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# Determination of Protein Secondary Structure by Fourier Transform Infrared Spectroscopy: A Critical Assessment†

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The ultimate goal of structural studies of proteins is to gain insight into protein three-dimensional structure at high-resolution level. This can often be accomplished by the application of techniques such as X-ray crystallography or multidimensional nuclear magnetic resonance (NMR) spectroscopy. However, high-resolution studies of proteins are not always feasible. For example, crystallographic studies require high-quality single crystals which for many proteins (e.g., the vast majority of membrane proteins) are not available. Furthermore, the question arises as to whether the relatively "static" structure in single crystals adequately represents the protein conformation in a complex and dynamic environment of living cells. There is a growing realization [e.g., Martinek et al. (1989)] that in vivo most proteins act in an *interfacial* environment where they form dynamic complexes with biological membranes, nucleic acids, polysaccharides, or other proteins. Aqueous buffers, from which protein crystals are usually grown, do not necessarily mimic well the conditions of protein functioning in vivo. NMR offers a somewhat better flexibility in studying protein structure in "biologically relevant" environments. However, the interpretation of NMR spectra of larger proteins is very complex, and the assignment of interproton distances generated by the NMR experiment is not always feasible; at present the technique is restricted to small proteins of less than approximately 15–20 kDa.

The practical limitations encountered in high-resolution structural studies of proteins stimulate continual progress in the development and improvement of "low-resolution" spectroscopic methods (e.g., circular dichroism, various branches of vibrational spectroscopy) which provide global insight into the overall secondary structure of proteins without being able to establish the precise three-dimensional location of individual structural elements. One of such techniques is Fourier transform infrared (FT-IR) spectroscopy. Infrared spectroscopic studies of proteins have advanced greatly over the past decade, and now the technique experiences a rapid growth in popularity. One particular advantage of the FT-IR method is its apparent easiness and rapidity of acquiring high-quality spectra from very small amounts of proteins (~100 μg). Furthermore, infrared spectroscopy allows studies of proteins in a variety of environments, including optically turbid media. This renders the technique uniquely well-suited to probe the structure of membrane proteins as well as of protein assemblies, i.e., the systems which are notoriously difficult to study by other spectroscopic methods.

Despite a well-recognized conformational sensitivity of protein infrared bands, the analysis of the spectra in terms of protein secondary structure is not straightforward and presents serious conceptual and practical problems. It is felt by the present authors that some of these difficulties and potential pitfalls are often not fully realized by the practitioners of the technique. Particularly controversial is the "quantitative" aspect of the infrared method. It was claimed that some types of analysis of infrared data can provide highly accurate quantitative estimates of the secondary structure content, with a standard deviation as low as 2–3% with respect to the corresponding X-ray structures. However, the general validity of such claims still remains to be verified. In the following we will discuss briefly the methodologies currently used to analyze infrared spectra of proteins. We shall critically assess the validity of the different approaches and point out potential

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sources of error which have to be recognized to prevent fallacious interpretation of the spectroscopic data.

## FROM AMIDE BANDS TO PROTEIN SECONDARY STRUCTURE

Infrared spectra of proteins and polypeptides exhibit a number of so-called amide bands which represent different vibrations of the peptide moiety. Current understanding of such spectra is based largely on normal coordinate analysis which was pioneered by Miyazawa and associates (Miyazawa et al., 1958; Miyazawa, 1967) in the milestone work on *N*-methylacetamide and was subsequently extended to study more complex systems. Detailed description of various approaches used in theoretical analysis of vibrational spectra of peptides and proteins, as well as of the difficulties and limitations of normal-mode calculations, may be found in the excellent review of Krimm and Bandekar (1986). Normal-mode calculations have proven remarkably successful in analyzing vibrational spectra of relatively simple peptides. A notable illustration of the power of normal-mode analysis of amide band spectra is the study of Naik and Krimm (1986a,b) on gramicidin A, which allowed determination of the structure of this peptide antibiotic in a variety of environments. Normal-mode calculations have also provided a theoretical basis for understanding empirical spectra–structure correlations of more complex systems such as large heteropolypeptides and proteins. However, despite continual progress, the application of rigorous theoretical analysis to the study of such complex systems is in its infancy.

Of all the amide modes of the peptide group, the single most widely used one in studies of protein secondary structure is the amide I. This vibrational mode originates from the C=O stretching vibration of the amide group (coupled to the in-phase bending of the N—H bond and the stretching of the C—N bond) and gives rise to infrared band(s) in the region between approximately 1600 and 1700 $cm^{-1}$. The major factors responsible for conformational sensitivity of amide bands include hydrogen bonding and the coupling between transition dipoles [e.g., Krimm and Bandekar (1986) and Bandekar (1992)]. The transition dipole coupling leads to the splitting of the amide I mode. The magnitude of this splitting depends on the orientation and distance of interacting dipoles and thus provides information about geometrical arrangements of peptide groups in a polypeptide chain. The relationship between the position of the amide I band and the type of secondary structure may be best recognized by analyzing infrared spectra of simple homopolypeptides that fold into well-defined and often homogeneous (i.e., purely $\alpha$-helical or purely $\beta$-sheet) structures. However, in contrast to homopolypeptides, proteins usually fold into complex three-dimensional structures which consist of a variety of domains containing polypeptide segments folded into different types of secondary structure. Since each of these conformational entities contributes to the infrared spectrum, the observed amide I band contours are complex composites: they consist of many overlapping component bands that represent different structural elements such as $\alpha$-helices, $\beta$-sheets, turns, and nonordered or irregular structures. The fundamental difficulty encountered in the analysis of such composite band contours arises from the fact that the width of the contributing component bands is usually greater than the separation between the maxima of adjacent peaks. As a consequence, the individual component bands cannot be resolved and/or identified in the broad contours of experimentally measured spectra, and the extraction of structural information encoded in these infrared bands requires extensive mathematical manipulation of the experimental data. The methods currently used to analyze the infrared spectra of proteins can be classified into two categories: those based on band narrowing and decomposition of the amide I band contour into its underlying components (sometimes referred to as "frequency-based" approaches) and those based on the principle of "pattern recognition".

*Approaches Based on Band Narrowing.* A widely used approach to extract information on protein secondary structure from infrared spectra is linked to the computational procedure of Fourier deconvolution. This procedure, sometimes referred to as "resolution enhancement", decreases the widths of infrared bands, allowing for increased separation and thus better identification (visualization) of the overlapping component bands present under the composite band contour (Kauppinen et al., 1981). Increased separation of the overlapping bands can also be achieved by calculating the $n$th derivative of the absorption spectrum, either in the frequency domain or through mathematical manipulations in the Fourier domain (Cameron & Moffatt, 1987). A distinct advantage of the Fourier deconvolution method is that it introduces less distortion to the spectra. In particular, it does not affect the integrated intensities (areas) of the individual component bands.

For the discussion of the theoretical aspects of Fourier deconvolution and Fourier derivation as applied to complex infrared band contours, the reader is referred to original reports (Kauppinen et al., 1981; Cameron & Moffatt, 1987). Despite their apparent simplicity, the procedures of band narrowing present a number of experimental problems which are not fully appreciated by some investigators and thus lead to frequently encountered errors. In particular, the practitioner of the technique should be aware that both Fourier deconvolution and derivation procedures amplify the noise significantly. Therefore, the degree of band narrowing (the resolution enhancement factor in Fourier deconvolution or the degree of derivation in derivative spectroscopy) is limited by the signal-to-noise ratio of the spectrum. Great care should be exercised to avoid "overdeconvolution", for the amplified noise can be easily misinterpreted as a real band. Another commonly encountered problem is related to the presence of atmospheric water vapor which gives rise to narrow absorption bands in the region overlapping the amide I mode. While these bands are often very weak (or even "invisible") in the original spectrum, because of their relative sharpness compared to that of the amide bands they become disproportionally amplified upon Fourier deconvolution or derivation. These features may appear in the resolution-enhanced spectrum as artifacts that are indistinguishable from the real components of the protein amide band. Therefore, every effort should be made to eliminate water vapor by careful purging of the spectrometer with dry air or nitrogen. If necessary, residual water vapor absorption may be compensated by spectral subtraction. As a practical guideline, the absence of artifacts arising from the amplification of noise or water vapor bands should be carefully verified by examining the resolution-enhanced spectrum in the region where no protein infrared bands are expected.

Artifacts may also appear as a result of the presence of highly infrared absorbing contaminants or counterions that are not an integral part of the peptide or protein. Particular care should be exercised in studies with synthetic peptides. They often contain trifluoroacetate which is introduced during the purification. This counterion gives rise to a strong IR

band around 1673 $cm^{-1}$ (Surewicz & Mantsch, 1989), and it is not uncommon to erroneously include this band as part of the amide I mode.

A critical step in the interpretation of infrared spectra of proteins is the assignment of the amide I component bands to different types of secondary structure. This assignment is guided by theoretical calculations and by spectra–structure correlations established experimentally for model peptides and proteins of known three-dimensional structure. The general tendency, particularly in quantitative curve-fitting analysis, has been to assign in a unique way the individual bands resolved in the amide I region to well-defined types of secondary structure. However, theoretical analyses [e.g., Krimm and Bandekar (1986) and Torii and Tasumi (1992)], as well as an increasing number of experimental observations, call for caution in applying the empirical rules for unique structural assignment of the amide bands of proteins. For example, recent model calculations on the amide I bands of globular proteins (Torii & Tasumi, 1992) have demonstrated that the spectral contributions of structural elements other than $\alpha$-helices and $\beta$-sheets spread over the wide wavenumber region. Infrared absorption of these elements cannot be separated into individual contributions of turns and unordered structures.

Amide I bands centered between approximately 1650 and 1658 $cm^{-1}$ are generally considered to be characteristic of $\alpha$-helical structures, an assignment supported by theoretical calculations and experiments with a large number of $\alpha$-helical peptides and proteins. However, recent studies (Surewicz et al., 1988; Prestelski et al., 1991a; Wilder et al., 1992) have drawn attention to proteins which show bands in the 1650–1658-$cm^{-1}$ region but for which no appreciable $\alpha$-helical structure could be detected by other experimental techniques. At least in some cases (Prestelski et al., 1991a,b; Wilder et al., 1992), bands around 1655 $cm^{-1}$ were attributed to large loop structures rather than to $\alpha$-helices. Another complication associated with the uniqueness of the assignment of "$\alpha$-helical bands" is presented by the observation that for a small number of proteins known to be largely $\alpha$-helical the major amide I component is shifted to wavenumbers somewhat below 1650 $cm^{-1}$ (Trewhella et al., 1989; Jackson et al., 1991). This shift may be caused by unusual amide group–solvent interactions in these proteins or (if the spectra are measured in $D_2O$) by the accessibility of $\alpha$-helices to essentially complete NH/ND exchange. Helical structures of another type, $3_{10}$ helices, are much less common and have been less well studied by infrared spectroscopy. In the spectrum of cytochrome $b_5$, the band around 1639–1640 $cm^{-1}$ was assigned to this structure (Holloway & Mantsch, 1989). However, synthetic $\alpha$-aminobutyric acid-containing peptides, which are known to form $3_{10}$ helices, show strong amide I bands at 1662–1663 $cm^{-1}$ (Kennedy et al., 1991). A general problem encountered in the assignment of $\alpha$-helical structures is that their amide I bands differ only slightly from those of random or unordered structures. The problem can often be remedied by recording the spectra in $D_2O$. The shift of the amide I band associated with backbone deuteration is usually much faster and more extensive for the unordered peptide segments than for $\alpha$-helices.

Amide groups in $\beta$-pleated sheets give rise to highly diagnostic bands between approximately 1620 and 1640 $cm^{-1}$ (Krimm & Bandekar, 1986; Susi & Byler, 1987), although in some cases "$\beta$-bands" are shifted even below 1620 $cm^{-1}$ (Surewicz & Mantsch, 1988). For many proteins more than one component is observed in the 1620–1640-$cm^{-1}$ region. This multiplicity reflects differences in hydrogen-bonding strength as well as differences in transition dipole coupling in different $\beta$-strands and is of potential diagnostic value. However, no correspondence has been established so far between various $\beta$-bands and specific types of $\beta$-structure (Susi & Byler, 1987). Although bands below $\sim$1640 $cm^{-1}$ seem to be present in the spectra of all $\beta$-structure-containing proteins and peptides, at issue is the unambiguity of the assignment of these bands in the spectra of unknown proteins. Especially disconcerting is the presence of weak bands around 1630 $cm^{-1}$ in the spectra of a few proteins which by crystallographic criteria contain very little or no $\beta$-structure. It has been proposed (Byler & Susi, 1986) that these bands may represent short extended segments that do not form sheets in a generally defined sense. However, recent model calculations for myoglobin (Torii & Tasumi, 1992) indicate that such an interpretation is not necessarily correct. These calculations suggest that bands below 1640 $cm^{-1}$ may also originate from vibrational motions of $\alpha$-helices. Parallel and antiparallel $\beta$-structures are in principle distinguishable as only the the latter are characterized by a large splitting of the amide I mode caused by interstrand interactions (transition dipole coupling) (Krimm & Bandekar, 1986). However, in the spectra of proteins identification of the high-frequency "$\beta$-component", characteristic of antiparallel sheets, is often hampered by the overlapping of this band with contributions from turns and unordered structures (Torii & Tasumi, 1992).

Numerous attempts have been made to extract quantitative information on protein secondary structure from the resolution enhancement-guided analysis of amide I band profiles. Byler and Susi (1986) proposed a procedure which involves curve fitting of deconvolved amide I band contours as a linear combination of individual component bands by iterative adjustment of heights, widths, and position of these bands. The resulting fractional areas of the bands assigned to different types of secondary structure (i.e., $\alpha$-helices, $\beta$-sheets, turns, and irregular structures) were assumed to represent percentages of these structures in a given protein. This procedure was claimed to provide highly accurate estimates of protein secondary structure and was adopted, with various modifications, by other investigators [e.g., Surewicz and Mantsch (1988) and Goormaghtigh et al. (1990)]. However, more recent studies indicate that this approach is not without serious drawbacks, and some cautionary remarks are warranted.

(i) While the curve-fitting analysis could have indeed provided accurate (i.e., closely corresponding to X-ray data) estimates of the secondary structure of some selected "model" proteins [e.g., Byler and Susi (1986)], the lack of uniqueness in band assignment (see above) raises serious doubts as to the general validity of this method.

(ii) An assumption implicit in this type of quantitative analysis is that the molar absorptivities of the amide I bands assigned to different conformers are equal. This assumption has not been adequately tested and appears to be not always correct. An illustrative (though possibly somewhat extreme) example is provided by poly(L-lysine), for which molar absorptivities of different conformers may differ by as much as 30% (Chirgadze et al., 1973; Jackson et al., 1989; Mantsch et al., 1989).

(iii) Mathematically, the curve-fitting analysis of amide band contours contains an element of subjectivity (initial choice of input parameters) and uncertainty. The result of fitting with a large number of adjustable parameters ($3n + 1$, where $n$ is the number of component bands) is not necessarily unique.

(iv) One version of the method proposed fitting of band areas in second-derivative spectra (Dong et al., 1990). This

introduces an additional source of error due to subjectivity and uncertainty in the definition of the baseline. Furthermore, since derivation does not preserve the integrated areas of individual components, we feel that any type of analysis based on fitting of derivative spectra should be discouraged.

The difficulties highlighted above clearly point to serious limitations and shortcomings of the curve-fitting analysis as a generally valid method to assess quantitatively the "absolute" content of protein secondary structure. However, methods based on band narrowing, if used cautiously, provide a sensitive diagnostic tool for monitoring, in relative terms, the nature of changes in the conformation of the protein backbone.

*Approaches Based on Pattern Recognition.* Recently, a number of closely related methods have been proposed that avoid spectral deconvolution and which are based on the use of a calibration matrix of infrared spectra of proteins of known (i.e., determined by X-ray crystallography) secondary structure. This type of data treatment is conceptually very similar to that used in the analysis of circular dichroism or Raman spectra of proteins (Compton & Johnson, 1986; Williams, 1983). Using the partial least-squares method, Dousseau and Pezolet (1990) obtained best results when both amide I and amide II regions were used to generate the calibration set and when it was assumed that the secondary structure of proteins is composed of four types: ordered helix, disordered helix, $\beta$-sheet, and undefined conformation. The distinction between ordered and disordered helices was found to be necessary in order to account for the observation that vibrational frequencies of the amide groups in short helices are different from those in extended helical structures. Thus, the first two and the last two residues in an $\alpha$-helical segment were considered to form a disordered helix while the remaining residues form an ordered helix. Lee et al. (1990) employed the methodology of factor analysis and multiple linear regression. In this case, best results were obtained when only amide I bands of normalized infrared spectra of "known" proteins were used to construct the calibration matrix. The most recent addition in this category is the approach of Sarver and Kruger (1991), who adopted the matrix method originally developed to analyze far-UV CD spectra (Compton & Johnson, 1986).

All of the above methods avoid certain difficulties associated with the frequency-based approaches. In particular, they eliminate the element of subjectivity implicit in resolution enhancement procedures and do not require that individual component bands be assigned to different types of secondary structure. However, these methods are not without shortcomings. Normalization procedures used in the partial least-squares and factor analysis methods are based on the assumption of equal absorptivity of amide bands, regardless of the type of secondary structure (see above). More importantly, the pattern recognition approaches do not address the fundamental problem of the lack of uniqueness in the relationship between amide bands and the types of secondary structure. These approaches are related to the use of a bank of reference spectra of proteins of known three-dimensional structure. The analysis of the unknown protein may be successful only as long as the spectral features characteristic of this protein can be recognized in the spectra of the calibration set. Any unusual structural property of the protein under study may result in an erroneous estimation of the secondary structure, even though the experiment is carefully performed and the mathematical treatment is formally correct. Finally, the results of any quantitative analysis (using either pattern recognition or resolution enhancement methods) may be significantly distorted by the contribution into the amide I region of the spectrum of the amino acid side-chain absorption. Detailed analysis of infrared absorption bands of individual amino acid residues may be found in the papers of Chirgadze et al. (1975) and Venyaminov and Kalninin (1990). Amino acids with by far the highest absorption coefficients in the 1600–1700-$cm^{-1}$ region are asparagine and glutamine. Other residues that give rise to infrared bands overlapping the amide I region include side chains of arginine and, to a lesser extent, tyrosine, aspartic acid, glutamic acid, and lysine. Quantitative contribution of side-chain absorption to the spectrum in the amide I region depends on the amino acid composition of a particular protein. The typical contribution may be estimated as 15–20% of the total band intensity between 1600 and 1700 $cm^{-1}$, although it may be higher if the protein is particularly rich in amino acids such as Asn or Gln. Ideally, the side-chain group absorption should be subtracted from the spectrum prior to the analysis of amide bands in terms of protein secondary structure.

Another difficulty may arise from the greatly diminishing accuracy of the pattern recognition methods when applied to the spectra of proteins in $D_2O$. Infrared studies in $H_2O$ require subtraction of a strong water band which overlaps the amide I region. Digital subtraction of this strong band, although in principle possible (Powell et al., 1986; Dousseau & Pezolet, 1989), may introduce an additional source of error and is particularly difficult for membrane-associated proteins.

## CONCLUSIONS AND FUTURE DIRECTIONS

The advances in infrared spectroscopic instrumentation, coupled with the development of increasingly sophisticated methods of numerical data analysis, have now evoked very high expectations from infrared spectroscopy as a universal tool to analyze, in a quantitative manner, the secondary structure of proteins. However, experience shows that the methodologies that are presently used for the quantitative estimation of protein secondary structure content from infrared spectra are not without serious shortcomings. Limitations in the quantitative assessment of protein secondary structure appear to be common to all low-resolution spectroscopic techniques, including circular dichroism. Since the potential sources of error in the circular dichroism and infrared spectroscopic methods are different, these two techniques are highly complementary and, if at all possible, should be used in conjunction.

Despite limitations in assessing quantitatively absolute protein secondary structure, infrared spectroscopy provides a good tool for monitoring, in relative terms, even subtle changes in the conformation of the polypeptide backbone. Selected examples of the types of protein conformational changes that can be probed by the infrared method include conformational transitions induced by ligand binding (Trewhella et al., 1989; Kennedy et al., 1990; Surewicz et al., 1990) and those brought about by changes in environmental conditions such as temperature (Muga et al., 1991), pressure (Wong & Heremans, 1988), or pH (Fraser et al., 1991). The technique has proven particularly valuable in studies of membrane-associated proteins and peptides which are often not amenable to other spectroscopic measurements [for recent reviews, see Chapman et al. (1989) and Surewicz and Mantsch (1990)]. The added advantage of infrared spectroscopy in studies of the latter systems is its ability to probe, through the measurement of linear dichroism in macroscopically oriented samples, the average orientation of membrane-associated helices or $\beta$-sheets [e.g., Rothschild et al. (1980) and Nabedryk et al. (1988)]. Polarized infrared studies of membrane-associated proteins

and peptides are particularly powerful when measurements are performed in the attenuated total reflection (ATR) mode (Braiman & Rothschild, 1988; Brauner et al., 1987; Cornell et al., 1989; Fringeli et al., 1989).

Further improvements in the sensitivity of detecting changes in protein conformation are likely to occur as a result of a more general use of the strategy of difference infrared spectroscopy. In this approach, spectra of the protein in two different states are measured. These spectra are subsequently subtracted to yield a difference spectrum that represents only a particular structural change, with the bulk protein absorption being removed. Since the difference signal is usually very weak compared to the background absorption due to bulk protein and the solvent, reproducible results can often be obtained only if the spectra to be subtracted are recorded in a rapid succession and the protein functional change is triggered in a "noninvasive" manner, i.e., with minimal sample manipulation and without removing it from the spectrometer. The above requirements have so far restricted this methodology largely to probing chromophoric proteins in which transitions between different states are triggered by light (e.g., bacteriorhodopsin, rhodopsin) (Rothschild et al., 1987; Braiman & Rothschild, 1988). However, recent reports demonstrate that the strategy of difference spectroscopy can be extended to study a much larger class of proteins. Examples of noninvasive triggering methods that can be used to generate protein difference spectra include application of electrochemical potential (for studying redox enzymes) (Moss et al., 1990), photolytic release of caged ligands (Buchet et al., 1991; Barth et al., 1991; Gorne-Tschelnokow et al., 1992), and voltage gating (for studying membrane channels). Furthermore, a recent study with nicotinic acetylcholine receptor incorporated into planar multilamellar lipid films (Baenziger et al., 1992) indicates that highly reproducible difference infrared spectra may be obtained when the membrane film is immobilized on the germanium plate and the protein function is triggered by sequentially flowing a buffer either with or without the water-soluble ligand. This suggests that infrared difference spectroscopy should find an increasingly wide range of applications as a sensitive tool for probing ligand-induced conformational changes in receptors and other membrane proteins.

Another promising approach is linked to the introduction of isotopic labels at specific sites of peptides or proteins. The most useful appears to be the substitution of $^{13}C$ for $^{12}C$. Since for a localized amide I mode the replacement of a $^{12}C$ amide with a $^{13}C$ amide reduces the amide I frequency by 35–40 $cm^{-1}$, comparison of the spectra of labeled and nonlabeled molecules should allow the identification and analysis of the amide I bands that originate exclusively from the labeled site. This approach should thus increase considerably the information content of infrared spectra, by allowing the location of particular secondary structure within the polypeptide chain.

Another potentially useful isotopic substitution is $^{15}N$ for $^{14}N$. While this substitution has relatively little effect on the frequency of the amide I band, it results in a significant shift of the amide II band. The amide II mode is particularly useful for studying hydrogen–deuterium exchange. Uniform $^{15}N$ labeling has been proposed for obtaining structural information about protein–protein interaction through the measurement of the rate of hydrogen–deuterium exchange of one protein in the presence of the other (Haris et al., 1992).

The strategy of isotopically enhanced infrared spectroscopy, originally proposed by Krimm and Bandekar (1986), has recently been used by Tadesse et al. (1991) to locate different elements of secondary structure in conformationally heterogeneous model peptides. This approach was subsequently extended, through the analysis of the effect of isotopic substitutions on the transition dipole coupling, to identify individual amino acid residues in antiparallel $\beta$-sheets in fibrillar proteins (Halverson et al., 1991). Infrared spectroscopic studies with isotopically labeled peptides provided a basis for developing structural models for the $\beta$-amyloid protein associated with Alzheimer's disease (Halverson et al., 1991) and for the islet amyloid polypeptide (Ashburn et al., 1992). Although still in the developmental stage, the methodology of isotope-edited infrared spectroscopy is of a great potential value. It should open up new avenues in conformational analysis of proteins and peptides in solution as well as in biophysical studies of protein–ligand, protein–membrane, and protein–protein interactions (Haris et al., 1992).

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