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# 'Random coil' $^1$H chemical shifts obtained as a function of temperature and trifluoroethanol concentration for the peptide series GGXGG

Gene Merutka*, H. Jane Dyson** and Peter E. Wright**

*Department of Molecular Biology, The Scripps Research Institute, 10666 North Torrey Pines Road, La Jolla, CA 92037, U.S.A.*

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**Summary**

Proton chemical shifts of a series of disordered linear peptides (H-Gly-Gly-X-Gly-Gly-OH, with X being one of the 20 naturally occurring amino acids) have been obtained using 1D and 2D $^1$H NMR at pH 5.0 as a function of temperature and solvent composition. The use of 2D methods has allowed some ambiguities in side-chain assignments in previous studies to be resolved. An additional benefit of the temperature data is that they can be used to obtain 'random coil' amide proton chemical shifts at any temperature between 278 and 318 K by interpolation. Changes of chemical shift as a function of trifluoroethanol concentration have also been determined at a variety of temperatures for a subset of peptides. Significant changes are found in backbone and side-chain amide proton chemical shifts in these 'random coil' peptides with increasing amounts of trifluoroethanol, suggesting that caution is required when interpreting chemical shift changes as a measure of helix formation in peptides in the presence of this solvent. Comparison of the proton chemical shifts obtained here for H-Gly-Gly-X-Gly-Gly-OH with those for H-Gly-Gly-X-Ala-OH [Bundi, A. and Wüthrich, K., (1979) *Biopolymers*, **18**, 285–297] and for Ac-Gly-Gly-X-Ala-Gly-Gly-$NH_2$ [Wishart, D.S., Bigam, C.G., Holm, A., Hodges, R.S. and Sykes, B.D. (1995) *J. Biomol. NMR*, **5**, 67–81] generally shows good agreement for CH protons, but reveals significant variability for NH protons. Amide proton chemical shifts appear to be highly sensitive to local sequence variations and probably also to solution conditions. Caution must therefore be exercised in any structural interpretation based on amide proton chemical shifts.

## Introduction

Although the chemical shift of a resonance is highly sensitive to the local chemical environment of the nucleus, it remains one of the most difficult NMR parameters to interpret in structural terms. This is because of the very large number of factors that can influence the chemical shift, including, but not limited to, the presence of nearby aromatic, charged or polar groups. Such influences can be accounted for in folded proteins, where theoretical predictions of chemical shifts are beginning to be made with some success (Ösapay and Case, 1991). Many attempts have been made to calibrate empirically the structure-based chemical shifts in proteins of known structure for which chemical shift data are available (Dalgarno et al., 1983; Pardi et al., 1983; Szilágyi and Jardetzky, 1989; Williamson, 1990; Wishart et al., 1991). These methods rely heavily on comparison with so-called 'random coil' chemical shifts, that is, the chemical shifts of peptide protons in the absence of conformational preferences for secondary structure in solution. These baseline values have also been used qualitatively to estimate the conformational preferences for secondary structure in peptides (Shin et al., 1993). As attempts are made to quantitate secondary structure in peptides from chemical shift data (see, for example, Merutka et al. (1993) and Rizo et al. (1993)), it becomes important to re-evaluate the baseline chemical shifts for a wide range of solution conditions.

* Present address: Trimeris Inc., Two University Place, Durham, NC 27707, U.S.A.
**To whom correspondence should be addressed.

The random coil parameters that are used are generally those of Bundi and Wüthrich (1979a), which were determined using 1D methods for a single set of peptides with sequence H-Gly-Gly-X-Ala-OH, at pH 7 and 35 °C. These conditions are not necessarily relevant to peptide work, which is often done at significantly different (usually lower) temperatures and pH values. A pH dependence of random coil shifts for some members of the same set of peptides has been published (Bundi and Wüthrich, 1979b), and a partial set of peptides has been examined to establish the effects of temperature and urea concentration on chemical shift (Jimenez et al., 1986). Sets of $^{13}C$ random coil chemical shifts have been published for this peptide series (Richarz and Wüthrich, 1978), and $^{15}N$ chemical shifts have been reported more recently (Braun et al., 1994). The nature of the constant residues in these peptides is also of concern. The C-terminus is an alanine residue, which has a preference for backbone dihedral angles in the α-region of conformational space. While not expected to cause the formation of secondary structure per se in such a short peptide, it is possible that by biasing the backbone and side-chain dihedral angles adjacent to the variant residues at position 4, some systematic error may be induced. This has indeed been noticed for the peptide $CF_3CO$-Gly-Gly-Tyr-Ala-$OCH_3$ (Wüthrich and De Marco, 1976). No complete data set has been published for the temperature coefficients of peptide amide protons under 'random coil' conditions. This is a serious lack, since it is essential to correct any baseline amide proton chemical shifts according to the temperature used in the particular study.

Because of the incompleteness of previous studies, and to convince ourselves that the C-terminal alanine residue was not affecting the chemical shifts, we have undertaken a 2D NMR study of a related set of peptides, H-Gly-Gly-X-Gly-Gly-OH in water solution at pH 5.0 at a variety of temperatures, giving a set of random coil chemical shifts at a number of temperatures, as well as a complete set of 'random coil' temperature coefficients. In addition, a large number of recent peptide studies have used the solvent trifluoroethanol (TFE), which has been shown from CD studies to induce helix in some peptides. The extension of TFE studies to NMR has pointed out the need for chemical shift data in TFE. We therefore decided to include in the present study measurements of random coil shifts in various concentrations of TFE. The constitution of the set of peptides used is particularly suitable for the TFE study, since the sequence is one of the least likely to form helix in solution. $^{13}C$ chemical shifts have been reported for peptides in this series (Keim et al., 1973a,b,1974) and there is a recent study of the $^{13}C$ chemical shifts of the identical series of peptides under very similar conditions to those used in the present study (Thanabal et al., 1994). An extensive study of $^{1}H$, $^{13}C$ and $^{15}N$ random coil shifts for the peptide series Ac-Gly-Gly-X-Ala-Gly-Gly-$NH_2$ has been submitted by another group at the same time as this work (Wishart et al., 1994).

## Materials and Methods

Peptides were synthesized using Boc chemistry and PAM resin, employing the method of Schnölzer et al. (1992) as described previously (Merutka et al., 1993). Peptides were deprotected and cleaved from the resin using an HF:*p*-cresol mixture (9:1) at 273 K for 1 h. Crude peptide was extracted first with ether, then with a solution of 10% acetonitrile containing 0.1% trifluoroacetic acid, and subsequently lyophilized. NMR samples contained about 0.03 M peptide in 90% $H_2O$/10% $D_2O$ or deuterated trifluoroethanol/$H_2O$ mixtures and were adjusted to pH 5.0 ± 0.1. Deuterated trifluoroethanol (2,2,2-trifluoroethanol-$d_3$) was obtained from Cambridge Isotope Laboratories. Dioxane was added as an internal reference in all samples. For peptides containing alanine and leucine, a second internal standard was included, TSP (3(-trimethylsilyl)-1-propane-sulfonic acid) and DSS (2,2-dimethyl-2-silapentane-5-sulfonate), respectively.

CD spectra of samples in water and water–TFE mixtures were recorded as previously described (Waltho et al., 1993). All TFE concentrations are calculated as mole percent TFE. Peptide concentrations for CD spectroscopy were 10–20 μM.

NMR experiments were carried out on Bruker AMX500 (for 1D and TOCSY data) and AMX600 spectrometers (for ROESY data). Fast-TOCSY experiments (Marion et al., 1989; Merutka et al., 1993) were employed for resonance assignment, using two-step phase cycling with a presaturation delay of 1.8 s and 128 $t_1$ points. Spectral widths were commonly 7024 Hz, with 8K data points in $\omega_2$. ROESY experiments were obtained at 278 K with a presaturation delay of 1.8 s and a mixing time of 300 ms, using a spectral width of 6024 Hz, 8K data points in $\omega_2$ and 512 $t_1$ points. The probe temperature was calibrated using methanol by the method of Van Geet (1970).

Temperature coefficients were calculated for the amide protons of all peptides in $H_2O$ solutions and $H_2O$/TFE mixtures by standard methods, using chemical shift data from four and five temperatures, respectively, in the range 278–328 K.

## Results and Discussion

*Selection of peptides*

The peptide series GGXGG chosen for this study was selected in order to ensure maximum flexibility and conformational freedom for residue X, so that the chemical shift values obtained from our study would best reflect those to be expected in 'random' structures. A short peptide sequence was chosen, in order to minimize the

TABLE 1
AVERAGE CHEMICAL SHIFTS AND STANDARD DEVIATIONS FOR THE INVARIANT GLYCINE RESIDUES

| Residue | NH | $C^{\alpha}H$ (average)[a] | $C^{\alpha}H$ (aromatic peptides)[b] | | | $C^{\alpha}H$[c] |
|---|---|---|---|---|---|---|
| | | | Phe | Tyr | Trp | |
| $Gly^1$ | – | 3.89 ± 0.01 | 3.85 | 3.86 | 3.80 | 3.87 ± 0.05 |
| $Gly^2$ | 8.68 ± 0.05 | 4.04 ± 0.02 | 3.97 | 3.97 | 3.96 | 4.06 ± 0.04 |
| $Gly^4$ | 8.66 ± 0.07 | 3.97 ± 0.01 | 3.91/3.83 | 3.91/3.84 | 3.94/3.90 | – |
| $Gly^5$ | 8.07 ± 0.04[d] | 3.77 ± 0.01 | 3.73 | 3.74 | 3.64/3.54 | – |

Chemical shifts are given in ppm; T = 277.2 K, pH = 5.0.
[a] Excluding the values from the peptides containing aromatic residues.
[b] A single chemical shift entry indicates that the glycine $C^{\alpha}H_2$ resonances are degenerate; the chemical shift values for both glycine resonances are listed when these could be resolved.
[c] From Bundi and Wüthrich (1979a), at 308 K and pH 7.0.
[d] The average for the NH of $Gly^5$ does not include the chemical shifts for the phenylalanine, tyrosine and tryptophan peptides, which are 7.62, 7.63 and 7.29 ppm, respectively.

likelihood of formation of structured conformers in the ensemble, and the influence of end effects from the N- and C-termini was minimized by the presence of two residues on either side of the central residue X. While the presence of blocking groups at the termini would probably have been an advantage in minimizing end effects, the charges present in the unblocked peptides were considered necessary to ensure adequate solubility of all peptides in the series. The influence of the charged termini on the chemical shifts of the central residue X is very small, as demonstrated by the similarity of the chemical shifts of residues 2 and 4 (see results below); we can be confident that the results from the peptide series GGXGG represent as nearly as possible 'random coil' chemical shifts for all residues.

*Resonance assignments*

The resonances of the four invariant glycines show little variation between peptides, except where the variant residue is aromatic. The ROESY spectrum of peptide GGAGG was used as a basis for sequential assignment of the resonances of the glycine residues. Chemical shifts for these residues, averaged over all 17 nonaromatic peptides, are given in Table 1, together with the values obtained for the $C^{\alpha}H$ of $Gly^1$ and $Gly^2$ by Bundi and Wüthrich (1979a) and those for the peptides containing aromatic residues.

Complete resonance assignments were made for the variant residue X in each of the peptides H-Gly-Gly-X-Gly-Gly-OH (GGXGG) at 278 K, using 1D and fast-TOCSY spectra, and are shown in Table 2. The differences in pH and temperature between the present work

TABLE 2
RANDOM COIL CHEMICAL SHIFTS OBTAINED FOR RESIDUE X IN THE PEPTIDES GGXGG

| Residue | NH | $C^{\alpha}H$ | $C^{\beta}H$ | $C^{\gamma}H$ | $C^{\delta}H$ | Other |
|---|---|---|---|---|---|---|
| Ala | 8.67 | 4.34 | 1.41 | | | |
| Arg | 8.69 | 4.34 | 1.80, 1.90 | 1.67, (1.67)[b] | 3.22, (3.22) | 7.26 ($N^{\varepsilon}H$); 6.49,6.92 ($N^{\eta}H$) |
| Asn | 8.76 | 4.76 | 2.79, 2.88 | | | 7.03,7.74 ($N^{\delta}H$) |
| Asp | 8.61 | 4.63 | 2.70, 2.71 | | | |
| Cys[c] | 8.75 | 4.58 | 2.97, (2.97) | | | |
| Gln | 8.70 | 4.36 | 2.02, 2.15 | 2.39, 2.40 | | 6.97,7.69 ($N^{\varepsilon}H$) |
| Glu | 8.83 | 4.29 | 1.99, 2.09 | 2.34, (2.34) | | |
| Gly | 8.66 | 4.01, (4.01) | | | | |
| His | 8.79 | 4.77 | 3.19, 3.33 | | 7.31 | 8.59 ($C^{\varepsilon}H$) |
| Ile | 8.52 | 4.18 | 1.89 | 1.21, 1.49 | 0.88 | 0.93 ($C^{\gamma}H_3$) |
| Leu | 8.64 | 4.35 | 1.64, (1.64) | (1.64) | 0.89, 0.93 | |
| Lys | 8.67 | 4.32 | 1.79, 1.87 | 1.46, (1.46) | 1.68, (1.68) | 2.99, (2.99) ($C^{\varepsilon}H$); 7.59 ($N^{\zeta}H$) |
| Met | 8.73 | 4.52 | 2.03, 2.15 | 2.56, 2.63 | | 2.10 ($C^{\varepsilon}H_3$) |
| Phe | 8.61 | 4.62 | 3.06, 3.15 | | 7.28 | 7.38 ($C^{\varepsilon}H$); 7.32 ($C^{\zeta}H$) |
| Pro | – | 4.44 | 2.03, 2.30 | 2.06, (2.06) | 3.63, 3.67 | |
| Ser | 8.69 | 4.49 | 3.93, 3.95 | | | |
| Thr | 8.52 | 4.39 | 4.32 | 1.22 | | |
| Trp | 8.46 | 4.67 | 3.28, (3.28) | | | 10.20 ($N^{\varepsilon 1}H$); 7.27 ($C^{\delta 1}H$); 7.64 ($C^{\varepsilon 3}H$); 7.17 ($C^{\zeta 3}H$); 7.24 ($C^{\eta}H$); 7.50 ($C^{\zeta 2}H$) |
| Tyr | 8.59 | 4.56 | 2.98, 3.06 | | 7.15 | 6.85 ($C^{\varepsilon}H$) |
| Val | 8.51 | 4.13 | 2.12 | 0.95, 0.97 | | |

[a] Chemical shifts were obtained in 90% $H_2O$/10% $D_2O$ at 277.2 K, pH 5.0 and are referenced to dioxane (3.75 ppm).
[b] Numbers in parentheses are tentative assignments; two resonances are presumed to be overlapped.
[c] The peptide GGCGG was always kept acidic and the central cysteine residue is assumed to be reduced.

and that of Bundi and Wüthrich (1979a) do not cause large differences in the chemical shifts of most protons. The exceptions are obvious – all of the amide proton chemical shifts are changed by the difference in temperature, and the resonances of the acidic residues (histidine, aspartic acid and glutamic acid) shift due to the difference in pH, which alters their ionization state. In order to compare more closely the values obtained for the two sets of peptides, we calculated the differences between the values of Bundi and Wüthrich (1979a) for the peptides GGXA at pH 7.0 and 35 °C and the random coil chemical shifts obtained for the peptides GGXGG at pH 5.0 at the same temperature. These are plotted in Fig. 1.

The most significant differences are observed for the side-chain resonances of histidine, consistent with the difference in pH (7.0 vs. 5.0), which spans the $pK_a$ range of the histidine side chain. Only the $C^{\alpha}H$ resonances of aspartic acid, histidine and cysteine, and the $C^{\beta}H$ resonances of threonine, aspartic acid and tryptophan differ from those of Bundi and Wüthrich (1979a) by more than 0.1 ppm. The aspartic acid and histidine side-chain resonances appear to be affected by the difference in pH between the two studies, while the changes in the chemical shifts of cysteine are no doubt due to the difference in oxidation state between cysteine (this work) and a presumed disulfide-bridged dimeric species (Bundi and Wüthrich, 1979a). We thus conclude that our results resemble closely those of Bundi and Wüthrich (1979a) for the nonexchangeable resonances, any differences being ascribable to the two-unit difference in pH between the two studies and to a difference in the oxidation state in the case of cysteine.

Larger discrepancies are observed for the amide protons (Fig. 1). Differences of greater than 0.2 ppm are observed for the amide proton resonances of valine, asparagine, glutamic acid and cysteine; 0.1–0.2 ppm differences are seen for alanine, arginine, histidine, phenylalanine and tyrosine amides. The temperatures are identical; the pH of the previously published study is 2.2–5.0 for the amide protons (Bundi and Wüthrich, 1979a), and pH effects are only likely to be seen for aspartic acid, glutamic acid and possibly histidine over this pH range. Disregarding the result for the cysteine amide because of the difference in oxidation state mentioned above, we infer that, for the amide protons of valine, asparagine, alanine, arginine, phenylalanine and tyrosine, the sequence of the peptide must be contributing significantly to the estimated 'random' chemical shifts. The magnitude of these chemical shift differences can significantly affect interpretation of chemical shift changes in structural terms, since, for

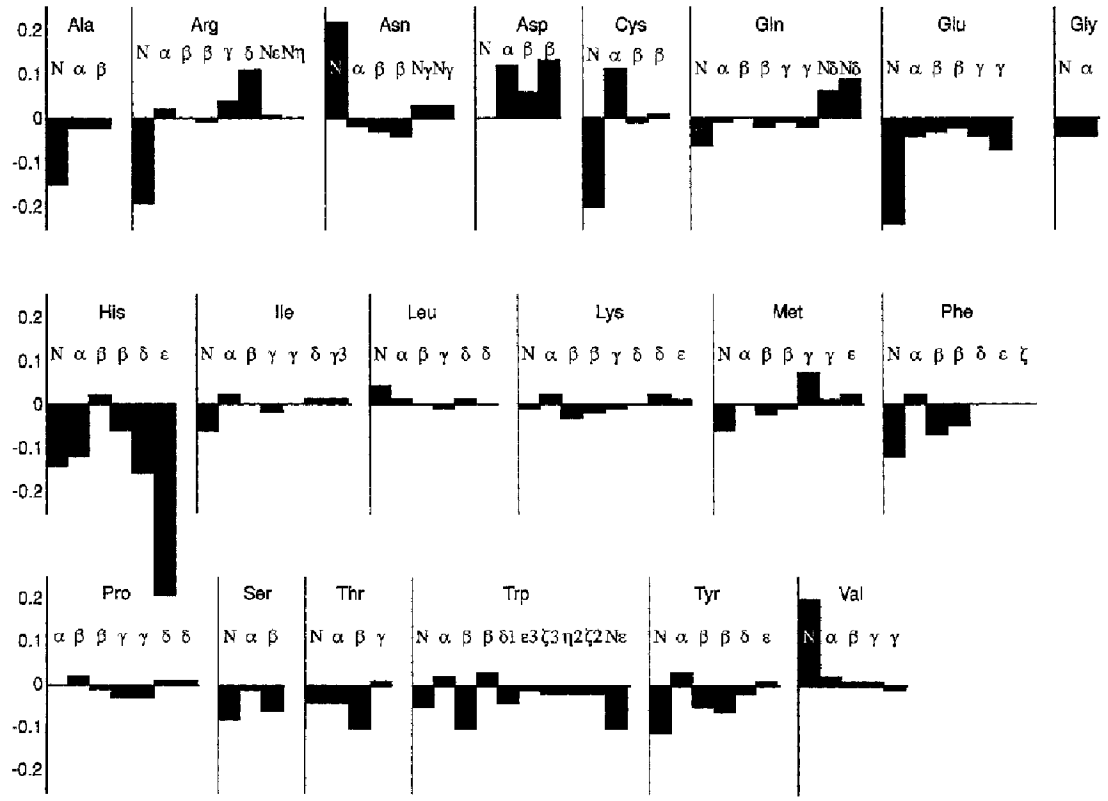


Fig. 1. Chemical shift differences (ppm) for each of the 20 naturally occurring amino acids, obtained by subtracting the values measured for the series GGXGG (308 K, pH 5.0, this work) from the corresponding values from the series GGXA (308 K, pH 7.0 for nonexchangeable protons, pH 2.2–5.0 for exchangeable protons, Bundi and Wüthrich (1979a)). Two values for geminal protons are included where these could be distinguished (for example, $C^{\beta}H$ of aspartic acid); where the two values could not be distinguished due to overlap or because of the complexity of the multiplets involved, a single value estimated at the center of the multiplet is used (for example, $C^{\alpha}H$ of glycine).

example, an ideal α-helix appears to have an amide proton upfield shift of 0.2 to 0.3 ppm (Wishart et al., 1991; Blanco et al., 1992), which is of the same order as the differences we observe from published 'random coil' values. While not claiming that our values are any more 'correct' than those of Bundi and Wüthrich (1979a), we believe that the choice of peptide sequence used in the two series may have significantly influenced the 'random' chemical shifts obtained, particularly for the amide protons. Differences of a similar order are observed for the temperature coefficients between the two series; we discuss our interpretation of the reasons for these and the above chemical shift differences in a later section.

A more recent tabulation of chemical shifts for the peptide series Ac-Gly-Gly-X-Ala-Gly-Gly-$NH_2$ (Ac-GGXAGG-$NH_2$) (Wishart et al., 1994) gives very similar values for the non-amide proton resonances to those in the present work. However, values for the amide proton resonances are widely at variance with our values for some residues. Generally, the amide proton chemical shifts of Wishart et al. (1994) are 0.2–0.3 ppm upfield of those obtained by us at the same temperature. Given the similarity in the non-amide proton chemical shifts between the two studies, it is hard to impute the difference in the amide proton chemical shifts to systematic errors in the measurements. There are several possible explanations for these NH chemical shift differences. The pH of the solutions and the temperature used for the comparison (298 K, as used by Wishart et al. (1994)) are identical. Three other possible explanations remain; firstly, the closer proximity of the residue X to the N- and C-termini in the series GGXGG compared to the series Ac-GGXAGG-$NH_2$ could produce the systematic downfield shift of the NH resonances. Several pieces of evidence argue that end effects are not the sole origin of the shift differences. Table 1 shows that the chemical shifts of the amide protons of $Gly^2$ and $Gly^4$ are virtually identical throughout the series and to each other, which implies that end effects are not significant even for these positions, adjacent to the terminal residues, and that such effects can therefore be neglected for the central position 3. We have also observed several examples of peptides where amide proton chemical shifts of residues that are one and two places removed from the C-terminus are essentially unaffected by extension or amidation of the peptide at the C-terminus (Dyson et al., 1988a,1992b), providing further evidence that the amide proton chemical shift of the residue in position 3 is unaffected by the unblocked C-terminus in either GGXGG or GGXA. (The amide proton resonance of the C-terminal residue itself in an unblocked peptide experiences an upfield shift (e.g. $Gly^5$ in Table 1).) The effects of N-terminal extension or acetylation are more variable; upfield shifts of residue 3 of between 0.07 and 0.2 ppm have been observed upon extension or acetylation at the N-terminus of a number of peptides (Dyson et al., 1992a,b; J.P. Waltho, H.J. Dyson and P.E. Wright, unpublished observations). However, comparison of our NH chemical shifts with those of Wishart et al. (1994) reveals differences ranging from 0.15 to 0.46 ppm (excluding the result for tryptophan, see below). The extent of this variation strongly suggests that the differences do not arise solely from the presence of the unblocked N-terminus in our peptides. The result for tryptophan is revealing: due to solubility problems, the peptide GGWAGG was studied by Wishart et al. (1994) in the unblocked form – the quoted NH chemical shift for tryptophan is 0.07 ppm upfield of our value (Table 2), significantly less than for the other members of the series, but significantly greater than, for example, any of the differences between CH resonances between the two series. The remaining differences between the two sets of measurements are: the presence of the alanine residue following residue X in the series Ac-GGXAGG-$NH_2$ used by Wishart et al. (1994) and the difference in solvent conditions, namely the addition of urea and phosphate to the solution by Wishart et al. (1994). We have already commented on the likelihood that the proximity of the C-terminal alanine affects the chemical shifts, especially those of the amide protons, measured by Bundi and Wüthrich (1979a) for the series GGXA. It is likely that these effects are also operating in the series Ac-GGXAGG-$NH_2$. Given the observed sensitivity of the amide proton resonances to TFE (see later), the difference in solvent conditions could also affect the results. We note, however, that urea alone appears to have little effect on amide proton chemical shifts (Jimenez et al., 1986; H.J. Dyson and P.E. Wright, unpublished observations). The variability of amide proton chemical shifts observed in the three series of 'random coil' peptides underscores the necessity for careful interpretation of amide proton chemical shifts in structural terms.

*Coupling constants*

We did not perform an exhaustive study of the coupling constants in the series of peptides GGXGG, although in principle this would be possible from the data. In many cases $^3J_{HN\alpha}$ coupling constants were difficult to quantitate, due to overlap of resonances even in the 2D spectra, but values that were obtained ranged from 5.5 Hz for alanine to 7.4 Hz for asparagine. $^3J_{\alpha\beta}$ coupling constants were calculated from the 1D spectra of the peptide GGFGG, and the values obtained were 6.6 and 7.7 Hz for the $C^{\beta}H$ resonances at 3.15 and 3.06 ppm, respectively, and 13.8 Hz for the $^2J_{\beta\beta}$ coupling constant. These can be compared to values of 5.6 and 10.3 Hz obtained for the $^3J_{\alpha\beta}$ coupling constants of the phenylalanine residue in GGFA (Bundi and Wüthrich, 1979a). The greater difference in the coupling constants in the latter case may well indicate some degree of ordering of the side chain of the phenylalanine.

*Temperature coefficients*

Changes in the amide proton chemical shift with temperature have been used to give some indication of hydrogen bond strength or formation (Kopple et al., 1969; Ohnishi and Urry, 1969). Amide proton temperature coefficients for residue X in the series GGXGG were estimated by plotting the linear relationship of the amide proton chemical shift as a function of temperature, at intervals of 10° over the temperature range 278 to 318 K. Changes in the chemical shifts of most side-chain protons are minimal (< 0.02 ppm), but the $C^{\alpha}H$ chemical shifts moved on average 0.02 ppm and as much as 0.05 ppm (for the $C^{\alpha}H$ of glutamic acid) downfield over this temperature range. The resonances of the primary amide side-chain proton of asparagine and glutamine, the $N^{\varepsilon}H$ of arginine and the indole NH of tryptophan also show a linear dependence on temperature. The $N^{\eta}H$ (guanidinium) protons of the arginine show a complex dependence on temperature, due to differences in exchange rates at the various temperatures; coefficients were not calculated for these resonances. The temperature coefficients obtained for the backbone and side-chain amide protons are shown in Table 3. In general, the coefficients for the backbone amide protons are rather large and negative (values < −6.5 ppb/K). The least negative value is for aspartic acid (−6.43 ± 0.21 ppb/K); all other temperature coefficients are considerably more negative than −7 ppb/K, the most negative being for tyrosine (−9.32 ± 0.24 ppb/K). The side-chain amides all have considerably less negative values, ranging from −3.04 ± 0.18 ppb/K for the arginine $N^{\eta}H$ to −6.08 ± 0.15 ppb/K for one of the glutamine side-chain amides.

The average amide proton temperature coefficients for the invariant glycine residues are −5.93 ± 0.77, −7.61 ± 0.48 and −6.57 ± 0.48 ppb/K for glycines 2, 4 and 5 respectively. Amide proton temperature coefficients for $Gly^5$ in peptides containing aromatic residues were lowered to −3.2 ppb/K (phenylalanine), −3.3 ppb/K (tyrosine) and −0.1 ppb/K (tryptophan). These results are consistent with the observed deviation of the $C^{\alpha}H$ chemical shifts of $Gly^4$ and $Gly^5$ and the $Gly^5$ NH chemical shifts for the aromatic peptides (although not with the $^3J_{\alpha\beta}$ coupling constants observed for phenylalanine in GGFGG) and suggest either some degree of conformational preference for the orientation of the aromatic side chain towards the carboxy terminus or some interaction with the C-terminal glycine. This effect has been observed previously for sequences Ar-Aa-Gly, where Ar is an aromatic residue and Aa is any amino acid, and has been ascribed to close contact between the aromatic ring and the glycine amide proton (Dyson et al., 1992a; Kemmink et al., 1993) and to aromatic–amide hydrogen bonding (Kemmink et al., 1993). In this work we show that this effect is present even in 'random' peptides, and is therefore probably due to an intrinsic property of the sequence, resulting from the bulky nature of the aromatic

TABLE 3
RANDOM COIL $^1H$ NMR TEMPERATURE COEFFICIENTS FOR THE AMIDE PROTONS OF RESIDUE X IN THE SERIES GGXGG IN WATER AT pH 5.0 (ppb/K)

| Residue | $-\Delta\delta/\Delta T$ | | |
|---|---|---|---|
| | Backbone NH | Side-chain NH | Other studies |
| Ala | 8.20 ± 0.21 | | 8.15 (pH 3.24)[a], 8.17 (pH 3.90)[a], 8.58 (pH 5.80)[a] |
| Arg | 7.64 ± 0.23 | 3.04 ± 0.19 ($N^{\varepsilon}H$) | 7.7 (pH 3.0)[b], 3.2 ($N^{\varepsilon}H$)[b] |
| Asn | 7.02 ± 0.09 | 5.57 ± 0.04, 4.80 ± 0.12 ($N^{\delta}H$) | 7.2 (pH 3.0)[b], 6.3, 5.4 ($N^{\delta}H$)[b] |
| Asp | 6.43 ± 0.21 | | 7.5 (pH 3.0)[b] |
| Cys | 7.36 ± 0.37 | | |
| Gln | 7.65 ± 0.10 | 6.08 ± 0.15 ($N^{\varepsilon}H1$ at 7.69 ppm)<br>4.99 ± 0.14 ($N^{\varepsilon}H2$ at 6.97 ppm) | 7.1 (pH 3.0)[b], 7.6, 5.9 ($N^{\varepsilon}H$)[b] |
| Glu | 7.01 ± 0.15 | | 6.94 (pH 3.24)[c], 6.60 (pH 3.90)[c], 6.45 (pH 5.80)[c], 7.1 (pH 3.0)[b] |
| Gly | 7.02 ± 0.09 | | 6.9 (pH 3.0)[b] |
| His | 7.49 ± 0.09 | | 7.1 (pH 3.0)[b] |
| Ile | 8.35 ± 0.21 | | |
| Leu | 8.42 ± 0.17 | | |
| Lys | 7.87 ± 0.20 | | |
| Met | 7.97 ± 0.17 | | |
| Phe | 8.12 ± 0.56 | | |
| Ser | 7.02 ± 0.14 | | |
| Thr | 7.40 ± 0.22 | | |
| Trp | 7.98 ± 0.10 | 4.03 ± 0.13 ($N^{\varepsilon 1}H$) | 6.6 (pH 3.0)[b], 3.4 ($N^{\varepsilon 1}H$)[b] |
| Tyr | 9.32 ± 0.24 | | |
| Val | 8.35 ± 0.21 | | |

[a] Bundi and Wüthrich (1979b); values are for $Ala^4$ of the peptide H-Gly-Gly-Glu-Ala-$OCH_3$.
[b] Jimenez et al. (1986).
[c] Bundi and Wüthrich (1979b).

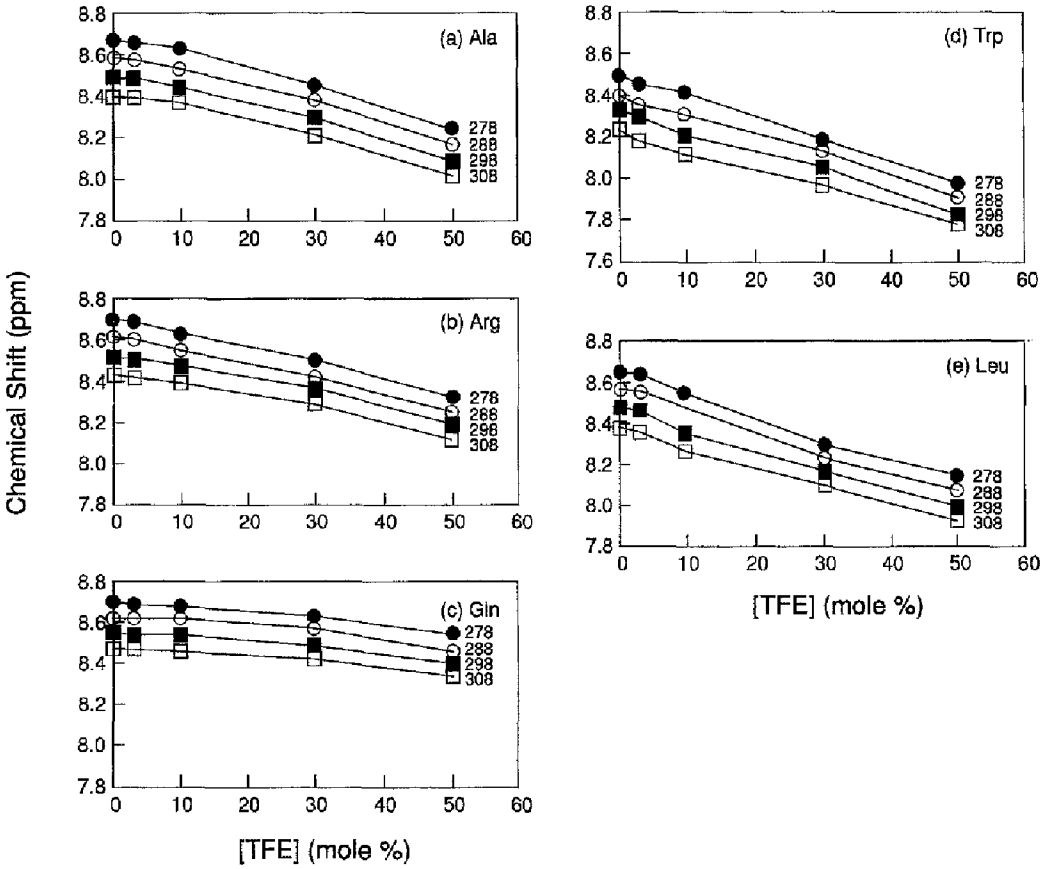


Fig. 2. Chemical shift of the amide proton of the variant residue as a function of TFE concentration at several temperatures. (a) Alanine in GGAGG; (b) arginine in GGRGG; (c) glutamine in GGQGG; (d) tryptophan in GGWGG; and (e) leucine in GGLGG.

ring and the absence of a side chain, with concomitant lack of steric hindrance, in the glycine residue. In spite of these interactions the chemical shifts of the aromatic amino acid protons determined for the series GGXGG are generally similar to those determined by Bundi and Wüthrich (1979a) for the series GGXA, where such an interaction does not occur, indicating that the chemical shifts for residue X (aromatic) can still be regarded as 'random coil'.

Amide proton temperature coefficients were obtained at three pH values for the peptide H-Gly-Gly-Glu-Ala-$OCH_3$ (Bundi and Wüthrich, 1979b), and for a total of eight peptides from the series GGXA at pH 3.0 (Jimenez et al., 1986). These values are included for comparison in Table 3. For the temperature coefficients available for comparison (Table 3), the largest differences occur for the amides of tryptophan, aspartic acid, glutamic acid and histidine. The latter three residues contain groups that titrate with $pK_a$ values in the range 3–5, which encompasses the difference in pH between the two studies. It therefore seems likely that the difference in the temperature coefficient can in this case be accounted for by the pH difference. These effects have been noted previously for glutamic acid (Bundi and Wüthrich, 1979b), although our results at pH 5.0 appear to be somewhat at variance with the published value at pH 5.80, and closer to that at pH 3.0 (Jimenez et al., 1986). The amide proton temperature coefficient for tryptophan at pH 5.0 appears to be quite widely at variance with the value obtained at pH 3.0 (Jimenez et al., 1986). In this case we cannot rule out an effect of the difference in sequence at the C-terminus – the C-terminal carboxyl group, which also titrates in the pH range 3–5, is only one residue removed from the tryptophan in GGWA, but in GGWGG it is two residues removed. We have already inferred from chemical shifts that the tryptophan side chain may be preferentially oriented towards the C-terminus; this may also influence the apparent pH effect observed on the tryptophan amide proton temperature coefficient.

*Quantitation of the effects of trifluoroethanol on chemical shift*

The solvent TFE has been used for a number of years to induce helical structure in peptides (Timasheff, 1970). There are many examples where TFE induces helix in peptides that have an intrinsic propensity for helix formation (Dyson et al., 1988b,1992a; Mammi et al., 1988; Nelson and Kallenbach, 1989; Yamamoto et al., 1990). Helical structures have also been shown to be induced by TFE in protein folding intermediates (Alexandrescu et al., 1993; Buck et al., 1993; Fan et al., 1993) and this solvent is commonly used to stabilize structures in peptides to fa-

cilitate calculation of the 3D structure (Clore et al., 1985; Breeze et al., 1991). The mode of action of TFE has been described as a differential solvation effect that depends on the highly hydrophilic nature of the peptide backbone (Nozaki and Tanford, 1971), but this mechanism may be too simple to explain all of the properties of peptides in TFE–water mixtures (Nelson and Kallenbach, 1989; Sönnichsen et al., 1992). The helix–coil equilibria in alcohol/water mixtures have recently been modeled using molecular dynamics (Brooks and Nilsson, 1993); these results also suggest that differential solvation causes the observed changes in helix composition in water–TFE mixtures.

Analyses of peptide structural changes by utilizing chemical shift changes in water/organic solvent mixtures (see, for example, Zhou et al. (1992)) have usually not taken into account the simple effects of the addition of solvent upon the chemical shift itself, irrespective of any structural change induced. We therefore investigated the effects of TFE upon the chemical shifts for selected peptides in the series GGXGG, representing a range of amino acid types. Data were obtained for peptides containing alanine, arginine, glutamine, leucine and tryptophan over a TFE concentration range of 0–50 mole percent. The $C^{\alpha}H$ chemical shift generally showed negligible change on addition of TFE; the $C^{\alpha}H$ of arginine moved about 0.04 ppm downfield. Changes of similar magnitude in the $C^{\alpha}H$ chemical shift were observed in an alanine tripeptide in a solution containing between 0% and 20% TFE (Nelson and Kallenbach, 1989). Little variation was observed in the chemical shifts of side-chain protons as a function of TFE concentration.

The change in amide proton chemical shift as a function of TFE concentration at a given temperature is not linear over this range (Fig. 2). It is not known at this stage what effect, if any, the solvent has upon the chemical shift reference (in this case, dioxane), which could contribute to the observed behavior. However, these results are valid as a reference set for the measure of the effect of TFE on chemical shifts, as long as the same chemical shift reference is used. At a given temperature, the addition of TFE results in an upfield shift of the amide proton resonances. Greater dependence of the amide proton chemical shift on TFE concentration is observed for the hydrophobic residues (alanine, leucine and tryptophan) than for the hydrophilic glutamine. The values for arginine are midway between these two extremes, perhaps an indication that both the long aliphatic side chain and the hydrophilic guanidinium group are influencing behavior in TFE solutions.

By analogy with amide proton temperature coefficients in water, hydrogen bonding in peptides can be examined by observing amide proton chemical shift changes as a function of solvent composition. Solvent-exposed amide protons will be affected more by a change in solvent composition than hydrogen-bonded and solvent-protected protons. Upon changing solvent from a good NH hydrogen bond acceptor, such as water, to a poor NH hydrogen bond acceptor, such as TFE, intramolecular hydrogen-bonded or solvent-shielded protons should remain unperturbed, whereas exposed protons should shift significantly upfield (Urry and Long, 1976). On the basis of extensive studies of gramicidin S, it has been suggested that an upfield shift indicates a decrease in hydrogen bonding with solvent (Pitner and Urry, 1972). On the other hand, increased hydrogen bonding of solvent to the backbone carbonyl group results in a downfield shift of the connected amide proton (Llinás and Klein, 1975; Kessler, 1982). Using a pentapeptide of elastin, solvent titration studies from poor proton donors (DMSO) to good proton donors (TFE or water) have also indicated that there is a significant downfield shift of the carbonyl carbon (Urry et al., 1974). This shift is slightly greater in transferring from DMSO to water than from DMSO to TFE, suggesting that peptide carbonyls are inherently more strongly hydrogen bonded to solvent in water than in TFE. It is most probable that the effects of hydrogen bonding of solvent to both backbone carbonyls and amide protons are operating in all cases. In the current study, the qualitative differences in the behavior of different peptides may indicate differences in the dominant effect of the addition of TFE. Implicit in this is the assumption that there are negligible conformational changes in the peptides with changes in solvent. Evidence that this assumption is correct is discussed in a later section.

TABLE 4
RANDOM COIL $^1H$ NMR TEMPERATURE COEFFICIENTS IN WATER/TFE MIXTURES AT PH 5.0

| Residue/amide | $\Delta\delta/\Delta T$ (ppb/K) for various TFE concentrations (mole %) | | | |
|---|---|---|---|---|
| | 3 | 10 | 30 | 50 |
| Ala | −7.92 ± 0.16 | −8.01 ± 0.23 | −7.28 ± 0.30 | −7.19 ± 0.27 |
| Arg | −7.37 ± 0.07 | −6.54 ± 0.29 | −7.01 ± 0.26 | −7.10 ± 0.09 |
| $N^{\epsilon}H$ | −2.30 ± 0.37 | −2.03 ± 0.26 | −1.84 ± 0.31 | −1.20 ± 0.15 |
| Gln | −8.02 ± 0.08 | −6.73 ± 0.10 | −6.82 ± 0.14 | −6.35 ± 0.54 |
| $N^{\epsilon}H1$ | −6.26 ± 0.44 | −6.08 ± 0.15 | −5.89 ± 0.37 | −5.80 ± 0.22 |
| $N^{\epsilon}H2$ | −5.34 ± 0.43 | −4.61 ± 0.05 | −4.70 ± 0.27 | −4.23 ± 0.36 |
| Trp | −8.94 ± 0.08 | −9.58 ± 0.41 | −6.82 ± 0.80 | −6.27 ± 0.26 |
| $N^{\epsilon}H$ | −5.22 ± 0.32 | −7.97 ± 0.57 | −3.50 ± 0.76 | −3.79 ± 0.76 |

Solvent effects also may complicate interpretation of amide proton temperature coefficients. The amide proton temperature coefficients at different TFE concentrations are shown in Table 4. Modest changes in temperature coefficient, generally close to the estimated error for these measurements (± 0.5 ppb/K), occur upon addition of TFE. No consistent trend in the temperature coefficient with increasing TFE concentration is observed. We conclude that the temperature coefficient in these 'random coil' peptides is largely independent of TFE concentration.

*Effect of solvent and temperature on the chemical shift reference*

The reference used in all of this work is the singlet resonance of internal dioxane, which is given a chemical shift of 3.75 ppm relative to that of TSP at 0 ppm. Previous studies (Bundi and Wüthrich, 1979a,b; Jimenez et al., 1986) used internal TSP, which is somewhat pH dependent (Bundi and Wüthrich, 1979b). Obviously, since all of the chemical shifts are referred to these resonances, the effects of TFE and temperature on the chemical shift of the reference must be assessed. References are generally chosen because of their negligible dependence on solution conditions and temperature, and the effects of pH and temperature on their resonances are generally well documented (Cross and Schleich, 1977; De Marco, 1977; Hoffman and Davies, 1988). The water resonance, for example, although convenient to use, is a poor reference because of its sensitivity to temperature and pH. The effects of TFE concentration on the water resonance are less well known. We observe that the resonance of dioxane in water solutions moves about 0.02 ppm upfield relative to those of TSP and DSS between 278 and 318 K, which is not enough to account for the differences seen in temperature coefficients between the previously published studies and our own. A 0.01 ppm upfield shift of the dioxane resonance relative to that of TSP is observed between TFE concentrations of 0% and 50% at constant temperature. The resonances of TSP and DSS show a small temperature dependence of their own (Hoffman and Davies, 1988).

*The peptides remain 'random', even in TFE*

While the peptides used in this study are in general unlikely to have any conformational preference for a structured conformation in water or TFE/water solutions, this assumption was tested by acquiring ROESY spectra of selected peptides under a variety of solvent conditions. The observation of certain NOE connectivities in ROESY spectra of short peptides has been extensively used in our laboratory as diagnostic evidence for the presence of structured conformers (Dyson et al., 1988a,1992a,b), and the connectivities to be expected if structured conformers are present are well known. The presence only of NOEs that are characteristic of unfolded (largely β) conformations is good evidence that the peptides are random and that there are no preferences for structured conformations. ROESY spectra were recorded for the peptides H-GGAGG-OH and H-GGEGG-OH at a number of TFE concentrations at 278 K. The low temperature generally makes the observation of structured conformers more likely (Dyson and Wright, 1991). No NOE connectivities characteristic of structured conformers were observed in these ROESY spectra. An example is shown in Fig. 3. Strong $d_{\alpha N}(i,i+1)$ cross peaks are visible for all residues; $d_{NN}(i,i+1)$ NOEs are extremely weak or absent, except at the C-terminus where such a connectivity is frequently

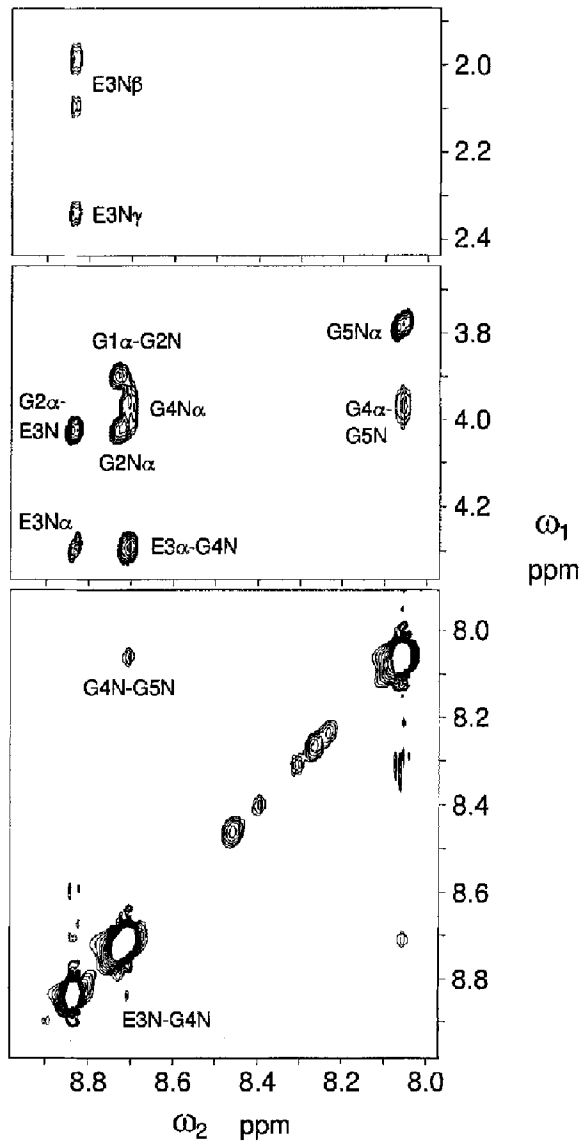


Fig. 3. Portions of a 600 MHz ROESY spectrum of the peptide H-Gly-Gly-Glu-Gly-Gly-OH in 90% $H_2O$/10% $D_2O$, pH 5.0, 278 K. Both positive (diagonal) and negative (off-diagonal) peaks are plotted without discrimination, and contour levels are identical in all sections of the figure. This peptide is one of the few for which the amide proton chemical shifts of residues 2, 3 and 4 are sufficiently well resolved that $d_{NN}(i,i+1)$ NOE connectivities could be observed if present.

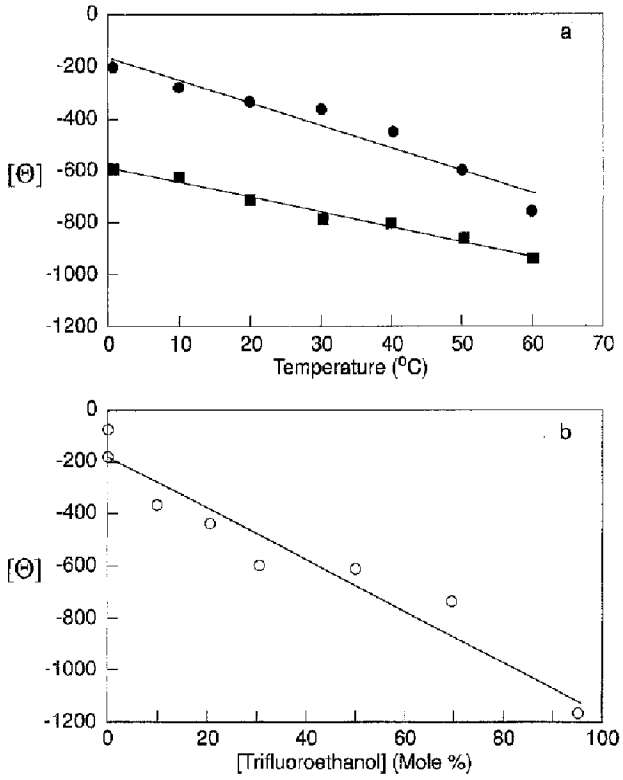


Fig. 4. The dependence of the CD molar ellipticity at 222 nm (Θ) for the peptide H-Gly-Gly-Ala-Gly-Gly-OH on (a) the temperature at two TFE concentrations, 0 mol % (filled circles) and 50 mol % (filled squares); and (b) the TFE concentration (mol %) at a temperature of 274 K.

seen, probably as a consequence of the proximity of the unblocked carboxyl group (Dyson et al., 1992a). No medium-range NOEs are seen. These results indicate that the peptides are present predominantly in the β-region of (ϕ,ψ) space.

Circular dichroism spectra were recorded for peptide H-GGAGG-OH at a number of temperatures and TFE concentrations. The spectra are characteristic of unfolded peptides, with a minimum below 200 nm and no significant minima at longer wavelengths. The ellipticity at 222 nm, characteristic of helix, shows a shallow linear dependence on temperature, as shown in Fig. 4, with slopes of −8.4 and −4.9 deg cm$^2$ dmol$^{-1}$/°C in solutions of 0 and 50% TFE, respectively. The ellipticity also shows a linear relationship with TFE concentration at 274 K (Fig. 4), with a slope of −9.6 deg cm$^2$ dmol$^{-1}$/mol % TFE. The absolute magnitude of the ellipticity change is very small (about 1000 deg cm$^2$ dmol$^{-1}$ between 0 and 100% TFE), suggesting that the observed dependences on temperature and TFE concentration may be in the nature of a baseline response to the addition of solvent or to temperature change. These results, together with those obtained from the ROESY spectra, strongly suggest that no preferred secondary structure is formed under the conditions of the study.

## Conclusions

In this study we have generated a unique set of random coil chemical shift values derived from the set of peptides H-Gly-Gly-X-Gly-Gly-OH, where X represents all 20 naturally occurring amino acids. After temperature differences are taken into account, chemical shift values are close to those previously determined (Bundi and Wüthrich, 1979a) for a similar set of peptides H-Gly-Gly-X-Ala-OH, with small differences that are probably related to the presence of alanine at the C-terminus of the latter set, and a resultant slightly nonrandom nature of the conformational ensemble. In addition, we have also measured a valuable reference set of amide and side-chain amide proton temperature coefficients, which will not only provide baseline values for the temperature coefficient itself, but can be used to obtain 'random' chemical shifts for amide protons at any temperature by interpolation. Comparison of the present data with other 'random coil' shift tabulations (Bundi and Wüthrich, 1979a; Wishart et al., 1994) serves to emphasize the extreme sensitivity of the amide proton resonances to environmental factors, including local sequence and solvent composition. The effects of the addition of the helix-promoting solvent TFE on random coil chemical shifts have also been evaluated. These results should be valuable for the interpretation of the structural implications of peptide and protein chemical shifts in water and water/alcohol mixtures.

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