Carbon 13 Nuclear Magnetic Resonance of Pentapeptides of Glycine Containing Central Residues of Aliphatic Amino Acids*

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SUMMARY

Pentapeptides containing either glycine, l-alanine, l-valine, l-leucine, or l-isoleucine as central residue in the pattern glycylglycyl-X-glycylglycine have been studied by proton-decoupled natural abundance 13C Fourier transform nuclear magnetic resonance spectroscopy. Resonances were assigned by comparison with the free amino acids and by detailed observation of changes in chemical shifts with pH over the range 1.32 to 10.43. The effect of varying the central residue on the chemical shifts of the flanking glycine residues was established. Small effects ascribable to incipient aggregation were noted, both in terms of chemical shifts and relaxation properties. Each resonance was categorized according to the degree of sensitivity to the state of protonation of the terminal groups, and the pK values at 20° for those groups were determined from the pH dependence of the most sensitive resonances. The sensitive terminal resonances undergo broadening which is maximum near the pK. Spin-lattice relaxation times, T₁, of protonated carbon nuclei were measured at two or more pH values in all cases except for the glycine pentapeptide. For each peptide Cα resonances showed gradients in increasing T₁ to each side from the central residue, indicative of contributions from segmental motion and internal rotation along the backbone in addition to the over-all rotational motion of the molecule. Internal rotational modes are expressed as well in the aliphatic side chains, especially by the most peripheral methyl groups for which spinning around the attaching bond is clearly prominent. Comparison of the anionic and cationic forms of the alanine, valine, and leucine peptides indicates that the rate of internal rotation of Cα of the NH₂-terminal glycine residues increases with deprotonation of the ammonium group.

The 13C nucleus acts in NMR experiments as a versatile and sensitive, nonperturbing probe of its physical chemical environment (2). Its chemical shift and relaxation properties offer a broad range of forms of chemical interpretation (3). NMR studies of a variety of compounds have illustrated that the 13C nucleus reflects not only the electronic features of its primary covalent linkage (4-6), but also the secondary influences of steric (7), electrostatic (8, 9), and solvent (10) perturbations.

13C NMR studies of proteins (2, 11-14) with emphasis on 13C enrichment (15-23) can provide, in principle, a wealth of information concerning the structural and functional relationships that describe an organized polypeptide system. In practice the interpretation of 13C NMR of proteins is hampered by a lack of fundamental information about small peptides to serve as model compounds. Presumably, the chemical shifts of the individual carbons which comprise amino acid residues in proteins can be described by a set of empirical terms that separately evaluate the effects of covalent linkage within the residue itself (9), as well as the effects of incorporating the residue into the primary polypeptide structure (24, 25), and the steric, electrostatic, hydrogen bonding, and solvent effects (18, 26, 27) arising from constraints imposed by the various levels of organization of the protein structure. Although determining the resonance positions for the individual amino acids in the various protonated forms (9, 24), (28) represents a necessary first step in describing the expected range and classification of 13C chemical shifts, such assignments describe only qualitatively the resonance profiles for native and denatured proteins (11-14). More appropriate models are offered by short peptides (24, 25, 28) in which the effects of incorporation into peptide linkage can be emphasized and other effects such as end group ionization, vicinal electrostatic and steric interactions, peptide conformation, and unusual solvent conditions can be isolated or minimized. The resulting assignments will serve as a reference for evaluating perturbations significant to protein structure.

Previous work in this laboratory on tri- and pentapeptides established that terminal ionization effects are negligible for the central residue of the pentapeptide representing the NH₂-terminal sequence of sperm whale myoglobin (24). The chemical shifts in proton decoupled spectra could be related to those of the constituent amino acids with systematic adjustments for incorporation into peptide linkages. Proton coupled spectra were taken in certain cases to confirm the assignments. Characteristic
chemical shifts of appropriate carbon nuclei were found to conform to theoretical titration patterns.

The present work is an extension of that study to a series of pentapeptides in which each common protein amino acid except cysteine is prepared as the central residue flanked by 2 residues of glycine in the general form Gly-Gly-X-Gly-Gly (29). Measurements of chemical shifts have been made over a range of pH to encompass all ionizations. In addition, spin-lattice relaxation times, $T_1$, have been measured. The present report deals with the aliphatic amino acids as central residues.

**EXPERIMENTAL PROCEDURE**

**Peptide Synthesis**—The amino acids L-alanine, L-valine, L-isoleucine, and L-leucine (Pierce Chemical Company) were converted to the respective t-BOC derivatives according to the method of Schnabel (30) by treatment with t-butyldiazocinate (Pierce). This procedure was also employed in the conversion of glycylglycine (Pierce) to the t-BOC form by reaction in dioxane-H$_2$O (1:1, v/v) at pH 9.5 for 9 hours. The product was recrystallized from hot ethyl acetate and melted at 131–132° (31). Pentaglycine was purchased from Cyclo Chemical Corp. and was used without further purification.

The pentapeptides were synthesized by the Merrifield solid phase technique (32) as outlined by Stewart and Young (33). A three stage procedure was employed whereby t-BOC-glycylglycine was initially coupled to the chloromethylated resin (Bio-Beads S-X2, 200 to 400 mesh, Bio-Rad) by reaction with triethylamine in absolute ethanol for 30 hours. The specific t-BOC-L-amino acid was then coupled to the resin-bound diglycine (3.5 meq of peptide per 7 g of resin) with N,N'-dicyclohexylcarbodiimide (Pierce), and finally t-BOC-glycylglycine was added to complete the synthesis by carbodiimide activation. The swelling solvent was methylene chloride and the t-BOC adducts were removed in 30 min by 50% trifluoroacetic acid-methylene chloride (v/v). A mole ratio of t-BOC amino acid or t-BOC-glycylglycine to resin-bound peptide of 2.5 to 1 was employed with coupling times of 2 hours. In the final stage t-BOC-glycylglycine was dissolved in methylene chloride-dimethylformamide, 4:1 by volume.

The finished resin-bound t-BOC-pentapeptides were dehocked and cleaved from the resin by bubbling HBr into an anhydrous trifluoroacetic acid suspension of the peptide-resin for 60 to 90 min. The HBr was passed through a scrubbing solution containing 2 g of resorcinol in 100 ml of anhydrous trifluoroacetic acid. The liberated peptide was collected in 250 ml of anhydrous trifluoroacetic acid and dried by rotoevaporation. The resulting oil was washed three times with 100 ml mixtures of methanol and water, 1:1 by volume. Finally, the peptide was dissolved in a minimum amount of water and extracted five times with equal volumes of absolute ether. The aqueous solution at pH near 2 was dried, and the peptide was stored in a desiccator at 4°.

Yields were approximately 80%.

Peptide aliquots were hydrolyzed by redistilled 6 N HCl in evacuated glass tubes at 110° for 24 to 72 hours. Analyses were obtained with a Beckman 121 amino acid analyzer. The expected mole ratio of 4:1 glycine to amino acid was obtained. The peptides were homogeneous on thin layer chromatography (Silica Gel GF, J. T. Baker) developed with 2-butanone-acetic acid-water, 2:6:5 v/v.

**Sample Preparation**—Each peptide, 0.5 to 1.0 g, was dissolved in a minimum amount of deionized, distilled water, approximately 2.5 ml, containing 1 to 2 drops of dioxane as an internal NMR standard. Measurements of $p$H were made before and after each NMR experiment on a Radiometer pH meter equipped with a GK2302C combination electrode. All pH measurements were obtained at 26°, and pH adjustments were made with either 6 N HCl or 5 N NaOH. Standard buffers at pH 4, 7, and 9 were used to calibrate the pH meter.

**$^1$H NMR**—Most experiments were performed on a "home-built," pulsed Fourier transform spectrometer equipped with a Varian 14.1 kG electromagnet operating at 15.08 MHz. A detailed description of this apparatus has been published elsewhere (11). Magnetic homogeneity and frequency settings were adjusted using neat ethylene glycol. All chemical shifts are reported upfield of CS$_2$ with the aqueous dioxane resonance located at 126.3 ppm and external neat ethylene glycol at 129.7 ppm. Chemical shifts for titration experiments were measured directly from locations in computer memory. In most cases the "observing window" was 250 ppm giving a spectral resolution of 0.1 ppm. In some cases the Varian XL-100-15 instrument was used as specified in the text. Partially relaxed Fourier transform spectra were obtained on the 15.08 MHz instrument by the Inversion-Recovery Method utilizing a 180°-90° pulse sequence (34). Temperature variation within a given relaxation set was ±1°. All spectra were obtained between 24.5 and 33.5° as indicated in the text. Unless otherwise stated the observing window was 125 ppm and recycle times were at least three times the longest measured $T_1$ values.

**Calculations**—The titration chemical shifts were fit to the Henderson-Hasselbalch equation using a computer program designed to minimize error by the Powell (35) method of conjugate directions. Each point in the fit was weighted according to the sum of the errors in spectral resolution and in pH, estimated to be 0.1 ppm and 0.02 pH units, respectively. The $p$K values reported by this method are accurate to the first decimal place.

Values for $T_1$ were obtained from computer analyses of the relaxed spectra as described elsewhere (11). Computed standard deviations fall in the range of 2.5 to 5%.

**RESULTS**

**General Characteristics of Spectra**—Illustrative spectra of the five pentapeptides containing an aliphatic central residue are presented in Fig. 1. The experimental conditions are described in the legend and are representative of those used for other experiments except as mentioned specifically below. With the exception of the spectrum in Fig. 1D for the leucine peptide which was obtained at pH 6.98, all these spectra were taken in the $p$H range 1.32 to 2.10. In each spectrum the dioxane resonance appears at 126.3 ppm upfield of CS$_2$. Downfield of dioxane the spectra show only the group of resonances representing the four peptide carbonyls and the terminal carboxyl carbon nucleus. In the upfield region all $C^\alpha$ resonances and those of the aliphatic side chains. Within each chemical class of protonated carbon nucleus the resonance area defined by signal height and line width generally reflects the numbers of resonating carbons.

Note should be taken of certain sources of variability of attenuation of resonance intensity in the spectra. For example, the development and construction of the $^1$H Fourier transform nuclear magnetic resonance equipment was supported in part by grants to Adam Allerhand from the National Science Foundation (GP-17966) and the Petroleum Research Fund (4559-AC5).

1 The abbreviations used are: t-BOC, tert-butyloxycarbonyl; T$_1$, spin-lattice relaxation time; NOE, nuclear Overhauser effect.
FIG. 1. Natural abundance 13C pulsed Fourier transform NMR spectra of pentapeptides of the type Gly-Gly-X-Gly-Gly containing different aliphatic amino acids as the central residue. Resonance positions are given as parts per million upfield from CS, using an internal dioxane reference located at 128.3 ppm. All spectra were obtained at 33.5° with complete, noise-modulated proton decoupling with 5-s recycle delay. A, glycylglycyl-glycylglycine, 0.05 M, pH 1.32, 4304 accumulations; B, glycylglycyl-L-alanine, 0.4 M, pH 2.10, 512 accumulations; C, glycylglycyl-L-valine, 0.4 M, pH 1.42, 512 accumulations; D, glycylglycyl-L-leucine, 0.4 M, pH 1.38, 512 accumulations; E, glycylglycyl-L-isoleucine, 0.05 M, pH 1.32, 4304 accumulations; F, glycylglycyl-L-isoleucyl-L-valine, 0.4 M, pH 1.38, 512 accumulations.

Table I

| Amino Acid       | Carbon | p5 peptide | p5 free | p5 peptide-p5 free |
|------------------|--------|------------|---------|--------------------|
| Glycine          | Cα     | 20.9      | 20.5    | 0.4                |
|                  | Cβ     | 20.3      | 20.3    | 0.0                |
| Alanine          | Cα     | 17.4      | 17.0    | 0.4                |
|                  | Cβ     | 142.3     | 142.0   | 0.3                |
|                  | Cγ     | 170.2     | 270.5   | 0.2                |
| Valine           | Cα     | 18.8      | 13.0a   | 0.0a               |
|                  | Cβ     | 155.0     | 156.1   | 0.1                |
|                  | Cγ     | 216.9     | 216.0b  | 0.7                |
| Leucine          | Cα     | 130.1     | 135.9   | 1.2                |
|                  | Cβ     | 175.4     | 176.0   | 0.6                |
|                  | Cγ     | 238.5     | 238.4   | 0.1                |
|                  | Cδ     | 215.0     | 215.0   | 0.0                |
|                  | Cε     | 217.1     | 217.0   | 0.0                |
| Isoleucine       | Cα     | 25.9      | 10.9    | 1.1                |
|                  | Cβ     | 133.1     | 135.1   | 1.0                |
|                  | Cγ     | 236.0     | 236.9   | 0.1                |
|                  | Cδ     | 236.5     | 236.0   | 0.0                |
|                  | Cε     | 256.5     | 256.3   | 0.0                |

Comparison of chemical shifts in amino acids and peptides

Chemical shifts of aliphatic amino acids in free dipolar form are compared with those incorporated as the central residue of a dipolar pentapeptide of the form Gly-Gly-X-Gly-Gly. Carbon types listed use the IUPAC-IUB nomenclature. All chemical shifts are expressed as parts per million upfield of CS.

*Gurd et al. (24).*

Relaxation times for carbonyl carbon nuclei in the pentapeptides are usually so long that it has been impractical as a rule to recycle slowly enough to avoid signal attenuation. Variations in the efficiency of 1H-decoupling may make a minor contribution to relative attenuation. Limitations in computer memory also restrict the comparison of intensities involving the narrow, non-protonated carbon resonances.

There is no evidence of significant differential broadening of the carbonyl or Cα resonances by the bonded 14N (36). Regardless of pH, no slow exchange effects are seen either in terms of sudden changes in signal to noise or with respect to the appearance of multiple resonances for the same carbon nucleus. As will be discussed further below, certain resonances that are sensitive to titration of the terminal residues undergo a broadening in the pH range near the pK of the titrating group. Chemical shift overlap is sometimes observed for the carbonyl nuclei of the glycine residues adjacent to the central residue, and is the rule for Cα of those residues so that a prominent landmark is recognizable in that region of each spectrum.

Spectral assignments have been made on the basis of comparison with the free amino acids and by following stepwise the effects of variations in pH, and also are supported by measurements of spin-lattice relaxation times. In some cases resolution has been expanded to define overlapping peaks. Complete 1H-decoupling was maintained throughout this work since assignments could be made without resorting to partial decoupling techniques. For convenience in illustrating the later discussion the assignments in Fig. 1 are identified by numbering the residues 1 to 5, starting at the Nα-terminal. Proceeding upfield in the carbonyl region the 5 residues fall in the case of the glycine peptide in the order 5, 4, 2, 3, and 1. In the case of the leucine peptide, the order is residues 3, 5, 4, 2, and 1, with 2 and 4 overlapped as mentioned above. With the leucine peptide the Cα of residue 5 is shifted enough at pH 6.98 that the order is 3, 5, 2, 4, and 1. With the glycine peptide Cα of residues 2, 3, and 4 all overlap, although the upfield shoulder of this band is clearly resolved into a single carbon resonance at 25.19 MHz. The side chain carbon nuclei fall progressively upfield according to their distance from Cα.

Chemical Shifts of Central Residues—For convenience in bringing out the systematic patterns the results for the varying central residue in the pentapeptides are presented first, followed by those of the flanking and terminal glycine residues. Table I compares the chemical shifts for the free amino acids involved,
obtained for the dipolar form in each case, with those for the central residues of the pentapeptides, the latter also in the dipolar form in each case. Columns are given for the respective chemical shifts $\delta_{\text{polyg}}$ and $\delta_{\text{free}}$, and for their difference, $(\delta_{\text{polyg}} - \delta_{\text{free}})$. The value of this difference reflects the effect of incorporating the amino acid into the peptide form. The effect varies for the different residues involved. With the exception of glycine as a central residue, the trends are consistent enough that the following average values are worth noting: $C^\beta$, +0.2; $C^\gamma$, +1.0; $C^\delta$, -0.2; $C^\varepsilon$, -0.2; $C^\zeta$, +0.4.

The relationships of the chemical shifts within the residues are consistent with known substituent effects (2, 4, 5, 9), excepting the recognized nonequivalence of the methyl groups in valine, leucine, and isoleucine (9, 24). Presumably the effects of near neighbors in the peptide sequence on the chemical shifts of the central residues are minimized by the choice of glycine for the flanking residues.

**Titration Effects**— Chemical shifts of the flanking and terminal glycine residues are presented conveniently in terms of their pH dependence. The variation of chemical shift of these glycine residue carbon nuclei with pH is summarized in Table II. The values at basic pH and at acid pH, $\delta_B$ and $\delta_A$, respectively, are shown, as well as the difference. Because of the wide separation of the titrating groups the deprotonation at one end of the molecule does not influence the chemical shifts at the other end.

In keeping with earlier results (24, 25) the largest values of $(\delta_B - \delta_A)$ with increasing pH are 8.4 and 3.1 ppm for the $C^\alpha$ and $C^\beta$, respectively, of the glycine residue 1, and $-3.2$ and $-2.15$ ppm for the $C^\gamma$ and $C^\zeta$, respectively, of glycine residue 5. The titration effects are not limited to the terminal residues, particularly for the cases of the $C^\zeta$ of residues 2 and 4. Notably the $C^\zeta$ of glycine residue 4 exhibits an upfield shift with deprotonation of the terminal carboxyl group. By expanding the resolution evidence of two peaks for the nearly identical resonances of $C^\zeta$ in residues 2 and 4 may be obtained in the midrange of pH, but the distinction is less than 0.05 ppm.

The fit of the chemical shifts to theoretical titration curves is illustrated in Fig. 2 for the glycine residues. The curve for the central carboxyl has been included in each case to emphasize the magnitudes of the shift range and the spectral overlap encountered in the individual titrations. Clearly the ionic strength was high enough in all cases that variations in it were without observable effect. The method of sample preparation was such that titrations were commenced from the acid rather than the neutral region. However, enough difficulty was encountered with solubility of most of these peptide solutions that the pH values were often approached from different sides, with attendant small irregularities in the trend of electrolyte concentration along any given titration curve. The insensitivity to variation in ionic strength of the $^{13}$C NMR spectra of such compounds under comparable conditions has been reported (24).

The pK values corresponding to the curves of Fig. 2 are collected in Table III. The average pK values computed by weighting according to the magnitude of $(\delta_B - \delta_A)$ are listed with the designation of the peptide, followed in the next column by the temperature of measurement. The pK values signaled

| Central Amino Acid | Temperature | $C_1^\beta$ | $C_2^\beta$ | $C_3^\beta$ | $C_4^\beta$ | $C_5^\beta$ | $C_1^\gamma$ | $C_2^\gamma$ | $C_3^\gamma$ | $C_4^\gamma$ | $C_5^\gamma$ |
|-------------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|
| Glycine           | 35.5        | 16.3 24.9  -8.6 | 20.8 20.9 s  -0.1 | 188.8 152.0 s  -3.5 | 150.25 150.2 s  0.0 | 149.8 151.6 s  -2.1 |
| Alanine           | 28.0        | 16.6 25.0  -8.4 | 21.3 21.7 s  -0.4 | 188.9 152.0 s  -3.1 | 150.25 150.3 s  -0.1 | 149.4 151.6 s  -2.2 |
| Valine            | 24.5        | 16.7 25.0  -8.3 | 21.1 21.3 s  -0.2 | 188.8 152.1 s  -3.3 | 150.45 150.4 s  0.0 | 149.5 151.6 s  -2.1 |
| Leucine           | 27.8        | 16.8 25.1  -8.3 | 21.0 21.5 s  -0.5 | 189.9 152.0 s  -3.1 | 150.45 150.4 s  0.0 | 150.25 150.3 s  0.0 |
| Isoleucine        | 33.5        | 16.5 25.1  -8.6 | 21.1 21.7 s  -0.6 | 199.5 151.2 s  -2.6 | 150.35 150.3 s  0.0 | 149.5 151.6 s  -2.1 |

* The $C^\gamma$ resonances of glycine residues 2 and 4 never differ by more than 0.05 ppm and are usually unresolved. These resonances are partially resolved near neutral pH and reflect titrationnal effects of the nearest terminal group.
by the various carbon nuclei are clearly coherent. As described previously (24), in the absence of overlapping titration ranges the pK values determined are those for the temperature of the pH measurements, here 26.0°. The observed values are in the expected range (24, 37). Several of the pK values in the table are designated with superscripts to show that although the results fit the respective pK value adequately the (δB − δA) value was too small for an accurate computation to be made. The magnitudes of these shifts were approximately 0.1 to 0.2 ppm. Those cases are indicated in which no evidence of pH dependence was seen. Note is also made of marginal effects of pH variation indicating very slight chemical shift changes either in a titration range or in the region of the pH.

As the resonances of the carbon nuclei of the terminal residues shift downfield with increasing pH broadening is observed, maximally near the pH corresponding to the pK. For the Cα resonances this broadening is less clearly made out than for Cβ because the titrating resonances near their pK are overlapped by the intense Cα resonance of the residues 2 and 4. However, the reduction of the peak maximum in this range may be observed even in the face of considerable overlap (see Fig. 1E). The broadening is clearly visible for Cα nuclei, especially that of residue 1. Even at one pH unit below the pK the broadening can be detected and there is usually a reduction in peak height of at least 20 to 30%.

The effects of incorporating the glycine residues into the terminal positions of the pentapeptide can be described in the same terms as were used above for the central residues (Table I). Taking the chemical shift values for dipolar glycine from Table I, and drawing on the values for the dipolar forms of the pentapeptides, it is seen that the incorporation produces a shift of +4.5 and −4.1 ppm, respectively, for Cα nuclei of the residues 1 and 5. As already pointed out, the incorporation into the central residue produces a shift of only ±0.4. The neighboring effect of the varying central residue in the pentapeptide series causes a variation in the corresponding shift differences for the respective flanking glycine residues 2 and 4, falling in the range of ±0.5 to 1.5 ppm. The magnitudes of these shift differences reflect the combined effects of the variable central residue and the other neighbors that are the two different terminal glycine residues. The small but notable differences in resonance position for the carboxyl of glycine residue 2 in these peptides at acid pH and of glycine residue 4 in alkali illustrate the magnitude of the “nearest-neighbor” effect of the central residue.

The analogous effects on Cα nuclei of glycine residues are smaller than those for Cβ: +0.85, −1.75, and −0.9 for the residues in positions 1, 5, and 2+4, respectively. The effect of variation in the nature of the central residue on Cα of residues 2 and 4 is small.

The resonance positions of nuclei of glycine residues reported here are consistent with effects predicted from studies of tri-peptides (9, 24, 25) and from substituent parameters (4, 5, 8).

Spin-lattice Relaxation Times, T1—T1 values were measured at more than one pH for each pentapeptide except pentaglycine. The conditions of measurement did not permit accurate determinations of the T1 values of the carboxyl carbon nuclei. The long T1 values encountered, of the order of 3000 to 7000 ms, require long periods of data collection for comparable accuracy. There are also experimental limitations on the accuracy of measurement for long T1 times (3, 38). Moreover, the mechanisms of relaxation of the nonprotonated carbon nuclei are not clear (3, 39, 40). For these reasons the results presented in Fig. 3 are limited to the protonated carbon nuclei, set out in a schematized form for ready identification of the individual backbone and side chain carbon nuclei. Following the style of a preliminary report (20), the relaxation times are written in at the location of the corresponding carbons in the structural scheme, and are expressed as NT1 values in which N is the number of directly bonded hydrogen atoms. This practice allows for the approximately additive effect of each hydrogen in contributing to the dominant Cα-H dipole-dipole relaxation mechanism (41). For ready identification of the charged state of the peptide each diagram shows the terminal amino and carboxyl groups in the appropriate ionized forms. Conditions of temperature and pH are given in Table IV.

The dominant dipole-dipole relaxation mechanism determines a dependence of NT1 on the effective rotational correlation time for the protonated nucleus in question (41, 42). Various com-
The pK values are calculated from the Henderson-Hasselbalch equation for chemical shift variations with pH of all 13C nuclei of pentapeptides of the form Gly-Gly-X-Gly-Gly. The central residues are aliphatic amino acids. The amino acid residues are numbered from 1 to 5 beginning at the NH2 terminus. Carbon types listed follow the IUPAC-IUB biochemical nomenclature. The pK values listed in parentheses are averages weighted according to the ranges of the dominant titrational shifts.

| Central Amino Acid | Temp., | C1, D | C2, D | C3, D | C4, D | C5, D | C6, A | C7, A | C8, A | C9, B | C10, Y4 | C11, Y3 | C12, Y2 | C13, Y1 |
|--------------------|--------|-------|-------|-------|-------|-------|-------|-------|-------|-------|---------|---------|---------|---------|
| Alanine            | 28.0   | 8.19  | 8.19  | a     | a     | b      | b      | 3.34  | 3.30  | a      | c       | c       | c       |         |
|                   | (3.32, 8.39) |       |       |       |       |        |        |       |       |        |         |         |         |         |
| Valine             | 24.5   | 8.14  | 8.12  | a     | a     | b      | b      | 3.31  | 3.35  | c      | d       | d       | d       |         |
|                   | (3.35, 8.13) |       |       |       |       |        |        |       |       |        |         |         |         |         |
| Leucine            | 24.5   | 8.14  | 8.12  | a     | a     | b      | b      | 3.48  | 3.53  | c      | d       | d       | d       |         |
|                   | (3.49, 8.16) |       |       |       |       |        |        |       |       |        |         |         |         |         |
| Isoleucine         | 27.0   | 8.17  | 8.18  | a     | a     | b      | b      | 3.58  | 3.59  | f      | g       | e       | e       |         |
|                   | (7.38, 8.15) |       |       |       |       |        |        |       |       |        |         |         |         |         |

a. Resonance tittrates with the ionization of the amino terminal residue, although the shift range is too small for accurate calculation of pK.

b. Resonance tittrates with the ionization of the carboxyl terminal residue, although the shift range is too small for accurate calculation of pK.

c. Resonance responds near the pK by moving upfield with increasing pH approximately 0.2 ppm, although the shift range is too small for accurate calculation.

d. Resonance responds near the pI by moving downfield with increasing pH approximately 0.2 ppm, although the shift range is too small for accurate calculation.

e. Resonance is insensitive to pK.

f. Resonance tittrates with the ionization of both the carboxyl and amino terminal residues although the shift range is too small for accurate calculation of pH values. The response is 0.1 ppm downfield for carboxyl ionization and 0.1 ppm upfield for the amino ionization.

g. Resonance tittrates with the ionization of both the carboxyl and amino terminal residues although the shift range is too small for accurate calculation of pH values. The response is 0.1 ppm upfield for the carboxyl ionization and 0.1 ppm downfield for the amino ionization.

The same pattern applies to the progression of carbon nuclei out the side chains.

The NT1 values for the majority of nuclei in a given peptide in this aliphatic series at neutral pH are lower than these for either pH extreme. This observation suggests that aggregation of these peptides tends to occur near neutrality. This behavior is consistent with the slight but unmistakable pH trends mentioned previously for the resonances of the central residues, as well as with the titrational properties of the terminal groups. It is also consistent with the observed decrease in solubility of these pentapeptides when predominantly in the neutral form. Interconversion among the peptide forms is fast, as judged by the resonance intensities and line widths.

The largest values of NT1 in Fig. 3 are observed for the methyl groups. Here the spinning motion about their axis of attachment is expected to make a major or dominant contribution. Comparison of the NT1 for methyl groups in Fig. 3 with those for the corresponding Cα shows in several cases a ratio approaching the theoretical limit of 9:1 deduced for a freely spinning methyl group attached to a large, rigid matrix tumbling isotropically with an appreciably longer correlation time (42). It is significant that the correlation is most obvious in those cases where aggregation is suspected, especially with the leucine and isoleucine peptides. In addition, the methyl NT1 values for the leucine peptide near pH 9.8 appear virtually insensitive to
Fig. 3. Spin-lattice relaxation times of protonated carbon nuclei in pentapeptides of the type Gly-Gly-X-Gly-Gly. The charged state of the peptide is indicated by the terminal amino and carboxyl groups in the appropriate ionized form. Experimental details are listed in Table IV.

Table IV

| Central Amino Acid | pH  | Temperature |
|--------------------|-----|-------------|
| Glycine            | 1.32| 31.3        |
| Alanine            | 1.50| 32.3        |
| Valine             | 1.83| 25.8        |
| Leucine            | 2.02| 25.8        |
| Isoleucine         | 1.38| 25.8        |

The values for the other nuclei increase over this temperature interval.

The three sets of results for the alanine peptide in Fig. 3 at pH 1.30, 6.87, and 9.80 offer the most clear-cut example of the effects of full ionization of the terminal groups. Deprotonation of the ω-amino group appears to increase NT1 for Ca of glycine residue 1. A small degree of aggregation may influence the values at neutral pH, but the comparison of the low and high pH forms appears secure in showing altered motion of Cα with complete deprotonation of the amino group of residue 1.

As mentioned in the preceding discussion of chemical shift response to deprotonation of terminal groups, the resonances of the responsive terminal glycine residues appear to broaden, going through a maximum line width near the pK and having minimal line width at least 2 pH units above or below the respective pK value. Integrated areas of resonances at the pH extremes correspond quite well with the number of nuclei involved. The line width of the NH-terminal Ca resonance appears to be significantly increased at pH 6.70 for the isoleucine pentapeptide compared with ~1% or 10. The line width was approximately 27 Hz at pH 6.70 compared with 7 Hz at low and high pH. Separate measurements of integrated areas in the presence and absence of complete 'H decoupling at 25.19 MHz at pH 6.70 showed that the apparent reduction of NT1 for the terminal Cα (Fig. 3) was accompanied by a reduction in the nuclear Overhauser effect (NOE) from 2.9 to 2.0, whereas all other protonated carbon resonances exhibited maximal NOE values.3 Integrated areas for NH-terminal Cα resonances in the other peptides at neutral pH was much less decreased.

Discussion

The choice of the pentapeptide model with the residue under study in the central position flanked by pairs of glycine residues was made with the purpose of having the simplest possible reference position for the central residue (44-46). This peptide design should yield a consistent comparison of chemical shifts

3 Because of instrumental limitations the NOE and NT1 measurements were made at different frequencies. If the relaxation mechanism involved is frequency dependent, the comparison will not be quantitatively valid (41).
of the various components of the different amino acid residues introduced in the central position. Such results are set out in Tables I and III. The good correlation observed between the Henderson-Hasselbalch equation and the titration profile of those resonances sensitive to the state of protonation of near neighbors (Fig. 2, Tables II and III) indicates that there are no strong interactions shown by these peptides that result in asymmetrical titration behavior (47). On the other hand, small effects were noted above involving very small changes in chemical shift of the central residues during titration. The interpretation of these marginal effects are caused by aggregation appear to be supported by the $T_1$ measurements (Fig. 3, Table IV). The chemical shifts and relaxation behavior are presumably sensitive here to a minor degree of aggregation, far too little to show systematic line broadening under the conditions of the experiments or to cause noticeable light scattering. One should be alert to the possibility that in the compact native protein structure the corresponding effects on chemical shifts resulting from bringing various residues into contact may be appreciably greater. Most of the other pentapeptides in this series, to be reported separately, are less amphiphatic and have presented fewer problems with solubility; such effects have been much less evident with them.

The observations on residues 2, 3, and 4 in the glycine pentapeptide show that, within limits, the chemical shifts of residues 2 and 4 are only moderately sensitive to terminal ionization effects, a point stressed more generally by the results of Christl and Roberts (25). As a result the pattern of variation of the central residue from one peptide to another can be used to follow the effects of the neighboring substitution on the glycine residues 2 and 4. Table II shows that in terms of chemical shifts these effects are small for $C^\alpha$ in those residues but are considerable for $C^\beta$, especially in residue 2. Not surprisingly all these aliphatic substituents have similar effects. To anticipate, somewhat larger effects have been observed in this way with other pentapeptides, and will be reported separately.

The characteristic pK values for these pentapeptides exhibit a greater variability for the terminal carboxyl group compared with the terminal amino group. This greater variability is not the result of ionic strength differences and cannot be explained strictly in terms of experimental temperature variations (see Table III) since the measured thermal coefficients over this temperature range are expected to be small (48). The carboxyl terminal pK values foIlow a weak trend toward elevated values as the size of the central residue increases.

The spin-lattice relaxation times, $T_1$, show meaningful trends within each peptide. Since the $^{13}$C-$^2$H dipolar mechanism is dominant in most cases and the extreme narrowing limit applies here, the values of NT$_1$ are related to a first approximation to the inverse of an over-all rotational correlation time, $\tau_R$ (42). The simplest relation is reserved for the case of isotropic tumbling (41, 42). In applications to small molecules (49) and indicate that $\theta$ and $\gamma$ methyl groups interact with the peptide backbone for any of these pentapeptides does not conform in the results of ionic strength differences and cannot be explained to the criterion of "isotropic rigidity" as would that for a cyclic peptide (27, 36-56), so that a direct interpretation of these NT$_1$ values in terms of specific correlation times for distinct motional modes since all these values are likely to be similar in magnitude. The effects of internal motion are much less evident for the side chain nuclei of the central residues, with the exception of the methyl groups.

Peptide association tends to reduce the NT$_1$ values of the nonterminal carbon nuclei by virtually the same percentage (Fig. 3). The terminal $C^\alpha$ nuclei are affected much less and the side chain terminal methyl groups are the least responsive of all the nuclei. Separate measurements of NT$_1$ values of the leucine peptide at pH 9.88 and 33°C, compared with those at pH 7.73 and 28.5°C illustrate that the backbone and side chain carbon nuclei, except for the methyl groups, show virtually an identical percentage increase in NT$_1$ with increased temperature (by approximately a factor of 2). Notably the methyl groups exhibit identical values of NT$_1$ regardless of temperature. These results suggest that all the carbon nuclei except those of methyl groups are influenced significantly by the over-all rotation of the molecule. The contribution of $\tau_R$ to $\tau_{eff}$ is marginally less at the terminal $C^\alpha$ carbons and is virtually absent at the methyl groups of leucine. Presumably the motion of the peripheral methyl groups is dominated by rotation about their axes of attachment to the side chain.

The trend of increased NT$_1$ values of the methyl groups with increased distance from the central $C^\alpha$ may be characterized by the ratio of the NT$_1$ value for the methyl group to the NT$_1$ value for the central residue $C^\alpha$. These ratios are approximately 4, 5, 9, 11 for the most peripheral methyl groups of alanine, valine, leucine, and isoleucine, respectively. The maximum ratio for a freely rotating methyl group attached directly to a rigid backbone undergoing isotropic reorientation is 9 (42). Clearly the backbone for any of these pentapeptides does not conform in its motional properties to the criterion of "isotropic rigidity" as would that for a cyclic peptide (27, 36, 52-56), so that a direct interpretation of these NT$_1$ values in terms of correlation times for internal motion is not strictly correct (42). However, theoretical calculations for dipeptide units containing glycine and alanine (57, 58), valine (59), leucine (60), and isoleucine (57) indicate that $\beta$ and $\gamma$ methyl groups interact with the peptide backbone, whereas the $\delta$ nuclei do not. These interactions have the consequence of restricting the available rotational angles of the backbone $\phi$ and $\psi$ angles and also serve to restrict the configurations of the associated methyl groups. Our trends in NT$_1$ ratios are consistent with these predictions and compare satisfactorily with results from x-ray analysis of lysozyme, myoglobin, and $\alpha$-chymotrypsin (61-63). This evidence for the dominance of the rotational motion of the most peripheral methyl groups about their axes of attachment carries with it the implication of relatively less active rotational motion about the bonds leading out the side chains.

4 V. Glushko, F. R. N. Gurd, P. Keim, P. J. Lawson, R. C. Marshall, J. S. Morrow, A. M. Nigen, and R. A. Vigna, unpublished results.
Comparison of the NT1 values of the respective pentapeptides in the acid and alkaline forms (Fig. 3) illustrates that the values for the NH3-terminal Cα nuclei are much increased at high pH compared with those for all other protonated carbon nuclei. Since the NOE is maximum for these carbons, the 13C-H dipolar mechanism is dominant. These results show that the NH3-terminal Cα undergoes a change in rotational motion with deprotonation of the amonium group. The simplest interpretation is that the rate of internal rotation of the NH3-terminal Cα increases with deprotonation of the amonium group. This response is consistent with the expected reduction in solvation of the neutral amino group compared with the positively charged ammonium function (84). Similar features have been observed in studies of the molecular dynamics of glycylglycine (65). The interpretation for the Cα of the carboxyl terminal is less clear-cut, since the indicated increases in NT1 at high pH approach experimental error.

The trends listed in Table II illustrate that the Cα of glycine residue 4 shifts upfield with deprotonation of the terminal carboxyl group. In contrast to all other resonances sensitive to terminal ionization, this shift is downfield with deprotonation. Similar upfield shifts have been observed for the carbonyl of the amino acid units directly attached to the carboxyl terminal residue in di- and tripeptides (24, 25). Such shifts have been interpreted to reflect possible contributions from protonated forms of the peptide bond carbonyl at low pH. The suggested mechanisms include full protonation of the peptide bond (66–68), hydrogen bonding with solvent (69) or intramolecular hydrogen bonding between the peptide carbonyl and the terminal protonated carboxyl group (69). Alternatively, these upfield shifts may be evidence for the formation of a cis-gauche conformation at neutral pH stabilized by an electrostatic interaction between the terminal carbonylate and ammonium groups (70). Our results are not compatible with most of these features. Proton charging of peptide bonds is unlikely under the present conditions (66). Since the upfield titration effect is observed at only one of four peptide bonds, nonspecific hydrogen bonding with solvent appears unlikely. The upfield shift is not evidence for a folded conformation stabilized by electrostatic forces since the responsive carbonyl resonance should shift downfield with deprotonation of the NH3 terminus. Our results tend to favor attributing this titration effect either to charge or steric effects associated with the state of protonation of the terminal carboxyl group or to hydrogen bonding at the peptide carbonyl directed by the terminal protonated carboxyl group.

For the single case of the isoleucine pentapeptide at pH 6.70, the measurements of T1, line width, and NOE most likely point to an additional relaxation mechanism, competing with the 13C-H dipolar effect and coming into play near the pK of the terminal ammonium group. Spectral broadening has been observed in this laboratory for a 13C-enriched glycine adduct attached to sperm whale myoglobin (19) and for the Cα of aspartic acid in pentapeptide form. The trends in line width discussed specifically for the isoleucine pentapeptide and observed generally for all the pentapeptides reported here are similar to trends which occur in the line width for the α-Cα protons of polyamino acids during helix-to-coil transitions (71). Transition exchange rates calculated from the α-Cα proton line widths are much slower than those based on other techniques (72). Proton exchange rates calculated from the α-Cα proton line widths of model peptide systems in which the peptide bond is protonated by strongly acidic organic solvents (66, 67) are similar to the values reported from the NMR studies of helix-to-coil transitions induced by increased amounts of strong organic acids (68, 71). A rate constant determined (71, 73) from the corrected line width of the Cα of glycine residue 1 of the isoleucine pentapeptide falls in the range associated with proton exchange rates (66, 67, 74, 75) in the peptide systems described above. The characteristics of this effect in other pentapeptides will require the study of isolated resonances over the entire pH range at different resonance frequencies.

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