Hydrogen Bonding Networks in Proteins As Revealed by the Amide $^1J_{NC'}$ Coupling Constant

Nenad Juranić,* Predrag K. Ilich, and Slobodan Macura

Contribution from the Department of Biochemistry and Molecular Biology, Mayo Graduate School, Mayo Clinic and Mayo Foundation, Rochester, Minnesota 55905

Received August 1, 1994[⊗]

Abstract: A regular dependence of the $^1J_{NC'}$ coupling constant on the nature of amide group hydrogen bonding is observed in the model compound, *N*-acetylglycine. Hydrogen bonding of the amide oxygen increases the coupling constant, whereas hydrogen bonding of the amide hydrogen decreases it. This establishes the $^1J_{NC'}$ coupling constant as a useful probe for amide group hydrogen bonding in proteins. From measured $^1J_{NC'}$ coupling constants in human ubiquitin, characteristic sequences of the coupling constant are observed which correlate with the protein secondary structure. In the α-helix, the coupling constant does not vary much (15.3 ± 0.5 Hz). In β-sheets, variations are larger (15.5 ± 1.5 Hz), except in the central region of the parallel and antiparallel β-sheets, where the coupling constant exhibits rather regular values. In this region, significant correlation of the coupling constants within the lines of interstrand N−H···O=C hydrogen bonding is observed, indicating a cooperative polarization of peptide bonds in the H-bonding network. The largest change of the coupling constant is found within reverse turns. At position 2 of reverse turns the coupling constant has the lowest values (13.9 ± 0.8 Hz), whereas at position 4 the values are highest (16.6 ± 0.6 Hz). This change of the coupling constant within the three residues of reverse turns is caused by specific hydrogen bonding of amide groups in the reverse turns. The result indicates that the intraprotein N−H···O=C hydrogen bonds of the main-chain amide groups are weaker than the hydrogen bonds of these groups to water.

Introduction

Hydrogen bonding in macromolecules is highly cooperative and most likely contributes substantially to protein function.[1] Accordingly, characterization of H-bonding networks in proteins is very important for the study of protein structure, dynamics, stability, and folding and of interactions between the protein and other biomolecules. The principal methods for studying hydrogen bonding in solution are spectroscopic, particularly infrared (IR), Raman, and nuclear megnetic resonance (NMR) spectroscopy.[1] However, to study a complex H-bonding network in biologic macromolecules in solution, spectroscopy must allow individual groups involved in H-bonding to be distinguished. Such resolution presently is afforded only by NMR spectroscopy. The most common approaches are use of ^{1}H, ^{13}C, and ^{15}N NMR, which all show a chemical shift dependence on H-bonding.[2] For example, amide ^{1}H chemical shifts are well established as a probe for H-bonding in proteins.[2] However, the relationship of chemical shift to H-bonding is able to reveal only a part of the complex nature of H-bonding.[1] New NMR probes of H-bonding would therefore be of considerable value. In this respect, the nuclear spin−spin coupling between directly bonded nuclei in the amide group, $^1J_{NC'}$, has shown promise.[3]

Complete isotope labeling with ^{13}C and ^{15}N is a standard procedure for the determination of protein structure by NMR spectroscopy.[4] With isotopic enrichment, the measurement of the spin−spin coupling constant between otherwise insensitive nuclei such as ^{13}C−^{15}N becomes feasible.[5] Interest in the determination of coupling constants in proteins is traditionally related to sensitivity of that parameter to molecular conformation.[6] In that context Bax and co-workers considered use of ^{15}N−^{13}C coupling constants in proteins.[5] In *Staphylococcal nuclease* they found that the one-bond $^1J_{NC'}$ coupling constant tends to be smaller for structured regions (α-helices, β-sheets) than for unstructured terminal ends. No correlation between the magnitude of the $^1J_{NC'}$ coupling constant and the nonplanarity of the peptide bond was found. Some relevance of the peptide backbone ψ angle was noted, because the highest values of the coupling constant were measured mainly in the residues for which the ψ angle was near zero. The involvement of amide groups in the H-bonding network of the protein was not considered. However, sensitivity of the $^1J_{NC'}$ coupling constant to the H-bonding of the amide group was demonstrated a long time ago.[3a,b] The effect of H-bonding on the $^1J_{NC'}$ coupling constant involves the polarization of π electrons in the multiple covalent bonds of the amide (peptide) group. The polarization results in the rehybridization of electronic orbitals and shortening of the C−N bond and lengthening of the C=O and N−H bonds.[1b] Rehybridization of orbitals and shortening of the C−N bond are then reflected in the value of the coupling constant. Therefore, the $^1J_{NC'}$ coupling constant may prove to

* Author to whom correspondence should be addressed.

[⊗] Abstract published in *Advance ACS Abstracts*, December 1, 1994.

(1) (a) Luck, W. A. P. In *The Hydrogen Bond*; Schuster, D., Eds.; North Holland Publishing Co.: Amsterdam, The Netherlands, 1976; Vol. II. (b) Jeffrey, G. A.; Saenger, W. *Hydrogen Bonding in Biological Structures*; Springer-Verlag: Berlin, 1991. (c) Gerothanassis, I. P. *Prog. NMR Spectrosc.* **1994**, *26*, 171−237.

(2) (a) Schwyzer, R.; Ludescher, U. *Helv. Chim. Acta* **1969**, *52*, 2033−2040. (b) Llinas, M.; Klein, M. P. *J. Am. Chem. Soc.* **1975**, *97*, 4731−4737. (c) Wagner, G.; Pardi, A.;Wüthrich, K. *J. Am.Chem. Soc.* **1983**, *105*, 5948−5955. (d) Llinas, M.; Wilson, M. D.; Klein, M. P. *J. Am. Chem. Soc.* **1977**, *99*, 6846−6850. (e) Wishart, D. S.; Sykes B. D.; Richards, F. M. *J. Mol. Biol.* **1991**, *222*, 311−333. (f) Zhou, N. E.; Zhu, B-Y.; Sykes, B. D.; Hodges, R. S. *J. Am.Chem. Soc.* **1992**, *114*, 4320−4326.

(3) (a) Berger, S. *Tetrahedron* **1978**, 3133−3136. (b) Walter, J. A.; Wright, L. C. *Tetrahedron Lett.* **1979**, *41*, 3909−3912. (c) Juranić, N.; Ilich, P. K.; Macura, S. *35th Experimental Nuclear Magnetic Resonance Conference*, April 1994, Pacific Grove, CA, Book of Abstract, p 110.

(4) Markley, J. L. *Methods Enzymol.* **1989**, *176*, 12−64.

(5) Delaglio, F.; Torchia, D. A.; Bax, A. *J. Biomolecular NMR* **1991**, *1*, 439−446.

(6) Bystrov, V. F. *Prog. NMR Spectrosc.* **1976**, *10*, 41−81.

0002-7863/95/1517-0405$09.00/0

be more useful as a probe for hydrogen bonding of amide groups in proteins than for peptide backbone conformation.

In studying the conformational dependence of the $^1J_{NC'}$ coupling constant of amides, Berger[3a] observed only a small difference in the coupling between *cis* and *trans* amides but a large increase in the coupling constant in amides protonated at the carbonyl oxygen. This was supported in the study by Walter and Wright[3b] of the H-bonding effects on $^1J_{NC'}$ coupling constants in cyclohexapeptides. The latter study also established that the effect of carbonyl oxygen protonation is more pronounced in amide groups which form the intramolecular H-bond through the amide hydrogen. From these studies it appears that $^1J_{NC'}$ coupling is influenced by H-bonding not only when bonding takes place via the amide oxygen but also through the amide hydrogen. What is not known is the relative importance and the direction of the influence of the two modes of amide hydrogen bonding on the coupling. This needs to be resolved in order to understand the effect of H-bonding on the coupling constant in proteins.

In the present work,[3c] we have studied the effect of H-bonding on the $^1J_{NC'}$ coupling constant in a simple model compound, *N*-acetylglycine. The focus of the study was on the two main events in amide–solvent interaction: H-bonding of the amide NH group to basic solvents (dimethyl sulfoxide [DMSO], dimethylformamide [DMF], pyridine) and H-bonding and protonation of the amide carbonyl by protic solvents (trifluoroacetic acid [TFAA], trifluoroethanol [TFE]).[2a,b] The patterns of changes observed in these systems were than applied to the interpretation of the coupling constant in proteins. Results of the measurements of $^1J_{NC'}$ coupling constants in human ubiquitin are presented and considered together with those reported for *Staphylococcal nuclease*.[5]

Materials and Methods

Uniformly labeled ^{15}N- and ^{13}C-enriched human ubiquitin was purchased from VLI research (Wayne, PA) and used without further purification. An NMR sample (2 mM in protein, pH = 4.1) was prepared by dissolving human ubiquitin in 25 mM acetic acid-d_4 solution in a 5% D_2O/95% H_2O mixture.

All NMR experiments were performed at 500 MHz on a Bruker AMX spectrometer. Two-dimensional spectra [$^1H-^{15}N$]HMQC-NOESY (mixing time 300 ms) and [$^1H-^{15}N$]HMQC-TOCSY (spin-lock time 64 ms) were recorded with standard pulse sequences.[7] The spectral width was 11 ppm in the acquisition domain and 77 ppm in the evolution domain. For each of 512 t_1 values, 160 scans were recorded with a repetition time of 0.7 s. The HSQC $^1H-^{15}N$ correlation spectrum, with $^{13}C_\alpha$ decoupling during the evolution period and ^{15}N decoupling during the acquisition period, was recorded by the sequence reported by Delaglio *et al.*[5]

Results

Model Study of *N*-Acetylglycine. The effect of pH on the $^1J_{NC'}$ coupling constant in *N*-acetylglycine is presented in Figure 1. The coupling undergoes an increase on the order of 30% when pH is lowered below zero in the solvent mixture of TFAA and water. The extremely high acidity of the solution at which the coupling constant changes merely indicates that at these pH values the amide oxygen is protonated and the N=C double bond has been formed. The result parallels our previous finding on the effect of amide oxygen protonation on the five-bond proton–proton coupling across the peptide bond, $^5J_{\alpha\alpha'}$.[8]

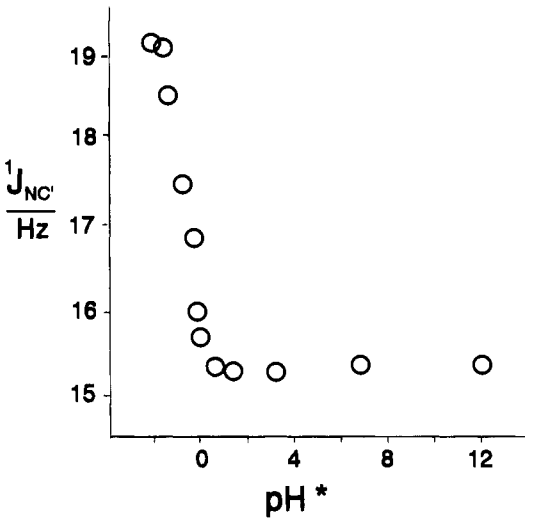
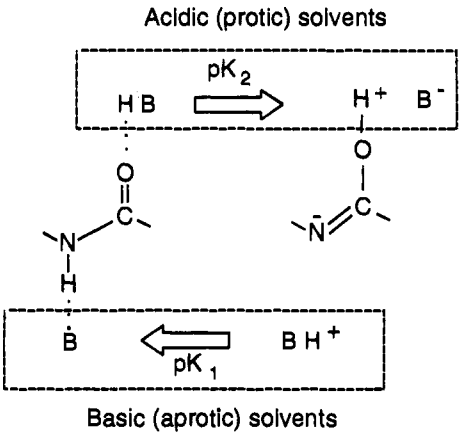


Figure 1. pH dependence of the $^1J_{NC'}$ coupling constant in *N*-acetylglycine. No correction of pH for the D_2O admixture (5%) was done.

Scheme 1



Strongly acidic protic solvents form H-bonds primarily with the amide oxygen, whereas basic aprotic solvents form H-bonds with the amide NH group.[2a,b] The process may be correlated with the corresponding acidity constants of the solvents (Scheme 1). The $^1J_{NC'}$ coupling constant in *N*-acetylglycine is found to increase with the proton-donating ability of protic solvents (pK_2) and to decrease with the proton-attracting ability of aprotic solvents (pK_1) (Figure 2). The same applies to amphoteric solvents (water, alcohols).

Amide group H-bonding is also observable by the amide proton NMR chemical shift.[2] Strong H-bonding results in a downfield shift, but the shift does not distinguish well between amide CO and amide NH hydrogen bonding.[2b] In this respect, the response of the $^1J_{NC'}$ coupling constant is more straightforward. This is shown in the plot of the $^1J_{NC'}$ coupling constant *versus* the amide proton chemical shift for *N*-acetylglycine in various solvents (Figure 3). For the strongly H-bonded (protonated) amide oxygen, the amide proton resonance is shifted downfield, into the same region as for the strongly H-bonded amide hydrogen. However, the $^1J_{NC'}$ coupling constant monotonously increases from predominantly NH to predominantly CO hydrogen bonding of the amide group. The plot also shows that the amide proton chemical shift is relatively more sensitive to NH hydrogen bonding, whereas the coupling constant is relatively more sensitive to CO hydrogen bonding.

It is of interest to compare the $^1J_{NC'}$ coupling constant of *N*-acetylglycine dissolved in water (Figure 3, ●) and in

(7) Gronenborn, A. M.; Bax, A.; Wingfield, P. T.; Clore, G. M. *FEBS Lett.* **1989**, *243*, 93–98.
(8) Juranić, N.; Likić, V.; Parac, T.; Macura, S. *J. Chem. Soc., Perkin Trans. 2* **1993**, 1805–1810.

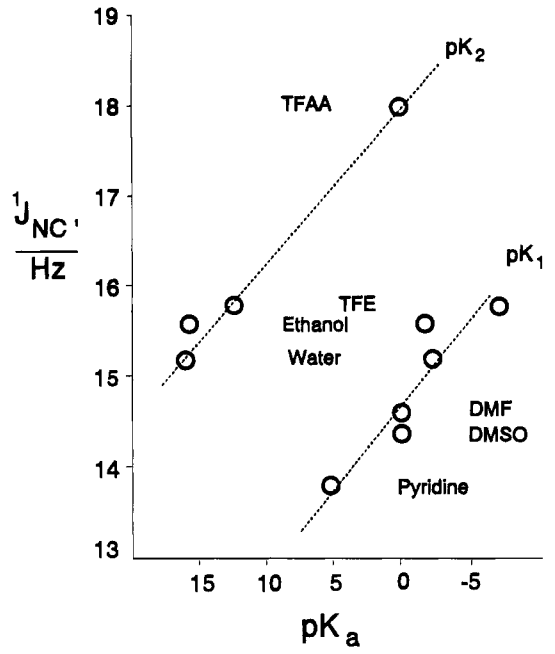


Figure 2. Dependence of the $^1J_{NC'}$ coupling constant in *N*-acetylglycine on acidity constants of various solvents. pK_1 and pK_2 are defined in Scheme 1; DMF = dimethylformamide; DMSO = dimethyl sulfoxide.

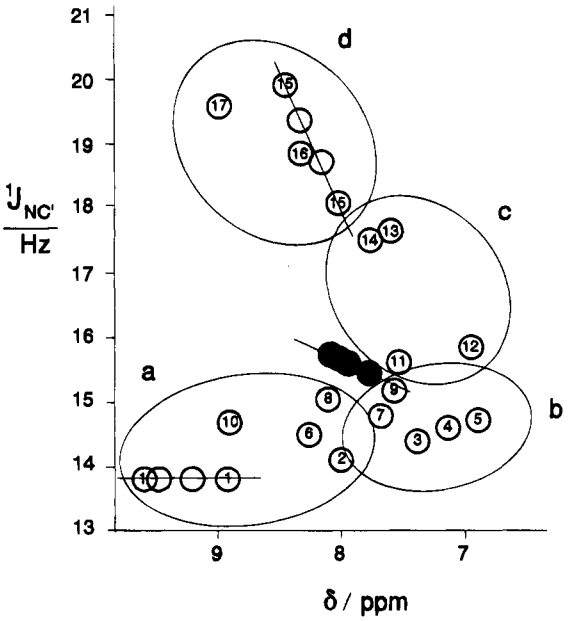


Figure 3. Correlation between the $^1J_{NC'}$ coupling constant and the amide proton chemical shift for *N*-acetylglycine in various solvents: (●) water (points for temperature region 175−323 K), (1) pyridine (temperature region 243−295 K), (2) dimethylformamide/pyridine (1:1 v/v), (3) dimethyl sulfoxide, (4) dimethylformamide, (5) dimethyl sulfoxide/acetonitrile (2:8 v/v), (6) *N*-methylacetamide, (7) ethanol, (8) *N*-methylformamide, (9) acetic acid, (10) pyridine/water (9:1 v/v), (11) methanol, (12) trifluorocetic acid, (13) trifluoroethanol/trifluoroacetic acid (1:1), (14) trifluoroacetic acid/water (6:4), (15) trifluoroacetic acid (temperature region 263−295 K), (16) trifluoroacetic acid/water (8:2), (17) trifluoroacetic acid/water (9:1 v/v).

N-methylacetamide (point 6). In the latter solvent, an inter-amide hydrogen bond is formed, analogous to that found in the structured region of proteins. Inter-amide H-bonding produces a lower coupling constant than H-bonding of the amide group to water.

The temperature dependence of the coupling constant for *N*-acetylglycine in TFAA, water, and pyridine is also presented in Figure 3. These are characteristic cases in which the amide group is mainly protonated at oxygen (TFAA), or H-bonded through both amide NH and CO groups (water), or H-bonded mainly through an amide NH group (pyridine). The result shows that in the extreme case of amide oxygen protonation, the dependence is quite large (about 0.05 Hz/K). However, for an amide group in water (both NH and CO hydrogen bonded), the dependence is only slight and disappears for the case of hydrogen-bonded NH. The amide proton chemical shift dependence on temperature is similar in all three cases: the shift decreases with temperature.[2]

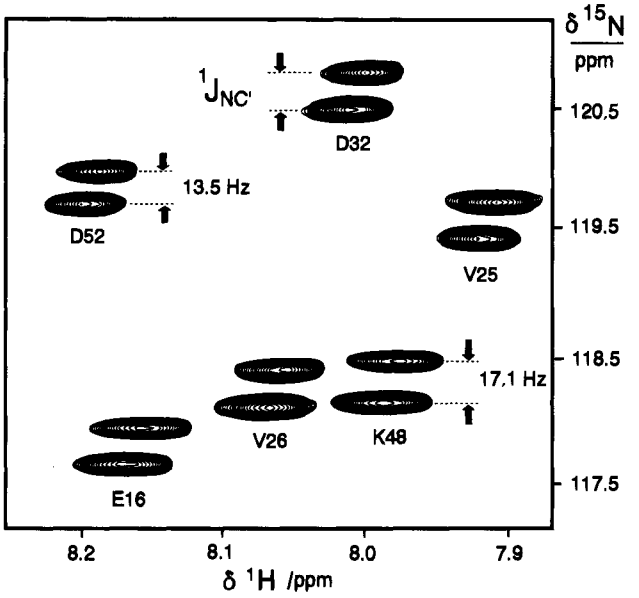


Figure 4. Region from the ^{1}H−^{15}N HSQC spectra of a 2 mM solution of human ubiquitin in 25 mM aqueous acetic acid-d_4 at 303 K. The $^{13}C_\alpha$ resonances were decoupled by a soft (80 μs) π-pulse in the middle of the evolution period. Acquisition times were 170 (t_2) and 64 ms (t_1). For the WALTZ decoupling during acquisition, a 2.5 kHz radiofrequency field was used. Water resonance was presaturated during delay time between scans (0.6 s) using a 30 Hz radiofrequency field. Data were processed with strong Lorentzian to Gaussian filtering in the evolution domain and zero filled to yield digital resolution of 0.2 Hz per data point.

$^1J_{NC'}$ Coupling Constant in Human Ubiquitin. Assignment of the proton signals in the amide region of human ubiquitin was done by analysis of [^{1}H−^{15}N]HMQC-TOCSY and [^{1}H−^{15}N]-HMQC-NOESY spectra and by use of the reported assignments.[9] All d_{NN} NOE connectivities in α-helices, β-sheets, and reverse turns were identical to those reported before.[9] The $^1J_{NC'}$ coupling constants were determined from the HSQC ^{1}H−^{15}N correlation spectrum (Figure 4), with $^{13}C_\alpha$ decoupling during the evolution period and ^{15}N decoupling during the acquisition period. Measurements at 7, 30, and 50 °C showed a small decrease in the value of the coupling constant with increasing temperature. The decrease amounted to about 5% for the temperature region. This is essentially the result expected from the study on *N*-acetylglycine. Because of an experimental error of about 0.2 Hz, an analysis of finer variations from residue to residue in temperature dependence of the coupling constant was not attempted at this stage. The best conditions for measurement of the coupling constants were at 30 °C, and

(9) (a) Weber, P. L.; Brown, C. S.; Mueller, L. *Biochemistry* **1987**, *26*, 7282−7290. (b) Di Stefano, D. L.; Wand, A. J. *Biochemistry* **1987**, *26*, 7272−7281.

(10) Kumar V-K.; Bugg, C. E.; Cook, W. J. *J. Mol. Biol.* **1987**, *194*, 531−544.

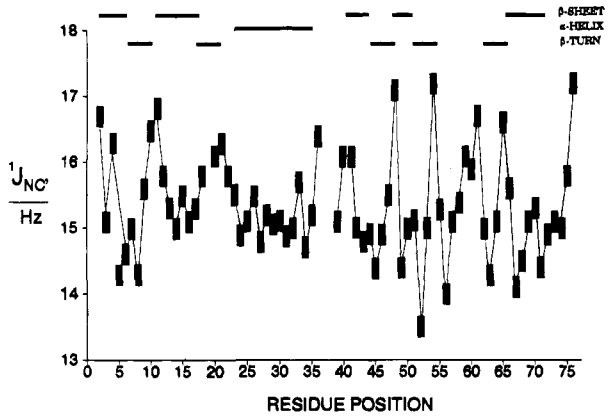


Figure 5. The $^1J_{NC'}$ coupling constants in human ubiquitin. Proline residues (19, 37, 38) were not observed. The coupling constants are numbered according to the residue that provided amide nitrogen in the peptide bond.

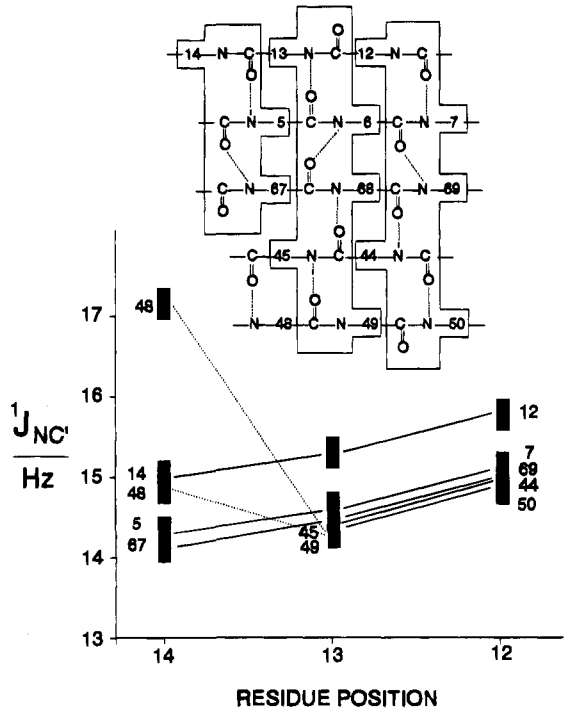


Figure 6. The $^1J_{NC'}$ coupling constant in the region of β-sheets with the extended network of intraprotein hydrogen bonding.

they are reported for this temperature only (supplementary material).

The coupling constants in human ubiquitin ranged from 13.5 to 17.2 Hz, depending on the position of the residue in the sequence (Figure 5). In the α-helix, variations in the coupling constants were small (15.3 ± 0.5 Hz) and comparable to those in the α-helix of *Staphylococcal nuclease* (14.8 ± 0.4)[2] taken at 35 °C.

In the β-sheets, variations in the coupling constants were larger (15.5 ± 1.5 Hz). In the central region of the mixed β-sheets, where the H-bonding network is spread over five strands, the coupling constant exhibited rather regular change (Figure 6).

The widest range of the coupling constant values was found within **reverse turns.** About 80% of the extreme values were in residues occupying reverse turn positions 1 to 5. Among them, the most regular behavior was exhibited by the coupling constants in residues at positions 1 to 4. The dependence of the $^1J_{NC'}$ coupling constant on the position of the peptide bond

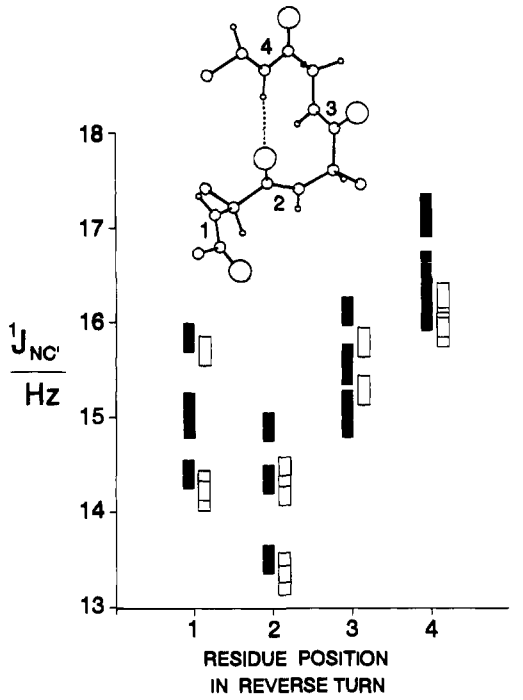


Figure 7. Dependence of the $^1J_{NC'}$ coupling constant on the peptide bond position in the reverse turns of human ubiquitin (■) (turns involve residues 7–10 (turn type I), 18–21 (I), 37–40 (III), 45–48 (III′), 51–54 (I), 62–65 (II)) and *Staphylococcal nuclease*[2] (□) (19–21 (I), 27–30 (I′), 83–86 (I), 94–97 (I′), 137–140 (II′).

in human ubiquitin and *Staphylococcal nuclease* reverse turns is shown in Figure 7. The smallest coupling was found in the residues at position 2, whereas the highest was in the residues at position 4 of the turn. There was no overlap in the coupling constant values between the two positions.

Finally, high values of the coupling constant were also observed at the beginning (16.8 Hz for residue 2) and at the end of the protein sequence (17.2 Hz for residue 76).

Discussion

Model Study. Investigation of the effect of amide–solvent interaction on the $^1J_{NC'}$ coupling constant in *N*-acetylglycine revealed a remarkable sensitivity of the coupling to amide H-bonding. The largest change in the value of the coupling constant, an increase of 40%, occurs when a basic aprotic solvent (pyridine) is replaced by a strongly acidic solvent (TFAA). This is related primarily to the change of the C–N bond order, caused by amide oxygen H-bonding and protonation. The pH dependence of the coupling constant (Figure 1) shows that formation of the N=C double bond accounts for most of the coupling constant change. The effect of amide NH hydrogen bonding on the coupling constant is smaller, because the replacement of weakly basic aprotic solvent (acetonitrile) by the strongly basic pyridine (Figure 3, points 5 and 1) decreases the coupling constant by ~10%. The most important practical consideration is that dependence of the $^1J_{NC'}$ coupling constant on the nature of amide H-bonding is rather regular. H-bonding of the amide oxygen increases the $^1J_{NC'}$ coupling constant, whereas H-bonding of amide NH decreases it. This response of the coupling constant to amide H-bonding is qualitatively comparable to that found for peptide ^{13}C carbonyl chemical shifts.[2d] However, the chemical shift is more sensitive to NH hydrogen bonding whereas coupling is more sensitive to CO hydrogen bonding.

The $^1J_{NC'}$ coupling constant alone emerges therefore as a good probe of amide H-bonding, but it is even more useful when

taken in combination with the amide proton chemical shifts. Together, these parameters enable recognition of different modes of amide H-bonding (see Figure 3): (a) strong H-bonding of the NH group induces a low $^1J_{NC'}$ coupling constant (<15 Hz) and a downfield amide proton chemical shift; (b) weak H-bonding of the amide group induces a medium $^1J_{NC'}$ coupling constant (~15 Hz) and an upfield amide proton chemical shift; (c) strong H-bonding of the amide oxygen induces a high $^1J_{NC'}$ coupling constant (>16 Hz) and a low-field to intermediate-field chemical shift of the amide proton; and (d) protonation of the amide oxygen induces a high $^1J_{NC'}$ coupling constant (>18 Hz) and a downfield chemical shift of the amide proton. An analysis of amide H-bonding in proteins in terms of these two parameters is straightforward because both the coupling constant and the chemical shift may be determined selectively for each residue in a single HSQC $^1H-^{15}N$ correlation experiment.

The dependence of the $^1J_{NC'}$ coupling constant on temperature offers another distinction between amide oxygen and amide hydrogen H-bonding of amides. The temperature dependence is large for the case of amide oxygen H-bonding and very small for the case of amide hydrogen H-bonding (Figure 3). This can be explained by disruption of H-bonds by temperature. The raise of temperature shifts the equilibrium from H-bonded to H-unbounded states of amide groups, thus weakening H-bonding as seen by the $^1J_{NC'}$ coupling constant. The temperature shift of the equilibrium is larger for the amide oxygen H-bonding because this H-bonding is stronger then H-bonding of the amide hydrogen.[1] The larger change of the amide oxygen H-bonding with temperature and the larger dependence of the $^1J_{NC'}$ coupling constant on the amide oxygen H-bonding make the temperature effect on $^1J_{NC'}$ far more pronounced in the amide oxygen H-bonding solvents than in the amide hydrogen H-bonding solvents (Figure 3).

It should be stressed that the $^1J_{NC'}$ coupling constant reflects the electronic polarization in the C–N bond induced by H-bonding. Therefore, many individual effects of directionality, bifurcation, and strength[1] of the H-bond cannot be unraveled from the $^1J_{NC'}$ coupling constant.

Proteins. The coupling constant values in the two proteins (13.1–17.2 Hz) are well within the expected range of changes induced by amide H-bonding. We have also considered the possible influence of the peptide backbone ψ angle.[5] In both proteins, the highest values of the coupling are measured mainly in peptide bonds preceded by a small ψ angle. However, the small ψ angle **does not cause** the high coupling constant, because there are many cases of small ψ angles being connected with intermediate values of coupling constants (Figure 8).

In relation to the effects of H-bonding on the $^1J_{NC'}$ coupling constant in proteins, the most intriguing is the sharp change of the constant within reverse turns (Figure 7). In most reverse turns there is characteristic 1←4 hydrogen bonding.[1] Also, reverse turns occur on the protein surface, exposed to water. Therefore, in reverse turns H-bonding may differ greatly from one main-chain amide group to another. A comparative analysis of the $^1J_{NC'}$ coupling constant and amide proton chemical shift should reveal differences. A plot of the coupling constants *versus* the proton chemical shift for positions 2 and 4 of reverse turns is presented in Figure 9. This plot shows that the hydrogen of the amide in position 2 is strongly H-bonded, because it exhibits the high field amide proton shift combined with a low $^1J_{NC'}$ coupling constant. In contrast, the hydrogen of the amides in position 4 is weakly H-bonded, characterized by the low-field shift. However, the large coupling constant at position 4 signifies considerable H-bonding of the amide oxygen. Taken together, this indicates strong H-bonding of solvent (water)

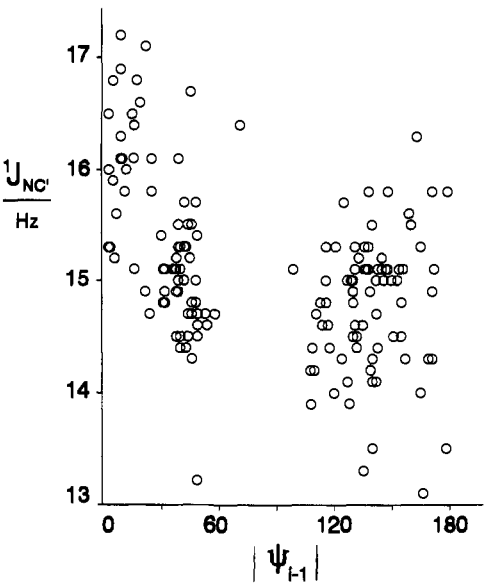


Figure 8. Correlation of the $^1J_{NC'}$ coupling constant with the ψ angle of the preceding residue: (○) human ubiquitin; (□) *Staphylococcal nuclease.*[2]

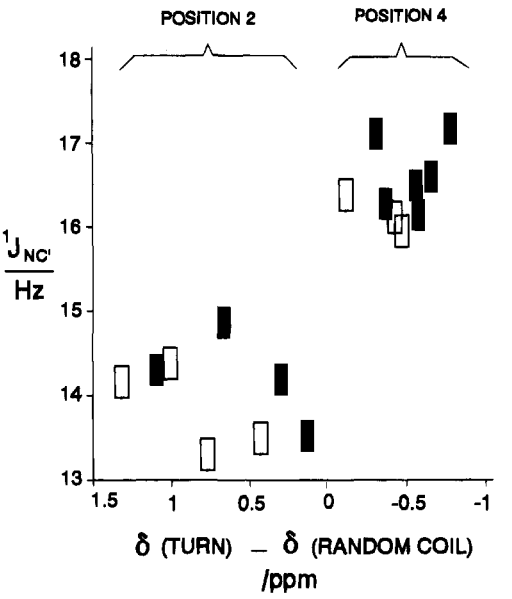


Figure 9. Dependence of the $^1J_{NC'}$ coupling constant on amide proton chemical shift (relative to the random coil value) for amide groups in positions 2 and 4 of the reverse turns of human ubiquitin and *Staphylococcal nuclease.* The turns are the same as in Figure 8.

molecules to the exposed amide proton at position 2 and the exposed amide oxygen at position 4. It also implies that the intrapeptide (1←4) hydrogen bond is considerably weaker than hydrogen bonds with water. This result is important because it provides the first experimental evidence of the weaker intraprotein main-chain amide H-bonding relative to main-chain amide H-bonding to water. The same has been expected from the model studies on the relative strength of amide–amide and amide–water hydrogen bonds,[11] which indicated that amides form stronger intermolecular hydrogen bonds with water than with other amides.

(11) (a) Eberhardt, E. S.; Rains, R. T. *J. Am. Chem. Soc.* **1994**, *116*, 2149. (b) Klotz, I. M.; Franzen, J. S. *J. Am. Chem. Soc.* **1962**, *84*, 3641.
(12) (a) Serrano, L.; Fersht, A. *Nature* **1989**, *342*, 296. (b) Lyu, C. P.; Zhou, H. X.; Jelveh, N.; Wemmer, D. E.; Kallenbach, N. R. *J. Am. Chem. Soc.* **1992**, *114*, 6560–6562.

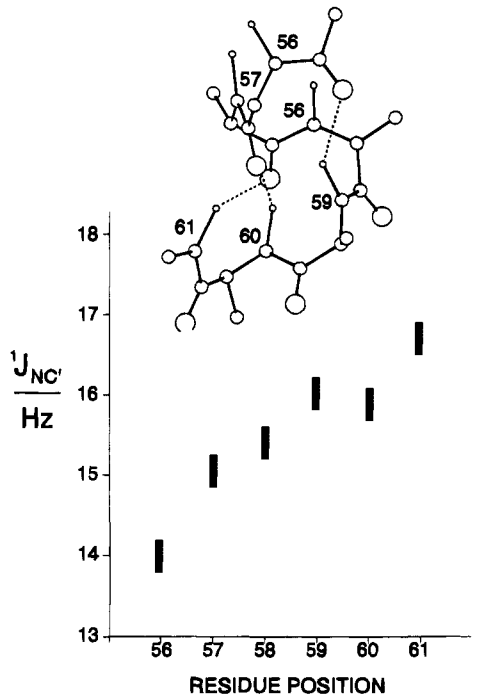


Figure 10. The $^1J_{NC'}$ coupling constant in the short helix (residues 56–61) of human ubiquitin.

The conclusion on the relative strength of the hydrogen bonds presented here should be taken with some caution, because the $^1J_{NC'}$ coupling constant and amide proton chemical shift changes do not directly reflect changes in H-bond energy.

In the structured regions of proteins (α-helix, β-sheets), intraprotein H-bonding of amide groups is dominant. Therefore, an intermediate and almost constant value of the coupling constant is observed in longer α-helices. Similar, and regular, values of the coupling constant are also observed in the region of β-sheets for which intraprotein H-bonding is extended over three or more strands (Figure 6). In such cases, a significant correlation between the coupling within each line of interstrand H-bonding is observed. For instance, the coupling constant along the line of residues 12–7–69–44–50 is generally higher than that along the line 13–6–68–45–49. If the line of interstrand H-bonding is interrupted, the correlation is lost, as happens in the line 14–5–67–46–48, which is cut short because residue 46 is part of the β-turn. This shows that $^1J_{NC'}$ is sensitive to cooperative polarization of the H-bonding network along the lines of interstrand NH···O=C hydrogen bonds.[1b]

Finally, we may remark on the coupling constants in the secondary structure of human ubiquitin involving residues 56–61, which was described in an ambiguous way as overlapped reverse turns, or as a short helical turn.[9,10] Our values of the $^1J_{NC'}$ coupling constant in residues 56–61 (Figure 10) exhibit a pattern distinct from both the reverse turn and the α-helix patterns, but the cause for the observed pattern is basically the same as in the reverse turns. Residues 56–58 provide amide CO and 59–61 amide NH for intraprotein H-bonding. Thus, the first three NH donors and the last three CO acceptors in the helix cannot be satisfied by main-chain peptide groups; they are H-bonded by water.[12] Strong H-bonding with water decreases the coupling constant at the protein H-bonding donors and increases the coupling constant at the acceptors of the water-exposed residues of the helix.

Because of a possible dependence on other parameters (type of side chain, local geometry) that are yet to be assessed, the peptide $^1J_{NC'}$ coupling constant is of limited applicability, at present, for characterization of individual H-bonds. Perhaps the only exception is the H-bonding in reverse turns where the extreme coupling constant values were found. However, if the cooperative character of H-bonding is taken into account, the peptide $^1J_{NC'}$ coupling constant is a sensitive probe for identification of H-bonding networks. The ability to identify the elements of secondary structure and within them chains of H-bonding may have several important consequences for the characterization of proteins in solution. As demonstrated by the examples presented, the $^1J_{NC'}$ coupling constant can be used to identify reverse turns unambiguously. Of course, for real application a much larger data base of the established correlations would be needed, as well as better theoretical understanding of the relationship among H-bonding, C–N bond polarization, amide hydrogen exchange, and the $^1J_{NC'}$ coupling constant.

Conclusion

We have shown that the amide group $^1J_{NC'}$ coupling constant is a valuable probe for amide H-bonding in proteins. Combined with the amide proton chemical shift, it enables recognition of different modes of amide H-bonding. Analysis of $^1J_{NC'}$ coupling constants in proteins revealed the characteristic variations of coupling constants in α-helices, β-sheets, and reverse turns, which were found to be caused by H-bonding patterns of the main-chain amide groups. Specific change of the coupling constant in reverse turns indicated that intraprotein amide–amide H-bonding is weaker than H-bonding of the amide groups with water. These findings are important for study of protein folding and stability. The coupling constant values also reflect a cooperative polarization of the H-bonding network in the protein.

Acknowledgment. We thank Professor Franklyn G. Prendergast for usefull suggestions and for critical reading of the manuscript.

Supplementary Material Available: Table of $^1J_{NC'}$ coupling constants and ^1H and ^{15}N chemicals shifts in human ubiquitin (2 pages). This material is contained in many libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.

JA942537G