

# Application of computerized infrared and Raman spectroscopy to conformation studies of casein and other food proteins

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Received 14 September 1987

Revised 7 December 1987

Accepted 8 December 1987

*Key words:* Spectroscopy, computerized; IR spectroscopy; Raman spectroscopy; Protein structure-function relationship; Conformation study

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## SUMMARY

The ability of modern biotechnology to produce new or modified proteins has outpaced current understanding of the relationship between protein structure and protein function. Resolution-enhanced infrared spectroscopy and Raman spectroscopy are excellent non-destructive techniques for investigating the secondary structure of proteins under a wide variety of conditions. The techniques yield rapid, reliable estimates of the proportion of helical structure,  $\beta$ -strands, and turns of proteins in solution, as gels, or as solids. These methodologies can also detect subtle variations in protein conformation that frequently occur upon change of the biomolecular environment. In particular, it is possible to study structural changes which arise from alterations in pH, ionic strength, nature of solvent, and from interactions with other molecules or ions, such as another protein or  $\text{Ca}^{2+}$  ions. The first part of this paper will briefly review various important aspects of the techniques. The subsequent part describes application to structural problems of casein and other food proteins.

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## INTRODUCTION

To understand the function of proteins and enzymes requires detailed knowledge of the molecular architecture of these complex biomolecules [15]. The conformation, or secondary structure, of a protein is a critical determinant of its functional properties, biologically [22] as well as in processed food products.

Infrared (IR) and Raman spectroscopy are sensitive methods for examining biomolecular structure at the submolecular level. These complementary spectroscopic techniques provide structural information on groups of atoms which comprise the biomolecule as a whole, indicating how the atoms are bonded to each other and the nature of their geometric arrangement. Equally important is the use of IR and Raman spectroscopy to examine the intra- and intermolecular interactions in which specific structural groups are engaged.

Although neither IR nor Raman spectroscopy

can, at present, provide detailed knowledge concerning bond lengths and bond angles of large molecules, both methods provide information about the overall molecular conformation imposed upon proteins by intra- and intermolecular hydrogen bonding, amino acid sequence, hydrophobic interactions, and other factors. The frequencies and intensities of IR and Raman bands of proteins are quite sensitive to rather small structural variations, such as changes caused by altering the molecular environment, e.g. pH and/or solvent composition. X-ray and neutron diffraction techniques, which provide more precise atomic parameters than the spectroscopic procedures, are limited to investigations of single crystals and can be, in addition, quite slow and tedious. Molecular spectroscopy, by contrast, can be used to study structural characteristics of lyophilized powders, solutions, gels and colloids, as well as crystalline samples, and in certain cases even particular molecular species within a whole cell [4,14].

For proteins – as distinct from synthetic polypeptides containing only one or two kinds of amino acid residues – each characteristic IR and Raman band is generally a broad composite that consists of overlapping components representing vibrational transitions associated with  $\alpha$ -helical sections,  $\beta$ -strands, turns, and aperiodic regions [32]. These components usually cannot be resolved by conventional spectroscopic techniques because their inherent widths are greater than the instrument resolution. Until recently, both IR and Raman spectroscopy therefore served essentially as qualitative tools for conformational studies of proteins [18], although some semi-quantitative applications have been attempted [6,13,19,21].

Since about 1983, several methods have been proposed to estimate protein conformation (secondary structure) quantitatively by means of Raman [1,2,26,29–31] or Fourier-transform infrared (FTIR) [3,24] spectroscopy. One method involves analyzing the intensity distribution across the amide I bands ( $1700\text{--}1620\text{ cm}^{-1}$ ) of the IR or Raman spectra of proteins of known structure to determine how this intensity distribution correlates with the percentage of  $\alpha$ -helices,  $\beta$ -structure, turns

or any other type of secondary structure present [1,29–31]. Once proper correlations have been established, the structure of unknowns can be investigated. An alternative method relies on the empirical observation that the broad amide I band (either IR or Raman) is actually composed of individual component peaks whose band areas are proportional to the amounts of different types of secondary structure present in the protein. In order to estimate the areas of individual components, the composite band is first resolved into components by Fourier deconvolution; the areas of the resolved components are then determined by a suitable curve-fitting technique (see below). This method was first developed using Fourier deconvolved FTIR spectra of proteins in  $\text{D}_2\text{O}$  solution [3,24]. Recently, the same method was successfully applied to Raman spectra of proteins in the solid state (either lyophilized or crystallized) [2,26]. A third approach involves the second derivative spectrum of a protein in the region of interest [23]. The second derivative spectrum exhibits peaks with a considerably reduced band width, thus again increasing the apparent resolution. To date, however, this technique has been used only for qualitative studies.

In this paper we will focus on deconvolved and second derivative IR and Raman spectra, and discuss some recent qualitative and quantitative applications of these techniques to elucidate protein structure. In the case of IR spectroscopy, the reliable and reproducible application of resolution enhancement techniques became feasible with the development of modern, computerized FTIR interferometric spectrometers. (In the past, only dispersive instruments were generally available. Their sensitivity and signal-to-noise (S/N) ratio was not sufficient to permit application of deconvolution or second derivative techniques.)

Fig. 1 illustrates the wealth of spectroscopic information hidden within the inherently broad IR bands of a typical globular protein, triose phosphate isomerase, in  $\text{D}_2\text{O}$  solution. The upper curve represents the original spectrum, the middle curve gives the deconvolved spectrum, the bottom curve the second derivative of the original spectrum. In

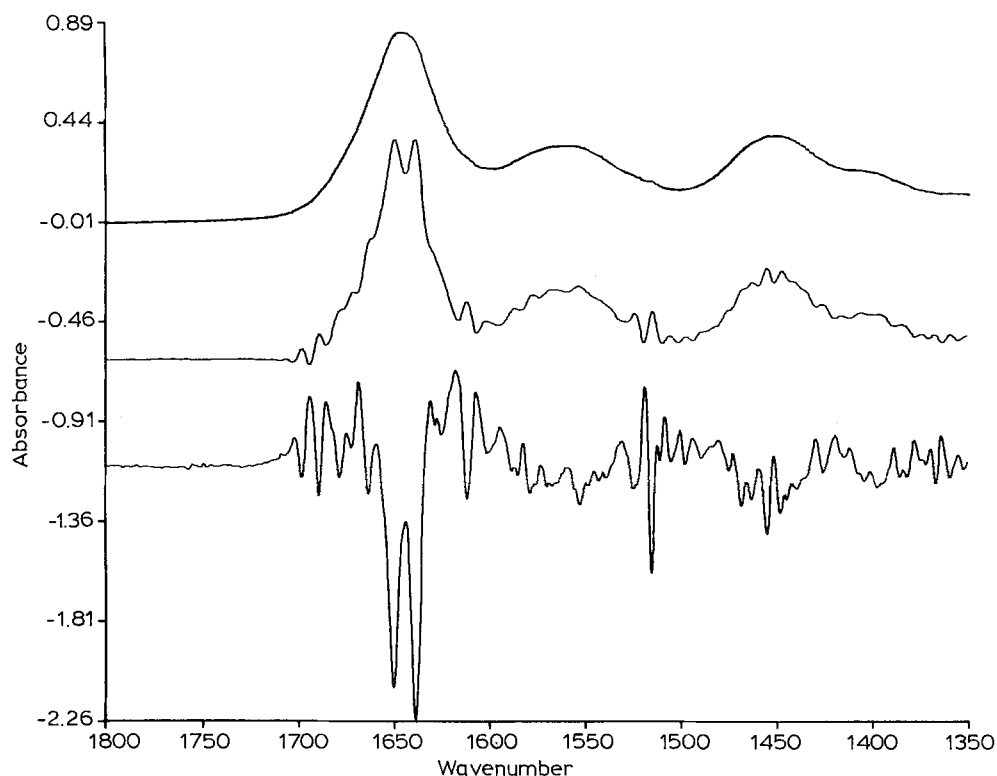


Fig. 1. FTIR spectra of triose phosphate isomerase (5% w/v in  $D_2O$ ). Upper curve: original spectrum; middle curve: deconvolved spectrum; lower curve: second derivative of the original spectrum.

the particular case of the amide I band (ca.  $1650\text{ cm}^{-1}$ ), the fine structure is due to the  $C=O$  stretching vibrations of different peptide groups. Each class of secondary structure ( $\alpha$ -helix,  $\beta$ -structure, turns, and aperiodic segments) gives rise to  $C=O$  component bands at different frequencies. In the following sections we will discuss how this new information can be used both qualitatively and quantitatively in protein structure studies. Susi and Byler [24] have given a brief review of the basic theory of Fourier transform interferometry and of the mathematical basis of derivative spectroscopy as well as Fourier deconvolution techniques. More detailed information on each of these subjects can be obtained by consulting references cited therein.

Fig. 2 illustrates the application of deconvolution, combined with curve fitting, to the FTIR spectra of ribonuclease S, concanavalin A, elastase and

lysozyme. Fig. 3 gives corresponding information about the Raman spectra of (A) immunoglobulin G, (B) serum albumin, (C) carbonic anhydrase, and (D) ribonuclease S. The curves given by crosses are the digitized experimental FTIR or Raman spectra after deconvolution. The individual peaks denoted by solid lines are the sub-bands determined by iteratively fitting the experimental curves with Gaussian components. The solid lines which closely fit the deconvolved experimental data are the summations of these components. It is evident that by using the combination of deconvolution and curve fitting the original broad amide I band can be resolved into a number of components with well-defined frequencies, shapes, and band areas. The application of second derivative spectroscopy as a complementary technique was illustrated in Fig. 1.

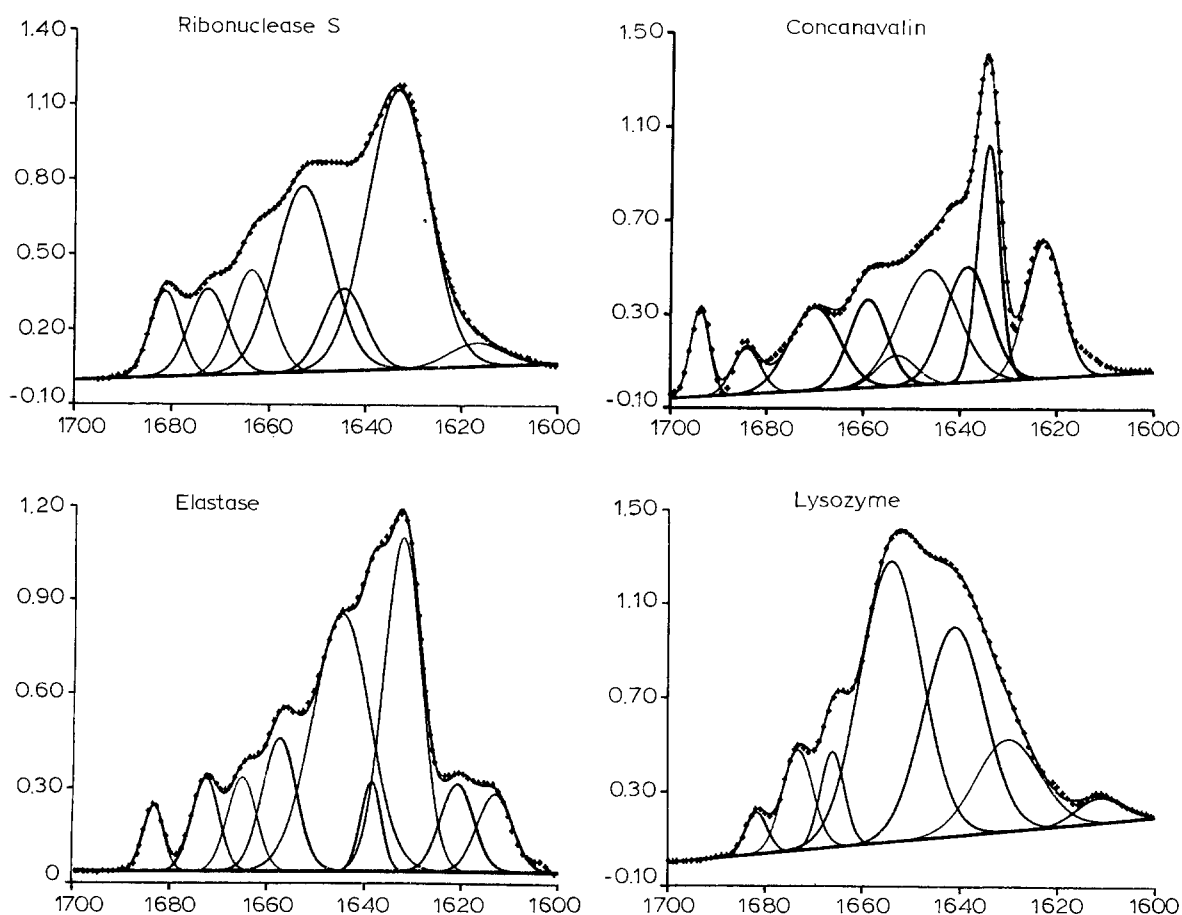


Fig. 2. Deconvolved IR amide I bands of four proteins as 5% (w/v) solutions in  $D_2O$  (++++). Individual Gaussian components determined by iterative curve-fitting and their sum are given by the solid curves (—). Relative intensity in absorbance units is plotted versus wavenumber ( $cm^{-1}$ ). (A) ribonuclease S; (B) concanavalin A; (C) elastase; (D) lysozyme (hen egg white). (From Ref. 3.)

## DEUTERATION AND CHOICE OF PROPER SOLVENT

Both IR and Raman spectra can, in principle, be obtained from samples in the solid state or in solution. IR spectroscopy in aqueous solution has always been very difficult because water absorbs strongly throughout much of the mid-IR region (ca.  $4000-400\text{ cm}^{-1}$ ). Particular problems are encountered in the conformationally important amide I region (ca.  $1700-1620\text{ cm}^{-1}$ ) because of the very strong HOH bending mode which occurs around  $1644\text{ cm}^{-1}$ . When FTIR first became available, it was thought that the increased sensitivity would render IR spectroscopy of proteins effortless even

in  $H_2O$  solution. Instead of the earlier 'differential methods' [27], subtraction of the solvent spectrum from the solution spectrum could now be easily accomplished in order to obtain the spectrum of the pure solute. Unfortunately, neither the subtraction nor the differential procedures are as straightforward as they first appear. Whenever a solute is present, changes occur in the frequency, width, and height of the water band. When strong solvent bands overlap those of the solute (as in the case of the HOH bending mode of water and the protein amide I band) residual features of the intense solvent bands often remain after subtraction and may distort the weaker solute bands of interest.

Koenig and Tabb have tabulated a number of

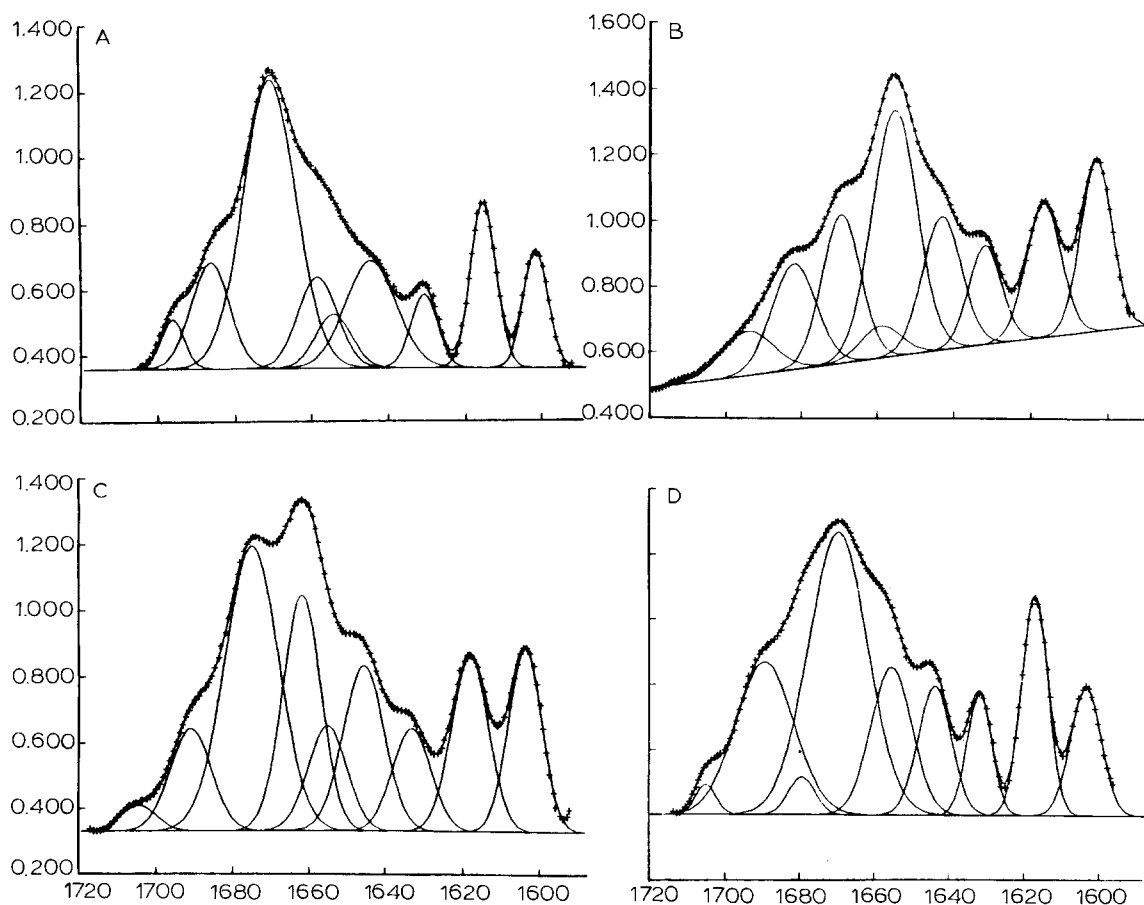


Fig. 3. Deconvolved Raman amide I bands of four lyophilized proteins (undeuterated) (+ + + + +). Individual Gaussian components determined by iterative curve-fitting and their sum are given by the solid curves (—). Relative intensity in counts/s is plotted versus Raman shift in wavenumber ( $\text{cm}^{-1}$ ). (A) immunoglobulin G; (B) serum albumin (bovine); (C) carbonic anhydrase; (D) ribonuclease S. (From Ref. 26.)

observed IR frequencies for selected proteins in  $\text{H}_2\text{O}$  solution [9]. Some of their results strongly indicate considerable distortion of the observed protein bands by residual water bands. For example, the amide I band of ribonuclease A is reported to absorb at 1646 and 1656  $\text{cm}^{-1}$ . This protein is known to contain a high proportion of  $\beta$ -structure and should exhibit a strong amide I component closer to 1630–1640  $\text{cm}^{-1}$  [3]. Recent studies [7,16] suggest that a better value for the amide I band of ribonuclease A in  $\text{H}_2\text{O}$  solution is 1642 or 1643  $\text{cm}^{-1}$ , with an obvious shoulder near 1655  $\text{cm}^{-1}$ . Therefore, one can conclude that quantitative conformation studies are more easily and accurately accomplished with  $\text{D}_2\text{O}$  solutions even if FTIR in-

strumentation is used (instead of the older dispersive instruments). The same appears to be true for Raman spectra. Although the disturbing HOH bending mode is weak in the Raman effect, difficulties remain because in the Raman effect the amide I band is also weak and subtraction without distortion may again be very difficult [30].

If  $\text{D}_2\text{O}$  is used as a solvent instead of  $\text{H}_2\text{O}$ , one must ensure, if possible, that complete H,D exchange has taken place in the backbone amide groups. This is most easily accomplished by following the decrease in the intensity of the amide II band near 1550  $\text{cm}^{-1}$  as progressive deuteration shifts it to lower frequencies (ca. 1450  $\text{cm}^{-1}$ ) [27,28]. The amide I band also shifts upon deuter-

ation, but by a considerably smaller amount, usually by less than  $10 \text{ cm}^{-1}$ . Although questions sometimes arise regarding the frequencies of amide I components for proteins which have not undergone complete deuteration [7,16], following the described procedure (i.e., determining the extent of deuteration by the shift of the amide II band) leads to consistent quantitative results in any case. It should be added that attempts to achieve complete deuteration by heating the protein solution can run the risk of irreversible denaturation, because most proteins begin to unfold around  $50\text{--}60^\circ\text{C}$ .

#### APPLICATION OF SECOND DERIVATIVE FTIR SPECTROSCOPY

Fig. 4 shows a single isolated IR band, and its first and second derivatives. For an ideal Lorentzian band, the width of the second derivative is  $1/2.7$  of the width of the original band. The second derivative band center is unchanged in frequency, but the peak is now negative with two small positive side lobes.  $A''$ , the peak intensity of the second derivative band (in intensity units/ $\text{cm}^2$ ) is proportional to  $-A/2\sigma^2$ , where  $A$  is the peak height and  $\sigma$  is the half width at half height of the original band. The relative intensities of second derivative bands can thus be markedly different from those of the original peaks. In particular, narrow features in the original, even if weak, exhibit sharply increased intensities in the second derivative, relative to the peak intensities of second derivatives of broad bands. The original spectrum must therefore have

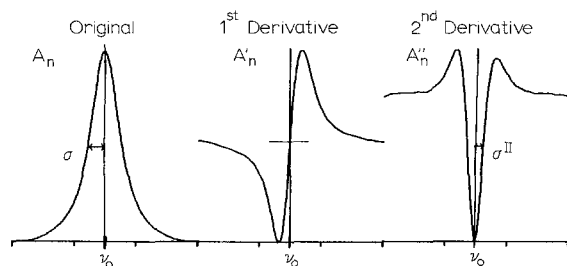


Fig. 4. Original, first derivative, and second derivative of an isolated empirical IR band ( $469 \text{ cm}^{-1}$  band of crystalline phenylalanine). (Maximum peak intensities were arbitrarily set equal.)

a high S/N ratio, otherwise the noise in the second derivative is amplified disproportionately and is likely to overshadow all but the strongest true absorption peaks.

The net effect of taking the second derivative of a broad band comprised of a number of overlapping peaks is to resolve the broad band into many of its original components. (See Figs. 1 and 5). Because the second derivative is accompanied by side-lobes, quantitative measurements of the intensities of individual components are very difficult. Second derivative spectra, nevertheless, provide an exceedingly useful 'fingerprint' of individual proteins and an effective qualitative means for following subtle changes in their secondary structure.

Fig. 5 provides an example of the usefulness of the technique. Fig. 5A shows the second derivative spectrum of hemoglobin from  $1800$  to  $1350 \text{ cm}^{-1}$ . Fig. 5B shows the same region for native  $\beta$ -lacto-

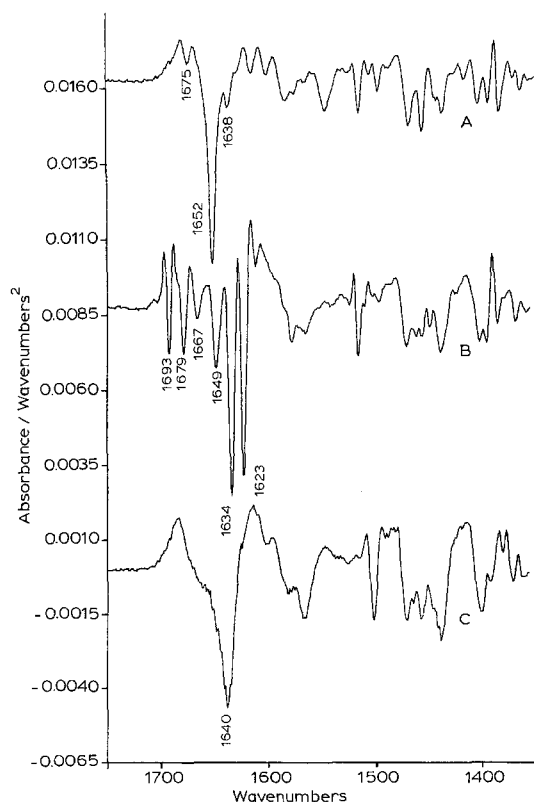


Fig. 5. Second derivative FTIR spectra of native hemoglobin (A), native  $\beta$ -lactoglobulin (B), and alkaline-denatured  $\beta$ -lactoglobulin (C). (5% solutions (w/v) in  $\text{D}_2\text{O}$ .) (From Ref. 23.)

globulin A and Fig. 5C, for alkaline-denatured  $\beta$ -lactoglobulin A. (All spectra are of 5% protein (w/v) dissolved in  $D_2O$ .) Hemoglobin, whose conformation is nearly 80%  $\alpha$ -helical, exhibits only one strong band at  $1652\text{ cm}^{-1}$  in the amide I region ( $1700\text{--}1620\text{ cm}^{-1}$ ). This band most likely results from vibrations of the helical coils of the proteins. X-ray studies show that  $\beta$ -lactoglobulin A (Fig. 5B)

has 47%  $\beta$ -structure but only about 7%  $\alpha$ -helix [17]. The two strong bands at  $1623$  and  $1634\text{ cm}^{-1}$  and the weak band close to  $1679\text{ cm}^{-1}$  are due to the  $\beta$ -strands [3]. The other features can also be assigned on the basis of earlier empirical and theoretical studies [3,24,28]; the peak at  $1649\text{ cm}^{-1}$  is probably an unresolved doublet resulting from a weak  $\alpha$ -helical band above  $1650\text{ cm}^{-1}$  and a band

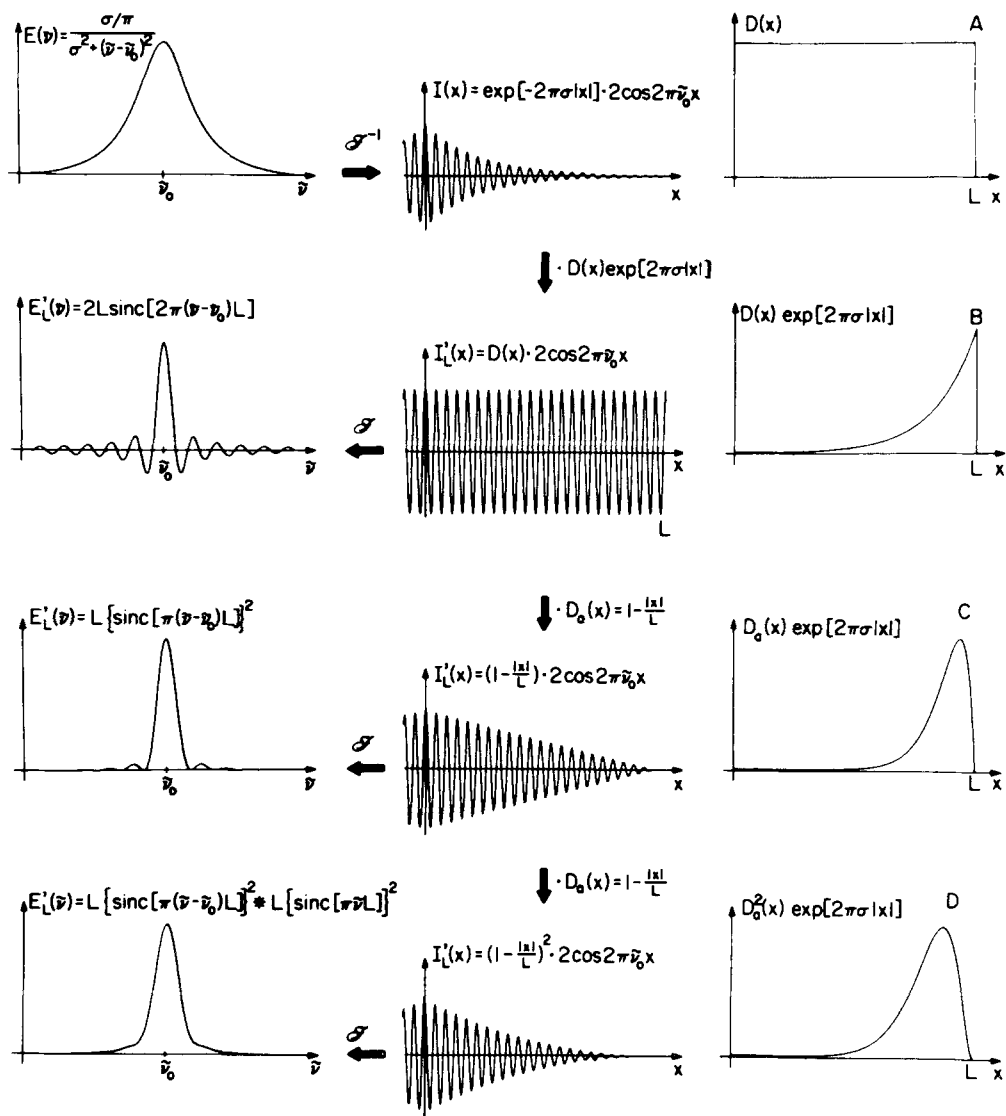


Fig. 6. Illustration of Fourier deconvolution and the effects of alternative apodization functions. (A) (left to right) Original band, Fourier transform, and 'boxcar' apodization; (B, C, D) deconvolution involving alternative apodization functions. B represents maximum band narrowing with the introduction of side-lobes; D represents the best compromise for the given set of conditions between optimal narrowing and the minimization of side lobes; C gives an intermediate case. (See Kauppinen et al. (Ref. 22) for more details.

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below  $1648\text{ cm}^{-1}$  due to aperiodic or 'random' segments. Bands at  $1667$  and  $1693\text{ cm}^{-1}$  are characteristic of different types of turns [3].

All fine structure in the amide I region ( $1700$ – $1620\text{ cm}^{-1}$ ) gradually disappears (Fig. 5C) when the pD of the  $\beta$ -lactoglobulin solution is raised to 13. When held for some hours under such conditions, the protein unfolds and is denatured [27,28]. Only one strong, broad band at  $1640\text{ cm}^{-1}$  (with a shoulder at ca.  $1665\text{ cm}^{-1}$ ) remains.

Note that the bands due to the amino acid side chains (which absorb mainly in the region between  $1600$  and  $1350\text{ cm}^{-1}$ ) are, as might be expected, much less affected by denaturation (compare Fig. 5B and C). One significant change is the decrease in frequency of the  $1515\text{ cm}^{-1}$  band associated with the ring stretching vibrations of tyrosine residues [3]. It is observed at  $1515\text{ cm}^{-1}$  in the native protein (Fig. 5B) and at  $1502\text{ cm}^{-1}$  at pD 13. This frequency shift is associated with the ionization of the hydroxyl group on the aromatic ring. The observations on the amide I band of the denatured protein clearly demonstrate that the fine structure observed in the second derivative of the amide I band of the native sample is real, and not simply noise or some artifact produced by the resolution enhancement process.

#### FOURIER DECONVOLUTION AND BAND FITTING OF FTIR SPECTRA

Fig. 1B shows the Fourier deconvolved FTIR spectrum of the protein triose phosphate isomerase, as compared with the original spectrum, given in Fig. 1A. In principle, deconvolution of a single band proceeds as indicated in Fig. 6. Fourier transformation of a single band yields a sine wave whose period is identical with that of the interferogram originally measured. When the original band has a Lorentzian or Gaussian shape the envelope of the sine wave is a slowly decaying exponential function. If this interferogram (sine wave) is multiplied by one or more appropriate functions (Fig. 6B–D) the rate of decrease in the sine wave envelope will be reduced. After Fourier transformation back into

the 'spectral domain', the width of the band will also have decreased. The procedure is more elaborate than can be easily summarized here; more detailed explanations of the theory and applications of deconvolution can be found elsewhere [3,8,24].

If several overlapping bands of ill-defined shape and width are involved, the procedure becomes much more complex and, to a degree, resembles an art more than an exact science [14]. The net effect (at least in favorable cases) is to resolve a broad, composite, band into its original components. This is illustrated for the amide I band of several proteins in Fig. 2. The areas of the resolved components can be fairly well estimated by curve fitting with Gaussian components. The components can be assigned to particular types of secondary structure by empirical comparison with spectra arising from proteins known to contain only one major component (e.g., the  $\alpha$ -helix of hemoglobin) [3,24] or with the help of theoretical calculations [10]. Table 1 lists such assignments, based on the study

Table 1

Characteristic IR frequencies and assignments for amide I band components ( $1700$ – $1620\text{ cm}^{-1}$ ) for 19 globular proteins in  $\text{D}_2\text{O}$  solution<sup>a</sup>

Mean frequency ( $\text{cm}^{-1}$ )	Assignment
$1623 \pm 3^b$	$\beta$ -Structure
$1630 \pm 4$	$\beta$ -Structure
$1637 \pm 3$	$\beta$ -Structure
$1645 \pm 4$	Unordered (aperiodic)
$1653 \pm 4$	$\alpha$ -Helix
$1663 \pm 4$	Turns
$1670 \pm 2$	Turns
$1675 \pm 5$	$\beta$ -Structure
$1683 \pm 2$	Turns
$1689 \pm 2$	Turns
$1694 \pm 2$	Turns

<sup>a</sup> Data for 17 proteins (carbonic anhydrase, carboxypeptidase, casein,  $\alpha$ -chymotrypsin, chymotrypsinogen, concanavalin A, elastase, immunoglobulin G,  $\alpha$ -lactalbumin,  $\beta$ -lactoglobulin A, lysozyme (hen egg white), papain, ribonuclease A, ribonuclease S, trypsin, trypsinogen, trypsin inhibitor (soybean)) are taken from Ref. 3; data for flavodoxin and triose phosphate isomerase are from Ref. 25.

<sup>b</sup> Maximum range of the observed frequency for each component.



Table 2

Estimated protein conformation by three independent methods<sup>a</sup>

Protein	% $\alpha$ -Helix			% $\beta$ -Structure			% Other <sup>b</sup>		
	R	FTIR	X-ray	R	FTIR	X-ray	R	FTIR	X-ray
Bovine serum albumin	39	47	–	32	28	–	29	25	–
Carbonic anhydrase	11	13	16	51	49	45	38	38	39
Immunoglobulin G	8	9	3	67	76	67	27	15	30
$\alpha$ -Lactalbumin	31	33	–	36	41	–	33	26	–
$\beta$ -Lactoglobulin A	10	10	7	54	50	47	36	40	46
Lysozyme	43	41	45	25	21	19	32	38	36
Ribonuclease A	21	21	22	50	50	46	31	29	32
Cytochrome <i>c</i>	–	51	49	–	34	10	–	15	41

<sup>a</sup> Raman (R), Ref. 26; FTIR, Ref. 3 (except for bovine serum albumin, this work); X-ray data, Ref. 12 (except for  $\beta$ -lactoglobulin A, Ref. 17).

<sup>b</sup> Includes turns and undefined or aperiodic segments.

of 19 proteins with known secondary structures [3,25]. The relative areas of the components provide a good indication of what percentage of peptide chain is folded into a given substructure in a given protein. When more than one band is associated with one type of substructure, the sum of the areas of all such bands must be used for this purpose. The procedure is essentially based on empirical observations but, nevertheless, yields quite acceptable results [3,24]. Table 2 gives some representative data. More are found in Ref. 3. In most cases the estimates for a particular structural component appear to be at least as good as those deduced from circular dichroism (CD) data (the prevailing method for such investigations), and in some cases better. IR methods have the additional advantage over other procedures in that practically any protein sample can be examined, regardless of turbidity, fluorescence and/or other disturbing factors.

If deconvolution of complex samples is carried out by the described procedure, extreme care must be exercised to avoid 'overdeconvolution', i.e., the use of too high values for the 'deconvolution parameters'. Two are needed: an assumed width of the original bands (full width at half height, FWHH) and a 'resolution-enhancement factor' *K*. Values of 13 cm<sup>-1</sup> and 2.4, respectively, have led to good results in this laboratory and elsewhere [3,32].

#### FOURIER DECONVOLUTION AND BAND FITTING OF RAMAN SPECTRA

Fig. 3 gives the deconvolved and curve-fitted Raman spectra of four proteins. The mathematical procedure and interpretation is analogous to the treatment of deconvolved FTIR spectra, as described above. A few pertinent facts must be kept

Table 3

Characteristic Raman frequencies and assignments for amide I band components (1700–1620 cm<sup>-1</sup>) for ten lyophilized globular proteins<sup>a</sup>

Mean frequency (cm <sup>-1</sup> )	Assignment
1632 ± 1 <sup>b</sup>	$\beta$ -Structure
1645 ± 3	Bound H <sub>2</sub> O
1656 ± 3	$\alpha$ -Helix
1661 ± 2	Undefined and/or turns
1672 ± 4	$\beta$ -Structure
1683 ± 3	Turns
1689 ± 2	Turns
1694 ± 3	Turns

<sup>a</sup> Data for carbonic anhydrase,  $\alpha$ -chymotrypsin, immunoglobulin G, insulin,  $\alpha$ -lactalbumin,  $\beta$ -lactoglobulin A, lysozyme (hen egg white), ribonuclease A, ribonuclease S, and serum albumin (bovine) from Ref. 26; all proteins are undeuterated.

<sup>b</sup> Maximum range of the observed frequency for each component.

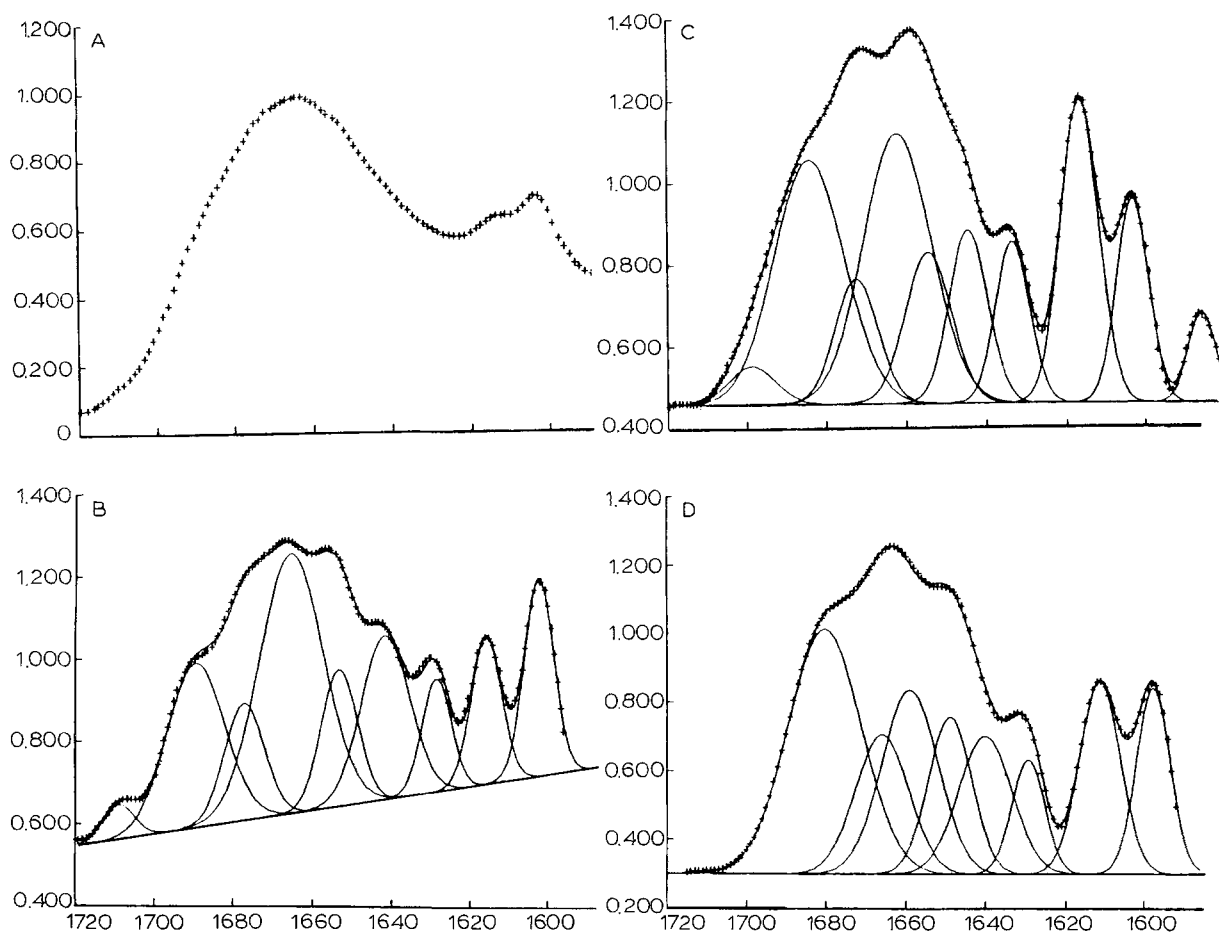


Fig. 7. Amide I Raman bands of lyophilized caseins. Relative intensity in counts/s is plotted versus Raman shift in wavenumber ( $\text{cm}^{-1}$ ). (A) Original spectrum of  $\beta$ -casein; (B) spectrum of  $\beta$ -casein, but now deconvoluted and curve-fitted; (C) deconvoluted and curve-fitted spectrum of  $\alpha_{s1}$ -casein; (D) deconvoluted and curve-fitted spectrum of whole bovine casein mixture ( $\text{Ca}^{2+}$  replaced by  $\text{K}^+$ ). (Note: the frequency scale in (D) is ca.  $+10 \text{ cm}^{-1}$  too high.) The two bands near  $1605$  and  $1615 \text{ cm}^{-1}$  result from aromatic side chains and not from peptide backbone vibrations. (From Ref. 2.)

in mind. First of all, the S/N ratio of Raman spectra is much lower (usually by a factor of at least ten) than the corresponding value of FTIR spectra. Resolution-enhancement techniques must therefore be applied more carefully and generally must be preceded by a mathematical smoothing of the data [26]. Secondly, high-quality Raman spectra are more easily obtained for proteins in the solid state than in solution; the opposite is true for IR spectra. The Raman data presented here are thus measured for proteins in the solid state. Finally, because of the change of state, shifts in frequency due to deuteration, and different effective selection rules [26],

assignments are not identical for the two spectroscopic techniques, i.e., the assignments for solid state Raman samples differ somewhat from the assignments listed in Table 1 for IR spectra of proteins in  $\text{D}_2\text{O}$  solution. Raman assignments based on a study of ten globular proteins [26] are reported in Table 3. Table 2 includes Raman results regarding quantitative estimations, calculated by a procedure quite analogous to the one described above for IR studies. Examination of the results presented in Table 2 reveals good agreement between the two spectral methods and published X-ray data.

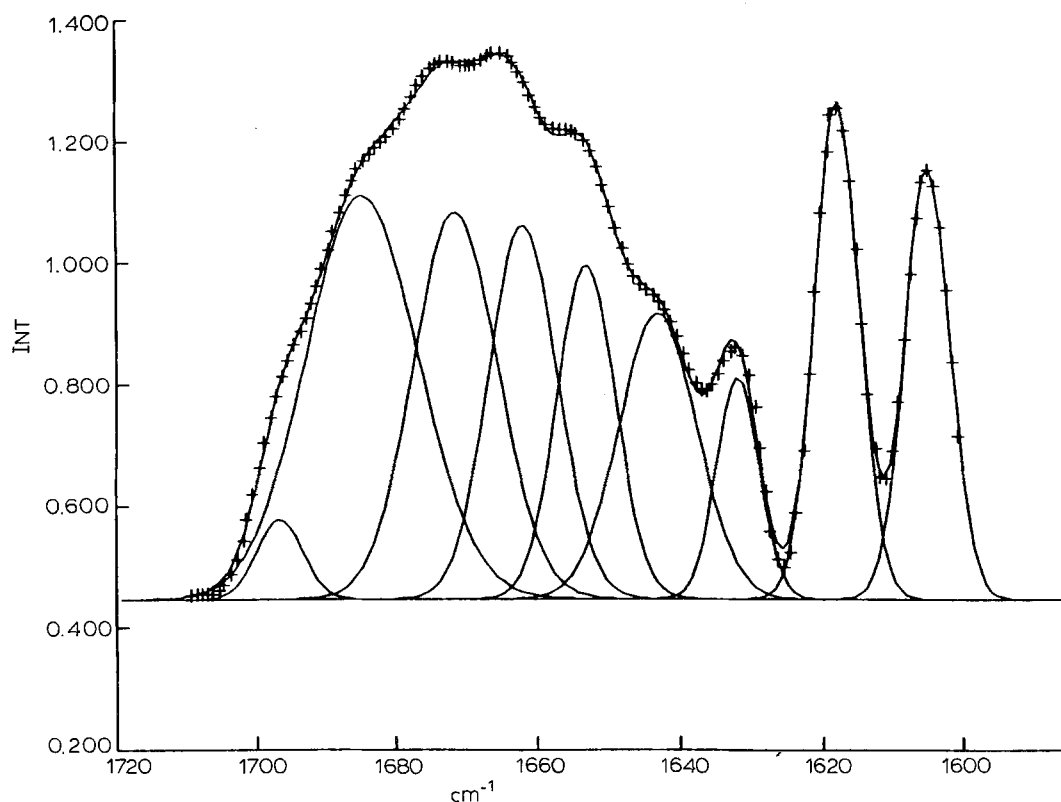


Fig. 8. Deconvolved and curve-fitted Raman spectrum of lyophilized whole bovine casein micelles containing typical amounts of  $\text{Ca}^{2+}$  (2.8% by weight). (Compare with the whole casein without  $\text{Ca}^{2+}$  in Fig. 7D.) The two low frequency bands result from side-chain vibrations, as in Fig. 7. (From Ref. 26.)

## RAMAN STUDIES OF CASEINS AND CASEIN MICELLES

Caseins represent a family of phosphoproteins which occur in skin milk as colloidal calcium phosphate complexes termed casein micelles [2,5]. The individual caseins are frequently regarded as largely 'structureless' proteins [5,27], but the deconvolved solid state Raman spectra of isolated  $\alpha_{s1}$ -casein and  $\beta$ -casein, as well as the spectra of lyophilized whole casein submicelles (no  $\text{Ca}^{2+}$ ) and micelles (with  $\text{Ca}^{2+}$ ), show some distinct fine structure. (By weight, whole bovine casein is about 40%  $\alpha_{s1}$ -, 40%  $\beta$ -, 10%  $\alpha_{s2}$ -, and 10%  $\kappa$ -casein.) Fig. 7 presents (A) the original amide I Raman band of  $\beta$ -casein, (B) the corresponding deconvolved spectrum of  $\beta$ -casein, (C and D) the deconvolved spectrum of  $\alpha_{s1}$ -casein and of whole casein submicelles ( $\text{K}^+$  only),

respectively. Fig. 8 represents the corresponding spectrum of freeze-dried casein micelles in the presence of  $\text{Ca}^{2+}$  ions. There appears to be about 10%  $\alpha$ -helical structure and approximately 20%  $\beta$ -structure in the two isolated caseins, but the fine structure of the spectrum of the two species is different, suggesting that their conformations are not identical [2]. The most interesting observations are concerned with the protein sections usually called 'random' or 'structureless'. These are clearly divided spectroscopically into two categories, one giving rise to bands in the  $1660 \text{ cm}^{-1}$  region, the other one in the region above ca.  $1680 \text{ cm}^{-1}$ . We tentatively call the first category 'undefined' or 'aperiodic' and the second one 'turns', in analogy with other proteins studied by this Raman method [26]. It is also interesting to observe that whole casein micelles in the presence of  $\text{Ca}^{2+}$  ions and submicelles

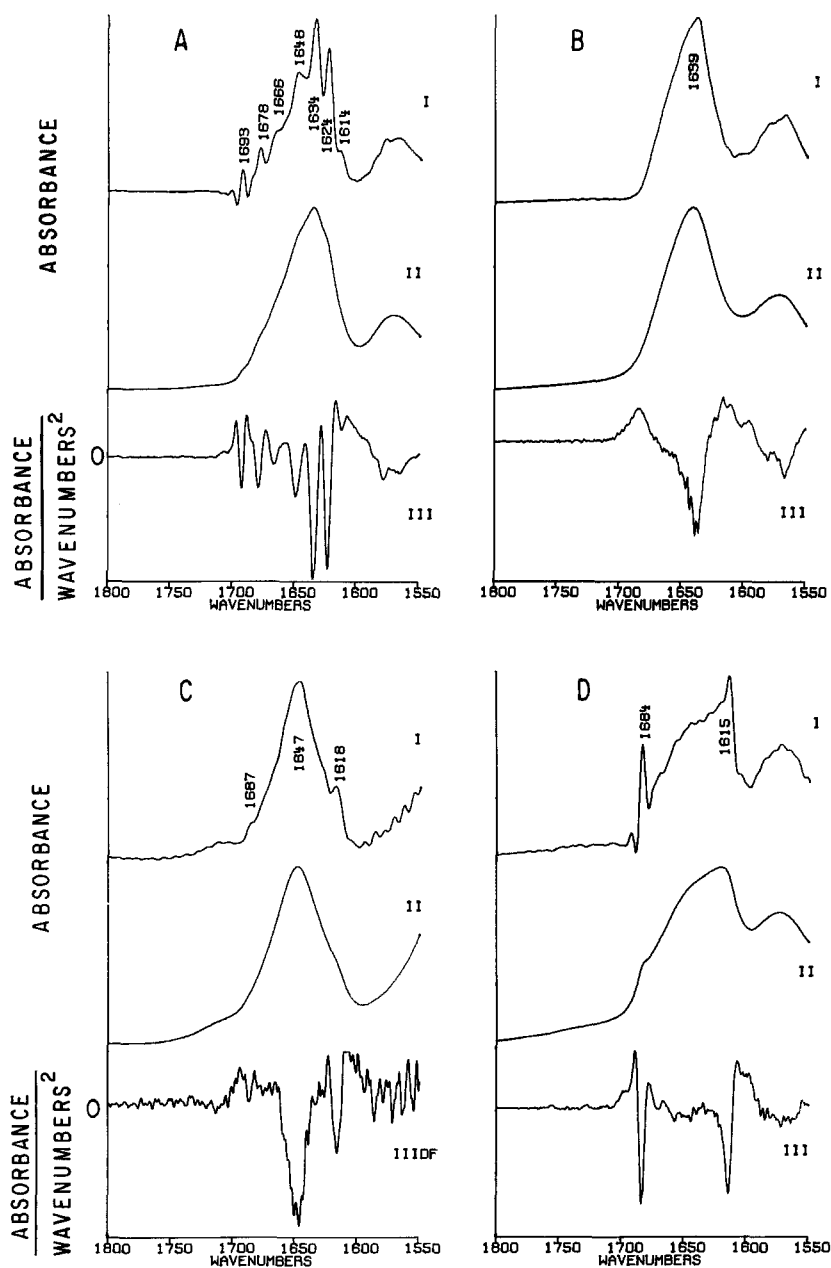


Fig. 9. FTIR spectra of  $\beta$ -lactoglobulin A in various solvents. (I denotes deconvoluted spectrum; II denotes original spectrum; III denotes second derivative of the original spectrum.) (A) native protein (5% w/v in  $D_2O$ , pH 7); (B) denatured protein (5% w/v in  $D_2O$ , pH 13); (C) denatured protein in 60%  $CH_3OD/40\%$   $D_2O$ , pH 4; (D) denatured protein in 40% isopropanol- $d/60\%$   $D_2O$ , pH 7. (From Ref. 20.)

in the presence of  $K^+$  ions appear to have more turns and  $\beta$ -structure compared to the isolated  $\alpha_s1$ - and  $\beta$ -caseins. The increase is at the expense of the amount of 'undefined' structure [2,26]. Because these caseins have never been crystallized and seem

to have relatively little periodic structure [5], no direct comparison with X-ray data of any homologous protein is possible. Thus, all conformational designations are more qualitative and have a more descriptive meaning for these proteins than for

other globular proteins yet studied by FTIR or Raman spectroscopy.

## SOLVENT DENATURATION OF PROTEINS AS STUDIED BY FTIR

Resolution-enhanced FTIR spectra of proteins in deuterated solvents provide a good method for studying the effect of different solvents on the structure of proteins. Denaturation can be defined as any departure from native conformation. This term, therefore, does not necessarily imply complete unfolding into a totally aperiodic or random coil. Instead, new types of secondary structures can even be introduced, some of which are different from any secondary structure observed in native proteins. The milk protein,  $\beta$ -lactoglobulin, furnishes a good example. The native form has a very high proportion of  $\beta$ -structure [3,17]; in methanol solution the protein appears to assume an essentially helical conformation [28]; in isopropanol solution [20] a new kind of extended structure, characterized by bands around 1685 and 1615  $\text{cm}^{-1}$ , is observed. In aqueous solution at high pH (above ca. 13) complete unfolding appears to take place, resulting in a spectrum similar to that for caseins. Figure 9 presents the amide I region of these four structurally different forms of  $\beta$ -lactoglobulin. To our knowledge no other spectroscopic technique has been used to study the effect of solvents on protein conformation in equivalent detail [20].

## PROTEIN-METAL ION INTERACTIONS

A comparison of Figs. 7 and 8 already showed that  $\text{Ca}^{2+}$  ions have but a limited effect on the amide I band of the Raman spectrum of whole casein. Casein, however, is unusual in that it forms micelles in the presence of calcium ions while, at the same time, retaining a very high proportion of 'undefined' structure. The interaction of trypsin with calcium ions presents distinctly different behavior. Fig. 10 gives the second derivative FTIR spectrum of trypsin (5% w/v in  $\text{D}_2\text{O}$  with 0.01 M NaCl) in

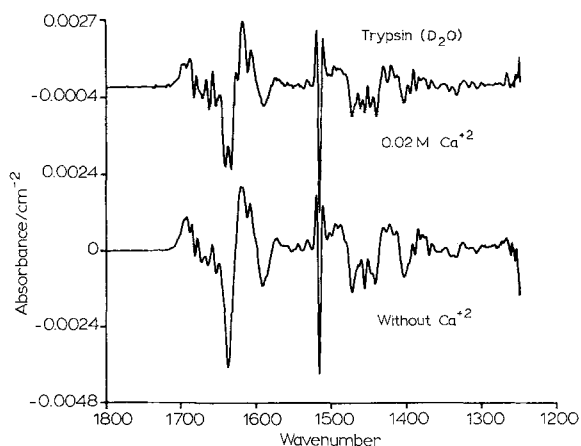


Fig. 10. Effect of  $\text{Ca}^{2+}$  on the second derivative spectrum of trypsin (5% w/v in  $\text{D}_2\text{O}$ ). Upper curve: enzyme in the presence of 0.02 M  $\text{Ca}^{2+}$ . Lower curve: enzyme with no  $\text{Ca}^{2+}$  present.

the presence of 0.02 M  $\text{Ca}^{2+}$  (upper curve) and with the  $\text{Ca}^{2+}$  removed by dialysis (lower curve). (Addition of  $\text{Mn}^{2+}$  or  $\text{Gd}^{3+}$  (also at 0.02 M) results in spectra nearly identical with that observed when  $\text{Ca}^{2+}$  is present.) Fig. 11 provides analogous information concerning the deconvolved FTIR spectra. The process appears to be reversible, i.e., if calcium ions are added to the calcium-free sample, the spectrum of the complex is again obtained. The total amount of  $\alpha$ -helix appears to remain about the same, but there is a noticeable decrease in  $\beta$ -strands and a comparable increase in 'aperiodic' structure when calcium is present, compared to the metal-free protein. This observation is in good agreement with the notion [11] that the calcium ions are embedded in a  $\beta$ -structure region of the protein which loses its regularity when the metal ions are present.

Resolution-enhanced FTIR spectroscopy thus constitutes a sensitive probe concerning conformational changes of proteins caused by protein-metal ion interaction. Another interesting example is provided by the interaction of the protein ferritin with  $\text{Fe}^{3+}$  ions. The protein is overwhelmingly in the  $\alpha$ -helical conformation in both the holo [3] and apo (D.M. Byler, unpublished data) forms, but removal of the ferric ions results in a marked narrowing of the characteristic  $\alpha$ -helix band centered close to 1650  $\text{cm}^{-1}$ . It appears that in the more highly associated halo form interactions with ferric ions

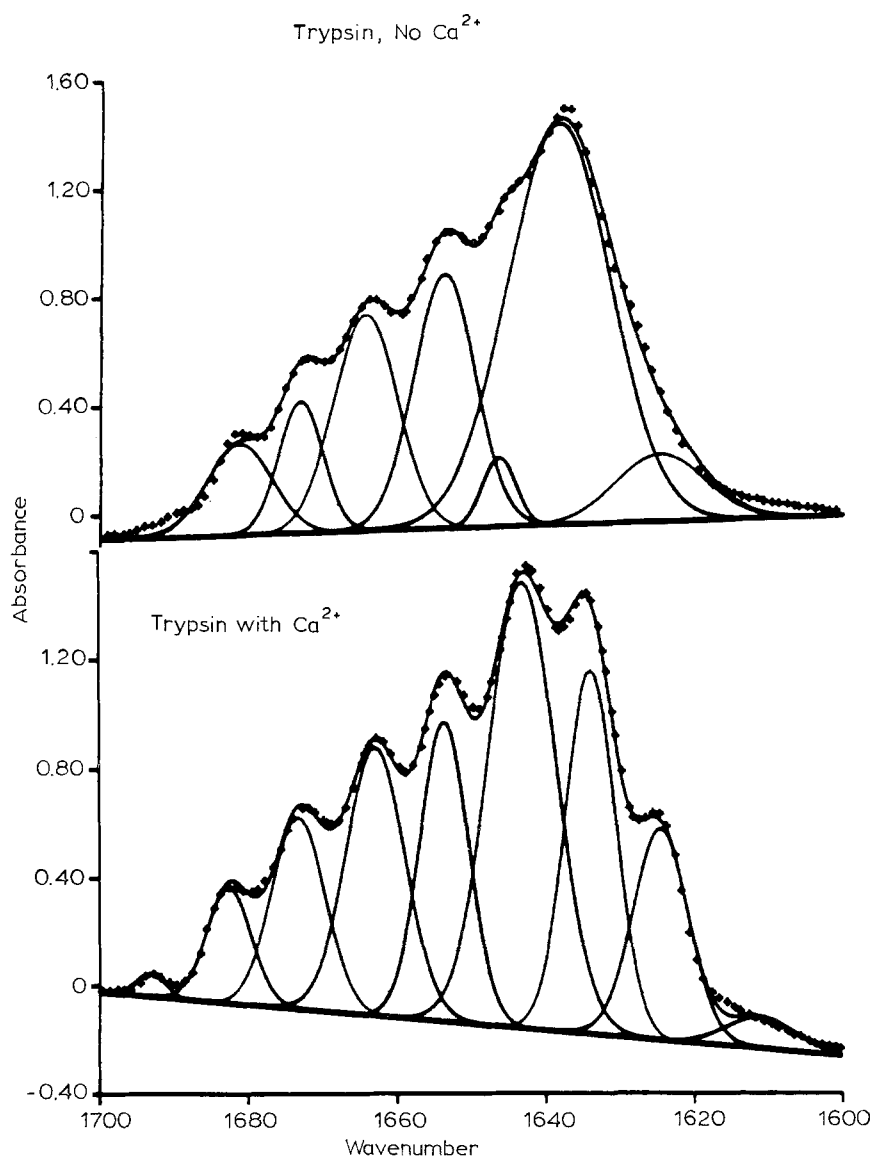


Fig. 11. Effect of  $\text{Ca}^{2+}$  on the deconvoluted and curve-fitted FTIR spectrum of trypsin (5% w/v in  $\text{D}_2\text{O}$ ). Upper curve: calcium-free trypsin. Lower curve: trypsin with 0.02 M  $\text{Ca}^{2+}$ .

result either in a loosening of the helical structure or in increased protein-protein interactions. In turn, one or both of these changes apparently induces a broadening of the characteristic helix band.

## CONCLUSION

In summary, resolution-enhanced vibrational (IR and Raman) spectroscopy is becoming an in-

dispensable tool for the study of the architecture of proteins and enzymes. Not only can this technique give qualitative information sometimes heretofore unavailable, but in many cases, when combined with the judicious use of iterative curve-fitting procedures, such spectra can provide a quantitative measure of the secondary structure present in a protein. Such spectra have proved invaluable for investigations of the conformation of proteins in so-

lutions, in gels and colloids, and as lyophilized solids, physical states for which X-ray diffraction is of but limited utility. In the case of gels, colloids, and solids, IR and Raman can provide useful information under conditions where the scattering of short wavelengths of light badly hampers the use of CD or optical rotatory dispersion. Finally, vibrational spectroscopy offers a non-destructive means for probing structural changes that result from variations in pH, ionic strength, the nature of solvent, and from molecular and ionic interactions.

## ACKNOWLEDGEMENT

The authors thank Janine N. Brouillette for preparing samples of trypsin, with and without  $\text{Ca}^{2+}$ , and for measuring their resolution-enhanced FTIR spectra.

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