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Protein conformational stability in the hydrofluoroalkane propellants tetrafluoroethane and heptafluoropropane analysed by Fourier transform Raman spectroscopy

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Abstract

Due to the inherent structural instability of proteins, development of chlorofluorocarbon (CFC) free metered dose inhalers (MDIs) containing these biomolecules is beset with numerous challenges. In assessing the conformation of proteins in any medium, both secondary and tertiary structures need to be elucidated. This study uses Fourier transform (FT-) Raman spectroscopy to probe protein conformational stability in hydrofluoroalkane (HFA) propellants. Assignments of molecular modes for lysozyme as a solid and in aqueous solution, and for the first time, HFAs tetrafluoroethane (HFA 134a) and heptafluoropropane (HFA 227) are given. The Raman spectra provided molecular structural information on the peptide backbone, disulfide bonds and C-C stretching vibrations of hen egg lysozyme, enabling the secondary conformation of protein in HFA propellants to be determined; structural integrity of this robust model protein was maintained in both propellants. These results demonstrate that FT-Raman may be a useful tool for the optimisation of protein MDI formulations.

Keywords: Protein; Stability; Hydrofluoroalkane propellant; Tetrafluoroethane; Heptafluoropropane; Fourier transform Raman spectroscopy

1. Introduction

As the native conformations of proteins tend only to be marginally stable (5–20 kcal/mol), their tertiary structure is very labile and consequently is easily disrupted (Pace, 1975). Maintaining the physical and chemical stability of such molecules is thus pivotal in formulation development. Loss of the native conformation may result in diminution or complete loss of biological activity.

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The inhalation route offers an appropriate method of delivering drugs directly to the lung. This route of drug delivery is also more acceptable for systemic delivery of peptide and protein drugs, which are typically administered by injection. There are reports on more than 40 peptides, proteins and genes which have been administered via the lung to animals, exploring the potential for local or systemic delivery. In addition several of these molecules including insulin (Patton and Platz, 1992), leuprolide (Adjei and Garren, 1990), the cystic fibrosis transmembrane regulator gene and recombinant human DNase (rhDNase; Aitken et al., 1992) have been administered to man. Currently rhDNase is the only product approved by the FDA for its use in the treatment of cystic fibrosis.

The conventional chlorofluorocarbon (CFC) propellants used to formulate metered dose inhalers (MDIs) may interfere with stratospheric ozone protection and all parties involved in the Montreal protocol agreed to phase out all CFCs by 2000. In 1998, the quantity of CFC gases allocated for use in medical aerosols constituted approximately 97% of the total CFC allocation in Europe (Commission of the European Communities, 1997).

Hydrofluoroalkanes (HFA) propellants do not contain chlorine, thus possess zero ozone depletion potential and their global warming potential is two- to 100-fold lower compared to CFCs (Gupta and Adjei, 1997). Presently, the only two HFAs approved for inhalation use are tetrafluoroethane (HFA 134a) and heptafluoropropane (HFA 227).

Pharmaceutical products undergo complete characterisation throughout the drug development process and for molecular characterisations infra-red (IR) spectroscopy is a well established tool. However, Raman spectroscopy offers an alternative method for elucidating the molecular state of systems and IR and Raman vibrational analysis provide similar but complementary data (Bugay and Williams, 1995). Raman spectroscopy has been a valuable technique for researching the primary and secondary structures of proteins as biological samples can be investigated in their natural (usually aqueous) environments. Good quality molecular data has been obtained on samples as varied as immunoglobulins (Qian and Krimm, 1992), viruses (Overman and Thomas Jr., 1998) and intact bovine lens proteins (Mathies and Yu, 1978).

For studies on peptides and proteins, Raman spectroscopy offers several key advantages over infra-red spectroscopy. First, Raman analysis requires essentially no sample preparation when compared with other spectroscopic techniques. In infra-red studies, samples are often dissolved in weakly absorbing solvents or ground in Nujol to form a mull. Apart from being labour intensive, the grinding process may lead to solid-state changes which can subsequently leads to spectral differences. Alternatively, if using attenuated total reflectance infra-red (ATR-IR) spectroscopy, casting a protein film can result in structural rearrangements or variations in the degree of hydration can induce spectral artifacts (Jackson and Mantsch, 1992). Second, Raman studies are not hampered by variations in sample morphology. Under physiological conditions, the polypeptide chain of a protein is folded into a specific conformation defined as the native state. This is the functional state of the protein in vivo. As water is a weak Raman scatterer, the interference from water seen in infra-red studies is minimised. This is particularly advantageous for biological samples since it allows studies of biomolecules in their native (aqueous state) and thus enables the conformational state of proteins in different environments (solid, aqueous solution and as a suspension in the HFAs) to be compared. Third, Raman spectra can be obtained non-invasively. The technique enabled analysis of HFA propellant systems in glass and polyethylene teraphthallate (PET) vials without requiring the need for high pressure cell facilities. Another important feature of the Raman technique is that spectral vibrational modes tend to be sharper when compared to those of an IR spectrum. Crowder and Lightfoot (1983) observed that IR bands arising from (C-F) stretching modes appeared to be broad and poorly resolved, in contrast to Raman bands which are sharp and well defined. However, the Raman technique is not as widely available as IR and it tends to have a lower limit of accurate detection.

Here we present the first study on the feasibility and difficulties of using FT-Raman spectroscopy as an analytical tool in assessing protein conformational stability in HFA propellants.

2. Materials and methods

2.1. Materials

Crystalline hen egg lysozyme was obtained from Boehringer Mannheim UK, East Sussex, UK. Tetrafluoroethane (HFA 134a, CH_2FCF_3) and Heptafluoropropane (HFA 227, CF_3CHFCF_3) were supplied by 3M Healthcare Ltd., Loughborough, UK. Where appropriate, the protein samples were dialyzed using Spectra/Por® DispoDialyzer® against a large volume of double distilled H_2O for 48 h.

2.2. Fourier transform Raman spectroscopy

FT-Raman spectra of protein powders, aqueous solutions of lysozyme and lysozyme suspended in HFA 134a and HFA 227 were obtained using a Bruker FRA-106 FT-Raman module mounted onto a Bruker IFS 66-FTIR optical bench. Solutions (7.5% w/v) were analysed in a 1 cm^3 quartz cuvette with a mirrored rear surface, solids were placed in stainless steel sample cups and HFA suspensions (7.5% w/w) were sealed in crimped PET and polypropylene coated glass pressurised vials. Sample excitation used a near infrared Nd^{3+}:YAG laser operating at a wavelength of 1.064 μm with a laser power was approximately 500 mW for solids and 800 mW for solutions and suspensions. All spectra were the average of 200 scans for solid samples and 4000 scans for solutions or suspensions at a resolution of 8 cm^{-1}. The FT-Raman module is equipped with a liquid nitrogen cooled germanium diode detector with an extended spectral bandwidth which covered the wavenumber range 3500–50 cm^{-1}. Wavenumber positions are correct to better than ± 1 cm^{-1}. The spectral response was corrected for white light. All experiments were performed at room temperature (25 ± 1°C).

3. Results and discussion

As the hydrofluoroalkane propellants are potentially explosive, they are usually contained within shatter proof vials; either plastic coated glass or polymer vials are generally used. Fig. 1(a) shows the FT-Raman spectrum over the wavenumber range 3250–250 cm^{-1} for a PET vial and Fig. 1(b). shows the equivalent spectrum of a polypropylene coated glass vial and of a polypropylene coated glass vial with a window from which the polypropylene protective layer has been removed (i.e. only the glass vial). There is a marked difference between the spectrum of the coated vials (either PET or polypropylene) and the coated glass vial with the window. PET and polypropylene are both strong Raman scatterers and hence give sharp intense vibrational peaks. Preliminary data revealed that the strong signal arising from the use of PET or polymer coated glass vials in spectroscopic analysis of protein molecules obscured the main spectral regions of interest from the protein; the amide I (~ 1660 cm^{-1}), amide III (~ 1240–1275 cm^{-1}), disulphide bonds (~ 507 cm^{-1}) and C-C stretching modes (~ 900 cm^{-1}) could not be discerned from peaks arising from the vials. Attempts to subtract the signal arising from the polymer from that of the protein in the vials were unsuccessful resulting in very poor quality Raman spectra of the proteins. In sharp contrast, there is little background scattering from glass, which is well known to give a very weak Raman effect. Spectral subtraction of the glass signal from the protein within the glass vial was relatively straightforward as described below.

Most data cited in the literature on HFAs relates to environmental issues or aerosol delivery properties of MDIs formulated with the propellants. To our knowledge no vibrational assignments have been reported. In Fig. 2(a, b) are FT-Raman spectra over the 3250–250 cm^{-1} range for heptafluoropropane (HFA 227) and tetrafluoroethane (HFA 134a), respectively, collected in a glass vial with a window as described above. Assignments of the bands in the spectra for tetrafluoroethane (HFA 134a) and heptafluoropropane (HFA 227) consistent with the

observed vibrational modes were made and the main molecular vibrations are summarised in Table 1 and Table 2, respectively. For most alkyl-halides, the C-X (X = F, Cl, Br, I) stretching frequencies seen in vibrational spectra are quite unique and are relatively easy to assign. However, C-F modes are exceptional. The C-F stretching modes tend to couple strongly with other vibra-

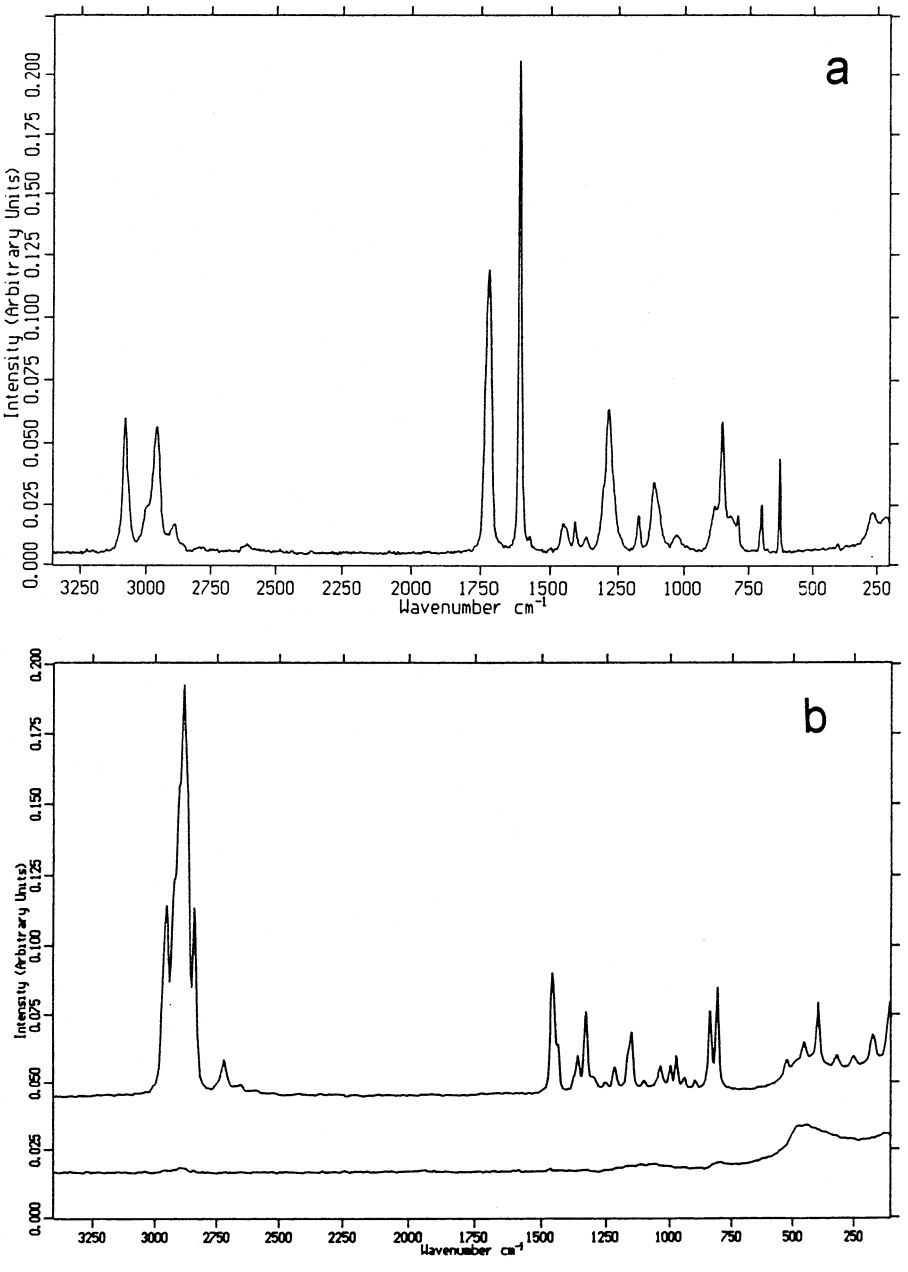

Fig. 1. FT Raman spectra of (a) PET vial over the wavenumber range 3250–250 cm^{-1} and (b) a plastic coated glass vial (upper trace) and a plastic coated glass vial with a window (bottom trace) over the 3250–250 cm^{-1} range.

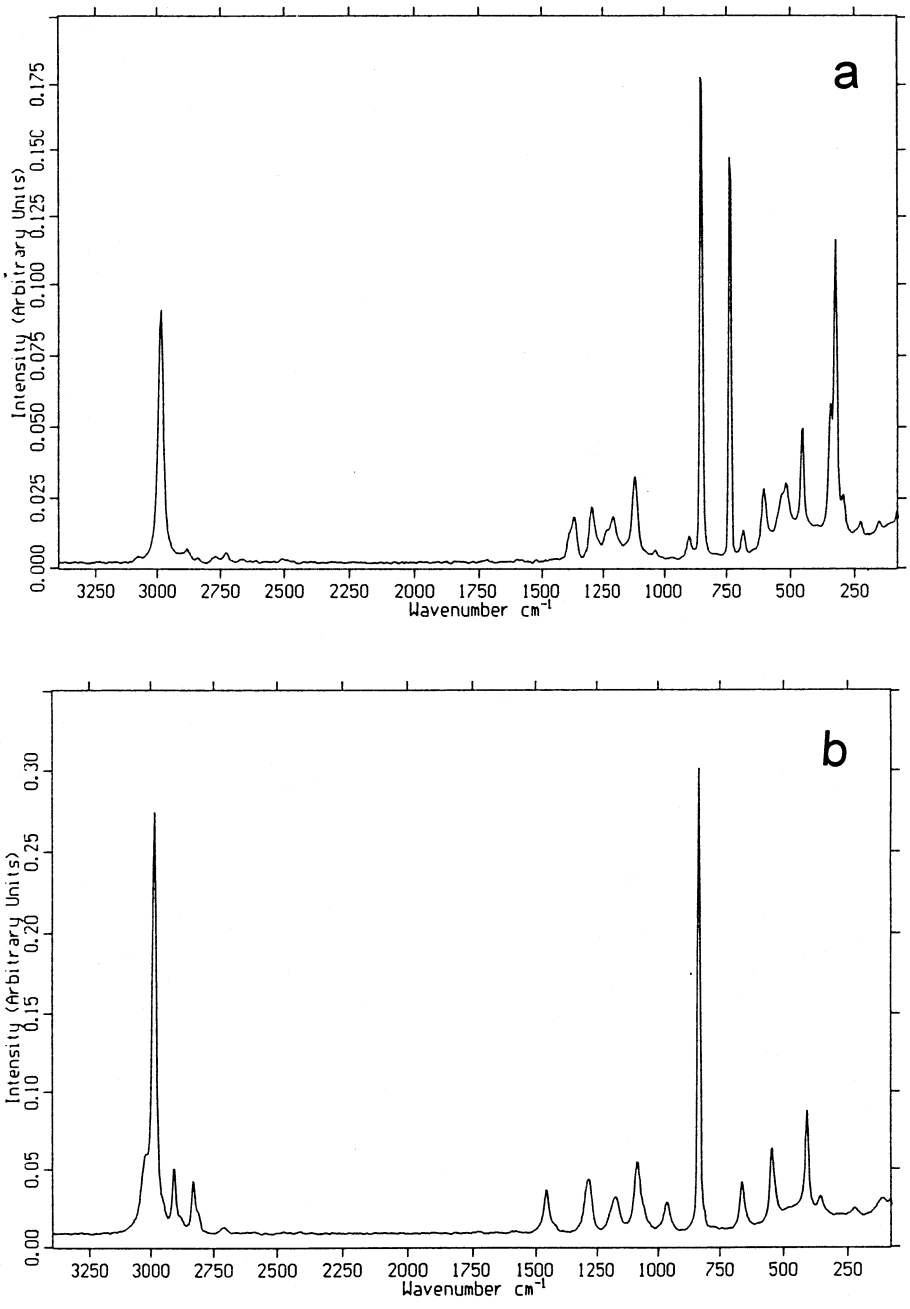

Fig. 2. FT Raman spectra of (a) heptafluoropropane (HFA 227) and (b) tetrafluoroethane (HFA 134a) over the wavenumber range 3250–250 cm^{-1}.

tional modes, especially C-C stretching vibrations, making accurate assignments complex. Furthermore, CF_2 and CF_3 moieties absorb in similar regions compounding the difficulty of band assignments. However, compounds containing $-CF_3$ have been studied extensively by vibrational

analysis, thus peak assignments and approximate description of vibrational modes for the Raman spectra of HFA 134a and HFA 227 have been given based on similar chemical entities (Berney et al., 1963; Perttilä, 1979; Francisco, 1984; Yadav and Singh, 1985).

The spectra shown in Fig. 2 are of good quality with a signal: noise ratio of greater than 200. The figure also shows that the propellants give relatively simple spectra with sharp vibrational modes. The unique nature of the C-F modes, absent from protein, offers the potential to subtract the propellant spectrum from that in which a protein is present; unlike infra-red spectroscopy, the intensity of Raman modes is directly related to the concentration of the scattering species thus allowing for linear spectral subtraction over the whole wavenumber range.

Fig. 3 illustrates the usual positions of characteristic bands of the amide I, amide III and C-C stretching vibrations for various secondary structures of proteins and polypeptides (Twardowski and Anzenbacher, 1994). Vibrations of the peptide bonds ('CONH') are termed 'amide' vibrations. The secondary structure of a protein can be determined by analysis of the amide band shape and position. The two most sensitive modes to conformation in the Raman spectrum are the amide I and amide III vibrational modes, which give prominent Raman bands that can be correlated with the structural properties of proteins. Proteins exhibiting α-helical structure typically show a strong amide I band around 1655 cm^{-1}, whilst proteins with a β-structure show an intense band around 1235 cm^{-1} The amide I stretching mode is a combination of the carbonyl stretching vibration ($\upsilon C=O$) and the N-H in-plane bending vibration (δN-H). Analysis of the amide I band to estimate the percentage protein secondary in terms of α-helix, β-strand and reverse turn content was developed by Williams et al. (1980). Similar correlation data have been used to determine the contribution of secondary structure in mixed

Table 1
FT Raman spectral assignments and approximate descriptions of the main vibrational modes for tetrafluoroethane (HFA 134a)[a]

Wavenumber (cm^{-1})[b]	Assignments and approximate description of vibrational modes[c]
2987s	υ (CH_2) Asym[d]
2908m	–
2832m	υ (CH_2) Sym[d]
2709w	–
1463m	δ (CH_2) scissoring
1290m	υ (CF_3)
1180m	υ (CF_3)
1089m	ρ (CH_2)
969m	(C-H) o.p. bend
839s	υ (C-C)
665m	δ (CF_3)
548m	δ (CF_3)
410m	ρ (CF_3)
357w	ρ (CF_3)
228w	ρ (CF_3)

[a] Data are wavenumber positions of bands in a representative spectrum resulting from 4000 scans at a resolution of 8 cm^{-1}.
[b] s, strong; m, medium; w, weak; sh, shoulder.
[c] υ, stretch; δ, deformation; ρ, rock.
[d] Asym, asymmetric; Sym, symmetric.

Table 2
FT Raman spectral assignments and approximate descriptions of the main vibrational modes for heptafluoropropane (HFA 227)[a]

Wavenumber (cm^{-1})[b]	Assignments and approximate description of vibrational modes[c]
2987s	υ (CH)
2730w	υ (CH)
1375m	δ (CH)
1301m	(C-H) i.p. bend
1213m	υ (C-F) Asym
1124m	υ (CF_3)
905w	(C-H) o.p. bend
857s	υ (C-C)
688w	δ (CF_3) Sym[d]
741s	υ (C-F) Asym[d]
607m	δ (CF_3) Sym
519m,sh	δ (FCF_2)
455m	ρ (CF_3)
345w	δ (CF)
326s	–
229w	ρ (CF_3)

[a] Data are wavenumber positions of bands in a representative spectrum resulting from 4000 scans at a resolution of 8 cm^{-1}.
[b] s, strong; m, medium; w, weak; sh, shoulder.
[c] υ, stretch; δ, rock; ρ, deformation.
[d] Asym, asymmetric; Sym, symmetric.

proteinaceous systems (Anigbogu, et al. 1995). The amide III band is also conformationally sensitive, arising predominantly from the N-H bending (δN-H). However there is often overlap of the band frequencies of the amino acid side chains and the vibrations of the amide group. This is particularly noted in the amide III region which presents as a broad peak in contrast to the amide I band which is a single strong peak. Therefore, it is not always possible to deduce a simple relationship between such a frequency and the backbone conformation. Lord and Yu (1970) consider that the peaks of the amide III band may be correlated to the various structural components of lysozyme; i.e. the α-helix, β-sheet and random coil arrangements.

Fig. 4 shows the 'fingerprint' region (1800–400 cm^{-1}) FT-Raman spectra of lysozyme in the solid state and in aqueous solution (7.5% w/v), respectively, both spectra show the characteristic bands

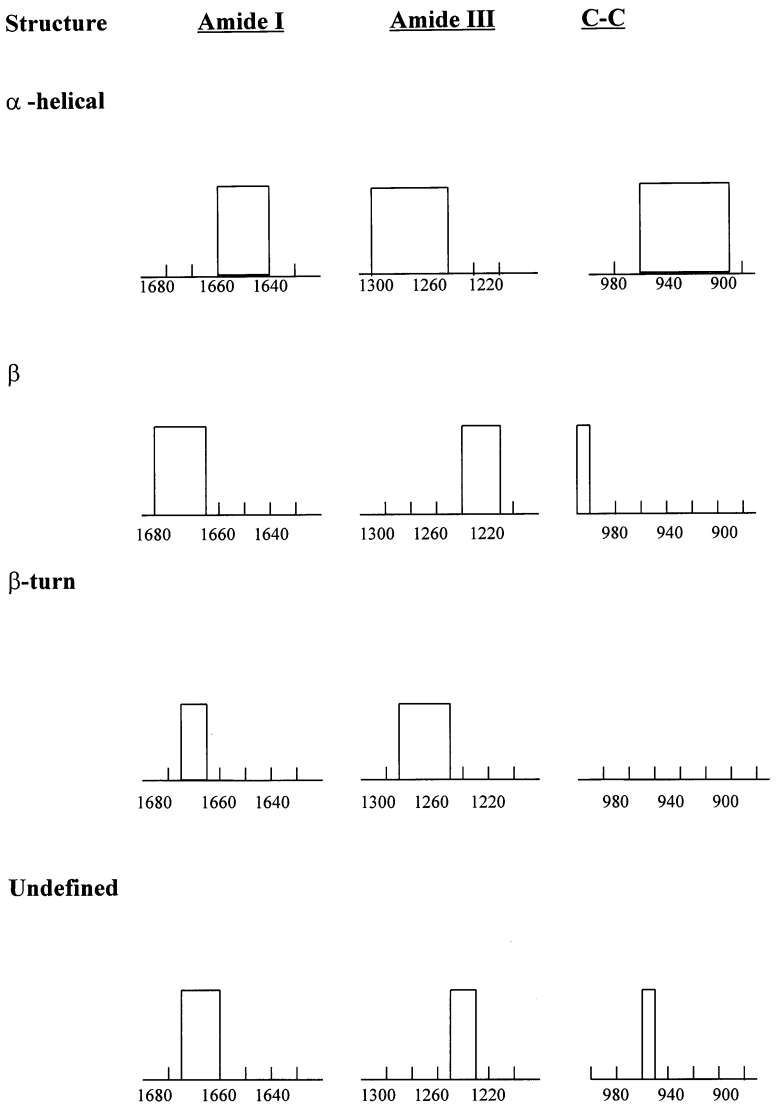

Fig. 3. Positions of bands of amide I, amide III and C-C vibrations for various secondary structures.

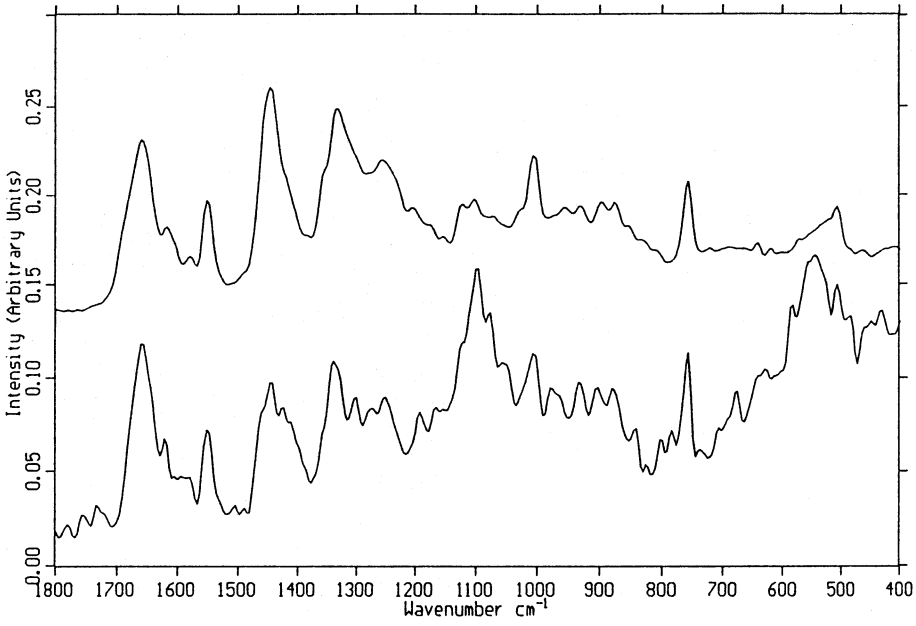

Fig. 4. FT Raman spectra of lysozyme in the solid state (upper trace) and lysozyme in aqueous solution (bottom trace) over the wavenumber range 1800–400 cm^{-1}.

for a protein with an α-helical structure. Lysozyme is a protein exhibiting α-helical structure, with a low amide I band at 1660 cm^{-1} and high amide III band between 1264 and 1300 cm^{-1}. Other spectral regions of interest include the vibrational modes of the disulphide bonds. When a polypeptide chain is cross-linked with a covalent bond, such as a disulphide bond between two cysteine residues, the conformational entropy of the system is decreased, thus enhancing the stability of the protein. The S-S vibrational stretching band lies in the 500–550 cm^{-1} region and the prominent stretching frequency of the S-S band in lysozyme occurs at ~ 507 cm^{-1}, which corresponds with a *gauche-gauche-gauche* conformation.

Lord and Yu (1970) assigned many of the vibrational modes observed in the solution phase for lysozyme, using Raman spectra obtained from mixtures of constituent amino acids to aid data interpretation. Table 3 lists peak assignments for the FT-Raman spectra of lysozyme solution and lysozyme solid, based on those of Lord and Yu. A sharper more defined Raman spectrum of lysozyme in the solid state compared to the solution is observed as there is no interference arising from a solvent background. Raman detector technology is such that limits of detection by this technique tend to be worse than those in infra-red spectroscopy, therefore it is necessary to use relatively high concentrations of protein to detect and produce well resolved Raman bands.

Fig. 5(a) provides FT-Raman spectra of a HFA 227 suspension of lysozyme and a HFA 134a suspension of lysozyme using PET vials, respectively, over the wavenumber range 1800–400 cm^{-1} and Fig. 5(b) shows the equivalent spectra over the range of 1800–400 cm^{-1} for the HFA 227 suspension of lysozyme and HFA 134a suspension of lysozyme using plastic coated glass vials with windows. The Raman signal arising from the respective HFAs (Fig. 2(a, b)) and the PET or glass have been subtracted from the above data; FT-Raman spectra were collected for the propellants in the vials (no protein) and this signal was scaled to the most intense propellant peaks, 857 and 839 cm^{-1} for HFA 227 and HFA134a, respectively, and then subtracted from the data obtained for lysozyme in the propellants within the vials.

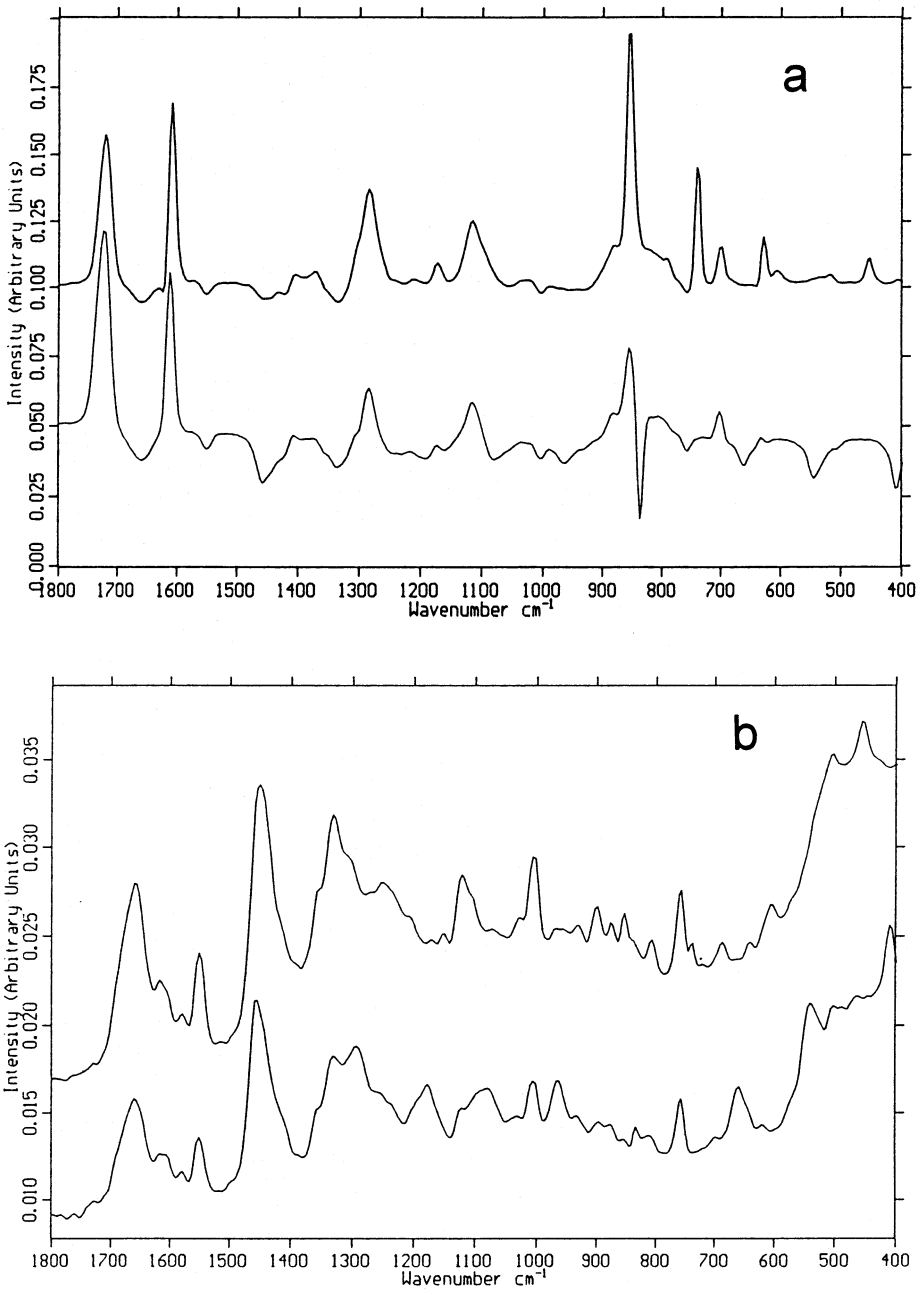

Fig. 5. FT Raman spectra of (a) HFA 227 suspension of lysozyme using a PET vial (upper trace) and HFA 134a suspension of lysozyme using a PET vial (bottom trace) over the wavenumber range 1800–400 cm^{-1} and (b) HFA 227 suspension of lysozyme using a glass vial (upper trace) and HFA 134a suspension of lysozyme using a glass vial (bottom trace) over the 1800–400 cm^{-1} range.

Fig. 5(a) highlights the importance of correct sampling technique. Due to the strong PET spectrum, background subtraction was problematic and failed to reveal the presence of the lysozyme in either propellant, which could lead to misleading conclusions. However, the FT-Raman data accumulated from the glass vial with the window clearly shows the structural integrity of lysozyme in both HFA34a and HFA 227. Here, the subtraction routine is greatly improved as there is only a weak Raman signal from the glass to remove. Subtle spectral changes result when the lysozyme is suspended in the hydrofluorocarbons (Fig. 5(b)) compared with the aqueous or solid samples (Fig. 4). No change in the amide I region of lysozyme (1660 cm^{-1}) when suspended in the HFAs results, as a strong signal, nearly identical to the spectra obtained for lysozyme in the solid and aqueous state is observed. Similarly, the C-C stretching modes (υC-C) around 900 and 930 cm^{-1} show good agreement with the spectra obtained in Fig. 4. The amide III regions loses definition in both HFA suspensions, indicating some difference between the backbone conformation of lysozyme in an non-aqueous environment and in the native state (aqueous). A similar observation was observed when α-lactalbumin was lyophilised (Yu, 1974) and ribonuclease A dehydrated (Yu et al., 1972). This may arise as a result of less uniform hydrogen bonding in the peptide backbone and a change in the local environment of amino acid side chains. No significant differences in the spectra, between the lysozyme in the solid and aqueous states compared to the lysozyme suspended in HFAs, as shown by the amide I (~ 1660 cm^{-1}), disulphide bonds (~ 507 cm^{-1}) and C-C stretching modes (~ 900 cm^{-1}) are evident.

Table 3
FT Raman spectral assignments and approximate descriptions of the main vibrational modes for lysozyme in solution and solid state[a]

Wavenumber (cm^{-1})[b]		Assignments and approximate description of vibrational modes[c]
Solution	Solid	
1660s	1659s	υ (C = 0) amide I (α-helix)
1618m	1621sh	Try, Tyr, Phe
1551s	1552s	Tyr
1446sh	1448s	δ ($C-H_2$)
1340s	1334s	Tyr, δ ($C-H_2$)
1275w	–	δ (C-N) amide III
1253w	1257sh	δ (C-N) amide III
–	1128	υ (C-N)
1079w	–	υ (C-N)
1007s	1008s	Phe
934m	932m	υ (C-C)
906m	899m	υ (C-C)
880m	877m	Try
758s	758s	Try
507m	507sh	S-S

[a] Data are wavenumber positions of bands in a representative spectrum resulting from 4000 scans at a resolution of 8 cm^{-1}.
[b] s, strong; m, medium; w, weak; sh, shoulder.
[c] υ, stretch; δ, deformation.

4. Conclusion

The investigation has shown for the first time that FT-Raman spectroscopy is a valuable tool with great potential for investigating the conformational stability of biomolecular structures in HFA propellants. Assignments of the molecular modes for lysozyme in the solid, solution and for HFA 134a and HFA 227 have been elucidated. Whilst lysozyme largely exhibits structural stability in HFA 134a and HFA 227, there may be some minor alterations to the conformational order due to a somewhat altered local environment around the protein.

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