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The Use and Misuse of FTIR Spectroscopy in the Determination of Protein Structure

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Abbreviations: CD, circular dichroism; FTIR, Fourier transform infrared; NMR, nuclear magnetic resonance.

ABSTRACT: Fourier transform infrared (FTIR) spectroscopy is an established tool for the structural characterization of proteins. However, many potential pitfalls exist for the unwary investigator. In this review we critically assess the application of FTIR spectroscopy to the determination of protein structure by (1) outlining the principles underlying protein secondary structure determination by FTIR spectroscopy, (2) highlighting the situations in which FTIR spectroscopy should be considered the technique of choice, (3) discussing the manner in which experiments should be conducted to derive as much physiologically relevant information as possible, and (4) outlining current methods for the determination of secondary structure from infrared spectra of proteins.

KEY WORDS: infrared spectroscopy, proteins, secondary structure, quantitation.

I. INTRODUCTION

As the "second part of the genetic code" proteins play a pivotal role in living organisms, facilitating metabolism, communication, transport, and the maintenance of structural integrity. The diversity of functions of proteins is matched only by the diversity of protein structure, each protein being uniquely and exquisitely designed to fulfill its role. A true understanding of proteins can only be achieved by exploring the relationship that exists between the unique structure adopted by a protein and its function. To this end, a variety of techniques have been applied to the elucidation of the three-dimensional structure of proteins, ranging from prediction based on the sequence and physico-chemical properties of the constituent amino acids (Chou and Fasman, 1978; Fasman, 1989) to precise methods for the identification of atoms and the determination of their molecular coordinates, such as NMR spectroscopy (Braun, 1987) and X-ray diffraction (Wlodawer et al., 1982).

Fourier transform infrared (FTIR) spectroscopy has emerged as a useful tool for the characterization of protein secondary structure with a precision lying between that of the purely predictive and the molecular coordinate approaches (Susi and Byler, 1986; Byler and Susi, 1986; Surewicz and Mantsch, 1988a; Jackson et al., 1989a; Arrondo et al., 1993; Jackson and Mantsch, 1993). An explosive growth in both the applications of FTIR spectroscopy and the number of laboratories that utilize the technique has occurred over the last 5 to 10 years, in part fueled by significant advances in instrumentation, methodology, and the relatively low cost of the required instrumentation, but also due in no small part to the apparent success associated with recent applications.

However, there are many potential pitfalls for the unwary investigator that are frequently manifested in the literature. As with advances and growth in the application of all instrumental methods, a point is reached where it becomes necessary to pause and evaluate the status of the technique, the significance of the applications, and to ask what the future holds. In this review we critically assess the application of FTIR spectroscopy to the characterization of protein secondary structure. Rather than attempt to give an exhaustive

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review of the infrared literature, we stress examples that illustrate general principles. Where appropriate, review articles are cited in which the interested reader may find more detailed information.

II. DETERMINATION OF PROTEIN STRUCTURE FROM FTIR SPECTRA — GENERAL PRINCIPLES

That IR spectroscopy could provide information on the secondary structure of proteins was first demonstrated by Elliot and Ambrose (1950), Ambrose and Elliot (1951), and Elliot (1954), who showed that an empirical correlation existed between the frequency of the so-called amide I and amide II absorptions of a protein and the predominant secondary structural motif within the protein as determined by X-ray diffraction studies. These studies demonstrated that proteins that were known to be predominantly α-helical in structure exhibited amide I and II absorptions in the spectral range 1652 to 1657 and 1545 to 1551 cm^{-1}, respectively, in aqueous solution, while proteins with a predominantly β-sheet structure exhibited similar absorptions at 1628 to 1635 and 1521 to 1525 cm^{-1}. These initial observations were later extended to proteins in ^2H_2O solution (Susi et al., 1967; Timasheff et al., 1967). That such an empirical correlation exists is not surprising given the nature of the amide I and II modes. According to the descriptions of amide modes derived by Miyazawa and co-workers in the 1950s and 1960s (Miyazawa et al., 1956; Miyazawa et al., 1958; Miyazawa, 1960, 1961, 1962) and Krimm and colleagues (Jakes and Krimm, 1971a,b; Abe and Krimm, 1972a,b; Krimm and Bandekar, 1986), the amide I absorption contains contributions from the C=O stretching vibration of the amide group (about 80%) with a minor contribution from the C–N stretching vibration, while the amide II absorption appears to be significantly less "pure", arising from N–H bending (60%) and C–N stretching (40%) vibrations. Based on these descriptions, the exact frequency of the amide I and II absorptions would be predicted to be influenced by the strength of any hydrogen bonds involving amide C=O and N–H groups. In proteins, each of the amide groups is involved in a secondary structure of some type: either a helix, an extended sheet, or one of the aperiodic secondary structures. Because each of these secondary structural motifs is associated with a characteristic hydrogen bonding pattern between amide C=O and N–H groups, it is to be expected that each type of secondary structure will give rise to characteristic amide I and II absorptions. It is this separation of amide absorptions that underlies the determination of protein secondary structure by IR spectroscopy.

Empirically, most workers have found the amide I absorption to be more useful for protein secondary structure determination than the amide II absorption, probably due to the fact that it effectively arises from only one of the amide functional groups, in contrast to the amide II mode.

It has been suggested that protein secondary structure may also be determined based on analysis of the amide III absorption (Kaiden et al., 1981; Anderle and Mendelsohn, 1987; Singh et al., 1991; Singh et al., 1993). The amide III absorption is generally described as arising predominantly from C–N stretching vibrations coupled to N–H in-plane bending vibrations, with weak contributions from C–C stretching and C=O in-plane bending (Bandekar, 1992). In addition, there is often significant mixing with the CH_2 wagging vibrations of side chains. Therefore, the amide III appears to be a less well-defined vibrational mode, with contributions from the various vibrations involved varying between proteins (Krimm and Bandekar 1986). Thus, characterization of protein structure using the amide III presents problems in that the exact nature of the information obtained and its relation to the secondary structure of proteins is not fully understood (Hsu et al., 1976; Lord, 1977).

In addition to problems associated with the nature of the amide III vibration, problems arise when using the amide III absorption for characterization of protein secondary structures due to the fact that the amide III absorption is found in a spectral region associated with a number of other absorptions, including those arising from CH_2 wagging vibrations. Failure to account for these vibrations will lead to incorrect assignment of absorptions.

A more rigorous approach to the assignment of amide absorptions to discrete secondary structures than the purely empirical approach has been pioneered by Krimm and co-workers (reviewed in Krimm and Bandekar, 1986), who developed force-field treatments of amide vibrational modes in order to predict the IR absorption frequencies of protein secondary structures. While this approach has met with considerable success, it is, unfortunately, limited in its application at the present time due to the inability to account for the effect of dielectric constant and solvent interactions on amide absorptions. Nevertheless, this work provides a thorough theoretical explanation for many experimental observations and represents a valuable adjunct to the empirical approach.

We have recently suggested an approach that lies between the empirical and force-field treatment approaches, which explains the empirical IR correlations by correlation of dihedral angles, hydrogen bond strengths, C=O group electron density, and amide I frequencies for the various secondary structures (Jackson and Mantsch, 1991a). In short, the dihedral angles of a polypeptide chain determine the chain geometry, which dictates the length and direction of hydrogen bonds involving amide C=O and N–H groups. Variations in the length and direction of hydrogen bonds result in variations in the strength of the hydrogen bond for different secondary structures, which in turn produces characteristic electron densities in the amide C=O groups, resulting in characteristic amide I frequencies. The stronger the hydrogen bond involving the amide C=O, the lower the electron density in the C=O group and the lower the amide I absorption appears. Using this approach, one would predict the lowest amide I frequency to occur for extended polypeptide chains, such as those found in denatured proteins. In such extended chains, intramolecular hydrogen bonding is not possible. However, the extended nature of the chains allows very close alignment of neighboring chains, which favors the formation of extremely strong intermolecular hydrogen bonds. The formation of these very strong hydrogen bonds would be expected to produce a correspondingly low amide I maximum. This is indeed what is observed, an intense amide I feature being observed at 1610 to 1628 cm^{-1} for denatured, aggregated proteins (Clark et al., 1981; Byler and Purcell, 1989). If the dihedral angles are changed such that formation of intramolecular hydrogen bonds is possible with the polypeptide chains still in an extended state, the classic antiparallel β-sheet is formed. Steric constraints mean that the strength of the intramolecular hydrogen bonds stabilizing the β-sheet are not as strong as the intermolecular hydrogen bonds stabilizing the extended aggregate, and the amide I frequency is expected to be increased. Again, this is what is observed, the amide I maximum for proteins known to be predominantly antiparallel β-sheet in structure being observed at 1630 to 1640 cm^{-1}. For ϕ,ψ angles of -53°, -52°, the C=O group of residue n is positioned so as to be able to form a linear hydrogen bond with the N–H group of residue n + 4, forming the α-helix. The length of the hydrogen bond formed in the α-helix will be slightly longer (and so weaker) than that in an antiparallel β-sheet, and the resulting amide I frequency will be further increased. This is indeed the case; predominantly helical proteins such as myoglobin exhibit an amide I maximum at around 1648 to 1658 cm^{-1}. Protein amide C=O groups that are not involved in hydrogen bonds, either with solvent or with amide N–H groups, have been shown from studies of proteins and peptides in DMSO (which contains no hydrogen bond donor) to absorb at 1666 cm^{-1} (Jackson and Mantsch, 1991b). It should be stressed that this frequency may not represent the stretching vibration of a completely noninteracting amide C=O, as it is possible that weak dipole-dipole interactions occur between the amide C=O and solvent S=O groups. However, as such dipole-dipole interactions are weaker than hydrogen bonding interactions, any such interactions would be expected to produce only a small reduction, on the order of a few wavenumbers, in the frequency of the C=O stretching absorption.

If non-hydrogen-bonded amide C=O groups absorb at 1666 to 1670 cm^{-1}, the question immediately arises of the assignment of amide I features at frequencies greater than 1670 cm^{-1}, features that are consistently seen in all proteins. If the protein under investigation contains antiparallel β-sheet structures, then one of the absorptions above 1670 cm^{-1} can be assigned to a high-fre-

quency β-sheet component that arises from transition dipole coupling (Miyazawa, 1960; Moore and Krimm, 1975). The precise position of this absorption is difficult to determine, although it is usually found at a frequency 50 to 70 cm^{-1} higher than the main β-sheet component. Other absorptions are more difficult to assign, but we believe that they arise from sterically constrained non-hydrogen-bonded amide C=O groups within turns. The amide group is generally considered to be planar ($\omega = 180°$), due to resonance and tautomerism, which results in the C–N bond assuming partial double-bond character due to electron flow from the C=O group. Distortion of the amide planarity ($\omega = 180°$) in sterically constrained turn structures would result in flow of electrons back into the C=O group, causing an increase in frequency above that normally seen for free amide C=O groups. Thus, we assign absorptions above 1670 cm^{-1} not attributable to transition dipole coupling in antiparallel β-sheets to sterically constrained non-hydrogen-bonded amide C=O groups, such as those expected to occur in many types of turns. In addition, the dielectric constant of the medium may be expected to have some effect on the position of amide absorptions, due to variations in weak solvent-solute interactions (such as weak dipole-dipole interactions). High-frequency amide C=O absorptions may therefore also arise from non-hydrogen-bonded C=O groups in a low-dielectric constant environment (i.e., the protein interior).

Combining all three approaches, we may develop the correlations shown in Table I. It should be stressed that these correlations are guidelines only, and while they will prove to be adequate for IR spectroscopic studies on most proteins and peptides, there are proteins and peptides that contain secondary structures that absorb outside the frequency range given in the table. Examples include the helical configurations of poly-L-lysine (~1630 cm^{-1}; Jackson et al., 1989b) and bacteriorhodopsin (1662 cm^{-1}; Lee et al., 1985). Such discrepancies may result from an unusual degree of solvent-protein interaction, distortion of structural elements, or other factors. However, whatever the cause of such discrepancies, the infrared spectroscopist should not forget that these correlations can and should be superseded by common sense and information obtained from other sources (Wilder et al., 1992).

TABLE I
Correlations between Common Protein Structures and Amide I Frequency

Structure	Amide I frequency (cm^{-1})
antiparallel β-sheet/ aggregated strands	1675–1695
3_{10}-Helix	1660–1670
α-Helix	1648–1660
Unordered	1640–1648
β-Sheet	1625–1640
Aggregated strands	1610–1628

III. RESOLUTION ENHANCEMENT

For a real protein, the situation is much more complex than that discussed above, which assumes the presence of only one major structural motif. All proteins contain more than one secondary structural motif, and, consequently, give rise to more than one amide absorption. Unfortunately, the width and separation of these absorptions is such that they overlap and produce a composite, often featureless absorption profile. Some information may be obtained by an analysis of the frequency of the composite amide I maximum and any visible shoulders. However, changes in the position of such a composite absorption can prove deceptive. Such frequency shifts may be caused by true frequency shifts of component bands, or by variations in the relative intensities of component bands. For example, a shift to lower wavenumber of a composite absorption may indeed mean that one or more of the underlying absorptions has shifted to a lower frequency. Alternatively, such a shift may be produced by an increase in the intensity of a low-frequency component, resulting in a redistribution of intensity without any frequency shifts. Analysis of the composite band alone cannot distinguish between these two possibilities and could lead to misinterpretation of spectral shifts (Hübner et al., 1990). Deduction of structural parameters, and particularly changes in these parameters, from the relatively

featureless amide I band alone is therefore of limited use.

A number of mathematical techniques, such as Fourier self-deconvolution (FSD) and derivation, have been developed that allow visualization of overlapping bands following manipulation of the spectrum (Kauppinen et al., 1981a,b; Cameron and Moffatt, 1984; Griffiths and Pariente, 1986; Cameron and Moffatt, 1987; Mantsch and Moffatt, 1993). FSD is the most widely used of these mathematical methods and the general principles of the technique, plus possible pitfalls for the unwary, are discussed. For a more detailed discussion the interested reader is referred to the original articles (Kaupinnen et al., 1981a,b; Cameron and Moffatt, 1984, 1987).

Any IR absorption can be considered to arise from the convolution of a delta function that has position but no width and a Lorentzian that has width but no position to produce a Lorentzian with both position and width. In the Fourier domain, this is expressed as the multiplication of the Fourier transform of the delta function (a cosine, the period of which is the frequency of the delta function) with the Fourier transform of the Lorentzian (a decaying exponential, the rate of decay of which is determined by the width of the Lorentzian) to produce an exponentially decaying cosine. As we have said, the rate of decay of the cosine is determined by the width of the Lorentzian — the wider the Lorentzian, the more rapid the rate of decay. Or conversely, narrow bands are characterized by slowly decaying exponential cosines in the Fourier domain. In theory, therefore, it is possible to reduce the IR bands to delta functions that have no width but maintain their frequency characteristics by deconvolving the correct Lorentzian from the absorption profile (hence the term Fourier self-deconvolution, referring to the removal of the intrinsic shape of the absorption band). This is achieved by multiplying the Fourier transform of the absorption band (our exponentially decaying cosine) by the correct increasing exponential to regenerate the corresponding cosine. The inverse Fourier transform then gives the delta function.

Of course, in practice we never produce the delta function as we are usually dealing with many decaying cosines superimposed on each other, each of which is decaying at a different rate. Rather, what is attempted is to simply reduce the rate of decay of the underlying cosines by multiplication with an increasing exponential and so reduce the width of the corresponding absorptions. Unfortunately, the choice of the correct increasing exponential is subjective, and a poor choice of deconvolution parameters will produce poor results. If the deconvolution parameters are chosen such that the rate of increase of the exponential corresponds to a band width greater than the width of the absorptions being studied, the result will be sidelobes at the edges of the absorption bands, which can lead to problems with visualization of weaker neighboring bands. On the other hand, if the chosen band width is too small, the absorption bands will appear unaltered and no additional information will be gained.

In addition to the above caveats, it should be remembered that FSD also reduces the width of water vapor absorptions and enhances noise, producing very sharp peaks with even minimal deconvolution; noise and water vapor can very quickly become a problem in deconvolved spectra. Thus, FSD should only be performed on spectra with a high signal-to-noise ratio and a low contribution from water vapor.

The effect of water vapor on deconvolved spectra is shown in Figure 1. It can readily be seen that after correct application of FSD (Figure 1c) a number of bands are apparent that are not apparent in the original absorption spectrum (Figure 1a). For this reason FSD is often termed resolution enhancement. However, this terminology is misleading; resolution is an instrumental parameter that cannot be increased after a spectrum is recorded. Procedures such as FSD should therefore more correctly be referred to as band-narrowing procedures. Figure 1b demonstrates that a number of additional absorptions are introduced into the spectrum due to the presence of trace amounts of water vapor. Obviously, assignment of these features to amide modes will result in an incorrect structural determination. A spectrum may be judged to be water vapor-free if no sharp absorptions are seen between 1700 to 1800 cm^{-1}. Inclusion of at least part of this spectral region in illustrations will aid readers in determining the extent of possible water vapor contributions to

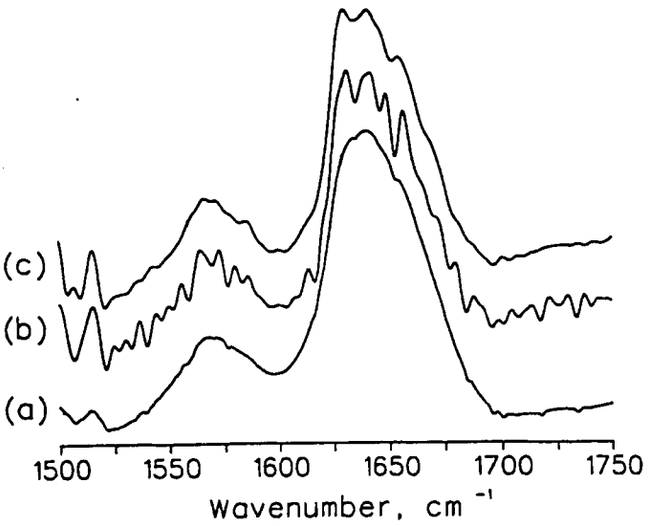

FIGURE 1. The effect of water vapor on deconvolved protein spectra: (a) original absorbance spectrum with inadequately compensated water vapor; (b) spectrum shown in (a) following deconvolution (k = 2.0, HW = 12); (c) same spectrum as in (a) with deconvolution performed after residual water vapor has been interactively subtracted.

deconvolved spectra, and for this reason should be considered good practice for feature-rich spectra.

The effect of noise on deconvolved spectra is illustrated in Figure 2. In this figure we present the region between 1000 and 1200 cm^{-1} of the spectrum of a thin film of mylar, a common polymer, as an illustrative example. However, the same discussion applies to protein spectra. In Figure 2a we present a deconvolved spectrum of mylar with a poor signal-to-noise ratio. Considerable fine structure is apparent in this spectrum. However,

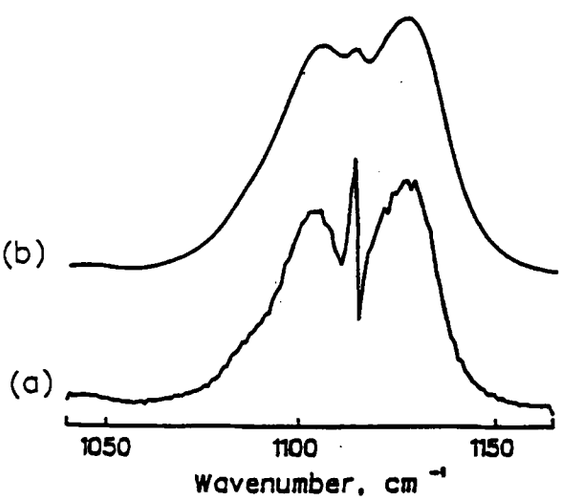

FIGURE 2. Effect of deconvolution on spectra with low (a) and high (b) signal-to-noise ratios (k = 3.25, HW = 6).

deconvolution of a high-quality spectrum (Figure 2b) showed these features to be irreproducible; in other words, a direct consequence of the noise present in Figure 2a.

In addition to FSD, calculation of spectral derivatives is often used to aid in the visualization of overlapping absorptions. The caveats that apply to FSD are also applicable to derivation. In addition, derivation suffers from the fact that relative integrated intensities are not maintained. Furthermore, significant edge effects are possible in derivative spectra (Moffatt and Mantsch, 1992). If the spectrum has a significant slope at the edges of the region to be subjected to derivation, distortions will be introduced at the edges of the derivative spectrum. This is easily avoided by choosing a wider spectral region than is desired for analysis and ignoring any features at the edges of this extended region. It can be seen that special care is needed in the interpretation of derivative spectra. Ideally, spectra should be subjected to both derivation and FSD and only features present in both derivative and deconvolved spectra should be assigned in order to avoid artifacts due to data processing.

IV. IS FTIR SPECTROSCOPY THE RIGHT TECHNIQUE TO USE?

Having established that FTIR spectroscopy can indeed provide information concerning protein secondary structure, and having determined how we can extract this information from the spectrum, it is often pertinent to ask, what information does one hope to obtain about a protein and is FTIR spectroscopy the best method to obtain this information (or indeed can FTIR spectroscopy provide any information)? The nature of the information that can be obtained by FTIR spectroscopy concerning protein secondary structure is highlighted later in this review. It is sufficient to mention at this point that if precise information concerning the relative positioning of individual functional groups in three-dimensional space is required, FTIR spectroscopy is not the technique of choice and the investigator should resort to X-ray crystallography or NMR spectroscopy.

It is more pertinent at this point to address the second question. While depending to a large extent on the information sought, the question of whether one should use FTIR spectroscopy should also be significantly influenced by our understanding of the comparative strengths and limitations of the many biophysical measurement methods that exist. NMR spectroscopy, for instance, can only presently be applied to proteins with a molecular weight of less than 20,000 Da. In addition, NMR spectroscopy cannot be readily applied to the study of proteins in a membrane environment due to line-broadening effects associated with motional restriction. The membrane environment also provides problems for CD spectroscopy, due to artifacts associated with light scattering. X-ray diffraction obviously requires the formation of good-quality crystals. IR spectroscopy is not limited by protein size (the largest water-soluble protein studied to date is fibrinogen [Azpiazu et al., 1992], which has a molecular weight of 340,000 Da) or the nature of the environment (many membrane proteins and peptides have been studied by FTIR spectroscopy, including mellitin [Lavialle et al 1982], photosynthetic reaction centers [Gerwert et al., 1988; He et al., 1991; Haris and Chapman, 1993], a variety of ion-transporting ATPases [Fringeli, U. P. et al., 1989; Chapman et al., 1989; Arrondo et al., 1987; Villalain et al., 1989; Barth et al., 1990] and bacteriorhodopsin [Krimm and Dwivedi, 1982; Lee et al., 1985; Breton and Nabedryk, 1989; Earnest et al., 1986; Earnest et al., 1990; Fahmy et al., 1991], perhaps the most widely studied protein, spectroscopically).

In addition to such factors as protein size and the nature of the protein environment, there are other, less intuitive factors that may have an effect on the decision of which technique is applied to the study of the protein. Many peptides and small proteins may rapidly interconvert between a variety of structural forms, that is, there exists a dynamic equilibrium. Such structural forms may not be detected by NMR spectroscopy if the lifetime of the structural forms is short (the intensity of the nuclear Overhauser effect is dependent on the length of time the chromophores are in contact; short lifetimes produce weak NOEs that may not be detectable) but may be detected by IR spectroscopy (Jackson and Mantsch, 1992a). In such an instance, an optical method such as IR

spectroscopy should be considered the technique of choice.

Therefore, it can readily be seen that there are many instances where FTIR spectroscopy may be considered the technique of choice, as few or no other techniques exist to adequately study the protein or peptide. Such instances include the analysis of membrane-associated proteins and peptides, large proteins, and smaller proteins and peptides that may exist in a rapidly interconverting dynamic equilibrium. Conversely, there are circumstances in which FTIR spectroscopy is perhaps only one of many methods available for characterization, and the decision of which technique an investigator uses is based on the problem being addressed, familiarity with the technique, and availability of instrumentation.

Having decided that FTIR spectroscopy is the technique of choice for the analysis of a particular protein, one must now decide how to make the measurements.

V. EXPERIMENTAL CONSIDERATIONS

A major advantage of FTIR spectroscopy for structural characterization is the lack of dependence on the physical state of the sample. Samples may be readily examined as aqueous or organic solutions, hydrated films, inhomogeneous dispersions, or solids, and proteins have been analyzed by FTIR spectroscopy in all of these physical states.

Information may be obtained from analysis of solid proteins either deposited on an IR-transparent substrate, pressed into a KBr pellet, or by using specialized techniques such as photoacoustic IR spectroscopy (Wang et al., 1994). However, the information obtained from such an analysis is of limited relevance due to the nonphysiological nature of the measurement. In addition, the structure of the protein or peptide under investigation may depend critically on the medium from which is was dried and the solvent history may not be known.

In addition to these traditional methods for the analysis of solid materials, a number of studies (Goormaghtigh et al., 1990) have been reported in which water-soluble proteins have been analyzed as films by attenuated total reflectance (ATR). In such studies, a small volume of dilute protein solution is allowed to dry on the surface of a (usually trapezoidal) crystal composed of an IR-transparent material of high-refractive index. IR light impinging on the crystal face is reflected along the interior of the crystal. Each time the IR radiation impinges on the crystal-sample interface the beam partially penetrates the sample. If the difference in the refractive indices of the sample and crystal is great enough, the radiation is reflected back into the crystal after being partially absorbed by the sample. In this way a spectrum of the sample may be recorded. This approach to the analysis of proteins raises a number of questions. Is there any interaction between the ATR crystal and the protein? Do differences in the nature of the ATR element (e.g., degree of hydrophobicity) affect the structure assumed by the protein in the solid state? Assuming that no significant interactions occur between the ATR element and the protein, the question of the relevance of spectra of proteins in the dry state remains. Attempts have been made to avoid this problem by passing a carrier gas saturated with water vapor over the film to hydrate the protein. However, it is unlikely that this procedure results in a hydration state similar to that seen in solution. This decreased hydration may have important structural and spectroscopic consequences (Jackson and Mantsch, 1992b). Figure 3a shows the FTIR spectrum of concanavalin A recorded in aqueous (^2H_2O) solution, while in Figure 3b and 3c we present the spectra of concanavalin A as a film hydrated with ^2H_2O vapor and as a dry film. It can be seen immediately that significant differences exist between the spectra of the protein in solution and as dry and hydrated films. In particular, the absorption at 1623 cm^{-1} in the solution spectrum of concanavalin A is absent from the spectrum of the dry film and much reduced in intensity in the spectrum of the hydrated film, suggesting that the presence of this band in solution spectra is in some way related to hydration of amide C=O groups, and that solvation of the protein amide groups in hydrated thin films of concanavalin A is reduced compared with the protein in solution.

In addition to variations in the hydration of important chromophores, significant structural rearrangements may be evident on drying of pro-

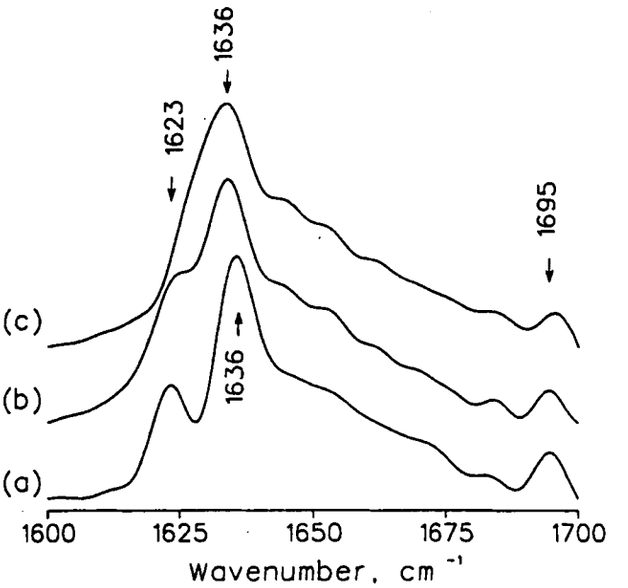

FIGURE 3. Deconvolved spectra (k = 1.7, HW = 13.5) of concanavalin A in solution (a), as a hydrated film (b), and as a dry film (c).

teins onto ATR elements (Jackson and Mantsch, 1992b). Figure 4 shows spectra of myoglobin in ^2H_2O solution and as a thin film hydrated with ^2H_2O vapor. In solution, myoglobin exhibits an amide I maximum at 1653 cm^{-1}, characteristic of a protein with a predominantly α-helical secondary structure. Minor absorptions at 1632 and 1672 cm^{-1} remain to be assigned, but most likely arise from turns. It is apparent that the intensities of these two minor absorptions are significantly increased in hydrated thin films of myoglobin and that the amide I maximum is shifted to lower

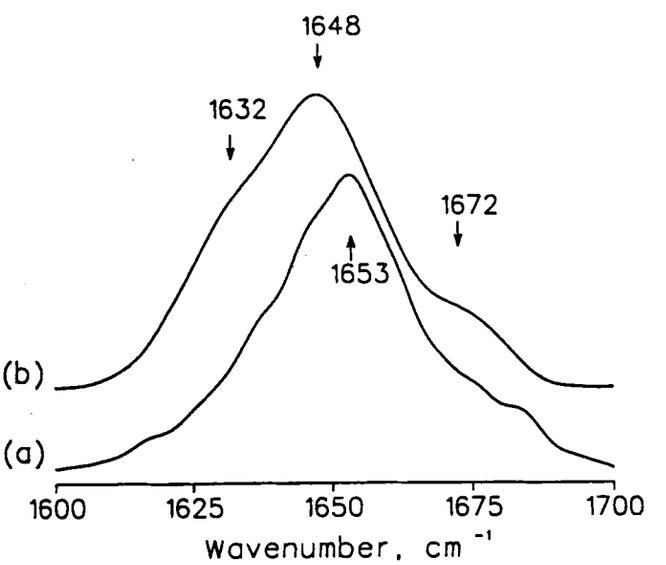

FIGURE 4. Deconvolved IR spectra (k = 1.7, HW = 13.5) of myoglobin in solution (a) and as a hydrated film (b).

frequency, indicative of significant structural rearrangements after drying and rehydration.

These two brief examples highlight the difficulties in obtaining spectra that accurately reflect the true solution structure of proteins using techniques such as ATR spectroscopy. Given that significant artifacts are readily introduced into ATR-IR spectra of proteins, we suggest that caution be exercised when conducting structural studies of water-soluble proteins by ATR-IR spectroscopy. In addition, as the amount of protein required to produce spectra with adequate signal-to-noise ratios from an ATR experiment is similar to that required for solution studies (about 10 to 50 μg), there seems to be little real advantage to using ATR methods for the analysis of water-soluble proteins.

Organic solvents have proven to be a popular medium for the structural characterization of proteins and peptides. There are four main reasons for the study of proteins and peptides as solutions in organic solvents. First, it has been suggested that organic solvents such as trifluoroethanol (TFE) and chloroform may act as membrane mimetics, that is, they mimic the environment of lipid membranes. Second, it may be difficult to form a true solution or even a homogeneous dispersion of hydrophobic peptides in aqueous solutions. Third, many NMR and CD spectroscopists use TFE and dimethyl sulfoxide (DMSO) as solvents for hydrophobic peptides, and it is often of interest to compare results obtained by FTIR spectroscopy to those obtained by NMR and CD spectroscopy. Finally, manipulation of the solvent can facilitate studies of protein unfolding and refolding.

We have shown that the use of organic solvents to solubilize and unfold proteins and peptides and as a membrane mimetic is fraught with problems (Jackson and Mantsch, 1991b; Jackson and Mantsch, 1992c). The problems associated with these approaches are highlighted by discussions of the effects of halogenated alcohols on protein secondary structure.

Despite the fact that the dielectric constants of trifluoroethanol and the interior of biological membranes are very different (26.6 and 1, respectively) and that TFE contains a potent hydrogen bond donor, TFE is widely used as a membrane mimetic solvent. However, we have shown that dissolution of a large number of proteins, encompassing a wide range of secondary structures, in TFE results in pronounced alterations in the secondary structure of the proteins deduced from FTIR spectra as evidenced by the appearance of an amide I maximum at 1656 cm^{-1}, a frequency characteristic of a protein with a predominantly α-helical secondary structure (Jackson and Mantsch, 1992c). Whether this structure truly represents the structure that would be adopted in a membrane environment can only be shown by performing studies of the peptide or protein in a membrane environment. Therefore, it is perhaps more suitable to perform the initial experiments in a membrane environment rather than in TFE.

Perhaps more importantly, significant structural effects are possible even in aqueous mixtures containing very low concentrations of halogenated alcohols. While spectra obtained at room temperature in the presence of trace amounts of halogenated alcohols appeared to be identical to those obtained in pure water, the denaturation temperature of concanavalin A was reduced by almost 20°C, suggesting a significant destabilization of the protein even at very low concentrations of TFE. In addition, at intermediate concentrations of halogenated alcohol, unfolding and aggregation could be detected for all proteins that we studied.

Structural perturbations were also apparent when proteins were dissolved in DMSO and DMSO/^2H_2O mixtures (Jackson and Mantsch, 1991b). In pure DMSO, the presence of a strong hydrogen bond acceptor (S=O) resulted in the disruption of all intramolecular hydrogen bonds, and the appearance of an amide I absorption characteristic of free amide C=O groups. In DMSO/^2H_2O mixtures containing low concentrations of DMSO, as in similar halogenated alcohol/^2H_2O mixtures, thermal destabilization was found. At higher concentrations, unfolding and aggregation was apparent.

Studies involving the dissolution of proteins in organic solvents can be seen to require great care. For this reason, the majority of FTIR spectroscopic studies of proteins are carried out in aqueous solution/suspension. Standard absorption/transmission techniques are readily applicable to aqueous protein solutions and membrane dispersions. Such methods were initially limited in their

applications to protein studies due to the strong O–H bending absorption of water at 1644 cm^{-1}, which obscures the amide I band. Digital subtraction routines running on low-cost PCs removed this problem, allowing subtraction of a water spectrum (or buffer spectrum) from the spectrum of the protein solution to generate the spectrum of the protein. Adequate subtraction will only be obtained if the spectra of the protein solution and water (or buffer) are recorded at the same temperature, due to the strong dependency of water absorptions on temperature. To this end, most workers prefer to record spectra in a thermostated cell. Unfortunately, problems still remain when studying proteins dissolved in water or aqueous buffers due to the high molar absorptivity of the O-H bending vibration of water and problems associated with detector linearity. At high absorbance values, all IR detectors suffer from problems of nonlinearity, which results in distortions of band shapes and relative intensities of absorptions. This limits the pathlength that may be used in studies of protein dissolved in water to 10 μm or less. Longer pathlengths result in significant distortions of the water absorptions, making subtraction impossible. This, unfortunately, requires relatively high protein concentrations to achieve adequate signal-to-noise ratios (20 to 50 mg/ml). In addition to a reduction in pathlength, some benefits may be gained in recording aqueous spectra by the correct choice of detector. While mercury-cadmium-telluride (MCT) detectors are favored by many workers because of faster spectral acquisition rates compared with deuterated triglyceride sulfate (DTGS) detectors (20 kHz compared with 5 kHz), DTGS detectors have the advantage of an extended linear range (i.e., are linear at higher absorbance values than MCT detectors) and may be the detector of choice under certain circumstances.

An alternative approach is to use ^2H_2O (D_2O) as a solvent. The O–^{2}H bend is shifted some 400 cm^{-1} to lower frequency as compared to the O–H bend, thus leaving a useful window free of absorptions in the amide I region of the spectrum. Much longer pathlengths may then be used (50 μm is standard, although pathlengths of 100 μm or more may be used), which allow a significant reduction in the concentration of samples (0.1 to 1 mg/ml). In addition to these advantages, the rate and extent of exchange of amide protons for deuterons may provide valuable information concerning the degree of solvent exposure and strength of hydrogen bonds involving amide N–H groups. Deuteration also allows more complete assignment of absorptions arising from secondary structure. In particular, dissolution of proteins in ^2H_2O allows discrimination between α-helical and unordered secondary structures. Absorptions arising from α-helices and unordered structures are found at approximately the same frequency in water. However, the shift seen on deuteration of unordered secondary structures is much greater than that seen on deuteration of helical secondary structures (10 cm^{-1} compared with 5 cm^{-1}), resulting in the appearance of discrete absorption for the two structural motifs in ^2H_2O solution.

From this brief discussion it is clear that although FTIR spectroscopy is not limited by the nature of the sample, the nature of the sample strongly affects the outcome of the experiment. In other words, IR spectra can be obtained from any sample, but the result obtained depends very strongly on the physical state of the sample. Samples are best studied in the environment that most closely mimics the physiological environment of the protein under investigation (while this may seem self-evident, it is unfortunately a fact that is often overlooked). Membrane-associated proteins and peptides should be studied as aqueous protein-lipid dispersions to avoid structural artifacts associated with the use of membrane mimetic solvents. In addition, the use of protein-lipid dispersions of defined composition allows the effects of lipid acyl chain length, headgroup, unsaturation, and phase on protein secondary structure to be assessed. For water-soluble proteins, samples are best analyzed as adequately buffered aqueous solutions (in ^2H_2O, H_2O, or preferably both) rather than solids or hydrated films.

VI. QUALITATIVE ESTIMATION OF PROTEIN STRUCTURE FROM FTIR SPECTRA

Qualitative information concerning the structure of proteins may be obtained from IR spectra by a number of means. This information may be

of a macroscopic nature, relating to relatively gross structural characteristics such as compactness of protein folding or the degree of penetration of protein structures into a membrane. Such information may be obtained by following the kinetics of hydrogen-deuterium exchange. On the other hand, this information may be more microscopic in nature, relating to the environment of particular amino acid side chains as deduced from the position of their infrared absorptions. Studies of this kind may be of special use in the investigation of protein denaturation. More commonly, the information obtained lies between these two extremes and relates mainly to the types of secondary structure present within a protein and how these secondary structures are affected by external perturbations such as ligand binding and thermal manipulations. Of course, it is possible to obtain information at all three levels from any experiment, although, unfortunately, to date most investigators have not made full use of the information contained within their IR spectra.

A. Macroscopic Information

A useful illustration of the information that can be obtained by studying amide protein exchange rates is the elucidation of subtle structural differences between ribonuclease A and S (Haris et al., 1986). Ribonuclease A and S differ only in the cleavage of the peptide bond between amino acids 20 and 21 in ribonuclease S. Not suprisingly, the FTIR spectra of ribonuclease A and S in aqueous buffer are virtually identical, indicative of proteins with predominantly β-sheet secondary structures, in agreement with X-ray diffraction studies. Immediately after dissolution of the proteins in deuterated buffer, the spectra of the two proteins are again virtually identical. While many amide protons have undergone exchange, as indicated by a reduction in the integrated area of the amide II absorption, a residual amide II absorption of significant intensity remains, suggesting that a number of amide N–H groups are involved in strong hydrogen bonds and/or are buried within the hydrophobic protein core, and as such are not readily accessible to exchange. The relative intensity of the residual amide II absorption is approximately the same for the two proteins, again suggesting significant structural homology. However, after prolonged exposure to deuterated buffer, the relative intensities of the residual amide II absorptions of the two proteins are significantly different, indicating that ribonuclease S has undergone significantly more deuteration than ribonuclease A. This in turn implies that ribonuclesase S contains secondary structures with amide N–H groups involved in weaker hydrogen bonds than ribonuclease A or that these amide N–H groups are more accessible to solvent. Either possibility suggests that some subtle structural differences exists between ribonuclease A and S that may only be detected by studying the deuteration profiles of the proteins.

In addition to providing information concerning subtle differences between homologous water-soluble proteins, deuteration studies have also been used to examine solvent accessibility of amide protons in membrane-embedded proteins (Earnest et al., 1990). Studies on the human erythrocyte glucose transporter have proven particularly interesting (Jung et al., 1986, Alvarez et al., 1987a). The human erythrocyte glucose transporter is, as its name implies, a membrane-embedded protein responsible for the transportation of glucose across the erythrocyte membrane. Although this protein may be easily purified, little is known concerning its structure and mechanism of action.

From a structural point of view, the protein appears to be predominantly α-helical (confirmed by IR spectroscopy, the amide I maximum being seen at 1657 cm^{-1}) with most of the protein mass embedded within the membrane. Functionally, the most important question remains, how does the protein transport a highly polar molecule such as glucose through the hydrophobic membrane? One possibility would be for the membrane-embedded helical structures of the protein to form the walls of a water-filled channel, through which glucose could pass with relative ease. This hypothesis has been tested by examination of the hydrogen-deuterium exchange kinetics of the protein. As most of the protein mass appears to reside within the membrane, most amide protons should not be readily accessible to the solvent and should exchange at very low rates. However, if a water-filled channel lined by polypeptide chains does indeed exist, then the number of amide protons in

contact with the solvent should be greater than anticipated. Infrared spectroscopic assessment of hydrogen-deuterium exchange indicated that almost 40% of amide protons exchange within 1 min of suspension in ^2H_2O buffer (Jung et al., 1986), and that 80% of protons have exchanged after 1 h (Alvarez et al., 1987a), implying that up to 80% of amide protons must be in contact with solvent. This result strongly implies the existence of a water-filled pore. Similar studies in the presence of excess D-glucose found a reduced rate of proton-deuteron exchange, suggesting a transient blocking of the pore during transport or a significant compaction of the protein structure.

By far, the greatest use of FTIR spectroscopy in the study of proteins is the deduction of the major types of secondary structure present within proteins and evaluation of the gross structural consequences of ligand binding. Studies of this type have been performed on an enormous variety of proteins and peptides, with widely different aims, ranging from attempts to define the active conformation of peptide hormones to attempts to understand the mechanism of ion transport across biological membranes.

A wide variety of water-soluble and membrane-associated pore-forming peptides, peptide hormones, and neurotransmitters have been studied by FTIR spectroscopy in attempts to define their physiological (active) conformation, including somatostatin (Jackson and Mantsch, 1992a), angiotensin (Surewicz and Mantsch, 1988b), substance P (Choo et al., 1994), galanin (Wennerberg et al., 1994), dynorphin (Renugopalakrishnan et al., 1988), bombesin (Erne and Schwyzer, 1987), mellitin (Lavialle et al., 1982; Brauner et al., 1987; Sui et al., 1994), the magainin family of antibiotics (Jackson et al., 1992d), alamethicin (Haris et al., 1988), and colicin E1 (Rath et al., 1991). A general trend that appears from studies such as these is that most water-soluble peptides studied to date are structureless in aqueous solution at neutral pH. Ordered secondary structures could generally be induced by manipulation of pH or interaction with the appropriate membrane environment (or solvents such as TFE). Many of these studies are described in recent reviews (Arrondo et al., 1993; Jackson and Mantsch, 1993).

Variations in protein secondary structure as a function of lipid composition can also be assessed by IR spectroscopy (see Mendelsohn et al., 1984; Fernadez-Ballester et al., 1994 and references therein). The studies of Mendelsohn et al. (1984) highlight one of the major advantages of IR spectroscopy, the ability to provide information concerning two or more components of multicomponent systems. For example, for the ternary system of Ca^{2+}ATPase reconstituted in a binary lipid mixture comprising acyl chain deuterated DPPC and nondeuterated DOPC, Mendelsohn et al. (1984) were able to demonstrate that the protein preferentially partitioned into a DOPC-rich phase, by analysis of the temperature dependence of the CH and C^2H stretching absorptions of the lipid components in the presence of protein. In addition, information concerning structural modifications of the protein was obtained. In principle, therefore, it is possible to obtain information concerning the structural properties and interactions of each component in a ternary mixture. Such studies are impossible by any other biophysical method (with the exception of Raman spectroscopy, a technique closely related to IR spectroscopy). Unfortunately, studies of this type have been regrettably few to date, and this is an area that should be more actively pursued as it allows proteins to be investigated in more physiologically relevant (multicomponent) systems.

In addition to protein/peptide-lipid interactions, peptide-peptide, peptide-protein, and protein-protein interactions are of importance in biological systems, for example, in the aggregation of the β-A4 peptide in Alzheimer's disease (Fraser et al., 1991; Fabian et al., 1993). Unfortunately, the precise details concerning the structural perturbations of the individual peptides or proteins as a consequence of these interactions have proven impossible to study by IR spectroscopy due to the overlap of the two amide I absorptions. A technique recently described by Haris et al. (1992) and Zhang et al. (1994) known as isotope edited IR spectroscopy should change this situation. In brief, the technique involves complete isotopic labeling of one of the species under investigation. This isotopic substitution results in a shift of the amide absorptions to lower frequencies, in principle allowing the amide absorptions from two species to be observed simultaneously. As might be expected, ^{15}N labeling produced only minor changes in the amide I band contour due to the

relatively low C–N stretching contribution to the amide I vibration. On the other hand, the amide II absorption is shifted by almost 20 cm^{-1}. Unfortunately, this still does not leave a clear "window" for studies on the amide vibrations of an additional protein or peptide. However, complete labeling with ^{13}C results in a much greater shift of the amide I vibration, by approximately 55 cm^{-1}, leaving the traditional amide I region of the spectrum absorption free. It then becomes possible to study the interaction of a labeled protein and a nonlabeled peptide or peptide.

Zhang et al. (1994) recently used this approach to probe the conformational changes associated with binding of nonlabeled synthetic peptides resembling the calmodulin-binding domains of myosin light chain kinase and caldesmon to ^{15}N and ^{13}C-labeled calmodulin. These studies showed that the amide I frequency for the unlabeled peptides was increased from 1644 cm^{-1} (indicative of unstructured peptides in ^2H_2O solution) to higher frequency, indicative of the formation of helical structures. The shift was smaller for the caldesmon fragment than for the myosin light chain kinase fragment, indicating induction of less helical structure in the peptide. This finding is in agreement with the weaker affinity of the caldesmon peptide for calmodulin. Only minor changes were detected in the structure of calmodulin. This technique should have great potential for the study of protein-protein interactions and protein-DNA interactions (DNA also exhibits C=O absorptions in the amide I region of the spectrum).

While the interactions of protein and peptides with other proteins is of great importance in biology (e.g., binding of peptide hormones to receptors, activation of proteins by other proteins or peptides), the majority of ligand-protein interactions involve interaction of proteins with simpler substrates such as metal ions or neurotransmitters. The spectroscopic consequences of many such interactions have been studied, including those involved in activation of proteins (Jackson et al., 1991; Dong et al., 1994), transportation of ions across membranes (Arrondo et al., 1987; Villalain et al., 1989), ligand binding to specific receptors (Fernadez-Ballester et al., 1992), binding of substrates and cofactors (Belasco and Knowles, 1980, Alvarez et al., 1987b), or binding of substances to transport or storage proteins (Alben and Bare, 1978; Nara et al., 1994; Sampath et al., 1994).

B. Microscopic Information

Absorptions arising from amino acid side chains have long been thought of as annoying interferences by biological IR spectroscopists. Contributions from aspartate, glutamate, arginine, tyrosine, lysine, and glutamine are seen in the amide I and II regions of the spectrum (Chirgadze et al., 1975; Venyaminov and Kalnin, 1990). However, rather than treat these absorbances as unwanted interferences, it is possible to make use of amino acid side chain absorptions to deduce information relating to protein structure. How this may be done is easily imagined when one realizes that a number of these absorptions show a dependence on environmental factors (e.g., solvation, dielectric constant).

Fabian et al. (1993, 1994) have used the dependency of the IR absorptions of amino acid side chains on environment to probe thermal denaturation of proteins. The intensity of the antisymmetric COO^- stretching vibration of aspartate (1585 cm^{-1}) and the position of the tyrosine ring vibration (around 1515 cm^{-1}) were measured as functions of temperature for ribonuclease T1 and were shown to be excellent monitors of protein unfolding. Plotting intensity of the aspartate band and position of the tyrosine band as a function of temperature produced sigmoidal curves from which the onset and Tm of protein unfolding could easily be deduced. Thus, changes in the microenvironment of amino acid side chains, resulting from changes in protein structure, translate into changes in frequency and intensity and can be used to monitor protein unfolding and refolding.

Information relating to changes in the microenvironment of amide C=O and N–H groups produced by structural perturbations may also be probed by FTIR spectroscopy, although this requires a more sophisticated approach (as a rule of thumb, approximately 5% of amide C=O groups must be perturbed before an appreciable effect on

the amide I absorption profile can be detected by standard data analysis methods).

Such structural perturbations may be induced by biological "triggers", for example, the binding of a physiological ligand, absorption of photons, or an electrochemical event. In experiments of this type, a spectrum of the protein in the non-perturbed state is recorded. The same sample of the protein is then perturbed (e.g., by the photolytic release of a caged substrate such as ATP, which then binds to an ATPase [Barth et al., 1991], by illumination of a light-transducing protein such as bacteriorhodopsin with light [Nabedryk and Breton, 1986; Diller et al., 1991; Fahmy et al.,1991], or by the oxidation or reduction of a redox protein such as cytochrome oxidase [Moss et al., 1990] by the application of a potential difference) and the spectrum of the perturbed protein is recorded, often as a function of time. The ratio of the two spectra may then be taken to produce a spectrum that corresponds to the difference between the two states. This procedure is often termed reaction-induced difference spectroscopy (RIDS) and has been reviewed by Braiman and Rothschild (1988), Rothschild (1992), and Mäntele (1993).

The first examples of RIDS involved the use of light as a trigger to probe the differences between structural intermediates of light-transducing proteins such as bacteriorhodopsin and rhodopsin (Rothschild et al., 1983; Siebert and Mäntele, 1983; Earnest et al., 1986). Such studies have been able to identify specific amino acid residues taking part in proton transfer and structural rearrangements of the retinal chromophore in response to illumination. Many of these early studies were carried out at low temperature (~80 K) in order to capture the protein in the required intermediate state long enough to obtain spectra. Rapid scan (Braiman et al., 1987), step scan (Uhmann et al., 1991), and stroboscopic (Souvignier and Gerwert, 1992) IR methods allow such studies to be conducted at more physiological temperatures, and provide greatly improved temporal resolution. The use of picosecond lasers has improved temporal resolution even further, and the photodynamics of light-sensitive proteins can now be studied at room temperature on a picosecond time scale (Moore et al., 1988; Anfinrud et al., 1989; Diller et al., 1991). A discussion of the exciting work continuing in this very active field is outside the scope of this review, and the interested reader is referred to the references cited above and reviews by Braiman and Rothschild (1988) and Rothschild (1992).

Reaction-induced differences are most easily studied when the protein under investigation is a light-transducing protein. Hence, light-sensitive proteins are the earliest and most widely studied proteins using RIDS. Unfortunately, if only light-transducing proteins are amenable to study, the technique is of limited benefit. However, it has been shown that it is also possible to produce reactively induced difference spectra by binding ligands to proteins. This is best illustrated by the work of Barth et al. (1990) and Buchet et al. (1992), who have studied the catalytic cycle of the Ca^{2+}ATPase by RIDS. In this case, structural rearrangement of the protein was stimulated by photolytic release of the ligand, ATP, from a non-reactive precursor form. Barth et al. (1990) were able to detect spectral changes related to photolysis of the caged ATP, hydrolysis of ATP by the Ca^{2+}ATPase, and changes in the protein itself. The changes in protein structure were small, absorbance changes in the amide I region being less than 2% of the total amide I absorbance. Similarly, Buchet et al. (1992) found only small changes in the amide I and amide II regions of the spectrum in response to photolytically released Ca^{2+}. These results indicate that no major rearrangement of the protein secondary structure occurs on phosphorylation of and Ca^{2+} binding to the Ca^{2+}ATPase.

As an alternative to photolytic release of substrates, Marrero and Rothschild (1987a,b) and Baenziger et al. (1992a,b; 1993) proposed a methodology utilizing ATR spectroscopy of films of membrane proteins. In this methodology, a spectrum of the protein film in its resting state may be acquired with a ligand-free buffer flowing over the film. A spectrum of the activated protein is recorded from the same film, but this time the necessary ligand is added to the buffer flowing over the film. These authors utilized this technique to describe structural changes in the nicotinic acetylcholine receptor associated with binding of acetylcholine and a number of antagonists. These studies showed that subtle differences in

the structure of this large protein (MW = 300,000 Da) could reproducibly be detected by RIDS. These changes reflected rearrangements of the polypeptide backbone and changes in the environment and protonation state of amino acid side chains.

Electrochemically induced difference spectroscopy is perhaps the least utilized of the RIDS methods. The principles underlying electrochemical RIDS are well illustrated by the studies of Mäntele and co-workers (Moss et al., 1990; Schlerth and Mäntele, 1992; Schlerth and Mäntele, 1993). Studies investigating the redox-linked changes in the structure of cytochrome c were used to verify the applicability of the technique (Moss et al., 1990). This study again demonstrated that absorbance differences of less than 1% of the total amide I absorption could readily and reproducibly be detected in reduced-oxidized protein difference spectra. For a small protein such as cytochrome c (104 amino acids), this can be seen to represent perturbation of a very small number of amide C=O groups. Differences relating to the protein amide N–H groups and amino acid side chains could also be detected. In particular, a sharp differential peak was observed at around 1516 cm^{-1}, a frequency characteristic of tyrosine absorptions. This would suggest perturbation of the environment of a tyrosine residue, resulting in a shift in the absorption maximum. Consistent with this interpretation, X-ray diffraction studies have suggested a perturbation of Tyr67 in the reduced protein. Thus, RIDS can provide important structural information even when structural perturbation is limited to relatively few amide C=O groups and a single amino acid side chain. That these differences are indeed real is supported by the fact that they are reproducible and that the oxidized-reduced and reduced-oxidized difference spectra are mirror images of each other.

A more sophisticated study was carried out to compare the effect of the redox transition of the structure of myoglobin and hemoglobin (Schlerth and Mäntele, 1992). This study reported subtle perturbations of the secondary structure of myoglobin, amino acid side chains, and the heme moiety itself. For hemoglobin the changes were similar but of much greater magnitude, suggesting conformational changes in all four heme moieties of hemoglobin. In addition, the larger redox-linked signals observed for hemoglobin when compared with myoglobin was suggested to be consistent with the greater shift in helix F away from the heme pocket in hemoglobin on reduction.

It can readily be seen that RIDS can provide important structural information at the microscopic level, revealing perturbations of secondary structural elements and amino acid side chains related to ligand binding. Alternative methods are required when the difference between two protein states cannot be induced by a triggering event, as in the case of point mutations. In this case, spectra of the two protein states must be recorded independently under carefully controlled conditions and subtracted from one another (Fabian et al., 1994). In RIDS, direct ratioing of spectra is possible because both spectra originate from the same sample, and the protein concentration and pathlength are identical. In the case of point mutations, interactive subtraction of two spectra matched as well as possible with respect to concentration, temperature, and pathlength must be performed. Correct subtraction is judged by the appearance of a flat baseline in regions that contain no absorptions. A sloping baseline indicates over- or undersubtraction. This approach is obviously more subjective than RIDS, but under correctly controlled conditions can provide valuable information. Fabian et al. (1994) demonstrated structural differences due to point mutations by the application of such difference techniques. Spectra of wild-type ribonuclease T1 and three mutants were normalized using the spectra of the unfolded state of each protein as internal intensity standards. Subtraction of each of the mutant spectra from the wild-type spectra therefore resulted in difference spectra that contained positive and negative features resulting from differences in structure due to the mutations. Changing Trp59 to tyrosine, in particular, was found to cause changes in the amide I region of the spectrum. Although small, these differences pointed to variations in the β-sheet and helical secondary structures in the various mutants studied.

This technique has also been used to detect more gross structural changes associated with protein denaturation (Yamamoto and Tasumi, 1988; Sosnick and Trewhella, 1992).

VII. QUANTITATIVE ESTIMATION OF PROTEIN STRUCTURE FROM FTIR SPECTRA

A large proportion of reports on the IR spectroscopic assessment of protein structure include a quantitative estimate of secondary structure in some form. The most common method of quantitation of protein secondary structure involves curve fitting of the amide I (Ruegg et al., 1975; Susi and Byler 1986; Byler and Susi, 1986; Surewicz and Mantsch, 1988; Villalain et al.,1989) or occasionally the amide III (Singh et al., 1991; Singh et al., 1993) band. As an initial step, deconvolution or derivation is performed to obtain an estimate of the number of discrete absorptions that make up the complex amide I band profile. This estimate of the number of components plus estimates of their width, height, and shape are then used as input parameters in an iterative least squares routine that attempts to reproduce the experimentally obtained amide I band profile by varying these parameters. (Curve fitting is also often performed on deconvolved spectra, as the individual components are easier to visualize, which in turn makes a visual check of the fit easier.) However, it has been shown by Goormaghtigh et al. (1990) that the relative integrated areas of the component bands is affected by the deconvolution parameters used. For example, after curve fitting the IR spectrum of papain, Goormaghtigh et al. found the helix content to be either 29 or 35%, depending on whether the chosen half-width was 20 or 30 cm^{-1} for the same resolution enhancement factor (k = 1.8). Keeping the half-width constant (20 cm^{-1}) and varying the resolution enhancement factor produced similar variations, resulting in helix estimates of 29% for k = 1 and 35% for k = 1.8.) The integrated area under each band after the final iteration is calculated as a percentage of the total amide I area, and this value taken to be the percentage of the particular secondary structure present in the protein. Of course, there are a number of assumptions implicit in this approach that are not necessarily justified (Surewicz et al., 1993). Each of these assumptions is discussed in turn.

The first assumption is that the number and positions of the IR bands that make up the amide I band, as obtained from deconvolution or derivation, is an accurate reflection of the real number of components. Of course the "real" number of individual amide I band contours is the number of chromophores (number of amide groups) present in the protein. Obviously, the positions of the absorptions arising from each of these chromophores can never be known, except perhaps for very short peptides, and one must use a smaller number of composite absorptions arising from amide C=O groups in similar environments.

In addition to an estimate of the number of amide absorptions, curve fitting routines require an assumption of band shape (i.e., Lorentzian, Gaussian, or mixed). For simple molecules, IR absorptions are usually assumed to be Lorentzian in shape. However, this assumption does not necessarily hold for larger, complex molecules such as proteins, and the shape of IR absorptions arising from proteins is less than clear. In fact, it is not clear whether all protein secondary structures give rise to the same band shape, or to what extent environmental effects are important in determining band shape. It is probable that dielectric constant and hydrogen bond strength will have effects on band shape.

Assuming that the real number and line shape of these composite absorptions is known, the next step is to assign each absorption. Two possible sources of error are inherent in this step. First, it is assumed that the absorptions arise from amide C=O groups involved in secondary structures. In fact, significant absorptions in this region of the spectrum may arise from amino acid side chains, including tyrosine, phenylalanine, glutamine, arginine, and lysine (Chirgadze et al., 1975; Venyaminov and Kalnin, 1990). In proteins containing significant amounts of these amino acids a nontrivial contribution from the side chains to the amide I band envelope is to be expected (10 to 15% of the total intensity of the amide I band may in fact arise from side chains). Failure to take side chain contributions into account will result in under- or overestimation of some secondary structures. It has been suggested that this problem can be surmounted by subtraction of the spectra of the relevant side chains from the amide I band contour based on absorption profiles determined from amino acids or polypeptides (Venyaminov and

Kalnin, 1990). However, this approach assumes that the molar absorptivity, position, and shape of the side chain absorption are the same in proteins as they are in amino acids and simple peptides. Given the tremendous variation in environment (solvent exposed, buried within the protein hydrophobic core), variations of pKa with environment, and the possibility for hydrogen bond and salt bridge formation, this assumption would appear to be naive. Subtraction of "standard" absorptions based on amino acids and peptides is therefore likely to introduce artifacts into spectra and led to additional errors in quantitation.

A second source of error lies in the assignment of particular amide I absorptions to specific secondary structures. This is typified by examination of the spectrum of myoglobin (Figure 4a), a protein known to be predominantly α-helical in structure, with little or no β-sheet structure present. IR spectra exhibit an amide I maximum at 1653 cm^{-1}, characteristic of proteins with predominantly helical secondary structures. However, a strong shoulder at 1632 cm^{-1} may be taken as evidence of the presence of a significant amount of β-sheet secondary structures, in marked contrast to X-ray diffraction data. If one assumes that the high-resolution structure obtained by X-ray diffraction from crystals of myoglobin is representative of the structure of the protein in solution, then assignment of the observed amide I bands in accordance with the empirically determined assignments discussed above leads to an incorrect structural determination. The absorption suggestive of β-sheet secondary structures in fact most likely arises from turns within the protein.

Unfortunately, situations similar to that described above, in which assignments are not clear, are not uncommon. In particular, absorptions arising from turn structures are difficult to assign, due to the different hydrogen bonding characteristics of the various types of turn. Mantsch et al. (1993) recently undertook a systematic investigation of the IR characteristics of β-turns, which revealed that β-turns give rise to a number of amide absorptions, a low-frequency absorption (1645 to 1630 cm^{-1}) attributed to C=O groups involved in hydrogen bonds that stabilize the turn, and a high-frequency absorption (1660 to 1680 cm^{-1}), which may arise from C=O groups that are not hydrogen bonded and are sterically constrained. In addition, it is possible that absorptions arising from turns may be influenced strongly by the dielectric constant of the environment. A recent study of the peptide antibiotic valinomycin provided evidence for significant spectral shifts associated with changes in the dielectric constant of the medium (Jackson and Mantsch, 1991c). Valinomycin is a cyclic peptide with alternating ester and amide linkages. In chloroform solution, the peptide is known to adopt a "bracelet structure" characterized by six identical β-turns formed between amide C=O and N–H groups. The six ester C=O groups are non-hydrogen bonded and are seen at 1754 cm^{-1}. In DSMO solution, the frequency of the ester C=O is reduced to 1743 cm^{-1}. As DMSO contains no hydrogen bond donors, this shift to lower frequency is not related to hydrogen bond formation, and must result from the increase in the dielectric constant of the solvent (any weak dipole-dipole interactions that may occur between solvent and the peptide would not be expected to produce such a large shift in the C=O stretching frequency). This is supported by the appearance of the ester C=O absorption at an intermediate frequency in chloroform/DMSO mixtures. Similar differences in dielectric constant may be expected to exist between the surface of a protein and the protein core, which would be expected to result in different amide I frequencies for turns in these locations, making assignment of absorptions from turns difficult (it should also be realized that similar shifts related to dielectric constant may be seen for other secondary structures).

A number of weak features are usually seen between 1670 and 1700 cm^{-1} in IR spectra of proteins, due to the presence of absorptions from turns and the high-frequency β-sheet component, which arises as a result of transition dipole coupling (Miyazawa, 1960). The complexity of this region of the spectrum, coupled to the unpredictability of the absorption maximum of the high-frequency turn absorptions, makes it difficult to correctly assign the series of weak features seen above 1660 cm^{-1}, which will lead to problems in quantitative analysis.

Perhaps the most important assumption involved in curve-fitting procedures is that the molar absorptivities of amide C=O groups involved in different secondary structures are the same. More

simply put, it is assumed that the degree and strength of hydrogen bonding to an amide C=O group does not affect the amount of energy required to stimulate the C=O stretching vibration. This is important for the final stage of curve fitting, in which the area of each fitted band is expressed as a percentage of the total area of the amide I envelope. For this to produce values that correlate directly with the percentage of a particular secondary structure present within the protein, the molar absorptivities of C=O groups giving rise to each of the fitted bands must be equal, or some weighting function must be applied to each band to normalize molar absorptivities. For simplicity, it is generally assumed that molar absorptivities do not vary between secondary structures. However, there are reasons for doubting this assumption.

Poly-L-lysine has proven to be a useful model for proteins in many studies, as it can be induced to adopt three distinctly different conformations, depending on pH and thermal treatment. Early CD experiments have shown that at low pH, when all lysine side chains are fully protonated, polylysine adopts an unordered conformation due to charge repulsion between the side chains. Elevation of the pH to around 10 results in deprotonation of the side chains, abolition of the charge repulsion, and adoption of an α-helical configuration. Subsequent heating of the sample to 60°C results in the formation of structure giving rise to a CD spectrum characteristic of β-sheet structures. IR spectra of these states are characterised by amide I maxima at 1640 cm^{-1} (unordered), 1630 cm^{-1} (helix), and 1612 cm^{-1} (β-sheet). Interestingly, the frequencies of both the ordered secondary structures fall outside the frequency ranges empirically found to be characteristic of these structures in proteins (see Table 1). For the helical structures, this may be the result of increased solvent interaction, resulting in the formation of many three-center (bifurcated) hydrogen bonds involving amide C=O and N–H groups and water molecules. In addition, intrachain coupling effects may become important in such regular polymers. The structure formed after heating polylysine has IR absorptions that are highly characteristic of aggregated peptide chains held together by strong intermolecular hydrogen bonds. Thus, rather than the typical β-sheet structures that this form of polylysine has often been suggested to adopt based on CD spectra, the IR spectrum suggests that the structure adopted is more likely to consist of extended strands of polylysine stabilized by intermolecular hydrogen bonds. Therefore, strictly speaking, this spectroscopic signature is characteristic of quaternary structure rather than secondary structure.

The discrepancy between early CD and IR results can easily be explained when one realizes that CD spectroscopy is sensitive to backbone conformation, while IR spectroscopy is sensitive to the degree and strength of hydrogen bonding to amide C=O groups. Thus, the extended chains found in standard β-sheet secondary structures and in the aggregates found in polylysine may be expected to produce similar CD signatures. However, the strength of the hydrogen bonds required to stabilize the two structures is different, and so distinctive IR signatures are seen.

Regardless of whether the structures present within polylysine are "standard" secondary structures, it is obvious that the conformation of polylysine can be readily manipulated, which provides us with a useful model for the study of variations in molar absorptivity with structure. We have shown using this model that variations of up to 30% in the integrated area of the amide I absorption occur as the structure of the peptide is altered, providing direct evidence that there is a strong dependency of the molar absorptivity of polylysine on the degree and strength of hydrogen bonds formed with amide C=O groups (Jackson et al., 1989b). This raises a number of important questions. Does such a dependence of molar absorptivity exist for proteins as well as for simple homopolypeptides? Is the variation in molar absorptivites the same in all proteins? Does the amino acid composition of the protein or peptide affect the molar absorptivities of secondary structures? In what way is this dependence affected by factors such as dielectric constant (does a membrane-embedded helix have the same molar absorptivity as a water-soluble helix)? These are questions that we are not able to answer at this time, but they have pronounced implications for the quantitation of protein secondary structure by IR spectroscopy.

In addition to curve fitting of the amide I absorption, quantitation of protein secondary struc-

ture is also attempted by some workers based on the relative areas of absorptions in derivative spectra (Dong et al., 1990; Caughey et al., 1991; Dong et al., 1994). This approach suffers from many of the problems inherent in curve fitting of amide modes, including the assumption of equal molar absorptivities of secondary structures, correct assignment of absorptions, and the presence of interfering absorptions from amino acid side chains. In addition, quantitation of protein secondary structure based on derivative spectra presents a number of unique difficulties.

Application of derivative routines results in gross modifications of band shapes, resulting in the presence of large side lobes on either side of intense absorptions. The presence of such side lobes makes it difficult, if not impossible, to deduce the true limits of these absorptions in derivative spectra.

In addition to problems defining a baseline in derivative spectra, significant differences between the relative intensities of absorptions in absorbance and derivative spectra exist. This is clearly illustrated in Figure 5, which shows the spectrum of a complex lipid-protein mixture in the N–H stretching (amide A) and C-H stretching region. The original absorbance spectrum is shown in Figure 5a and can be seen to consist of at least seven absorptions of varying intensity and width. After derivation (Figure 5b), significant narrowing of the four relatively narrow absorptions between 2800 and 3000 cm^{-1} is apparent, leading to increased band separation and enhanced visualization of the overlapping absorptions. However, the very broad N–H absorption at 3300 cm^{-1} is significantly reduced in relative intensity in the derivative spectrum. Whereas in the original spectrum this broad feature is the most intense absorption in this spectral range, it becomes one of the weakest absorptions in the derivative spectrum. Furthermore, variation in the derivation parameters results in significant variations in the relative intensities of the absorptions. This is a major disadvantage of derivative calculations. Relative intensities in derivative spectra are dictated to a large extent by the width of the absorption in the original spectrum, narrow absorptions being preferentially enhanced at the expense of broader bands. In other words, relative integrated intensities of absorptions are not retained after application of derivative routines. This should perhaps be intuitively obvious, given the nature of derivative routines. Rather than being based on intensities, second-derivative methods are by definition based on measurement of the rate of change of absorbance values, and the rate of change of absorbance values is obviously greatest for narrow bands. Clearly, this presents a problem when attempting to determine protein secondary struc-

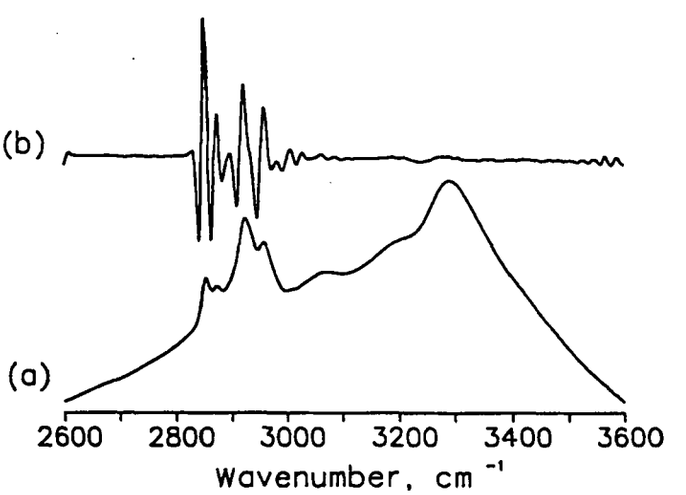

FIGURE 5. IR spectra of a complex lipid-protein mixture in the N–H and C-H stretching region of the spectrum: (a) original absorbance spectrum; (b) smoothed fourth-derivative spectrum.

tures quantitatively from derivative spectra, and we strongly advise against this approach.

Other methods for the quantitative determination of protein secondary structure are being developed that require fewer assumptions and remove much of the subjectivity inherent in the methods discussed above. These methods are based on techniques such as singular value decomposition (Sarver and Krueger, 1991), factor analysis (Lee et al., 1990), and partial least squares analysis (Dousseau and Pezolet, 1990) of IR spectra, and are analogous to methods used for quantitation of CD spectra. In essence, protein spectra are broken down into a number of eigenspectra, or principal components, which represent the most important spectral information. These components are then compared with those calculated for a number of proteins of known composition. This comparison allows a relationship between the components and a particular aspect of secondary structure to be determined, and the importance, or weight, given to each component is related to the percentage of the secondary structure to which the components correspond present in the protein. In a closely related approach, spectra of proteins of known structure can be used to generate spectra characteristic of the various secondary structures. Quantitation is then performed by assuming that a spectrum of a protein to be analyzed is a linear superposition of the characteristic spectra (Pribic et al., 1993). In addition to the advantages outlined above, these methods allow different spectral regions to be combined in a single analysis. Thus, Pribic et al. (1993) demonstrated that an improved quantitation was achieved by combining IR spectra with CD spectra of the same proteins and using this extended spectrum for the analaysis.

Of course, for this approach to be valid, it is an absolute requirement that the data-base of reference proteins comprises sufficient proteins composed of the types of secondary structure likely to be encountered to allow a correlation between these structures and the principal components or eigenspectra to be determined. For example, if none of the reference proteins contain 3_{10} helices, then it will not be possible to identify the presence of 3_{10} helices in a protein of unknown composition, as its eigenspectra will not be known. Furthermore, it is unclear to what extent this approach will be complicated by the presence of unusual degrees of hydration or distortion of secondary structures, factors that will significantly affect the IR spectrum when compared with a non-perturbed, poorly hydrated protein of similar structure. In addition, structure is deduced for proteins in solution using reference spectra structures determined for crystals, thereby assuming identity of structure in solution and in the crystalline state. Perhaps the accuracy of such techniques can be improved by the use of data obtained from solution-phase NMR spectra. Finally, some of these computational approaches involve normalization of amide I absorptions, which assumes equal molar absorptivities for various secondary structures (Lee et al., 1990). As we have discussed earlier, there is empirical evidence that this is not the case.

However, despite these drawbacks, these computational approaches have a major advantage over other methods, namely, the operator does not have to assign absorptions, thereby removing a large element of subjectivity. Therefore, it is likely that quantitative structural determinations using this approach are among the more reliable obtained from IR spectroscopic data.

VIII. SUMMARY

In summary, FTIR spectroscopy has become an established tool for the structural characterization of proteins. Information concerning protein secondary structure ranging from gross aspects of protein conformation (solvent exposure, major secondary structural elements present) to very subtle rearrangements associated with ligand binding or point mutations can be obtained. However, as with all instrumental techniques, correct results can only be obtained if the investigator has a full understanding of the strengths and limitations of IR spectroscopy and designs experiments with these points in mind. Correct experimental design begins with the choice of protein to be studied. Examples of proteins that are good choices for IR spectroscopic studies include membrane associated proteins (and peptides), small proteins and peptides that exist in a number of rapidly interconverting states, and large water-soluble proteins. The environment of the sample should

be as physiologically relevant as possible; the use of organic solvents as membrane mimetics should be restricted. To obtain the maximum amount of information, proteins should be studied in both ^2H_2O and H_2O solutions. Correct application of band-narrowing techniques is crucial for a full interpretation of results and should only be performed on high-quality spectra (high signal-to-noise ratio and no water vapor contributions). Ideally, both deconvolved and derivative spectra should be calculated, and only absorptions visible in both spectra should be assigned. Assignments should be guided by current structure-frequency correlations, but common sense and knowledge obtained from other techniques are equally important. Although quantitative estimates of protein secondary structure are sought by many investigators, each of the techniques currently used suffers from significant shortcomings. Quantitative estimates based on derivative spectra are particularly suspect. The less-subjective quantitative methods based on pattern recognition algorithms appear to be more reliable, but they are not without problems. The major strengths of IR spectroscopy lie in the detection of relative differences in secondary and tertiary structure induced by external factors such as ligand binding and protein activation, and the ability to conduct studies on complex lipid-protein mixtures, areas that should be more actively pursued.

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