Chapter E3
Raman scattering spectroscopy

E3.1 Historical review and introduction to biological problems

1928
L. Mandelshtam and C. V. Raman independently discovered that a wavelength shift in a small fraction of scattered visible radiation depends on the chemical structure of the molecule responsible for the scattering. Raman was awarded the 1931 Nobel Prize in physics for the discovery of the phenomenon, which became known as Raman scattering or the Raman effect (Comment E3.1).

Comment E3.1
Mandelshtam–Raman phenomenon
In Russian scientific literature the effect is called the Mandelshtam–Raman phenomenon. We follow the more general usage and call it the Raman effect.

Raman scattering is inelastic light scattering that results from the same type of quantised vibrational transitions as those associated with IR absorption and occurs in the same spectral region. The Raman scattering and IR absorption spectra for a given molecule are often similar. Differences between the properties that make a chemical group IR or Raman active, however, make the techniques strongly complementary rather than competitive. Raman spectroscopy has the advantages of minimal or no damage to the sample, and relatively little interference from the water signal in aqueous solution or in vivo. The possibility of observing Raman spectra from crystals as well as from solutions in vitro or in vivo provides a very useful approach to relating molecular structures solved by X-ray crystallography and conformations in solution or in living cells.

Raman spectroscopy was not widely used by molecular biophysicists until lasers became available in the 1960s. Interference from fluorescence excited in the molecule studied or in sample impurities was also a deterrent to the general use of the method. This problem has now been largely overcome by the use of near-IR laser sources.

E3.2 Classical Raman spectroscopy

Classical or non-resonance Raman spectroscopy probes radiation scattering by vibrational modes, which have energies in the IR spectral region. The method has proven very useful in molecular biophysics for the determination of protein, nucleic acid and membrane conformational states.
Resonance Raman spectroscopy is discussed in Section E3.3. The resonance effect is between the scattered radiation and electronic transitions in the sample molecules. Since these transitions occur in the visible spectral region, which is also readily accessible by the conventional lasers commonly used for Raman spectroscopy, the method has provided considerable information on visual pigments, haem and other metalloproteins. The most studied chromophores are non-fluorescent so that the relatively weak Raman signals can be observed without contamination from broad fluorescence emission.

E3.2.1 Raman spectra

Figure E3.1 shows a portion of a Raman spectrum obtained by irradiating a sample of carbon tetrachloride with an intense beam from an argon ion laser (488.0 nm wavelength, corresponding to 20 492 cm\(^{-1}\) wave numbers). The emitted radiation is of three types: Stokes’ scattering, anti-Stokes’ scattering and Rayleigh scattering. The last, which corresponds to elastic scattering of wavelength exactly equal to that of the excitation source, is significantly more intense. The Stokes’ and anti-Stokes’ lines are inelastic scattering corresponding to quantum energy given to or taken away from the sample by the light beam, respectively. The x-axis in Fig. E3.1 is the wave number shift \(\Delta \nu\), defined as the difference in wave numbers (cm\(^{-1}\)) between the observed and excitation radiation. Note the three Raman peaks in the spectrum are found on both sides of the Rayleigh peak, with identical shift magnitudes. Stokes lines are at 218, 314 and 459 cm\(^{-1}\), wave numbers below the Rayleigh peak, while anti-Stokes’ lines occur at 218, 314 and 459 cm\(^{-1}\) above it. Quite generally, anti-Stokes’ lines are appreciably less intense than the corresponding Stokes lines, indicating a higher probability for the sample to emit inelastic radiation.
to be excited by the beam rather than the other way around. In practice, only the more intense, Stokes portion of a spectrum is analysed.

The appearance of Raman spectral lines of lower energy (longer wavelengths) than the excitation beam is analogous to the Stokes’ shifts found in a fluorescence experiment (Chapter D8); for this reason, Raman shifts to longer wavelengths were called Stokes’ shifts. We see in Section E3.2.2, however, that Raman and fluorescence spectra arise from fundamentally different processes, so that the application of the same terminology to both may be misleading.

E3.2.2 Frequency, intensity and polarisation

Raman spectroscopy is a form of vibrational spectroscopy. Like IR spectroscopy it is related to transitions between vibrational energy levels in a molecule. It differs from IR spectroscopy in that information is derived from light scattering whereas in IR spectroscopy information is obtained from absorption processes.

Let us assume a light beam of frequency $v_{\text{exp}}$ is incident upon a solution of molecules. The electric field $E$ associated with the radiation is described by the wave equation (see Chapter E1)

$$E = E_0 \cos (2\pi v_{\text{exp}} t)$$  \hspace{1cm} (E3.1)

where $E_0$ is the amplitude of wave. As the field propagates through the sample the electron charges respond to it. The dipole moment, $m$, induced in the electron cloud of a molecular bond by the oscillating electric field is given by

$$m = \alpha E = \alpha E_0 \cos (2\pi v_0 t)$$  \hspace{1cm} (E3.2)

where $\alpha$ is the polarisability of the bond.

In order to be Raman active (i.e. to be active in inelastic light scattering), the vibration of the atoms joined by the bond must be reflected favourably by polarisability changes in the bond electrons,

$$\alpha = \alpha_0 + (r - r_{eq}) \left( \frac{d\alpha}{dr} \right)$$  \hspace{1cm} (E3.3)

where $\alpha_0$ is the polarisability of the bond at the equilibrium internuclear distance $r_{eq}$ and $r$ is the internuclear separation at any instant. The change in internuclear separation varies with the frequency of the vibration $\nu_v$ as given by

$$r - r_{eq} = r_m \cos (2\pi \nu_v t)$$  \hspace{1cm} (E3.4)

where $r_m$ is the maximum internuclear separation relative to the equilibrium position. Substituting Eq. (E3.4) into Eq. (E3.3) gives

$$\alpha = \alpha_0 + \left( \frac{d\alpha}{dr} \right) r_m \cos (2\pi \nu_v t)$$  \hspace{1cm} (E3.5)

We can then obtain an expression for the induced dipole moment $m$ by substituting Eq. (E3.5) into Eq. (E3.2):
Recall from trigonometry that

\[ 2 \cos x \cos y = [\cos (x + y) + \cos (x - y)] \]

Applying this identity to Eq. (E3.6) gives

\[
m = \alpha_0 E_0 \cos (2\pi \nu_0 t) + E_0 r_m \left( \frac{d\alpha}{dr} \right) \cos (2\pi \nu_0 t) \cos (2\pi \nu_0 t) \]

(E3.6)

The first term on the right-hand side of Eq. (E3.7) represents Rayleigh scattering at the excitation frequency \( \nu_0 \). The second and third terms correspond, respectively, to the Stokes’ and anti-Stokes’ frequencies of \( \nu_0 - \nu_v \) and \( \nu_0 + \nu_v \). Here, the excitation frequency has been modulated by the vibrational frequency of the bond. It is important to note that Raman scattering requires that the polarisibility of the bond varies as a function of distance: i.e. \( \frac{d\alpha}{dr} \) in Eq. (E3.7) must be greater than zero for Raman lines to appear.

The energy shifts observed for a given bond in Raman experiments should be identical to the energies of its IR absorption band, provided that the vibrational modes involved are active toward both IR absorption and Raman scattering. Figure E3.2 illustrates the similarity of the two types of spectra. Several peaks with identical \( \bar{\nu}_0 \) and \( \Delta \bar{\nu} \) values exist for indene. However, the relative sizes of the corresponding peaks are frequently different, and certain peaks that occur in one spectrum are absent in the other.

The differences between a Raman and an IR spectrum are not surprising when it is considered that the basic mechanisms, although dependent upon the same vibrational modes, arise from processes that are mechanistically different. IR

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**Fig. E3.2** Comparison of Raman and IR spectra for indene. (Adapted from Skoog *et al.*, 1995.)
absorption is observed for vibrational modes which change the dipole moment of the molecule, while Raman scattering is associated with modes that produce a change in the polarisability of the molecule. Because of this fundamental difference in mechanism, the Raman activity of a given vibrational mode may differ markedly from its IR activity. For example, a homonuclear molecule such as nitrogen has no dipole moment either in its equilibrium position or when a stretching vibration causes a change in the distance between the two nuclei. Thus, absorption of radiation corresponding to the vibration frequency cannot occur. On the other hand, the polarisability of the bond between the two atoms varies periodically in phase with the stretching vibrations, reaching a maximum at the greatest separation and a minimum at the closest approach. A Raman shift is therefore observed at the frequency of the vibrational mode.

It is interesting to compare the IR and Raman activities of coupled vibrational modes such as those described for the carbon dioxide molecule (Section E3.3.1). In the symmetric mode, no change in the dipole moment occurs as the two oxygen atoms move away from or toward the central carbon atom; thus, this mode is IR-inactive (Fig. E3.3). The polarisability, however, fluctuates in phase with the vibration since distortion of the bonds becomes easier as they lengthen and more difficult as they shorten. Raman activity is associated with this mode. In contrast, the dipole moment of carbon dioxide fluctuates in phase with the antisymmetric vibrational mode. Thus, an IR absorption peak arises from this mode. On the other hand, as the polarisability of one of the bonds increases as it lengthens, the

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**Fig. E3.3** Selection rules for IR and Raman spectra using the carbon dioxide molecule as an example. For vibration to be IR-active, the dipole moment of the molecule must change. Therefore, the symmetric stretch in carbon dioxide is not IR-active because there is no change in the dipole moment. The asymmetric stretch is IR-active due to a change in dipole moment. For vibration to be Raman active, the polarisability of the molecule must change with the vibration motion (Altose et al., 2001.)
polarisibility of the other decreases resulting in no net change in the polarisibility. Thus, the asymmetric stretching vibration is Raman-inactive.

As in the example above, parts of Raman and IR spectra can be complementary, each associated with a different set of vibrational modes within a molecule. Other vibrational modes may be both Raman- and IR-active. For example, all of the vibrational modes of sulphur dioxide yield both Raman and IR peaks. Peak heights differ, however, because the probability for the transitions is different for the two mechanisms.

### E3.2.3 Raman spectrometers and Raman microscopes

In order to obtain the Raman spectrum of a protein or nucleic acid, one places the sample at the focal point of a focused laser beam. The scattered light is collected by a lens system and directed through a suitable monochromator to a photon detector. The Raman spectrum consists of a plot of the scattered intensity as a function of its frequency difference with the incident laser frequency. A band at a particular frequency in the Raman spectrum corresponds to a vibrational mode of that frequency. It is a fundamental principle of the Raman effect that the spectrum is independent of the incident laser frequency.

Three types of Raman spectrometer are in general use for the characterisation of protein or nucleic acid structure: (1) the classic laser Raman spectrometer, (2) the laser Raman microscope, and (3) the UV resonance spectrometer (Comment E3.2). The classic spectrometer uses a wavelength in the visible spectral region, and has the advantage that it puts no constraints on the sample, which may be a protein or nucleic acid powder, crystal, fibre or solution. Because these samples are transparent to visible light, there is little or no heating of the sample by the laser beam. Classical Raman spectra of protein or nucleic acid solutions have been obtained from 5–50 μl samples in a glass capillary mounted in a circulating water temperature controlled copper block, with ‘tunnels’ for the incident and scattered beams. The scattered light is collected at right angles to the incident beam. The concentration of the solution is typically 2.0–20 mg ml$^{-1}$, or about 20–100 mM in nucleotide or peptide groups.

A powerful advantage of Raman microscopy is that it allows a single protein crystal to be studied in situ in a hanging drop (Fig. E3.4). The Raman spectrum recorded from the crystal is caused only by the focal volume of the laser beam within the crystal, which is typically ∼20 μm in diameter and 50 μm in depth. In this volume, the protein concentration is about 50 mM, thus providing very high quality data. Any Raman scattering collected from the surrounding mother liquor makes a negligible contribution because protein concentrations there are less than 1 mM. Thus, Raman microscopy can be used to screen the properties of protein crystals in a hanging drop.
In general, Raman spectroscopy and Raman microscopy provide direct comparison of the properties of a molecule in solution and in a single crystal.

**E3.2.4 Protein secondary structure from Raman spectra**

The different conformations of polypeptide chains have remarkable differences in their Raman spectra. Table E3.1 presents a summary of some of the Raman active modes that are useful for obtaining information about the structure and dynamics of proteins. Of the modes listed in Table E3.1, one of those whose Raman spectrum is sensitive to conformation is the amide I mode. Thus, the amide I vibration changes from $1650 \pm 5 \text{ cm}^{-1}$ in the $\alpha$-helical forms in H$_2$O to $1670 \pm 3 \text{ cm}^{-1}$ in the $\beta$-sheet form in H$_2$O, indicating that the carbonyl group is more strongly polarised, i.e. has a higher dipole moment in the $\alpha$-helix than in the $\beta$-sheet. Hydrogen–deuterium exchange causes the frequency to decrease because of the increased mass of the deuteron. A frequency of $1632 \pm 5 \text{ cm}^{-1}$ was found for the $\alpha$-helical form in D$_2$O, whereas a frequency of $1659 \pm 3 \text{ cm}^{-1}$ was found for the $\beta$-sheet form in D$_2$O.

In the estimation of protein secondary structure content in terms of percentage helix, $\beta$-strand and turn, the amide I band is analysed as a linear combination of the spectra of the references proteins whose structures are known. The Raman spectra of globular proteins in the crystal and in solution are almost identical, reflecting the compact nature of the macromolecules. Thus one may use the fraction of each type of secondary structure determined in the crystalline state by the X-ray of diffraction studies to study protein in solution.
Table E3.1. *Main marker bands of proteins*\(^a\)

<table>
<thead>
<tr>
<th>Origin and frequency ((\bar{\nu} \text{ cm}^{-1}))</th>
<th>Assignment</th>
<th>Structural information</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Amide bands</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Amide I</strong></td>
<td>Amide C=O stretch coupled to N–H wagging</td>
<td>Strong band; hydrogen bonding lowers amide I frequencies</td>
</tr>
<tr>
<td>1655 (\pm) 5</td>
<td>–</td>
<td>(\alpha)-helix (H(_2)O)</td>
</tr>
<tr>
<td>1632 (\pm) 5</td>
<td>–</td>
<td>(\alpha)-helix (D(_2)O)</td>
</tr>
<tr>
<td>1670 (\pm) 3</td>
<td>–</td>
<td>Antiparallel (\beta)-related sheet</td>
</tr>
<tr>
<td>1661 (\pm) 3</td>
<td>–</td>
<td>(\alpha)-helix (D(_2)O)</td>
</tr>
<tr>
<td>1655 (\pm) 3</td>
<td>–</td>
<td>Disordered structure (H(_2)O)</td>
</tr>
<tr>
<td>1658 (\pm) 2</td>
<td>–</td>
<td>Disordered structure (D(_2)O)</td>
</tr>
<tr>
<td><strong>Amide III</strong></td>
<td>N–H in-plane bend, C–N stretch</td>
<td>Strong hydrogen bonding raise amide III frequencies</td>
</tr>
<tr>
<td>&gt;1275</td>
<td>–</td>
<td>(\alpha)-helix</td>
</tr>
<tr>
<td>1235 (\pm) 5</td>
<td>–</td>
<td>Antiparallel (\beta)-related sheet</td>
</tr>
<tr>
<td>983 (\pm) 3</td>
<td>–</td>
<td>Disordered structure (D(_2)O)</td>
</tr>
<tr>
<td>1245 (\pm) 4</td>
<td>–</td>
<td>Disordered structure</td>
</tr>
<tr>
<td><strong>Tyrosine doublet 850/830</strong></td>
<td>Resonance between ring fundamental and overtone</td>
<td>State of tyrosine</td>
</tr>
<tr>
<td><strong>Tryptophan 880/1361</strong></td>
<td>Indole ring</td>
<td>Ring environment</td>
</tr>
<tr>
<td><strong>Phenylalanine 1006</strong></td>
<td>Ring breathing</td>
<td>Conformation-insensitive</td>
</tr>
<tr>
<td><strong>Histidine 1409</strong></td>
<td>N-deuteroimidazole</td>
<td>Possible probe of ionisation state</td>
</tr>
<tr>
<td><strong>S–S</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>510</td>
<td>S–S stretch</td>
<td>Indication of conformational heterogeneity among disulphides</td>
</tr>
<tr>
<td>525</td>
<td>S–S stretch</td>
<td>\textit{Gauche–gauche–trans}</td>
</tr>
<tr>
<td>540</td>
<td>S–S stretch</td>
<td>\textit{Trans–gauche–trans}</td>
</tr>
<tr>
<td><strong>C–S</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>630–670</td>
<td></td>
<td>\textit{Gauche}</td>
</tr>
<tr>
<td>700–745</td>
<td></td>
<td>\textit{Trans}</td>
</tr>
<tr>
<td><strong>S–H</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2560–2580</td>
<td>S–H stretch</td>
<td>Environment, deuteration rate</td>
</tr>
</tbody>
</table>

\(^a\) Table adapted from Peticolas (1995).
In addition to the structural information on proteins that is obtained from the vibrations of the polypeptide backbone, the vibrations of the side-chains can also give valuable information on the environment of the side-chain and sometimes on the accessibility to protons and deuterons.

The state of tyrosine in proteins is often of great importance. The ratio of the intensity of the Raman bands at 850–830 cm\(^{-1}\) is sensitive to the environment of the phenolic –OH. This doublet is due to resonance between a ring-breathing vibration and an overtone of an out-of-plane ring-breathing vibration. A strong hydrogen bond from the phenolic hydrogen to a negative acceptor results in a low value of \(I_{850}/I_{830}\) (about 3:10), whereas hydrogen bonding to the phenolic oxygen from an acidic proton donor yields a higher value (about 10:30) for this ratio.

An example of the use of this technique was in the investigation of the buried tyrosines in fd filamentous phage coat protein (Fig. E3.5, Comment E3.3). The two tyrosines at positions 21 and 24 of the fd coat protein provide a potentially useful probe of the phage structure. Several peaks arising from the tyrosines are clearly evident in the Raman spectrum of the virus. The measured intensity ratio \(I_{850}/I_{830}\) is about 10:3. According to the above interpretation, such a ratio indicates that the tyrosine OH groups hydrogen bond with a very strong acidic proton donor. Further titration experiments showed that the tyrosines and their acidic donors in the filamentous phage fd are inaccessible to the solvent. One possible explanation of this fact is that the tyrosine OH groups are the recipients of hydrogen-bonded protons arising from fairly acidic donors, yet these acidic donors do not become titrated over the range pH 7–12.

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**Comment E3.3**

**Physicist’s box: fd filamentous phage**

The filamentous phage fd is a long thin structure of about 895 nm by about 6 nm. The phage particle contains about 2700 copies of a largely \(\alpha\)-helical coat protein. This phage is structurally similar to Pf1 phage which is discussed in Comment J2.5.

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**Fig. E3.5** Laser Raman spectra of the fd phage pellets at (a) pH 8.1 and (b) pH 12.0. The positions of the two tyrosine peaks are shown by arrows. Lack of a change in the \(I_{850}/I_{830}\) ratio is evident from comparison of the peak intensities (see text). (Dunker et al., 1979.)
E3.2.5 Protein conformational dynamics in solution and in crystals

Insulin provides an attractive model for protein conformational dynamics because of its wealth of prior structural characterisation. Insulin is a small globular protein containing two chains, A (21 residues) and B (30 residues). Stored in the pancreatic β cell as Zn$^{2+}$-stabilised hexamers, the hormone functions as a Zn$^{2+}$-free monomer. Classical crystallographic studies have focused on Zn$^{2+}$-stabilised hexamers (Fig. E3.6). Use of engineered insulin monomers and dimers enables

![Fig. E3.6](image1.png)

**Fig. E3.6** $T_6$ crystal structure of insulin (Protein Data Bank (PDB) identifier 4INS).

![Fig. E3.7](image2.png)

**Fig. E3.7** (a) Amide I region of the Raman spectra (1550–1750 cm$^{-1}$) of insulin in solution in the hexameric $T_6$, dimeric $T_2$ and monomeric $T_1$ states. The arrow indicates the amide I maximum; spectra are colour-coded as follows: continuous line, experimental data (black); broken line, fitted sum (light purple); dash-dotted line individual components (Gaussian lines) at 1657 cm$^{-1}$ (red), near sum at 1657 cm$^{-1}$.

(b)-(d) Amide I profile deconvolution 1611 cm$^{-1}$ (blue), at 1687 cm$^{-1}$ (green), and at 1585 cm$^{-1}$ (brown); and dotted line, residual (black). The peak at 1657 cm$^{-1}$ in the amide I profiles is due to contributions from α-helices, whereas the shoulder near 1680 cm$^{-1}$ is due to β-sheet and β-strands. Residues in disordered regions are expected to contribute weakly at 1648 cm$^{-1}$ and 1678 cm$^{-1}$. The unresolved doublet near 1610 cm$^{-1}$ (labelled $x$ and $y$) and the shoulder at 1585 cm$^{-1}$ are due to ring modes of phenylalanine and tyrosine. (Adapted from Dong et al., 2003.)
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Fig. E3.8 Structure of insulin fibrils formed by gentle agitation of the protein in 60% ethanol and 0.25 M NaCl at 35 °C: (a) electron micrograph of insulin fibrils; (b) Raman spectrum of insulin fibrils obtained using a Raman microscope. The amide I region can be fitted by a line centred at 1673 cm⁻¹ and to features centred at 1653 cm⁻¹ and 1630 cm⁻¹, respectively. The peak at 1673 cm⁻¹ is due to β-sheet formation. (Adapted from Dong et al., 2003.)

direct comparison of the structure and dynamics of a protein in different states of self-assembly.

Solution Raman spectra (1550–1750 cm⁻¹ region) of the T₆ hexamer, the engineered dimer designated T₂ and the engineered monomer, herein designated T₁, are shown in Fig E3.7. Strikingly, amide I profiles broaden and change in relative peak heights on disassembly of the protein. Linewidths of Raman bands associated with the polypeptide backbone exhibit progressive narrowing with successive self-assembly. A major increase in width at half-height (WHH) occurs upon dissociation of the aqueous T₆ hexamer (42.9 cm⁻¹) to the T₂ dimer (48.7 cm⁻¹); a further increase is seen in the T₁ monomer (55.1 cm⁻¹).

A further narrowing of linewidths of Raman bands associated with the polypeptide backbone occurs under fibril formation. Figure E3.8 shows the amide profile of the insulin fibrils. The peak at 1673 cm⁻¹ has a narrow WHH of 19.7 cm⁻¹. These results provide physical evidence for the flexibility of the isolated monomer and damping of fluctuations in native and pathological assemblies.

Additional information on conformational dynamics for insulin can be obtained from studies in a single crystal in situ in a hanging drop. The partial Raman spectrum of a single insulin crystal in its native conformation is shown in Fig. E3.9(a) and (c). Raman data for a single T₆ insulin crystal in the amide I region (Fig. E3.9(a)) are very close to the data in solution (Fig. E3.7(a)).

Dramatic changes occur when the reducing agent tris(2-carboxyethyl)phosphine is added to the mother liquor containing the crystal. The α-helical 1657 cm⁻¹ band (Fig. E3.9(a)) disappears and is replaced by a single β-sheet marker band at 1669 cm⁻¹ (Fig. E3.9(b)). The WHH of this band of 24 cm⁻¹ compares quite closely to that found for the fibrils. These findings show that
**Fig. E3.9** Partial Raman spectra of an insulin single crystal obtained by a Raman microscope: top: native crystal; bottom: β-transformed crystal after the reducing agent tris(2-carboxyethyl)phosphine has been added to the mother liquor. The peaks at 1615 and 643 cm\(^{-1}\) are due to tyrosine modes, and the peaks at 1604 and 621 cm\(^{-1}\) are due to phenylalanine ring modes. The ring modes are expected to only undergo small changes in intensity on secondary structure change and, thus, act as integral intensity standards. (After Zheng et al., 2004.)

**Fig. E3.10** Raman spectra of aqueous solutions of r(CGCGCG) \cdot r(CGCGCG) in the A-form (top), d(CGCGCG) \cdot d(CGCGCG) in the B-form (middle), and d(CGCGCG) d(CGCGCG) in the Z-form (bottom). The top and middle spectra were of samples dissolved in 0.5 M NaCl solution, whereas the bottom spectrum is from a sample dissolved in 6 M NaCl. The marker bands for the A-, B-, and Z-forms are shaded black. All of the spectra were taken with the argon laser line at 514.5 nm. (Adapted from Peticolas and Evertsz, 1992.)
native proteins in the crystalline phase can also undergo large-scale conversion to $\beta$-sheets as for solution state described above (Comment E3.4).

E3.2.6 Conformation of DNA

Some of the vibrational normal modes of DNA are conformationally sensitive. This leads to changes in frequencies and/or intensities of the Raman bands with changes in conformation. From a comparison of the Raman spectra taken from different crystals of DNA of known structure, a correlation between the frequencies and intensities of Raman bands and the conformation of the DNA has been established.

Figure E3.10 shows the Raman spectra of hexameric oligonucleotides containing only cytidine (C) and guanine (G). The spectra are of r(CGCGCG) in the A-form (top spectrum), d(CGCGCG) in the B-form, and d(CGCGCG) in the Z-form (bottom spectrum). The bands that change with conformation are highlighted in black. These are the marker bands for guanine, cytosine, and the sugarphosphate backbone as the DNA goes from the A- to the B- to the Z-form.

E3.3 Resonance Raman spectroscopy (RRS)

The central advantage of RRS is that when the wavelength of the exciting laser line is adjusted to coincide with that of an allowed electronic transition in a molecule, the intensities of certain Raman bands are enhanced relative to the off-resonance values. This technique is very selective, because only those vibrational modes that are related to the chromophoric part of the scattering molecule can be enhanced at resonance. This means that the vibrations of the chromophoric group can be

Comment E3.4

Amyloidogenic diseases

Protein unfolding and misfolding events that eventually lead to extensive $\beta$-sheet structure, are central to amyloidogenic diseases. Thus, native soluble proteins partially unfold and aggregate, possibly using a residual `$\beta$-sheet' domain as a nucleation motif. This leads to amyloid fibrillogenesis and the formation of highly insoluble fibrils (Booth et al., 1997).

Fig. E3.11 Illustration of the selective enhancement of vibrational modes in a resonance Raman spectrum. The UV–visible absorption spectrum of a *P. aeruginosa* azurin is shown together with two different Raman spectra that derive from laser excitation within the S(Cys) → Cu(II) charge transfer absorption band at 615 nm (647.1 nm, right) and away from the absorption (488.0 nm, left). (After Spiro and Czernuszewicz, 1995.)
monitored in biological macromolecules. If the chromophore is itself a site of functional activity, then RRS can provide structural information associated with reactivity.

This is illustrated in Fig. E3.11 which shows a dramatic increase in the intensity of the Cu-S(Cys) vibrational modes near 400 cm\(^{-1}\) as the excitation wavelength (647.1 nm) approaches that of the S \(\rightarrow\) Cu charge transfer electronic transition (~600 nm) in the blue cooper protein azurin.

Thus the correct identification of the vibrational modes showing RRS enhancement aids in the assignment of the resonant electronic transition and vice versa. Because vibrational frequencies are sensitive to the bond strength, the number of atoms, the geometry and the coordination environment, this technique provides information which is complementary to that obtained by X-ray crystallography and X-ray absorption spectroscopy.

### E3.4 Surface enhanced Raman spectroscopy (SERS)

Raman scattering is a very weak effect, with a cross-section between \(10^{-30}\) cm\(^2\) and \(10^{-25}\) cm\(^2\) per molecule. Such small Raman cross-sections require a large number of molecules to achieve adequate conversion rates from exciting laser photons to Raman photons.

SERS involves obtaining Raman spectra in the usual way on samples that are adsorbed on the surface of colloidal metal particles. The same phenomenon can be observed in the visible region (Comment E3.5). For reasons that are not fully

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**Comment E3.5** Plasmon resonant particles (PRP) for biological detection

The light scattering by nanometre-sized colloidal metal particles is dominated by the collective oscillation of the conduction electrons induced by the incident light. The specific colour (i.e. frequency band) of the scattered light is a function of the size, shape, and material properties of the particles. Silver or gold particles with diameters of 30–120 nm efficiently scatter light in the visible spectrum (Fig. (a)). The peak scattering wavelength of a particle is generally referred to as the PR peak (Fig. (b)). Under the same excitation conditions a PRP with diameter of 100 nm can have a brightness that is equivalent to >100 000 fluorescent molecules, and does not undergo photobleaching.

**Comment Fig. E3.5** (a) A colour photograph of three PRPs illuminated with white light. The particles were chosen so that their PR peak wavelength would be in the red, green, and blue, respectively. (b) Spectral curves for the three particles shown in (a). (Shultz, 2003).
understood, the Raman lines on the adsorbed molecules are often enhanced by a factor of $10^3$–$10^6$. When surface enhancement is combined with the resonance enhancement technique discussed in the previous section, the net increase in signal intensity is roughly the product of the intensity produced by each of the techniques. In some cases enhancement factors are much larger than the ensemble-averaged values derived from conventional measurements, in the order of $10^{14}$–$10^{15}$. Such large enhancement factors provide in single-molecule SERS effective Raman cross-sections which are of order of $10^{-16}$ cm$^2$ per molecule. These cross-sections are comparable to the effective fluorescence cross-sections of common laser dyes and as we will see in Chapter F4 are sufficient for single-molecule detection. A highly sensitive Raman imaging system with submicron spatial resolution can be constructed by combining atomic force microscopy with near-field optics (Chapter F2).

### E3.5 Vibrational Raman optical activity

Phenomena that are sensitive to molecular chirality include optical rotation of the plane polarisation of a linearly polarised light beam on passing through a solution of chiral molecules (with equal and opposite optical rotation angles for mirror image enantiomers) and UV circular dihroism (UV CD) where left and right circularly polarised UV light is absorbed slightly differently. Such ‘chiroptical’ techniques have a special sensitivity to the three-dimensional structure of chiral molecules and were discussed in Chapter E2. The importance of the newer vibrational optical activity methods is that they are sensitive to chirality associated with all the $3N - 6$ fundamental molecular vibrational transitions, where $N$ is the number of atoms, and therefore have the potential to provide more stereochemical information than UV CD, which measures optical activity associated with electronic transitions and so requires an appropriate chromophore.

#### E3.5.1 Vibrational circular dichroism (VCD)

Proteins with different folds give characteristic VCD shapes, which vary most for the amide I mode (C=O stretch), but are also easily detectable for the broader amide II and III (very weak) modes (N–H deformation and C–N stretch). Examples are shown in Fig. E3.12.

Surveys of protein spectra show that secondary structure determines the dominant contributions to the VCD shape. Analyses of amide I (proteins in D$_2$O, N–D exchanged), plus amide III and III VCD and ECD (circular dichroism of electronic transition in UV) or FTIR data all yield secondary structure at some level. The important aspects were that VCD sensed sheet and other structural elements (including turns) differently than did ECD, which in turn was superior for helix determination. Combining them gave better determination of all components, especially minimising the impact of outliers on the prediction scheme.
E3.5.2 Raman optical activity (ROA)

In the ROA technique a small difference in the intensity of Raman scattering is measured using right and left circularly polarised incident light. Interference between the waves scattered via the polarisability and optical activity tensors of the molecule yields a dependence of the scattered intensity on the degree of circular polarisation of the incident light and on a circular component in the scattered light.

ROA is described by the dimensionless circular intensity difference (CID),

$$\Delta = (I^R - I^L)/(I^R + I^L)$$

(E3.8)

as an appropriate experimental quantity, where \( I^R \) and \( I^L \) are the scattered intensities in right and left circularly polarised incident light (Comment E3.6).

Polypeptides and proteins

Vibrations of the backbone in polypeptides and proteins are associated with three main regions of the Raman spectrum. These are: the backbone skeletal stretch region \( \sim 870-1150 \text{ cm}^{-1} \) originating in mainly \( C_\alpha - C, C_\alpha - C_\beta \) and \( C_\alpha - N \) stretch coordinates; the amide III region \( \sim 1230-1310 \text{ cm}^{-1} \), which is often thought to involve mainly the in-phase combination of largely N–H in-plane deformation with the \( C_\alpha - N \) stretch; and the amide I region \( \sim 1630-1700 \text{ cm}^{-1} \), which arises mostly from the \( C=O \) stretch. The extended amide III region is particularly
Comment E3.6  Circular intensity difference

The theory of ROA shows that unlike the conventional Raman intensity, which is the same in the forward and backward directions, the ROA intensity is maximised in backscattering and is zero in forward scattering. The CID for right-angle scattering takes intermediate values. A given ROA signal-to-noise ratio can be achieved at least an order of magnitude faster in backscattering than in the (easier to implement) right-angle scattering geometry. These considerations led to the important conclusion that backscattering boosts the ROA signal relative to the background biomolecules in aqueous solution (Barron et al., 2000).

Important for ROA studies because the coupling between N–H and Cα–H deformations is very sensitive to geometry and generates a rich and informative ROA band structure. Bands in the amide II region ~1510–1570 cm⁻¹, which originate in the out-of-phase combination of largely the N–H in-plane deformation with a small amount of the Cα–N stretch, are not usually observed in the conventional Raman spectra of polypeptides and proteins but sometimes make weak contributions to the ROA spectra in H₂O solution that are generally enhanced in D₂O.

Poly(L-lysine) at alkaline pH and poly(L-glutamic acid) at acid pH adopt well-defined conformations under certain conditions and are able to support an α-helical conformation stabilised by both internal hydrogen bonds and hydrogen bonds to the solvent. Backscattered Raman and ROA spectra of these samples are shown in Fig. E3.13. There are many similarities between the ROA spectra of the α-helical conformations of the two polypeptides.

A good example of the ROA spectrum of a highly α-helical protein is that of human serum albumin shown in Fig. E3.14. The amide I ROA couplet centred at ~1650 cm⁻¹, which is negative at low wave number and positive at high, appears to be a good signature of α-helix in proteins and agrees with the wave number range of ~1645–1655 cm⁻¹ for α-helix bands in conventional Raman spectra.

The two polypeptides in α-helical conformations show similar amide I ROA couplets (Fig. E3.13), but shifted to lower wave number, which may be due to the...
particular side-chains or the absence of disordered structure which gives an amide I ROA couplet at high wave number. A positive ROA intensity in the range $\sim 870$–$950 \text{ cm}^{-1}$ also appears to be a signature of an $\alpha$-helix. Again the two $\alpha$-helical polypeptides show positive ROA bands in this region. The conservative ROA couplet centred at $\sim 1103 \text{ cm}^{-1}$, which is negative at low wave number and positive at high, may also be due to an $\alpha$-helix since a similar feature appears in the ROA spectra of the two $\alpha$-helical polypeptides. In addition to a positive ROA band near $\sim 1340$–$1345 \text{ cm}^{-1}$, an $\alpha$-helix also gives rise to positive ROA bands in the range $\sim 1297$–$1312 \text{ cm}^{-1}$.

As well as providing the signature of an $\alpha$-helix, the ROA spectra of the two polypeptides also provide signatures of $\beta$-structure. For example, jack bean concanavalin A in H$_2$O solution (Fig. E3.15) shows a negative ROA band at $\sim 1238 \text{ cm}^{-1}$ that is almost unchanged in D$_2$O which strongly suggests that it originates in the $\beta$-strands in the parallel $\beta$-sheet within the hydrophobic core.
Carbohydrates
Carbohydrates in aqueous solution are highly favourable samples for ROA studies, giving a rich and informative band structure over a wide range of the vibrational spectrum. The cyclodextrins (Section D5.4) are particularly interesting samples for ROA studies because they exhibit an enormous glycosidic ROA couplet centred at $\sim 918 \text{ cm}^{-1}$ as compared to that observed in the corresponding $\alpha$(1–4)-linked disaccharide D-maltose. An example the ROA spectrum of $\alpha$-cyclodextrin is shown in Fig. E3.16.

Nucleic acids
Although ROA studies of nucleic acids are still at an early stage, the results obtained so far are most encouraging with ROA being able to probe three distinct sources of chirality: the chiral arrangement of adjacent intrinsically achiral base rings, the chiral disposition of the base and sugar rings with respect to the C–N glycosidic link, and the inherent chirality associated with the asymmetric centres of the sugar rings.

The ROA spectra of polyribonucleotides are generally more intense and informative than those of the corresponding nucleosides since there are fewer degrees of conformational freedom in the polymers which reduces the tendency for cancellation of ROA signals from different conformers. The polyribonucleotide ROA spectra can be conveniently subdivided into three distinct spectral regions, each of which encompasses a well-defined structural subunit. The extent and location of these regions are highlighted in the ROA spectrum of poly(rA).poly(rU), which adopts an A-type double helix, presented in Fig. E3.17. The base stacking region $\sim 1550–1750 \text{ cm}^{-1}$ contains ROA patterns from base modes which are characteristic of the particular bases involved as well as being sensitive to the base stacking arrangement. The sugar–base region $\sim 1200–1500 \text{ cm}^{-1}$ is dominated by normal
modes in which the vibrational coordinates of the sugar and base rings are mixed. The ROA band patterns in this region appear to reflect the mutual orientation of the two rings perhaps together with sugar ring conformation. ROA bands in the sugar-phosphate region $\sim 900-1150$ cm$^{-1}$ originate in vibrations of the sugar rings and phosphate backbone. Since the C3'-endo furanose sugar pucker predominates in A-type double helices, the negative-positive-negative ROA triplet ($\sim 994, 1049, 1086$ cm$^{-1}$) observed in this region provides a clear marker for this particular conformation. Unfortunately the ROA spectra of naturally occurring DNAs and RNAs are more complicated than those of the polyribonucleotides discussed above.

**E3.6 Differential Raman spectroscopy**

In general, the protein structure and conformation should change upon ligand binding. These alterations are evident in the Raman difference spectrum, and signals from the protein should be present in the protein ligand-minus-protein difference spectrum. Figure E3.18 shows the Raman spectra of lactate dehydrogenase (LDH) with bound NADH (i.e. reduced nicotinamide adenine dinucleotide) (spectrum (a)) and LDH itself (spectrum (b)). Spectrum (d) demonstrates the difference spectrum between the LDH/NADH binary complex spectrum and that of LDH. The Raman bands in the difference spectrum are mostly from bound NADH, although some protein bands show up owing to the effect of NADH on the protein upon binding (see Fig. E3.18 caption for assignments). A completely dominant protein-ligand-minus-protein difference spectrum can be observed by the isotope editing technique (Comment E3.7).

As a rule of thumb, the Raman spectrum of a protein minus that of the perturbed protein (perturbed by bound ligand or mutated residue) is about 1% that of the protein itself. It requires a protein Raman signal with a signal-to-noise ratio in excess of 300:1. This requirement is consistent with the difference FTIR spectroscopic studies of proteins (Section E1.4.8).
Comment E3.7 Isotope editing

In many cases, the signal from a bound ligand is similar in size to, or smaller than, that arising from changes in protein structure. Moreover, some proteins are not stable without bound ligands. The strategy of isotope editing of the difference spectrum obviates these problems; here, isotopically labelled ligands are prepared, so that the frequencies of those modes that involve motions of the isotope are shifted. Thus, a subtraction of the labelled ligand from the unlabelled one yields a difference spectrum of positive and negative peaks of just those modes, and all others cancel (Manor et al., 1991).

E3.7 Time-resolved resonance Raman spectroscopy

The inherent scattering processes ($10^{-14}$ s) carries with it the implication that it is theoretically feasible to monitor events on the subpicosecond time scale.
Advances in laser technology (ultrashort pulses) and development of highly sensitive detectors have now made it possible to approach these theoretical limits. Time-resolved resonance Raman methods provide the capability to probe the precise structure of fleeting intermediates which evolve and decay, even on sub-picosecond time scales, throughout the course of a given biological process.

In describing the time-resolved Raman methods, we organise the treatment according to two distinctly different approaches for initiating the reaction. The first and more common approach applies to biological systems that are naturally responsive to light. In these cases, the reaction or process of interest can be initiated by a short pulse of light (called the pump pulse). Other (obviously most) biochemical systems are unresponsive to light pulses, and the reaction must be initiated by rapid mixing of the reactants.

E3.7.1 Light-initiated methods

Molecular systems that are susceptible to the light-initiated approach are those that undergo a sequence of structural changes on exposure to photolysis. There are a number of ways that the photolysis step can be accomplished. By far the most common method is to use pulsed lasers; however, several methods have been devised using continuous wave lasers, and these too are useful if it is not necessary to probe at very short times. Although these latter methods provide time resolution on the microsecond time scale, the approach using a pulsed laser permits investigation at the nanosecond, picosecond, and even sub-picosecond levels, the temporal resolution being determined by the widths of the laser pulses.

Fig. E3.19 The biological active centre in haemoglobin and myoglobin molecules. The filled black circles represent the haem skeletal atoms, and the black circles with white dots represent the peripheral groups of the haem. The cross-hatched circles represent the atoms of the proximal and distal histidine and the white circles with black dots a bound CO ligand. (Kincaid, 1995.)
**Haem proteins**

One of the most important and impressive applications of transient Raman studies deals with the investigation of the structural dynamics and mechanism of action of haem proteins (Comment E3.8) and, in first place, haemoglobin (Hb) and myoglobin (Mb) molecules (Comment E3.8). Hb and Mb, two of the first proteins to be structurally determined by X-ray crystallography, both contain five-coordinate, ferrous haems at the active sites as shown in Fig. E3.19. In both cases the haem is attached to the protein through a coordinate linkage to an imidazole side-chain of a histidine residue (the so-called proximal histidine). The distal side of the haem pocket, at which exogenous ligands bind, contains non-polar amino acid residues as well as an imidazole fragment of a histidine (the so-called distal histidine), which is known to be capable of forming a hydrogen bond with certain bound ligands, including dioxygen (Comment E3.9).

Figures E3.20 and E3.21 show the spectra of the deoxyHb photoproduct on the 7 ns time scale acquired with an excitation line of 416 nm and 532 nm, respectively. As was explained in the previous Chapter E1 under Soret band excitation (i.e. 416 nm), the totally symmetric lines ($v_2$, $v_3$, $v_4$) are strongly enhanced, whereas excitation at 532 nm (Q-band) provides strong enhancement of non-totally symmetric modes ($v_{10}$, $v_{11}$, $v_{19}$). In Figs. E3.20 and E3.21 the spectrum of the equilibrium deoxy species is presented along with that of the 7 ns photoproduct. It obvious that all of the features associated with the haem macrocycle appear at low frequencies in the photoproduct spectra relative to their positions in the spectra of deoxyHb at equilibrium. Careful comparison of the frequencies of these core-size markers (Comment E3.10) with a series

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**Comment E3.8  Haem proteins**

Haem proteins are a class of biomolecules which possess a haem group embedded in a single polypeptide chain. The reactivity of the haem group varies greatly from protein to protein depending on the nature of the active site environment provided by the polypeptide side-chains, and this variability gives rise to the remarkable functional diversity that characterises this class of proteins. For example, myoglobin (Mb) and haemoglobin (Hb) bind dioxygen reversibly and thus serve as O$_2$ storage (Mb) and transport (Hb) proteins. Various proxidases and catalases have different active site environments that impart a different reactivity to the haem group, namely, the ability to react to H$_2$O$_2$ to form highly oxidising intermediates in which the oxidation equivalents are localised on the haem (and in some cases on surrounding protein resides) and which are capable of oxidising various substrates. Still other haem proteins such as cytochrome c and cytochrome b have active site environments which render the haem unreactive to exogenous ligands, and these serve only as electron transfer proteins. Finally, one of the most important and frequently studied haem proteins is cytochrome-c oxidase, whose function is to catalyse the four-electron reduction of dioxygen to water.
Fig. E3.20  Soret band excited time-resolved Raman spectra of deoxy Hb and Hb*(photoproduct) with 416 nm, 7 ns pulses. Concentration: 0.1 mM; laser energy, 0.5 mJ per pulse; spectral slit width, 8 cm⁻¹; collection time 10 min; and repetition rate 10 Hz. (Kincaid, 1995). Inset: made ν₄ at ten times reduced scale. (Dasgupta and Spiro, 1986.)

Comment E3.9  Cooperativity of O₂ binding

Whereas the oxygen storage protein, Mb, consists of a single polypeptide chain possessing a proteome at its active site, the oxygen transport, protein, Hb, is actually a tetramer of four (two α and two β) subunits, each of which contains a haem group at its active site. The tetrameric nature of Hb permits interactions between the subunits which give rise to the phenomenon known as cooperativity. Thus the binding curve assumes a sigmoidal shape, leading to more efficient oxygen transport in the sense that it binds O₂ less readily at low O₂ levels (in tissues) but has a much higher affinity at high O₂ levels (in the lungs). This behaviour is consistent with the two-state theory first proposed by Monod in which cooperative behaviour is the result of the existence of a low affinity (the so-called T) state and a higher affinity (R) state; ligand binding to the T state induces a protein structural transition to the more reactive R state. The subsequent crystallographic confirmation of two distinct structures for the ligated (R) and non-ligated (T) forms provided a structural basis for the validity of the thermodynamic arguments. Though the existence of several other states is required to explain the precise details of the binding curves under some conditions, the two-state model remains a valid good approximation under most conditions of ligand binding, indicating that T–R transition is the most important factor for regulating Hb activity. (See also Comment A2.1.)

Fig. E3.21  Q-band excited resonance Raman spectra of deoxyHb and Hb* with 532 nm, 7 ns pulses. Concentration, 0.5 mM; laser energy, 1 mJ per pulse; spectral slit width, 5 cm⁻¹; collection time, 30 min; and repetition rate 10 Hz. (Adapted from Kincaid, 1995.)
Various resonance Raman modes provide information about different fragments of the haem group and its immediate environment. Two high-frequency modes near 1470 cm$^{-1}$ and 1610 cm$^{-1}$ are sensitive to the electron density in the haem $\pi$ orbitals. Other high-frequency modes (near 1470 cm$^{-1}$, 1610 cm$^{-1}$, 1540 cm$^{-1}$ and 1555 cm$^{-1}$) are called core-size markers and respond to changes in the size of porphyrine core (the distances between the pirrole nitrogen and the centre of the porphyrin core).

E3.7.2 Rapid-mixing methods

The great majority of biological systems of interest cannot be photo-initiated and for these cases it is necessary to rely on rapid mixing methods, in which the temporal resolution is dictated by the speed with which the reaction mixture can become homogeneous with respect to reactant concentrations. This mixing time is referred to as the ‘dead time’ of the mixing apparatus, and various designs have been developed to maximise the mixing efficiency of such devices by minimising the dead time.

All the rapid-mixing time-resolved Raman spectroscopy studies so far reported have employed conventional mixing devices that were developed for use with optical (absorption or fluorescence) detection, in which the observation chamber must necessarily be optically homogeneous. However, given the fact that Raman spectroscopy is based on a scattering phenomenon, novel approaches for rapid mixing have been proposed. The use of one such device is depicted in Fig. E3.22.

The essential task to be faced in rapidly and efficiently mixing two solutions is to force them to combine turbulently in a very small volume. In the device shown in Fig. E3.22 the two reactant solutions are fed through concentric tubes to a tip which has a platinum sphere positioned very close (10 $\mu$m) to the (~100 $\mu$m) orifice of the outer tube. In this way both solutions are forced to flow through the 10 $\mu$m circular passage formed by the sphere and the outer tube orifice. The mixing of the two solutions is extremely efficient (dead times as low as 20–40 $\mu$s). The progress of the reaction can be conveniently monitored merely by varying the distance between the tip of the outer tube and the probe laser. This approach
Fig. E3.23  Time-resolved Raman spectra of the reaction of deoxyhaemoglobin (0.25 mM in haem) with an oxygen-saturated buffer, pH 7.0, obtained using the mixing device shown in Fig. E3.22. The excitation line was 457 nm. (Adapted from Kincaid, 1995.)

is very useful in the study of haem proteins. For example, as shown in Fig. E3.23, the monitoring of the reaction of O$_2$ with deoxyhaemoglobin is followed over the first 1.2 ms by monitoring modes which appear at 1358 cm$^{-1}$ (Hb) and 1374 cm$^{-1}$ (Hb$^*$O$_2$).

E3.8 Checklist of key ideas

- Raman spectroscopy is a form of vibrational spectroscopy and is related to transitions between the different vibrational energy levels in a biological macromolecule; in this respect Raman spectroscopy is like IR spectroscopy.
- Raman spectroscopy differs from IR spectroscopy in that information is derived from light scattering whereas in IR spectroscopy information is obtained from an absorption process.
- Raman scattering is associated with normal modes that produce a change in the polarisability of the molecule, while IR absorptions are observed for vibrational modes that change the dipole moment of the molecule.
- Raman spectroscopy of biological macromolecules is conventionally divided into two types, resonance and non-resonance, on the basis of the proximity of the excitation radiation frequency to electronic transitions of the components of the sample.
- Non-resonance or classical Raman spectroscopy is very useful in the determination of protein, nucleic acid and membrane conformational states.
- Resonance Raman spectroscopy has provided considerable information on haem proteins and other metalloproteins and visual pigments.
The central advantage of resonance Raman spectroscopy is that when the wavelength of the exciting laser line is adjusted to coincide with that of an allowed electronic transition in a molecule, the intensities of certain Raman bands are enhanced relative to the off-resonance values.

When a molecule is placed near the surface of a particle of metal, such as silver or gold, the Raman signal intensity is enormously enhanced; this effect is called surface enhanced Raman scattering.

Raman spectroscopy and Raman microscopy provide direct comparison of the properties of a molecule in solution and in a single crystal.

In the estimation of protein secondary structure content in terms of percentage helix, \( \beta \)-strand and turn, the amide I band is analysed as a linear combination of the spectra of references proteins whose structures are known.

From a comparison of the Raman spectra taken from crystals of DNA of known structure, a correlation between the frequencies and intensities of Raman bands and the conformation of the DNA has been established.

Proteins with different folds give characteristic VCD shapes, which vary most for the amide I mode (C=O stretch) but are also easily detectable for the broader amide II and III (very weak) modes (N–H deformation and C–N stretch).

In the ROA technique a small difference in the intensity of Raman scattering is measured using right and left circularly polarised incident light.

As well as providing a signature of the \( \alpha \)-helix, some of the ROA spectra also provide signatures of \( \beta \)-structure.

The inherent scattering processes (\( 10^{-14} \) s) carries with it the implication that it is theoretically feasible to monitor events on the subpicosecond time scale; advances in laser technology (ultrashort pulses) and development of highly sensitive detectors have now made it possible to approach these theoretical limits.

Suggestions for further reading

Historical review and introduction to biological problems


Non-resonance Raman spectroscopy


**Resonance Raman spectroscopy**


**Vibrational Raman optical activity**


**Differential Raman spectroscopy**


**Time-resolved resonance Raman spectroscopy**