion may have a distribution of environments which broadens the spectral peaks or creates multiple peaks; some of these peaks may not occur within the spectral region normally asso-

ciated with disulfides. An additional factor making detection of disulfides difficult may be that the 500–550 cm^{-1} region lies on the descending slope of the Rayleigh line. The exact locations and intensities of disulfide peaks remain to be determined by future work.

Further work should also make analysis of secondary structure more quantitative, and relate conformation and other structural features of the chorion to specific morphogenetic stages, and to the proteins which correspond to each stage.

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