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Laser-Raman and infrared spectroscopic studies of protein conformation in the eggshell of the fish *Salmo gairdneri*

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Laser-Raman and infrared spectroscopic studies reveal abundant β-pleated sheet conformation in the eggshell proteins of the fish *Salmo gairdneri*. This secondary structure is the underlying molecular conformation, dictating the formation of the helicoidal architecture of the eggshell. Disulphide bonds crosslink the eggshell proteins of the fertilized eggs and are apparently found in *g-g-g* (*gauche-gauche-gauche*), *g-g-t* (*gauche-gauche-trans*) and *t-g-t* (*trans-gauche-trans*) conformation. There is no evidence for the existence of free sulphydryls. The tyrosines appear to act as hydrogen-bond acceptors, whereas the aromatic residues phenylalanine and tryptophan are also eggshell protein constituents.

Introduction

The helicoidal structure is a common architectural feature of several extracellular fibrous structures, including plant cell walls, vertebrate tendons, arthropod cuticles, insect and fish eggshells, etc., reviewed extensively in Ref. 1.

At the ultrastructural level, the bulk of a helicoidal, fibrous tissue, a biological analogue of a cholesteric liquid crystal [2], consists of helicoidally arranged parallel 'planes' or sheets of fibrils. Within individual planes, fibrils are oriented parallel to each other. Between successive planes the fibril direction rotates progressively, thus giving rise to a helix with its axis perpendicular to the planes.

Frequently, as in the case of some insect and fish eggshells [3,4], the main components of the fibrous helicoidal structure are proteins, which self-assemble to create spherical shells with extraordinary mechanical properties [4]. It would be of interest to know whether or not these helicoidal structures share underlying molecular conformations, with the ultimate goal of understanding the rules governing the formation of the helicoidal architecture at the molecular level.

Recently, we have proposed the twisted β-pleated sheet as the molecular conformation which dictates the organization of protein molecules into fibrils, and of fibrils into helicoidal proteinaceous eggshells [5,6]. Our proposal was mainly based on data obtained from studies of the silkmoth eggshell.

In this study, we provide experimental evidence indicating that the β-pleated sheet conformation

Abbreviations: *g-g-g*, *gauche-gauche-gauche*; *g-g-t*, *gauche-gauche-trans*; *t-g-t*, *trans-gauche-trans*.

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also predominates in the eggshell proteins of the fish *Salmo gairdneri*: approx. 98% of the *S. gairdneri* eggshell is protein and the remainder is a polysaccharide (Refs. 7–9 and references therein). In addition, we present useful information about the state of certain amino-acid residues of the proteins.

Methods

Sample preparation. Fertilized eggs of rainbow trout (*S. gairdneri*), were obtained from the Fish farm of the river Luros, supervised by the Greek Ministry of Agriculture. Samples, taken randomly, belong to the period following the eyed stage of embryonic development.

The eggs were cut in half with fine scissors and washed several times in distilled water, followed by 95% ethanol, to remove the newly formed embryo and other remnants. They were then thoroughly dried. The samples used for laser-Raman experiments were hemispherical half-eggshells.

Electron microscopy. Electron microscopic observations of the samples used for Raman spectroscopy were performed as described elsewhere [10].

Laser-Raman and infrared spectroscopy. Raman spectra were measured on a Ramanor HG25 Jobin-Yvon spectrometer. The 514.5 nm line of a Spectra Physics 165 Argon-ion laser, operating at 100 mW, was used for excitation. A 90° scattering geometry was employed, with the laser beam hitting the eggshell surface tangentially. The samples tried showed initially a strong fluorescent background, masking completely the Raman signal. Thus, prolonged (2–4 h) laser irradiation of the sample was necessary to reduce fluorescence and to measure reasonable Raman spectra. To further reduce the noise level, the spectra were recorded at a scanning speed of 10 cm^{-1}/min and a time constant of 2 s. The spectral resolution was 5 cm^{-1}.

Infrared spectra were recorded on a Fourier-Transform Bruker 113v, vacuum spectrometer. Each spectrum is the result of signal averaging of 100 scans at 2 cm^{-1} resolution. Samples were in the form of KBr pellets, containing about 2% (w/w) material, which was thoroughly ground in a vibrating mill, before mixing with KBr.

Results

Ultrastructure of the S. gairdneri eggshell

Fig. 1 shows, under low magnification, the ultrastructure of the eggshell of a fertilized *S. gairdneri* egg, which was used to obtain the laser-Raman spectrum presented in Fig. 2.

The radial canals which traverse the eggshell can clearly be seen (arrows). Their shape suggests a twisted ribbon structure [4], and implies a helicoidal architecture for the eggshell. Obvious, also, are the lamellar components of the helicoidal architecture (double arrows).

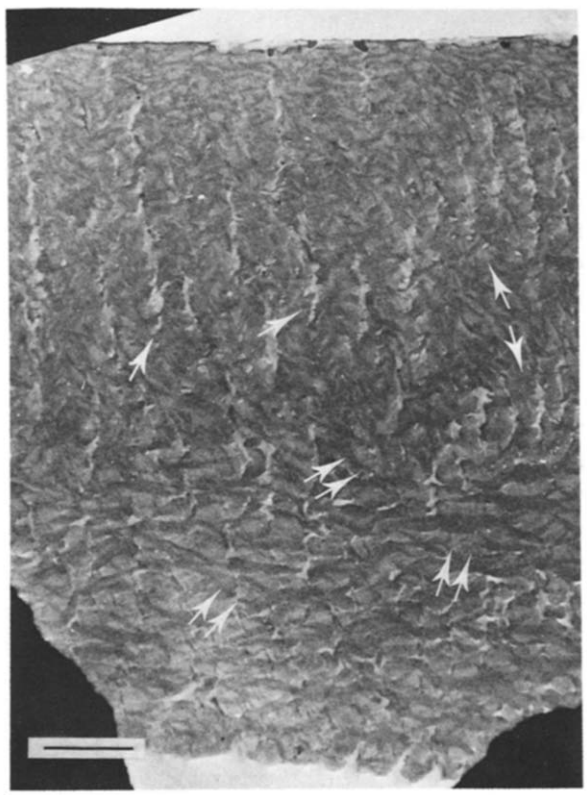

Fig. 1. Transmission electron micrograph of a thin section through an eggshell from a fertilized egg (belonging to the period following the eyed stage of embryonic development) of the fish *S. gairdneri*. Within the bulk of the eggshell, the radial canals (arrows) can be discerned, with the characteristic twisted ribbon structure [4], implying a helicoidal architecture for the eggshell. Obvious, also, are the lamellar components (double arrows) of the helicoidal eggshell. Bar is 5 μm.

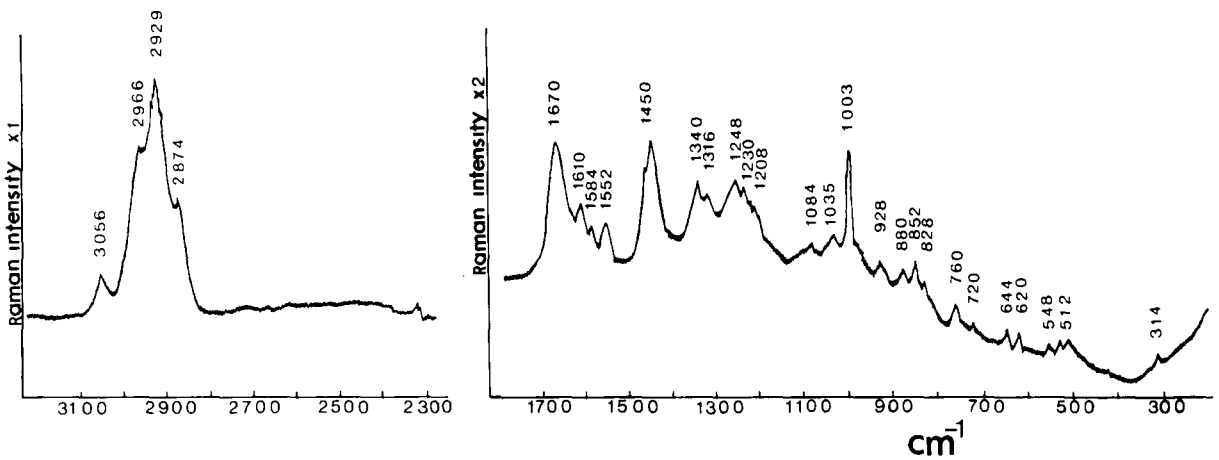

Fig. 2. Laser-Raman spectrum of the eggshell of the fish *S. gairdneri*. A 90° scattering geometry was employed, with the laser beam hitting the eggshell surface tangentially. Instrumental conditions: excitation wavelength, 514.5 nm; scanning speed, 10 $cm^{-1} \cdot min^{-1}$; time constant, 2 s; spectral resolution, 5 cm^{-1}; laser power at the sample, 100 mW.

Raman and infrared spectra: secondary structure of eggshell proteins

Table I gives the frequencies and our tentative assignments of the bands appearing in the laser-Raman spectrum (Fig. 2) of the *S. gairdneri* eggshell. Additional bands are resolved but not tabulated, because insufficient data are available for unambiguous assignments.

The infrared spectrum (Fig. 3) exhibits a wealth of information. However, in such a complicated proteinaceous system it is difficult to assign all absorption bands to certain vibrations of defined chemical groups. Therefore, we limit our attention to identifying bands suggesting a certain type of secondary structure.

Extensive theoretical and experimental studies have established that the frequencies of the diagnostic amide I, II and III bands in the Raman and infrared spectra are useful indicators of protein or polypeptide secondary structure [11–14].

In the Raman spectra, the bands at 1670 cm^{-1} (amide I) and 1248 cm^{-1} (amide III), can best be interpreted as resulting from abundant antiparallel β-pleated sheet structure in the proteins of the *Salmo* eggshell [11–13,15,22]. It is, perhaps, useful to draw attention to the analogy between the secondary structure of the *S. gairdneri* eggshell proteins and the β-sheet structure appearing in globular proteins, predominantly in the β-sheet

TABLE I

FREQUENCIES AND TENTATIVE ASSIGNMENTS OF BANDS IN THE LASER-RAMAN SPECTRUM OF THE *S. GAIRDNERI* EGGSHELL

Frequency (cm^{-1}) [a]	Tentative assignment
512	S–S stretch
525	S–S stretch
548	S–S stretch
620	Phe
644	Tyr
720	C–S stretch
760	Trp
828	Tyr
852	Tyr
880	Trp
1003 (+)	Phe, [C–C stretch (β-sheet)?]
1035	Phe
1208	Tyr, Phe
1230 (sh)	Amide III (β-sheet)
1248 (+)	Amide III (β-sheet)
1340 (+)	Trp
1450 (+)	CH_2 deformation
1552 (+)	Trp
1584	Tyr
1610 (+)	Tyr, Phe, Trp
1670 (+)	Amide I (β-sheet)
2800–3100 (+)	C–H stretch

[a] (+) indicates a strong peak.

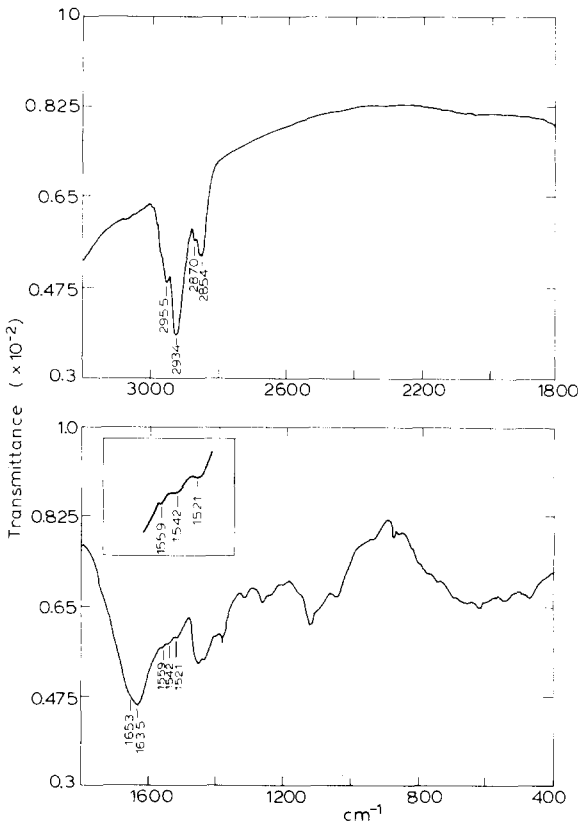

Fig. 3. Fourier transform infrared spectrum of the eggshell of the fish *S. gairdneri*. The spectrum is the result of signal averaging of 100 scans, at 2 cm^{-1} resolution. Samples were in the form of KBr pellets, containing about 2% wt. material, thoroughly ground in a vibrating mill, before mixing with KBr. Inset shows, at an expanded scale, the 1500–1600 cm^{-1} spectral region used for identifying the bands at 1521, 1542 and 1559 cm^{-1}.

conformation, such as the Bence–Jones proteins. It has been shown that a combination of an amide I band, at about 1670 cm^{-1}, and of an intense amide III band, in the region 1240–1250 cm^{-1}, indicates a particular form of β-sheet structure [22].

A reproducible shoulder, at 1230 cm^{-1}, may suggest that a portion of β-sheet structure is of the form appearing in synthetic homopolypeptides and in some fibrous proteins [11,12,15].

Supporting evidence for the abundance of the antiparallel β-pleated sheet conformation, in the proteins of the *S. gairdeneri* eggshell, is also the observation of a very intense absorption band at 1635 cm^{-1} (amide I), and of a weak band at 1521 cm^{-1} (amide II) in the infrared spectrum (Fig. 3 and inset). Bands at these frequencies, in the infrared spectrum, are characteristic of β-pleated sheet conformation [14].

Weak absorption bands at 1653 cm^{-1} (amide I) and at 1542 and 1559 cm^{-1} (amide II) in the infrared spectrum may indicate a proportion of unordered (coil) or β-turns, or α-helical structure [14]. We favour one of the first two alternatives, since the Raman spectrum (Fig. 2) shows no evidence characteristic of α-helical structure: the presence of α-helix is characterized by the absence of any strong features in the amide III region of a Raman spectrum, in the range 1230–1275 cm^{-1}, and by the appearance of bands in the range 1645–1660 cm^{-1} in the amide I region [11–13]. This obviously is not the case for the Raman spectrum of the *S. gairdneri* eggshell (Fig. 2).

Raman spectra: side chain environments

Bands in the 500–550 cm^{-1} region are typically associated with the S–S stretching mode of the C–C–S–S–C–C structural unit, of disulphide bonds [11–13]. Following Sugeta et al. [16], the bands at 512, 525 and 548 cm^{-1} may be assigned to S–S bridges in *g-g-g*, *g-g-t* and *t-g-t* conformation, respectively.

There is no evidence for the existence of free sulphydryls, as can be judged by the absence of bands in the 2530–2580 cm^{-1} spectral region, typically associated with the –S–H stretching mode (Ref. 15 and references therein).

C–S stretching vibrations from cystine (disulphide) and methionine groups in proteins lie normally in the 600–750 cm^{-1} region [11–13]. The C–S stretching frequencies depend upon the conformation about the C–C bond adjacent to C–S, a *gauche* rotamer yielding ν_{C-S} in the region 650–670 cm^{-1}, and a *trans* rotamer in the region 700–745 cm^{-1} (Ref. 17 and references therein). In this case (Fig. 2 and Table I) a band at 720 cm^{-1} is observed, probably indicating that the *trans* rotamers predominate.

Since there is evidence for the presence of methionines in the eggshell proteins [7,8], we conclude that the 720 cm^{-1} band may have arisen from the disulphides, and/or the methionines.

The intensity ratio of the tyrosine doublet at

850 and 830 cm^{-1}, $R = I_{850}/I_{830}$, is sensitive to the nature of hydrogen bonding, or to the state of ionization of the phenolic hydroxyl group [18]. Basically, if tyrosine functions as a strong hydrogen-bond donor to a negative acceptor, the ratio R is low, perhaps 0.3–0.5. Hydrogen bonding in which the phenolic oxygen serves as an acceptor yields a higher ratio [18]. In this work it was found that $I_{852}/I_{828} = 2.2$, which probably indicates that most tyrosines act as hydrogen-bond acceptors.

The peaks at 620 and 1035 cm^{-1}, clearly ascribable to phenylalanine [11–13], show that this aromatic amino acid is also present in eggshell proteins. The intensity ratio I_{620}/I_{644}, which may be used to estimate the ratio Phe/Tyr (Ref. 19 and references therein), was found to be approx. 1, in fair agreement with the amino-acid content of the *S. gairdneri* eggshell proteins [7,8]. The very intense and sharp band at 1003 cm^{-1} may also be attributed to phenylalanine, judging from its intensity and shape (Refs. 11–13 and references therein). However, a minor contribution to this band, probably arising from C_α–C′ and/or C_α–C_β stretching vibrations, characteristic of β-pleated sheet conformation, should not entirely be excluded [11–13].

The presence of another aromatic residue, tryptophan, may also be inferred by the presence of bands at 880 and 760 cm^{-1}, characteristic of tryptophan residues [11–13]. The absence of a spectral band at 1361 cm^{-1} probably suggests exposed tryptophans [20,21]. It is, perhaps, worth mentioning that the variation of the intensity of the 880 cm^{-1} band, as a function of pH, has been used successfully, in the case of the Bence-Jones proteins, to probe tryptophan environment, i.e., whether tryptophans are buried in the protein interior or exposed [22].

Discussion

The principles governing the self-assembly of protein molecules into helicoidal, proteinaceous, extracellular structures, biological analogues of cholesteric liquid crystals, should be based on simple rules of packing of protein molecules. This is because the helicoidal organization usually occurs at long distances from the secretory oocyte and epithelial cells [4,23]. Structures probably partially cooperating in the morphogenesis of the helicoidal stucture are the epithelial cells and the oocyte (their surfaces and microvilli secreting the eggshell proteins).

The packing of protein molecules or protein domains is known to depend on the interactions of secondary structure elements (α-helices and β-pleated sheets), constituents of the protein molecules or domains [24]. Therefore, the first step towards unraveling the modes of interaction of protein molecules, for the formation of helicoidal, proteinaceous eggshells, is the determination of eggshell protein secondary structure.

In the past two decades, laser-Raman spectroscopy has been empirically demonstrated to be a very powerful and reliable technique for the determination of protein secondary structure. It can also provide a wealth of useful information about the state of certain amino-acid residues, in proteins and protein systems, thus offering distinct advantages compared to infrared spectroscopy [11–13,17].

In many respects, proteinaceous eggshells are favourable systems for Raman spectroscopic studies; the advantages are summarized in our previous study [15].

For the silkmoth eggshell, serious difficulties were encountered in attempts to obtain samples suitable for infrared spectroscopy. This was due to the toughness, opacity and elasticity of the eggshells. Fortunately, this was not the case for the *S. gairneri* eggshell.

Our basic conclusion from the analysis of the Raman and infrared spectra is that β-sheets are a major part of the *S. gairdneri* eggshell protein secondary structure. The distribution of the phi and psi angles in the β-sheets appears to be rather narrow, since the amide I band at 1670 cm^{-1} is sharp: its half-width is approx. 45 cm^{-1}, compared to the 27 cm^{-1} observed in the very uniform silk fibroin, the 76 cm^{-1} observed in the less uniform β-keratin (Ref. 13 and references therein) and the 40 cm^{-1} observed in the silkmoth eggshell [15].

It is, perhaps, instructive to examine in some detail the structure of the Bence-Jones proteins [25], which apparently shares some similarities with the *S. gairdneri* eggshell protein structure, as can be judged by the similarities of their Raman

spectra (see Results). The basic structural unit of a Bence-Jones protein is an antiparallel twisted β-pleated sheet sandwich. The two twisted β-pleated sheets of the sandwich consist of three and four β-strands, respectively. The crystal structure is formed by the packing of such units, and this is probably one of the reasons for the relative rise of the amide III frequency, the other being the relative strength of the interstrand hydrogen bonds [22].

If these data are taken into account, together with the similarities between the Raman spectra of the Bence-Jones proteins and the *S. gairdneri* eggshell, they may lead us to the plausible conclusion that the molecular structure responsible for the formation of the helicoidal architecture of the *S. gairdneri* eggshell (which self-assembles from its constituent protein molecules) is the twisted β-pleated sheet. Nevertheless, this requires further experimental vefification.

Whether the disulphide bonds, apparently present in the eggshells of the fertilized *S. gairdneri* eggs, are intermolecular or intramolecular could not be inferred from the study of the Raman and infrared spectra alone. However, there is evidence [7,8] implying that several disulphide bonds are intermolecular, crosslinking eggshell proteins and contributing to the eggshell hardening and waterproofing process.

Apparently the disulphide bridges adopt all preferred conformations known to occur in solved protein structures [26], as can be judged by the presence of the bands at 512, 525 and 548 cm^{-1} (see Results).

The absence of free sulphydryls in the eggshell, suggested by the fact that there is no Raman scattering in the 2530–2580 cm^{-1} region, is further supported by the absence of any absorption bands in the 2550–2600 cm^{-1} region of the infrared spectrum, where the –S–H stretching bands typically appear [27].

To proceed in the elaboration of a detailed model of packing of protein molecules for the formation of the helicoidal architecture of the fish eggshell requires additional information about the amino-acid sequences of the eggshell proteins. Such data are not available yet. This procedure is well under way for the silkmoth eggshell (Ref. 5 and S.J. Hamodrakas, unpublished data), for which, in the last decade or so, the amino-acid sequences of several of its component proteins have been elucidated (Ref. 5 and references therein).

Further work is also needed to analyse the secondary structure quantitatively, and to relate conformation and other structural features (e.g., disulphide bonds and their exact time of formation) of the fish eggshell to well-defined morphogenetic stages.

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