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The ^{13}C chemical shifts of amino acids in aqueous solution containing organic solvents: Application to the secondary structure characterization of peptides in aqueous trifluoroethanol solution

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SUMMARY

The ^{13}C chemical shifts for all of the protonated carbons of the 20 common amino acid residues in the protected linear pentapeptide Gly-Gly-X-Gly-Gly have been obtained in water at low pH as well as in aqueous solution containing 10, 20 and 30% acetonitrile or trifluoroethanol. Dioxane was used as an internal reference and its carbon chemical shift value was found to be 66.6 ppm relative to external TMS in water. Comparison of the different referencing methods for ^{13}C chemical shifts in organic cosolvent mixtures showed that an external standard (either TMS or TSP capillary) was the most appropriate. In the present study, external TSP was chosen to define the 0 ppm of the ^{13}C chemical shift scale. When the difference in referencing the dioxane carbon resonance is taken into account, the carbon chemical shift values of the amino acids in aqueous solution are similar to those previously reported (Richarz and Wüthrich (1978) *Biopolymers*, **17**, 2133–2141; Howarth and Lilley (1979) *Prog. NMR Spectrosc.*, **12**, 1–40). The pentapeptides studied were assumed to be in a random coil conformation and the measured ^{13}C chemical shifts were used as reference values to correlate carbon chemical shifts with the secondary structure of two well-characterized peptides, bombesin and the 1–29 amino acid fragment of Nle^{27} human growth hormone-releasing factor. In both cases, the C^{α} chemical shifts exhibited a characteristic positive deviation from the random coil values, which indicates the presence of α-helices.

INTRODUCTION

The carbon chemical shifts of peptides and proteins can provide a wealth of information about the secondary structure and conformation. The NMR frequency of the carbon nucleus depends

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on its chemical (Levy and Nelson, 1972), electronic (Grant and Paul, 1964; Paul and Grant, 1964; Reynolds et al., 1973) and steric (Weigert and Roberts, 1967) environment. This implies that the carbon chemical shift of a given amino acid may be different depending on its position in the primary sequence of the peptide. Recent reports on the use of carbon chemical shifts as a structural diagnostic tool in proteins (Spera and Bax, 1991; Wishart et al., 1991; de Dios et al., 1993) and peptides (Lee et al., 1992; Reily et al., 1992) have increased the interest to determine the information available from these shifts. This interest in carbon chemical shifts is also paralleled by the increasing availability of ^{13}C chemical shift data for ^{13}C-labeled proteins.

In order to understand the influence of these structural effects on the carbon chemical shifts, it is necessary to have reference values for intrinsic carbon chemical shifts of each amino acid in the absence of any defined structure (e.g., 'random coil' shifts). Random coil carbon chemical shifts were reported for the 20 common amino acids in aqueous solution (Keim et al., 1973a,b, 1974; Richarz and Wüthrich, 1978; Howarth and Lilley, 1979) and dimethylsulfoxide (Grathwohl and Wüthrich, 1974). However, NMR studies on peptides are often carried out under a variety of conditions for which no reference values have been reported. For example, amide exchange rates or solubilities may dictate that the NMR studies be carried out at a lower pH. Also, organic cosolvents such as acetonitrile or trifluoroethanol (TFE) may be used either to increase the solubility of the peptide in aqueous solution or to increase the hydrophobic nature of the solvent to more closely approximate the biological conditions (Nelson and Kallenbach, 1986). Thus in an aqueous environment, an understanding of the effect of pH and additional organic solvents on the random coil shifts of amino acids is needed to evaluate the observed induced shifts by secondary structure.

Also critical to the use of carbon chemical shift changes to determine peptide secondary structure is an accurate and reproducible reference standard. Almost universally, the chemical shift of tetramethylsilane (TMS), either internally for organic solvents or externally for aqueous solvents, is used to define 0 ppm on the ^{13}C chemical shift scale. In aqueous solutions 3-trimethylsilyl[2,2,3,3,-^{2}H]propionate, TSP, is also used as an internal standard to define the 0 ppm carbon chemical shift. It is often more convenient to utilize a soluble secondary reference in aqueous solutions such as dioxane to indirectly reference shifts relative to external 0 ppm standard. When using such a secondary chemical shift standard, care must be taken such that the shift assigned accurately reflects its difference from the 0 ppm standard under similar experimental conditions.

Here, we report the random coil carbon chemical shifts for all the protonated carbons of X in the pentapeptide Gly-Gly-X-Gly-Gly (where X is each of the 20 common amino acids) in aqueous solution at low pH as well as in aqueous solution containing 10%, 20% and 30% v/v acetonitrile or TFE. External TSP was used to define the 0 ppm of the carbon chemical shift scale. We also report a careful study of the effect of these cosolvents on the chemical shift of a commonly used secondary reference, dioxane. Finally, we will correlate the C^{α} chemical shifts with the secondary structure of two well-characterized peptides, bombesin and 1–29 amino acid fragment of Nle^{27} substituted human growth hormone-releasing factor (Nle^{27}-hGHRF[1–29]NH$_2$), using these new reference values.

Abbreviations: HMQC, heteronuclear multiple-quantum coherence; HOBt, *N*-hydroxybenzotriazole hydrate; NOE, nuclear Overhauser effect; TOCSY, total correlation spectroscopy.

MATERIALS AND METHODS

Solid phase peptide synthesis

Bombesin and Nle^{27}-hGHRF[1–29]NH_2 were purchased from the Sigma Chemical Company as lyophilized powders and used without further purification. Gly-Gly-Gly-Gly-Gly was purchased from Research Plus, Inc. The pentapeptides (Gly-Gly-X-Gly-Gly, where X represents different amino acids) were prepared by solid phase peptide synthetic methodology. Cysteine was incorporated as the *S*-methyl derivative in the pentapeptide. All amino acids were of the L-configuration. To facilitate the synthetic strategy, five appropriately orthogonally protected N^{α}-Boc-L-amino acids in an equal molar mixture were coupled as the HOBt activated esters to a phenylacetamidomethyl (PAM) resin substituted with Gly-Gly. After completion of the synthesis the peptides were cleaved from the resin with hydrogen fluoride (HF) and simultaneously deprotected to yield four mixtures containing five different peptides each. The pentapeptide containing cysteine and the corresponding dimer was prepared independently. The peptide mixtures were used for NMR measurements without further purification.

Preparation of Gly-Gly-[Ala/Cys(Me)/Trp(For)/Ser/Glu]-Gly-Gly (Mixture 1). The title peptide mixture was prepared by a standard solid phase synthetic strategy (Stewart and Young, 1984) starting with 0.70 g of N^{α}-Boc-Glycine PAM resin on an Applied Biosystems 430A peptide synthesizer. After the coupling of N^{α}-Boc-glycine via the preformed symmetrical anhydride (2.0 mmol of N^{α}-Boc-glycine and 1.0 mmol of *N,N'*-dicyclohexylcarbodiimide) in *N,N*-dimethylformamide, an equal molar mixture of N^{α}-Boc-alanine, N^{α}-Boc-cysteine-*S*-methyl, N^{α}-Boc-tryptophan-N^{in}-formyl, N^{α}-Boc-serine-*O*-benzyl and N^{α}-Boc-glutamic acid-γ-benzyl ester (0.4 mmol each) was coupled as the individual HOBt-activated esters (2.0 mmol of *N,N'*-dicyclohexylcarbodiimide and 2.0 mmol of *N*-hydroxybenzotriazole). This was followed by incorporation of two additional glycine residues as above, removal of the terminal N^{α}-Boc-protecting group with 50% trifluoroacetic acid in dichloromethane and drying of the resulting resin under reduced pressure. The peptide was cleaved from the resin, and the side chain-protecting groups were removed by treatment with anhydrous hydrogen fluoride (HF, 9 ml) with anisole as a scavenger (1 ml) (60 min, 0 °C). The resin was washed with ethyl ether (3 × 25 ml) and extracted with 20% aqueous acetic acid (3 × 25 ml) and H_2O (3 × 25 ml). The aqueous fractions were combined and concentrated under reduced pressure. The peptide mixture was resuspended in H_2O (~50 ml) and passed through a C_{18} cartridge (20 cc, Analytichem International), and the absorbed peptide mixture was eluted with 70% acetonitrile, 0.1% trifluoroacetic acid (TFA) in water (30 ml), concentrated and lyophilized (122 mg). The peptide mixture was used without further purification.

Preparation of Gly-Gly-[Dip/Ile/Tyr/Gln/Asp]-Gly-Gly (Mixture 2). The title peptide mixture was prepared in a similar manner as Mixture 1, except that the following residues were incorporated: N^{α}-Boc-diphenylalanine (Josien et al., 1991; Chen et al., 1992), N^{α}-Boc-isoleucine, N^{α}-Boc-tyrosine-*O*-2-bromobenzyloxycarbonyl, N^{α}-Boc-glutamine, N^{α}-Boc-aspartic acid-β-benzyl ester (78 mg).

Preparation of Gly-Gly-[Leu/Met/His/Asn/Lys]-Gly-Gly (Mixture 3). The title peptide mixture was prepared in a similar manner as Mixture 1, except that the following residues were incorporated: N^{α}-Boc-leucine, N^{α}-Boc-methionine, N^{α}-Boc-histidine-N^{im}-tosyl, N^{α}-Boc-asparagine, N^{α}-Boc-lycine-N^{ε}-2-chlorobenzyloxycarbonyl (85 mg).

Preparation of Gly-Gly[Pro/Val/Phe/Thr/Arg]-Gly-Gly (Mixture 4). The title peptide

mixture was prepared in a similar manner as Mixture 1, except that the following residues were incorporated: N^{α}-Boc-proline, N^{α}-Boc-valine, N^{α}-Boc-phenylalanine, N^{α}-Boc-threonine-*O*-benzyl, N^{α}-Boc-arginine-N^{g}-tosyl (56 mg).

Preparation of Gly-Gly-Cys-Gly-Gly. The title peptide was prepared in a similar manner as Mixture 1, except that N^{α}-Boc-cysteine-*S-para*-methylbenzyl was incorporated (136 mg). The disulfide bridged cystine derivative was prepared by air oxidation of 10 mg of the monomer in 800 ml of ^2H_2O, pH 8.0, for 72 h.

NMR spectroscopy

NMR measurements were carried out using 15 mg of the amino acid mixture in 400 μl of ^2H_2O at 298 K. Dioxane (1 μl) and TSP (1 μl) were added as an internal reference for carbon and proton shifts, respectively. Appropriate amounts of deuterated acetonitrile or TFE were added to the aqueous solution to yield 10, 20 and 30% v/v organic solvent in ^2H_2O. In the case of bombesin and Nle^{27}-hGHRF[1–29]NH_2, 5 mg of the peptide was used. In all cases, the TFA salts of the peptides were used and pH of the aqueous solutions were in the range of 2.0–3.5.

All NMR experiments were recorded on a Bruker AMX500 spectrometer. The proton-decoupled ^{13}C NMR spectra were recorded at 125.76 MHz with a sweep width of 26 315 Hz over 4096 data points. TOCSY (Braunschweiler and Ernst, 1983) experiments were acquired into 512 t_1 blocks of 2048 t_2 data points each. A 7-kHz MLEV17 (Bax and Davis, 1985) spin-lock field was applied at the transmitter frequency (about 4.76 ppm) for 65 ms. A trim pulse of 2.5 ms was applied prior to the spin-lock pulse train. HMQC (Bax et al., 1983) experiments were acquired into 256 t_1 blocks of 2048 t_2 data points and a GARP-decoupling sequence (Shaka et al., 1985) was used for carbon decoupling during detection. In the ω_1 dimension a sweep width of 6800 Hz was used with the transmitter frequency set at 35 ppm, resulting in folding of aromatic resonances. The frequency sign discrimination in the ω_1 dimension was achieved by the time proportional phase incrementation (TPPI) method (Marion and Wüthrich, 1983) for the TOCSY experiments and by the States-TPPI hybrid method (Marion et al., 1989) for HMQC experiments. The data were zero-filled in both dimensions to the final matrix size of 2048 and 1024 real points. A 60°-shifted sine-square bell window function was applied to the free induction decay in both dimensions prior to Fourier transformation. A Gaussian multiplication function (GB = 0.01, LB = −1) was used in ω_2 dimension for the HMQC data. Proton chemical shifts were referenced with respect to internal TSP and carbon chemical shifts were referenced with respect to external TSP by setting internal dioxane to a calibrated value that depended on solution conditions.

RESULTS AND DISCUSSION

Dioxane is one of the most commonly used internal standard compounds to reference carbon chemical shifts in aqueous solutions. The carbon chemical shift of pure dioxane is reported to be 67.8 ppm relative to internal TMS and it is this value that is commonly used for dioxane as an internal reference in aqueous solutions (Wüthrich, 1976). Since the carbon chemical shift of dioxane is concentration dependent, it is necessary to obtain a reference shift under the conditions that are normally used with dioxane as the secondary internal reference compound. The carbon spectrum of 4 μl dioxane in 400 ml ^2H_2O, containing internal TSP and TMS as an external (capillary) reference was recorded. The carbon chemical shift of the external TMS signal was set

to 0 ppm (vide infra) and the dioxane peak was measured at 66.6 ppm. The resonance position of the dioxane carbon signal was independent of the capillary diameter used for external TMS. The 1.2 ppm difference in the carbon chemical shift between aqueous solution and pure dioxane should be taken into account for interpretation of carbon chemical shifts in aqueous solutions. The TSP carbon signal resonates at −2.8 ppm relative to external TMS. The relative positions of these signals are independent of pH in the range 2.0 to 7.0.

In order to facilitate the use of dioxane as an internal reference in various solvents and mixtures thereof, the effect of organic solvents on the dioxane carbon chemical shift in aqueous solution was investigated. The carbon spectra of dioxane in aqueous solution containing 30% acetonitrile and 30% TFE, respectively, were recorded. Since the dioxane peak position was different in these two solvent mixtures, when measured relative to external TMS, a systematic study was undertaken to check the effect of various methods of referencing the ^{13}C spectrum.

The absolute frequency corresponding to 0 ppm in a heteronuclear (X) spectrum can be calculated (Live et al., 1984; Bax and Subramanian, 1986) by multiplying the absolute proton frequency at 0 ppm by the ratio of gyromagnetic ratios, γ_x/γ_H, (0.25144950 in the case of ^{13}C). As previously reported (Live et al., 1984; Bax and Subramanian, 1986), the 0 ppm carbon frequency calculated by multiplying the proton 0 ppm frequency by 0.25144950 is in close agreement with the observed ^{13}C frequency of the TSP signal in water. However, in aqueous solution containing organic cosolvent, the TSP ^{13}C signal appears at markedly different frequencies than the signal calculated by this method. The direction of the shift depends on the solvent medium and in 30% TFE the TSP signal appears upfield (~74 Hz) and in 30% acetonitrile the TSP peak resonates downfield (~20 Hz) from the calculated 0 ppm carbon frequency. Thus the indirectly calculated 0 ppm carbon frequency may differ, depending on the cosolvent mixture, by as much as 0.6 ppm from the actual peak position.

Figure 1 shows the chemical shift of dioxane as a function of solvent mixtures, using different referencing methods. Since the chemical shift of an external standard cannot be influenced by solvent effects, direct referencing via a TMS or TSP capillary provides a reliable chemical shift standard regardless of solvent conditions (Fig. 1). However, introducing a capillary into NMR samples is often undesirable. As a second method, data were collected by setting the ^{13}C 0 ppm frequency relative to the TSP methyl protons, as described above. Since the absolute proton frequency of the TSP signal is solvent dependent, the calculated 0 ppm carbon frequency should be dependent on solvent conditions. This is evident in the case of dioxane ^{13}C chemical shift in different solvents (Fig. 1). The ^{13}C shift profile of dioxane at various concentrations of TFE is similar to that of observed with either external TMS or TSP capillary. But, in the case of acetonitrile, the dioxane ^{13}C chemical shift appears to be independent of the solvent concentration. This is due to the fact that there is an offsetting acetonitrile-induced shift of the 0 ppm TSP proton frequency and consequently in the calculated ^{13}C frequency. Thus, this method suffers from solvent effects. In a third approach, the internal TSP ^{13}C signal was set to 0 ppm. In this case, the apparent solvent-dependent chemical shift of dioxane was markedly different from either of the previous reference methods (Fig. 1). This is simply due to solvent-dependent ^{13}C shifting of the reference standard, TSP.

The results obtained with all three methods indicate that setting the external standard (either TMS or TSP capillary) signal to 0 ppm is the most appropriate method to reference the ^{13}C chemical shifts in organic solvent mixtures. Since the NMR studies on peptides and proteins are

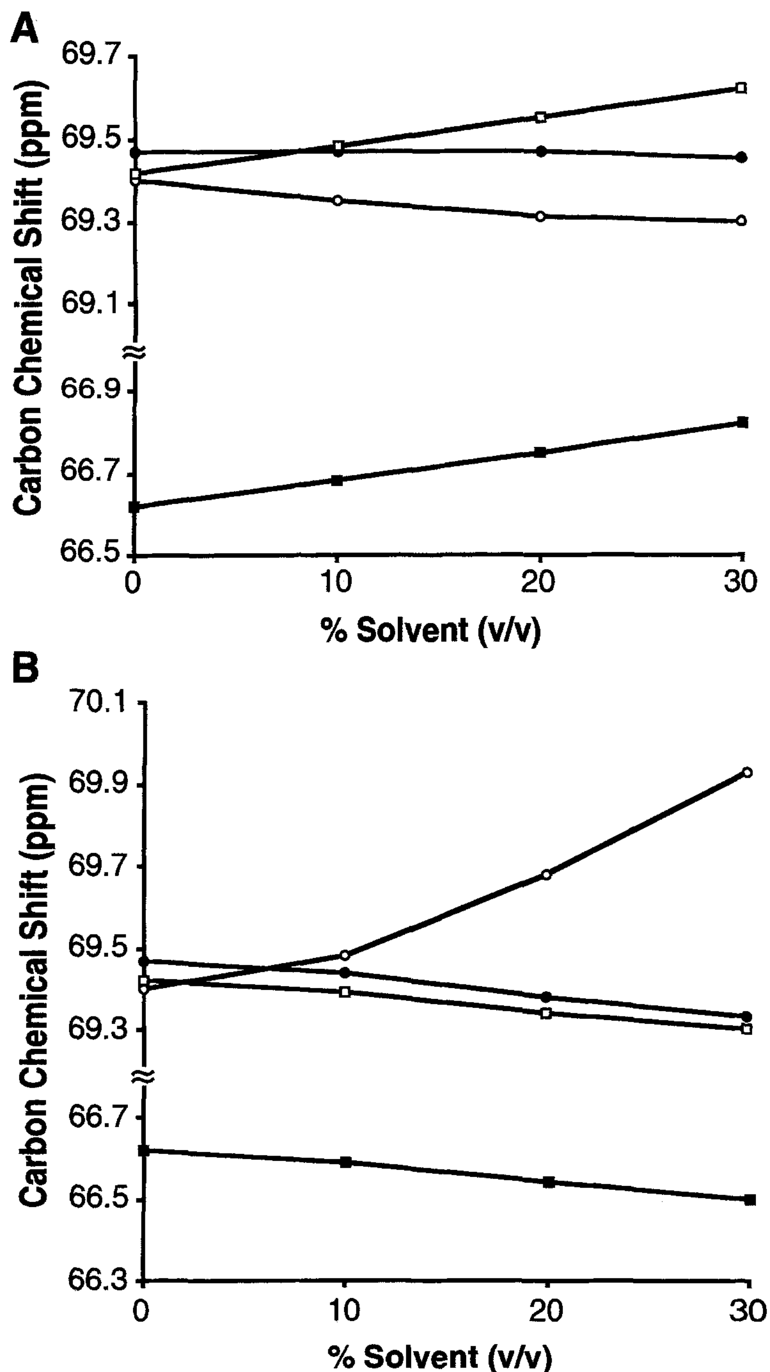

Fig. 1. Plot of dioxane carbon chemical shifts versus percent of added C^2H_3CN (A) and TFE-d2 (B). Various methods of referencing the ^{13}C chemical shift are shown. The external TMS signal was set to 0 ppm (-■-). The external TSP signal was set to 0 ppm (-□-). In both cases, the data show the linear response of these shifts as a function of cosolvent concentration within these limits. The carbon 0 ppm frequency was calculated by multiplying the internal TSP proton frequency by the ratio of gyromagnetic ratios, γ_x/γ_H, 0.25144950 (-●-) (see text). The internal TSP signal was set to 0 ppm (-○-).

often carried out in aqueous medium, it was decided to use an external TSP capillary to define the 0 ppm of the ^{13}C chemical shift scale. The carbon chemical shift of the external TSP signal is independent of the TSP concentration in the 1–100 mM range. In the present study, dioxane was used as the internal standard and was referenced relative to external TSP. The dioxane peak showed a marginal downfield shift in acetonitrile (Fig. 1A) and an upfield shift in TFE relative to its chemical shift in water (Fig. 1B). The linear dependence of the dioxane chemical shift as a function of cosolvent concentration allows the calculation of the internal reference value for dioxane at any intermediate cosolvent concentration.

Linear pentapeptides Gly-Gly-X-Gly-Gly where X represents the 20 natural amino acids were used to measure the carbon chemical shifts of individual amino acids without the influence of end-group effects. Since the peptides were prepared as mixtures, each containing five different pentapeptides, proton-detected 2D inverse experiments were used for the carbon assignments. As a result, chemical shifts for only the protonated carbons were obtained. The proton assignments for the individual spin systems of the peptide mixtures were obtained from the TOCSY experiments recorded in ^2H_2O at 298 K. The carbon assignments were then made from the HMQC spectra recorded on a 60–70 mM peptide mixture at natural abundance of ^{13}C nuclei. Data collected with one of the peptide mixtures at 5 mM concentration show that the carbon chemical shifts are not dependent on the peptide concentration. The C^α chemical shift for the central glycine residue in the pentaglycine peptide was obtained by comparing its ^{13}C NMR spectrum with that of Gly-Gly-X-Gly-Gly peptides, where X is any other amino acid other than glycine. Appropriate amounts of acetonitrile or TFE were added to obtain 10, 20 and 30% of the cosolvent in ^2H_2O and the internal dioxane peak was set to the appropriate value to reflect chemical shifts from external TSP. The chemical shift values for all of the protonated carbons of the 20 amino acids in Gly-Gly-X-Gly-Gly in aqueous solution and aqueous solution containing 10, 20 and 30% acetonitrile or TFE are listed in Table 1. In addition, values are reported for cysteine (free thiol), cystine (disulfide), and *S*-methyl cysteine. The carbon chemical shifts of the solvent signals are also listed in Table 1. When the difference in the reference value of the dioxane peak between the present work and the literature value (Richarz and Wüthrich, 1978) is taken into account, the carbon chemical shift values of all the amino acids in water are similar to those reported in the literature (Richarz and Wüthrich, 1978). The C^α chemical shift values obtained in this study are ~ 0.5 ppm larger than those reported by Richarz and Wüthrich. This is possibly due to the differences in the peptides used and the experimental conditions employed in both studies. In the present study, protected pentapeptides of the type Gly-Gly-X-Gly-Gly were used whereas Richarz and Wüthrich used protected tetrapeptides of the type Gly-Gly-X-Ala. The C^α chemical shift in *S*-methyl cysteine was similar to that observed for the cystine, but the C^β chemical shift was very much different from the values reported for either cysteine or cystine. When appropriate corrections were made to the internal dioxane chemical shift, addition of acetonitrile or TFE to the aqueous solution of the amino acids did not affect the carbon resonance positions and, as a result, the carbon chemical shift values in these organic cosolvents were only slightly different from the values obtained in water (Table 1).

Fluorinated alcohols such as TFE are often used as cosolvents to induce secondary structure in peptides. The data from the present study provides ^{13}C chemical shifts for amino acids in peptides of unordered structure at different solvent conditions which can be used as reference shifts in conformational studies of polypeptides in aqueous solution containing organic cosol-

TABLE 1
^{13}C CHEMICAL SHIFTS[a] FOR THE 20 COMMON AMINO ACID RESIDUES IN PROTECTED LINEAR PENTAPEPTIDES GLY-GLY-X-GLY-GLY IN ^2H_2O AND IN ^2H_2O CONTAINING ORGANIC COSOLVENTS AT 298 K

	^2H_2O	TFE			C^2H_3CN		
		10%	20%	30%	10%	20%	30%
Gly							
C^α	45.3	45.2	45.2	45.2	45.3	45.4	45.5
Ala							
C^α	52.8	52.8	52.8	52.8	52.8	52.8	52.9
C^β	19.3	19.3	19.1	19.1	19.5	19.5	19.6
Val							
C^α	62.9	62.9	62.9	63.0	62.9	63.0	63.0
C^β	32.7	32.7	32.6	32.6	32.8	32.8	32.9
C^γ	20.5, 21.2	20.4, 21.1	20.1, 21.0	20.2, 21.0	20.5, 21.5	20.5, 21.5	20.6, 21.5
Leu							
C^α	55.7	55.7	55.8	55.9	55.8	55.8	55.8
C^β	42.5	42.5	42.5	42.4	42.6	42.6	42.7
C^γ	27.1	27.1	27.1	27.1	27.3	27.3	27.5
C^δ	23.4, 24.9	23.5, 24.9	23.4, 24.8	23.3, 24.6	23.7, 25.1	23.7, 25.1	23.8, 25.3
Ile							
C^α	61.6	61.6	61.6	61.6	61.7	61.7	61.8
C^β	39.0	39.0	39.0	38.9	39.1	39.1	39.3
$C^{\gamma1}$	27.5	27.3	27.3	27.3	27.6	27.6	27.6
$C^{\gamma2}$	17.6	17.5	17.3	17.2	17.8	17.8	17.8
C^δ	13.1	13.1	13.0	12.8	13.4	13.4	13.5
Pro							
C^α	64.0	64.0	64.0	64.1	64.0	64.0	64.1
C^β	32.2	32.2	32.0	32.0	32.2	32.0	32.0
C^γ	27.4	27.3	27.2	27.1	27.3	27.2	27.1
C^δ	50.0	49.8	49.8	49.8	49.8	49.8	49.8
Ser							
C^α	58.6	58.6	58.5	58.5	58.7	58.7	58.8
C^β	64.0	64.0	64.0	63.9	64.1	64.1	64.3
Thr							
C^α	62.2	62.2	62.1	62.1	62.3	62.3	62.3
C^β	69.9	69.7	69.7	69.7	69.9	69.9	69.9
C^γ	21.7	21.6	21.5	21.4	21.8	21.8	21.9
Asp							
C^α	53.0	53.0	52.9	52.9	53.1	53.1	53.2
C^β	38.3	38.3	38.1	38.1	38.5	38.5	38.6
Asn							
C^α	53.4	53.4	53.4	53.4	53.5	53.5	53.6
C^β	39.0	38.9	38.9	38.9	38.9	38.9	39.3
Glu							
C^α	56.1	56.1	56.1	56.1	56.2	56.2	56.2
C^β	28.8	28.8	28.8	28.8	29.1	29.1	29.1
C^γ	32.8	32.8	32.6	32.8	33.0	33.0	33.1

TABLE 1 (continued)

	^2H_2O	TFE			C^2H_3CN		
		10%	20%	30%	10%	20%	30%
Gln							
C^α	56.3	56.3	56.2	56.2	56.4	56.4	56.4
C^β	29.6	29.6	29.5	29.5	29.7	29.7	29.8
C^γ	34.0	34.0	33.8	33.8	34.1	34.1	34.3
Lys							
C^α	56.8	56.8	56.8	56.9	56.9	56.9	56.9
C^β	33.1	33.0	33.1	33.1	32.9	32.9	33.3
C^γ	24.8	24.8	24.8	24.8	25.0	25.0	25.0
C^δ	29.1	28.9	29.0	28.9	29.1	29.1	29.3
C^ε	42.0	42.1	42.0	42.1	42.1	42.1	42.1
Arg							
C^α	56.6	56.6	56.7	56.7	56.7	56.7	56.8
C^β	30.9	30.9	30.8	30.8	31.0	31.0	31.0
C^γ	27.2	27.1	27.1	27.1	27.3	27.3	27.5
C^δ	43.3	43.5	43.3	43.3	43.5	43.5	43.6
Cys(SMe)							
C^α	55.8	55.7	55.7	55.7	55.9	55.9	55.9
C^β	37.6	37.6	37.6	37.5	37.8	37.8	38.0
CysH (Cysteine)							
C^α	58.5	58.6	58.7	58.8	58.6	58.6	58.7
C^β	28.1	28.2	28.3	28.3	28.0	28.0	27.9
(Cys)2 (Cystine)							
C^α	55.7	55.7	55.8	55.8	55.8	55.9	55.9
C^β	41.5	41.4	41.5	41.5	41.6	41.8	41.9
Met							
C^α	55.8	55.9	55.9	56.0	55.9	56.0	56.1
C^β	32.8	32.8	32.8	32.8	32.8	32.6	33.1
C^γ	32.1	32.3	32.1	32.1	32.1	32.3	32.5
C^ε	17.0	16.9	16.8	16.6	17.1	17.1	17.3
Phe							
C^α	58.3	58.3	58.3	58.4	58.3	58.4	58.5
C^β	39.7	39.7	39.6	39.6	39.6	39.8	39.8
C^δ	133.1	133.2	133.0	133.0	133.1	133.2	133.3
C^ε	132.8	132.9	132.8	132.7	132.8	132.9	132.9
C^ζ	131.5	131.5	131.3	131.3	131.5	131.6	131.6
Tyr							
C^α	58.3	58.3	58.3	58.2	58.2	58.4	58.5
C^β	39.0	39.0	39.0	38.9	38.9	39.1	39.3
C^δ	134.7	134.7	134.7	134.5	134.8	134.9	134.9
C^ε	119.6	119.6	119.6	119.3	119.7	119.6	119.6
His							
C^α	55.5	55.5	55.5	55.5	55.5	55.6	55.6
C^β	29.1	29.1	29.1	28.9	28.9	28.9	29.4
C^δ	121.3	121.4	121.3	121.3	121.6	120.8	121.7
C^ε	137.6	137.6	137.6	137.6	137.9	137.0	137.5

TABLE 1 (continued)

	^2H_2O	TFE			C^2H_3CN		
		10%	20%	30%	10%	20%	30%
Trp							
C^{α}	57.7	57.6	57.6	57.6	57.7	57.7	57.7
C^{β}	29.7	29.7	29.7	29.7	29.8	30.0	30.1
$C^{\delta 1}$	127.5	127.4	127.3	127.2	127.5	127.4	127.4
$C^{\zeta 3}$	124.9	124.8	124.8	124.7	125.0	124.9	124.9
$C^{\zeta 2}$	114.8	114.7	114.7	114.6	114.9	114.9	114.9
$C^{\epsilon 3}$	122.3	122.3	122.2	122.1	122.4	122.4	122.4
$C^{\eta 2}$	121.2	121.1	121.1	121.0	121.2	121.3	121.4
Dioxane peak	69.42	69.39	69.34	69.30	69.48	69.56	69.62
Solvent peak	–	–	61.70[b]	61.70[b]	3.37[d]	3.45[d]	3.52[d]
		127.45[c]	127.44[c]	127.43[c]			

[a] ^{13}C chemical shifts in ppm are referenced to internal dioxane at different cosolvent concentrations relative to external TSP.
[b] ^{13}C chemical shift of methylene carbon in TFE.
[c] ^{13}C chemical shift of trifluoromethyl carbon in TFE.
[d] ^{13}C chemical shift of methyl carbon in C^2H_3CN.

vents. Therefore, we examined two peptides, bombesin (Carver and Collins, 1990) and Nle^{27}-hGHRF[1–29]NH_2 (Clore et al., 1986), for which structural information has been reported.

Bombesin is a 14-residue peptide that exists in random coil structure in aqueous solution and a portion of the molecule adopts an α-helical structure upon addition of TFE (Carver and Collins, 1990). Recently, it was shown that deviation of the observed C^{α}-chemical shifts from that of random coil form of the peptide can be utilized to identify the location of the helix in bombesin (Reily et al., 1992). In that report, the α-carbon chemical shifts of bombesin itself in ^2H_2O were used for the random coil values to calculate the effective secondary shifts caused by the formation of a helical structure. This does not account, however, for the possibility of a small amount of residual secondary structure or for other anisotropic effects such as steric and end-group effects on these carbon chemical shifts in water or for purely solvent related effects. Also, in the case of bombesin with only 14 residues, it was possible to obtain the peak assignments in its random coil form but for the larger peptides the peak assignments will be more difficult to obtain due to the overlap of cross peaks (vide infra). Therefore, a proper structural analysis of peptides dissolved in solvent mixtures requires the use of random coil shifts derived under the same conditions as the study is to be done.

The plot of the difference between α-carbon chemical shifts of bombesin in 70% ^2H_2O/30% TFE (v/v) and the random coil values reported here (carbon secondary shifts) versus the primary sequence is shown in Fig. 2. The chemical shift difference between the α-carbon chemical shifts of bombesin in ^2H_2O and those of the random coil amino acids are also plotted in Fig. 2. Although this difference is expected to be zero, the observed small deviations underscore the importance of the primary amino acid sequence (any residual secondary structure) or other anisotropic effects on the α-carbon chemical shifts. The carbon secondary shifts in Fig. 2 are similar to those reported previously (Reily et al., 1992). In both cases the results show a similar envelope extend-

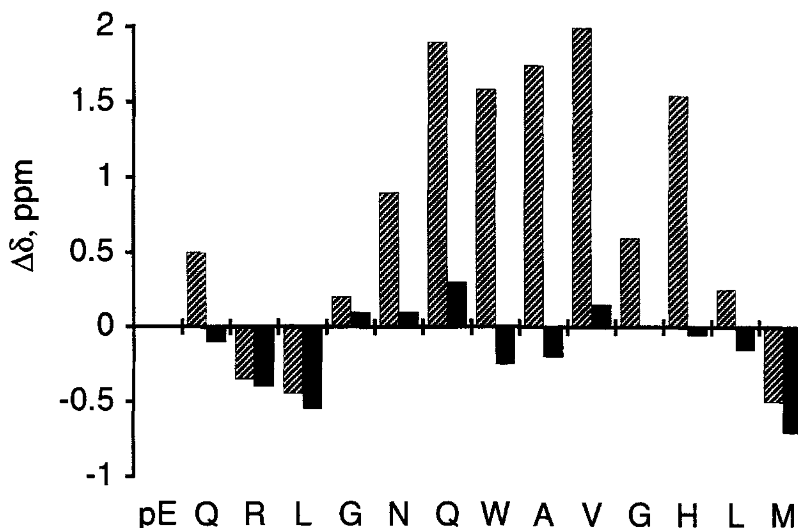

Fig. 2. Histograms showing the sequence specific deviation of the C^{α} chemical shifts of bombesin dissolved in 70% ^2H_2O/30% TFE (v/v) from the C^{α} random coil values, $\Delta\delta_{ppm} = \delta_{(observed)} - \delta_{(random\ coil)}$. pE in the primary sequence refers to pyroglutamic acid. The hatched bars represent the $\Delta\delta_{ppm} = \delta_{(observed)} - \delta_{(random\ coil)}$, where $\delta_{(observed)}$ refers to the C^{α} chemical shifts of bombesin in 70% ^2H_2O/30% TFE (v/v) and the $\delta_{(random\ coil)}$ refers to the C^{α} chemical shifts of amino acids in 70% ^2H_2O/30% TFE (v/v). The closed bars represent the $\Delta\delta_{ppm} = \delta_{(observed)} - \delta_{(random\ coil)}$, where $\delta_{(observed)}$ refers to the C^{α} chemical shifts of bombesin in 100% ^2H_2O and the $\delta_{(random\ coil)}$ refers to the C^{α} chemical shifts of amino acids in 100% ^2H_2O. Since there is no ^{13}C chemical shifts data available for pyroglutamic acid in 70% ^2H_2O/30% TFE, the C^{α} chemical shift of pE in bombesin was used as random coil value.

ing from residue 6 to 13, which has been reported as the α-helical part of the peptide (Carver and Collins, 1990). An exception to the expected trend is the small negative deviation of the secondary shifts in the N-terminus and for the last residue in the C-terminus when the random coil values in 30% TFE were used (Fig. 2). Although a negative deviation is characteristic of β-sheet structure (Spera and Bax, 1991), the small deviations (< 0.5 ppm) are difficult to interpret.

To examine the utility of this method further, another peptide, Nle^{27}-hGHRF[1–29]NH_2, was also investigated. It has been reported from NOE-derived results (Clore et al., 1986) in 30% TFE solution that Nle^{27}-hGHRF[1–29]NH_2 has two distinct regions of α-helix extending from residues 6 to 13 and 16 to 29. Carbon chemical shifts values for Nle^{27}-hGHRF[1–29]NH_2 in 30% TFE were obtained from the 2D HMQC spectrum recorded under the experimental conditions similar to those used in a previous report (Clore et al., 1986). Since the HMQC spectrum of Nle^{27}-hGHRF[1–29]NH_2 in ^2H_2O, where the peptide exists in a random coil structure, is not well resolved in the α-carbon region, it was not possible to assign all the α-carbon resonances in ^2H_2O. This necessitated the use of carbon chemical shifts of amino acids in 30% TFE (Table 1) as random coil values. The plot of the C^{α} secondary shifts, δ(observed)−δ(random coil), of Nle^{27}-hGHRF[1–29]NH_2 in 30% TFE versus the primary sequence is shown in Fig. 3. The plot shows two separate regions extending from residues 7 to 14 and 17 to 28 that considerably deviate from the random coil chemical shifts (secondary shifts) consistent with two α-helical domains. These results are in good agreement with the NOE-based NMR observations (Clore et al., 1986) of two α-helical regions extending from residue 6 to 13 and 16 to 29. The present results indicate that

Fig. 3. Plot of $\Delta\delta_{ppm}$, ($\delta_{(observed)} - \delta_{(random\ coil)}$), for the C^{α} carbons of Nle^{27}-hGHRF[1–29]NH_2 in 70% ^2H_2O/30% TFE (v/v), where $\delta_{(random\ coil)}$ refers to the C^{α} chemical shifts of amino acids in 70% ^2H_2O/30% TFE (v/v), versus residue type. X in the primary sequence denotes norleucine. The C^{α} chemical shift of leucine in 70% ^2H_2O/30% TFE was used for the random coil value of norleucine.

residue 29 is not part of the helix and the break between helices occurs at residues 15 and 16 rather than 14 and 15 (Fig. 3). At the N-terminus, the first 6 residues are largely unstructured and this is consistent with previous results (Clore et al., 1986). However, residues 3 to 6 show some secondary shift (0.7–1.4 ppm), possibly due to a transient helix in this region, which was not previously observed.

CONCLUSIONS

We have measured the carbon chemical shifts of all protonated carbons of the 20 common amino acids in aqueous solution and in aqueous solution containing acetonitrile or TFE as the cosolvent using the protected linear pentapeptide Gly-Gly-X-Gly-Gly. Carbon chemical shifts were referenced with respect to internal dioxane (69.4 ppm), which was calibrated against external TSP. External standard (either TMS or TSP capillary) was found to be the only reliable solvent-independent referencing technique. In the present study, external TSP was chosen to define the 0 ppm of the ^{13}C chemical shift scale. In the absence of organic cosolvent, indirect referencing to TSP protons (Live et al., 1984; Bax and Subramanian, 1986) provides a ^{13}C 0 ppm frequency that serendipitously coincides with internal TSP and is therefore a viable alternative when working in strictly aqueous solutions. The use of these values for random coil shifts was demonstrated with two model peptides, bombesin and Nle^{27}-hGHRF[1–29]NH_2. In both cases, the C^{α} chemical shifts exhibited a characteristic positive deviation from random coil values which is indicative of the presence of α-helix. For both bombesin and Nle^{27}-hGHRF[1–29]NH_2 as well as in proteins (Spera and Bax, 1991; Wishart et al., 1991), most of the amino acid residues in the α-helical conformations display positive C^{α} secondary shifts of 2.0 to 4.0 ppm. It is important to reference the carbon chemical shifts of peptides and proteins in the same way as the amino acids in a random coil, and any discrepancy in referencing can lead to over/underestimated secondary shift values, resulting in the misinterpretation of the secondary structure. It is advantageous to consid-

er these secondary shift analyses as complementary to circular dichroism or more rigorous NMR studies. In the case of circular dichroism, analysis of carbon secondary shifts provides additional information by localizing the secondary structure in the molecule. When used in conjunction with NOE-based structure determinations, secondary carbon chemical shifts will provide an accurate assessment of secondary structure in the early stages of a study and also will facilitate the spectral assignment process.

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