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A Method for Assigning Hydrogen Bonds Using Isotope Effects in Nuclear Magnetic Resonance and Infrared Spectroscopy

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Abstract: A method is described for assigning a hydrogen bonded or nonbonded character to each peptide NH in a molecule by obtaining the nmr and ir spectrum simultaneously at various time intervals throughout an H–D exchange. Resonance frequency shifts arising from the isotopic exchange occur in both spectra. The changes in the nmr spectrum are used to determine the amino acid residue to which an exchanged NH belongs and the concomitant changes in the ir spectrum are used to determine whether the exchanged NH was free or intramolecularly hydrogen bonded.

It is desirable to know the conformation of enzymes and oligopeptides in solution in order to relate changes in their conformation induced by chemical modifications to changes observed in their pharmacological properties and thereby obtain the structure *vs.* biological activity relationships. Spectroscopic techniques such as nuclear magnetic resonance (nmr) and infrared (ir) spectroscopy have been applied to the problem of obtaining the structure in solution.

One of the most important types of information sought is which NH groups are hydrogen bonded and which are not. In nmr studies there are two methods commonly used to obtain this information once the peaks in the nmr spectrum have been assigned to particular NH protons in the molecule.

The first method is to add D_2O to the solution and follow the rate of deuterium exchange of the NH to ND as manifested by a decrease in area of the NH peak in the ^1H nmr spectrum. This decrease occurs because H and D resonate at quite different frequencies in a fixed magnetic field. Hydrogen bonds are then ascribed to the slower exchanging NH protons. The use of isotopic exchange to obtain conformational information has been employed by Stern, Gibbons, and Craig in their measurements on Gramacidin S and by other researchers.[1-6] There are several problems associated with interpreting the results of such experiments. The method cannot determine whether protons which exchange slowly are hydrogen bonded or merely inaccessible to the D_2O and whether they exchange rapidly because they are nonhydrogen bonded or because they are in rapid equilibrium between two different conformations. In addition there is no intrinsic means of determining how long the half-life for the exchange should be in order to ascribe a hydrogen bond to that proton.

The second method is evaluation of the temperature dependence of the chemical shift ($d\delta/dT$) of an NH proton peak.[7-12] In this approach an NH proton peak which undergoes no upfield shift on heating ($d\delta/dT = 0$) is said to be intramolecularly hydrogen bonded while the molecule *N*-methylacetamide, which is used as a model for the nonintramolecularly bonded case, has a $d\delta/dT$ value of 6×10^{-3} ppm/°C in dimethyl sulfoxide (DMSO) or aqueous solution.[13] There are also problems associated with interpreting the results of this type of experiment. The method cannot determine whether a proton has a low $d\delta/dT$ value as a result of a lack of bonding to the solvent because the NH is intramolecularly hydrogen bonded or because the NH proton is inaccessible to the D_2O; nor does it contain any intrinsic means of determining how near zero the $d\delta/dT$ value of an NH group must be for it to be assigned to a bonded rather than a free state. In addition there is no explanation for the observed $d\delta/dT$ values less than zero[9] or greater than 6×10^{-3} ppm/°C[11] and no means to determine the effect on δ and therefore on $d\delta/dT$ of reorientation of nearby magnetically anisotropic groups.

Thus, each of the two nmr methods for establishing hydrogen bonded NH groups have inherent problems in the interpretation of the results. There is the additional difficulty that the results of the two methods are occasionally directly opposed to each other. For example, in valinomycin the NH group which only slowly exchanges on adding D_2O is found to have the larger rather than the smaller $d\delta/dT$ value.[5]

From ir studies there is a method commonly used to obtain information on the presence of hydrogen bonded amides. The data of Richards and Thompson[14] on bonded and nonbonded amides in CCl_4 and $CHCl_3$ show that a nonbonded CONHR group will have an NH stretching frequency about 3440 cm^{-1} while a hydrogen bonded NH in a CONHR moiety will occur about 3350 cm^{-1}. A number of researchers have examined the infrared spectra of oligopeptides to determine the occurrence of hydrogen bonding.[15-18] For example, the potassium complex of valinomycin in $CHCl_3$ solution shows only one band at 3309 cm^{-1} which led to a proposed structure in which all the NH groups were

hydrogen bonded,[16] However, there is a problem associated with the interpretation of the results of this type of experiment. If all the NH groups are either hydrogen bonded or nonhydrogen bonded so that only one band appears in the ir spectrum, then the bonded or nonbonded character of each NH can be determined as they are all the same, whereas in the more usual case, there is a mixture of bonded and non-bonded NH groups giving rise to several bands, and hence no information on which NH groups are bonded and which are nonbonded can be obtained.

The foregoing discussion illustrates the need for a better experimental method of assessing hydrogen bonding in proteins and peptides in solution. In the present paper we combine the nmr and ir methods and take advantage of the isotope effects in both forms of spectroscopy to develop a new and more powerful way of determining which NH groups are hydrogen bonded.

Experimental Section

Materials. *tert*-Butyloxycarbonylglycine 2,4,5-trichlorophenyl ester (Boc-Gly-tcp) was a gift from Dr. G. T. Young. Valinomycin and actinomycin D were purchased from Sigma Chemical Co., Kingston-upon-Thames, England. The nmr spectra of valinomycin[3] and actinomycin[2,4,5,19,20] matched those previously published. The deuterated chloroform ($CDCl_3$) used in these experiments was obtained from Ciba-Geigy Chemicals and was 99.9 mol % deuterium.

The nmr spectra were obtained on a Varian HR220 spectrometer at $19 \pm 1°$. In some instances the spectra were time averaged using a C1024 Multichannel analyzer. All chemical shifts are quoted as δ ppm from internal tetramethylsilane (TMS). The infrared spectra were obtained on a Perkin-Elmer Model 225 infrared spectrometer using AgCl windows and a Teflon spacer to produce a path length of 1 mm.

Results

The method employs the simultaneous observation of both the ir and nmr spectra throughout a H–D exchange. The first step is to use the usual means (spin decoupling, peak multiplicity, peak areas, chemical shifts, etc.) to assign each NH peak in the ^1H nmr spectrum to a proton in the oligopeptide. Both the nmr and ir spectra are obtained. A small aliquot of D_2O was added to the sample to give around a 5% v/v mixture with $CDCl_3$. Although the two solvents are not miscible, when they are in contact enough D_2O is present in the $CDCl_3$ to stimulate the exchange. The $CDCl_3$ soluble portion of the sample is divided and the nmr and ir spectra are obtained at the same time after which the separate solutions are recombined and brought into contact with D_2O so further exchange proceeds at the same rate for the entire sample. After a period of time the nmr spectrum will show the absence of a peak corresponding to an NH which has exchanged to ND, Since all the NH peaks in the nmr spectrum have been assigned to amino acid residues in the oligopeptide, the identity of the exchanged NH group is known. At this point in the exchange the ir spectrum will show a decrease in area in the amide NH stretching frequency region (about 3300 to 3500 cm^{-1}). The isotopic substitution of ND for NH results in a frequency shift and the ND stretching frequency occurs about 2500–2600 cm^{-1}.[21] By noting whether the decrease in area in the ir spectrum occurs at the hydrogen bonded stretching frequency ($\sim$3350 cm^{-1}) or the nonbonded frequency ($\sim$3440 cm^{-1}), the bonded or free character of the amino acid residue NH proton, whose identity is known from the effects observed in the nmr spectrum, is established.

The spectra of Boc-Gly-tcp in Figure 1 illustrate this experiment. Before exchange (0 min), the nmr spectrum shows the Gly-NH and $C^\alpha H$ at 5.06 and 4.23 ppm, respectively, and the ir spectrum shows a band about 3460 cm^{-1}
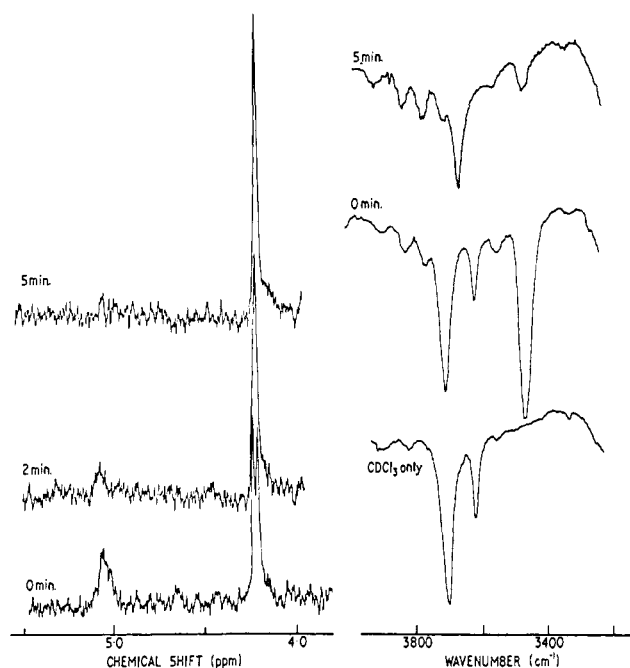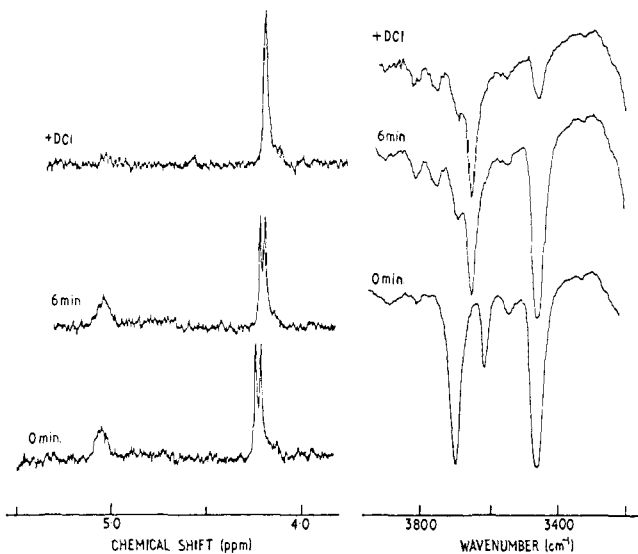

Figure 1. The nmr and ir spectrum of Boc-Gly-tcp (0.02 *M*) in $CDCl_3$ at the D_2O exchange times shown: 0 min corresponds to no added D_2O and 5 min is the spectrum 5 min after addition of D_2O.
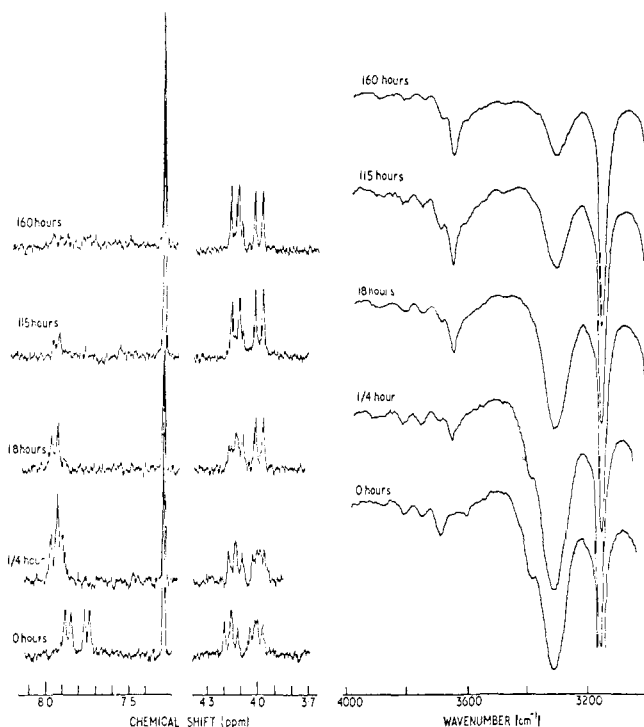
which indicates that an NH is nonbonded. Shortly after exchange (5 min), the nmr spectrum shows no NH peak and the $C^\alpha H$ peak is an apparent singlet as the ND splitting of the $C^\alpha H$ is much smaller. The ir spectrum shows a decrease in area at 3460 cm^{-1}. Coupling these two pieces of data, one learns that the exchanged NH was Gly and that it was nonhydrogen bonded.

Since the rate of exchange of the NH protons is both acid and base catalyzed, the presence of trace amounts of these materials in this unbuffered medium strongly affects the half-life of the exchange ($t_{1/2}$). It is unlikely that the rate constants (k) obtained will have the same absolute values in successive experiments although the values for each NH in the molecule will have the same relative relationship to each other. An example of the difference in rate of exchange possible on the same sample is seen in Figure 2. Here Boc-Gly-tcp hardly exchanges in the same time interval for which complete exchange occurred in Figure 1; however, a drop of 20% DCl in D_2O brought about complete exchange in 5 min.

The strength of this method is particularly apparent in studying compounds having several different types of NH present such as valinomycin and actinomycin. The nmr spectrum of valinomycin shows two NH peaks: one at 7.75 ppm (smaller *J*) and one at 7.86 ppm (larger *J* value). It has been found previously by use of ^{15}N labeling that in CCl_4 the peak having the larger *J* value is that of D-Val.[16] Thus, the 7.75-ppm peak is the NH of L-Val and the 7.86-ppm peak is the NH of D-Val. The $C^\alpha H$ peaks can be assigned by watching the change in multiplicities of the peaks during the exchange with the following result: the $C^\alpha H$ at 4.00 ppm is coupled to the NH at 7.75 ppm (L-Val) and the $C^\alpha H$ at 4.15 ppm is coupled to the NH at 7.86 ppm (D-Val). The ir spectrum of valinomycin shows a large band at 3315 cm^{-1} characteristic of a hydrogen bond and a shoulder at 3380 cm^{-1} characteristic of a weak bond or the free state. On adding D_2O/DCl the NH protons exchange to ND resulting in decreases in the area of the peptide NH peaks in the ^1H nmr and in the ir spectra (Figure 3). The NH peaks exchange at different rates. After 18 hr the NH
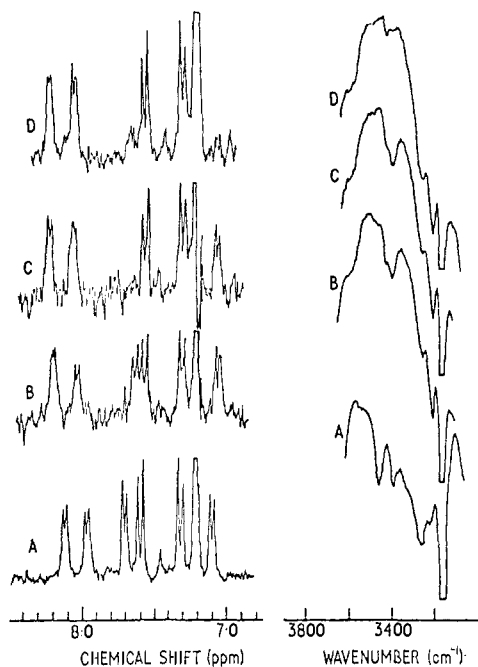
Figure 2. The nmr and ir spectrum of Boc-Gly-tcp (0.02 M) in CDCl$_3$ with the D$_2$O exchange times shown in a manner similar to Figure 1. An extra spectrum following addition of DCl is also shown.

Figure 4. The nmr and ir spectrum of actinomycin (0.008 M) in CDCl$_3$ with the exchange shown in a manner similar to Figure 1. The spectra labeled A correspond to 0 min and no added D$_2$O while those labeled B, C, and D correspond to increasing time and extent of exchange.

Figure 3. The nmr and ir spectrum of valinomycin (0.008 M) in CDCl$_3$ with the exchange times after addition of D$_2$O/DCl shown in a manner similar to Figure 1

and 8.12 ppm for the two Val. The ir spectrum of actinomycin shows bands between 3200 and 3500 cm^{-1} for the NH stretching frequencies of these amides and additionally the primary amine group on the chromophore. Thus the NH groups are a mixture of hydrogen bonded and nonbonded types. On adding D$_2$O (Figure 4B) the amino group will exchange first, resulting in only a negligible change in the nmr spectrum of the peptide NH peaks. However, there is a decrease in area of the ir spectrum both at 3460 and 3340 cm^{-1} which corresponds to the bands for the primary amine. On further exchange (Figure 4C) the NH peak at 7.71 ppm (Thr) in the nmr spectrum disappears and a corresponding decrease in the area of the peak at 3410 cm^{-1} (nonhydrogen bonded) in the ir spectrum occurs. Hence, one concludes that the NH of one Thr is nonbonded. The second NH to exchange (Figure 4D) is that of the other Thr residue (7.10 ppm and 3410 cm^{-1}), hence it also is not hydrogen bonded. The remaining two Val peaks both correspond to the remaining ir bands near 3230 cm^{-1} and therefore the NH group of both Val residues is hydrogen bonded.

Discussion

A method has been developed for assigning intramolecular hydrogen bonded or nonbonded character to each peptide NH in a molecule by obtaining the nmr and ir spectra simultaneously at various time intervals throughout an H-D exchange. Resonance frequency shifts arising from the isotopic exchange occur in both spectra resulting in the disappearance from their previously observed positions of the NH stretching frequency bands in the ir spectrum and the proton NH peak in the ^{1}H nmr spectrum. Observing the ir spectrum tells one whether the NH that has exchanged is bonded or free while observing the disappearance of the peaks in an assigned nmr spectrum yields the identity of the amino acid residue NH that has exchanged. By correlating the data simultaneously obtained from the two different forms of spectroscopy, one determines whether a particular

of D-Val remains in the ^{1}H nmr spectrum and the corresponding ir spectrum shows the band at 3315 cm^{-1}; hence, the NH of D-Val in this compound is hydrogen bonded. At the same time the NH of L-Val is virtually gone from the ^{1}H spectrum and the corresponding ir spectrum shows the diminishing of the shoulder at 3380 cm^{-1}. Hence, the NH of L-Val in some of the conformations of this molecule is either free or more weakly hydrogen bonded than the NH of D-Val as has been shown in Figure 6 of ref 16.

The nmr spectrum of actinomycin (Figure 4A) shows four NH peaks: 7.10 and 7.71 ppm for the two Thr and 7.96

NH in the molecule is or is not hydrogen bonded. Thus, the simultaneously observed isotope frequency shifts in nmr and ir spectra give more information than is available from adding together the information obtained separately from ir and nmr spectra.

This method then avoids the problems previously encountered in the separate ir or nmr spectroscopic approaches to determining the bonding character of NH groups since values of exchange rates ($t_{1/2}$) or temperature dependence of the chemical shift ($d\delta/dT$) obtained by nmr are no longer needed as criteria for NH bonding. Now that a more definite way of assigning hydrogen bonds has been established, the $t_{1/2}$ and $d\delta/dT$ values can be examined for the additional information which they contain. For example, if an NH group which is shown to be nonhydrogen bonded by the ir/nmr method also has a long $t_{1/2}$, then it may be in a hydrophobic pocket or shielded from the D_2O in the solvent. Further, if an NH group which has a large $d\delta/dT$ value is shown to be intramolecularly hydrogen bonded by the ir/nmr method, this may indicate that a backbone or side chain conformational change is occurring which affects the chemical shift.

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Synthesis and Some Pharmacological Properties of [1-α-Mercaptoacetic acid]-8-lysine-vasopressin and [1-γ-Mercaptobutyric acid]-8-lysine-vasopressin[1,2]

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Abstract: [1-α-Mercaptoacetic acid]-8-lysine-vasopressin and [1-γ-mercaptobutyric acid]-8-lysine-vasopressin, the 19- and 21-membered ring analogs of [1-deamino]-8-lysine-vasopressin, were prepared from two protected polypeptide precursors which had been prepared by the stepwise active ester method. Removal of the protecting groups was accomplished in NH_3 with Na, and oxidative cyclization was performed with CH_2ICH_2I. Both the 19- and 21-membered ring analogs showed significant changes in pharmacological activity as compared to [1-deamino]-8-lysine-vasopressin. [1-α-Mercaptoacetic acid]-8-lysine-vasopressin possessed 1.1 ± 0.1 units/mg of pressor activity, was inhibitory to oxytocin in the avian vasodepressor assay at $\bar{M} = 8.29 \times 10^{-8}$ ($pA_2 = 7.08$), and possessed 3.3 ± 0.3 units/mg of activity in the rat uterus assay. [1-γ-Mercaptobutyric acid]-8-lysine-vasopressin possessed 1.7 ± 0.3 units/mg of pressor activity and was inhibitory to oxytocin in the avian vasodepressor and rat uterus assays at $\bar{M} = 1.28 \times 10^{-8}$ ($pA_2 = 7.89$) and $\bar{M} = 1.80 \times 10^{-7}$ ($pA_2 = 6.74$), respectively.

A striking feature of the various octapeptide hormones which have been isolated from the posterior pituitary gland of mammals, birds, amphibians, and fish is the 20-membered disulfide ring. Several studies have been done on analogs of oxytocin (Figure 1) in which the ring size has been increased in various ways.[3-11] None of these compounds showed more than a slight degree of biological activity. One analog with a decreased ring size of 19 atoms showed a moderate degree of activity.[12] The synthesis of a 21-membered ring directly pertinent to the present communication was accomplished by the formal insertion of a methylene

group at position 1 to form [1-hemi-L-homocystine]oxytocin.[6] This analog showed no avian vasodepressor (AVD) or rat pressor activity and only a slight oxytocic activity. In this analog the substitution of the hemihomocystine for the hemicystine residue leads simultaneously to an increase in the size of the ring and in the separation of the free amino group from the disulfide bond. However, work with [1-hemi-D-cystine]oxytocin[13] which possesses extremely low activities had shown that the steric relationship of the free amino group at position 1 to the rest of the molecule may be critical. The synthesis of the highly potent 1-deamino-oxy-