# Side Chain Torsional Angles and Rotational Isomerism of Oxytocin in Aqueous Solution

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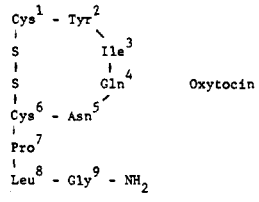
**Abstract:** Proton NMR spectra of oxytocin in aqueous solution over the temperature range 0–60 °C were measured, and the vicinal coupling constants and chemical shifts of $\alpha$ and $\beta$ protons and their temperature dependencies were derived. The possible fixed dihedral angles of the $C^{\alpha}$–$C^{\beta}$ bonds in the cystyl region of the ring and the populations of several interconverting staggered rotamers were calculated. The small temperature dependencies suggest that there are no marked changes in conformation over the temperature range studied. These results add to our knowledge of the dynamic conformation of oxytocin in solution and are useful in considering possible biologically active conformers.

The conformation of the peptide hormone oxytocin is believed to be relatively flexible in aqueous solution.[2] The number of conformers contributing to the overall dynamic conformation is probably small, however. This latter assertion is based on $^1$H NMR studies of the conformation of oxytocin in dimethyl sulfoxide,[3] on calculation of conformations based either on energy minimization or on statistical analysis of the dependence of conformation on adjacent residues,[4] on the relative rigidity of derivatives of oxytocin,[5] on the analysis of $^2$H and $^{13}$C relaxation times of oxytocin,[6] and on the apparent Stokes radius of the molecule.[7] If there is indeed a small number of interconverting conformers, then it is plausible that in the 20-membered ring formed by the first six residues only a limited number of the torsional angles show rotational isomerism. In addition, it is probable that rotational isomerism of side chains pendent to the ring will be restricted. Some information about values of torsional angles $\chi^1$ about the $C^{\alpha}$–$C^{\beta}$ bonds and their rotational isomerism on the NMR time scale may be obtained by measurement of the vicinal coupling constants about these bonds for the side chains of individual residues in the peptide.[8] Two of these side chains, those of the half-cystyl residues, are part of the 20-membered ring. We have derived those $H^{\alpha}$–$H^{\beta}$ coupling constants which may be obtained from 270-MHz proton spectra of the natural material without isotopic substitution over the temperature range 0–60 °C at pD 3.8. This paper summarizes the data obtained from these measurements and presents an interpretation of the vicinal coupling constants in terms of the conformation of the peptide.



Oxytocin

## Experimental Section

Proton NMR spectra were collected on a Bruker HX-270 spectrometer. Sample concentration was 40 mg of peptide/mL of $D_2O$. An exponential decay equivalent to 0.4 Hz in the frequency domain was applied prior to the transformation of the free-induction decay (the average of about 64 transients) in order to increase the signal-to-noise ratio at the expense of a slight loss in resolution. After Fourier transformation the 2703-Hz spectral width occupied 8192 memory locations. To compensate partially for the limited number of computer memory locations, peak positions were calculated from a three-point interpolation of a Lorentzian function. The pD was measured using an electrode equilibrated in $D_2O$ at 25 °C and calibrated using deuterated standards.[9] pD was adjusted to 3.8 using DCl. Sodium [2,2,3,3-$^2H_4$]-3-(trimethylsilyl)propionate (TSP) was added as an internal standard, from which all chemical shifts and resonance positions are reported in parts per million or hertz downfield. Analysis and simulation of spectra were performed by an implementation of LAOCN3,[10] or analysis as an ABX spin system was performed in accordance with previously published conventions.[11] In cases in which only the frequencies of the $\beta$ protons were observable, two possible solutions arise from the analysis. The solution used was selected on the basis that both vicinal couplings should be positive and <20 Hz. The assignments used were those of ref 5, which were confirmed by decoupling using the standard method of searching for multiplet collapse, or by double-resonance-difference spectroscopy.[12] The oxytocin sample used was synthetic,[13] had a uterotonic activity of 560 U/mg,[14] and showed a single spot in standard TLC systems before and after the experimental series.

## Results and Discussion

Figure 1A illustrates a typical observed spectrum in the range 2–6 ppm. The frequencies of the assigned transitions for the $\alpha$, $\beta$2, and $\beta$3 protons were analyzed to give chemical shifts and coupling constants. Figure 1B shows the result of using such derived values to calculate a simulation for the transitions of the $\alpha$ and $\beta$ protons of half-cystyls 1 and 6, tyrosyl 2, and asparaginyl 5. The derived chemical shifts and coupling constants of the $\alpha$ and $\beta$ protons of these and several other residues are presented in Table I. The temperature dependencies of these chemical shifts and coupling constants are also tabulated in Table I.

The use of vicinal coupling constants to investigate either a fixed conformation or rotational isomerism is well known.[8,15] At present, intuition rather than physical measurement is often used to decide whether rotational isomerism or a fixed angle pertains, and the appropriate form of analysis is then used. The values of $^3J(H^{\alpha}$–$H^{\beta})$, the vicinal coupling between $H^{\alpha}$ and $H^{\beta}$, for the half-cystyl residues 1 and 6 are related to the values of two of the torsional angles[16] of the cystyl link, $\chi_1^1$ and $\chi_6^1$. If the assumption is made that the two half-cystyl residues possess fixed torsional angles $\chi^1$, then their possible values can be derived graphically using an appropriate Karplus equation (Figure 2).[17] For half-cystyl 1, the value found (~−120°) is not significantly affected by the choice of NMR assignments to the $\beta$ protons, because the two coupling constants

**Table I.** Chemical Shifts and Coupling Constants for Some $\alpha$ and $\beta$ Protons of Oxytocin in $D_2O$ at pD 3.8[a]

| | Chemical shifts | | | Coupling constants | | |
|---|---|---|---|---|---|---|
| Residue | Proton[b] | Value at 25 °C,[c,d] ppm | Temperature coefficient,[e,f] ppm/deg × $10^4$ | Between protons[b] | Value at 25 °C,[c] Hz | Temperature coefficient,[e] Hz/deg × $10^3$ |
| Half-cystyl 1 | $\alpha$ | 4.274 (4.276) | 3.9 (0.4) | $\beta2$-$\beta3$ | −15.0 (−15.3) | 2 (3) |
| | $\beta2$ | 3.290 (3.291) | 5.8 (0.1) | $\alpha$-$\beta2$ | 4.6 (4.9) | 1 (2) |
| | $\beta3$ | 3.472 (3.473) | 4.4 (0.4) | $\alpha$-$\beta3$ | 5.1 (5.4) | 1 (4) |
| Tyrosyl 2 | $\alpha$ | 4.777 (4.775) | 4.4 (0.3) | $\beta2$-$\beta3$ | −14.2 (−14.2) | −1 (5) |
| | $\beta2$ | 3.009 (3.010) | 5.7 (0.2) | $\alpha$-$\beta2$ | 7.9 (7.8) | −4 (5) |
| | $\beta3$ | 3.167 (3.169) | −0.9 (0.2) | $\alpha$-$\beta3$ | 6.9 (7.0) | −1 (3) |
| Isoleucyl 3 | $\alpha$ | 4.025 (4.033) | 3.6 (0.2) | $\alpha$-$\beta$ | 6.5 (6.5) | −2 (1) |
| Glutaminyl 4 | $\alpha$ | 4.100 (4.110) | 4.8 (0.2) | $\alpha$-$\beta2$, $\beta3$[g] | 14.0 (14.0) | −3 (2) |
| Asparginyl 5 | $\alpha$ | 4.740 (4.742) | −2.1 (0.1) | $\alpha$-$\beta2$, $\beta3$[g,h] | 12.9 (13.4) | 4 (6) |
| | $\beta2$, $\beta3$ | 2.562 (2.562) | −3.9 (0.2) | | | |
| Half-cystyl 6 | $\alpha$ | 4.880 (4.878) | −1.1 (0.2) | $\beta2$-$\beta3$ | −14.3 (−14.5) | 6 (3) |
| | $\beta2$ | 2.972 (2.972) | 11.1 (0.3) | $\alpha$-$\beta2$ | 9.8 (9.6) | −20 (5) |
| | $\beta3$ | 3.234 (3.234) | −4.8 (0.2) | $\alpha$-$\beta3$ | 3.2 (3.4) | 11 (3) |
| Prolyl 7 | $\alpha$ | 4.448 (4.448) | 0.7 (0.2) | $\alpha$-$\beta2$, $\beta3$[g] | 13.4 (13.2) | −15 (8) |
| Glycyl 9 | $\alpha2$ | 3.934 (3.932) | −2.2 (0.3) | $\alpha2$-$\alpha3$ | −17.2 (−17.2) | −1 (1) |
| | $\alpha3$ | 3.850 (3.862) | 5.3 (0.6) | | | |

[a] pD at 25 °C. [b] No stereochemical assignments for $\alpha$ protons of glycyl 9 or $\beta$ protons of other residues have been made in oxytocin. Assignments here are arbitrary. [c] The measured value is given; the value in parentheses is that estimated from the regression. [d] Downfield from TSP. [e] This value is derived from a linear regression. The figure in parentheses is the standard error. [f] A downfield shift with increasing temperature is considered positive. [g] The sum of these coupling constants is derived from analysis of the $\alpha$ proton spectral region. [h] A difference in chemical shift of the two $\beta$ protons of asparginyl 5 cannot be detected at 270 MHz. Thus, individual couplings cannot be determined.

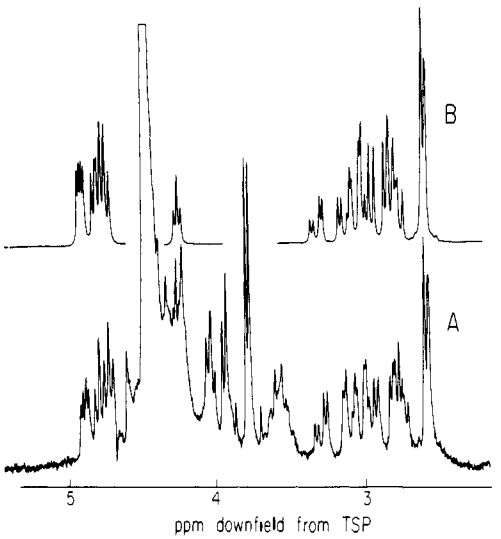



**Figure 1.** (A) Observed 270-MHz $^1H$ NMR spectrum of oxytocin in $D_2O$ at pD 3.8 and 50 °C. Chemical shifts are downfield with respect to TSP. pD was adjusted to 3.8 at 25 °C before raising temperature to 50 °C. The large peak around 4.5 ppm is the resonance of HDO. (B) Calculated simulation of the transitions of the $\alpha$ and $\beta$ protons of half-cystyls 1 and 6, tyrosyl 2, and asparginyl 5.

**Table II.** Possible Populations of Rotamers about $C^\alpha$-$C^\beta$ Bonds in Oxytocin in $D_2O$ at pD 3.8 and 25 °C[a]

| | Population[b] | | |
|---|---|---|---|
| Residue | I | II | III |
| Half-cystyl 1 | 0.15 | 0.20 | 0.65 |
| Tyrosyl 2 | 0.51 | 0.40 | 0.09 |
| Isoleucyl 3[c] | | 0.36 | |
| Glutaminyl 4[d] | | | 0.18 |
| Asparginyl 5[d] | | | 0.24 |
| Half-cystyl 6 | 0.72 | 0.28 | 0.00 |

[a] Populations ($p$) are calculated from vicinal coupling constants ($^3J$) by standard methods.[18] $p_1 = \{^3J(H^\alpha\text{-}H^{\beta2}) - {}^3J_g\}/\Delta^3J$, $p_{II} = \{^3J(H^\alpha\text{-}H^{\beta3}) - {}^3J_g\}/\Delta^3J$, and $p_{III} = 1 - p_1 - p_{II}$, where $\Delta^3J \equiv {}^3J_t - {}^3J_g$. $^3J_g$ and $^3J_t$, the vicinal coupling constants for $\alpha$ and $\beta$ protons in the gauche and trans conformations, respectively, are taken to be 2.60 and 13.56 Hz, respectively [K. G. R. Pachler, *Spectrochim. Acta*, **20**, 581 (1964)]. Note that $p_{III}$ is a function of the sum of vicinal coupling constants. [b] The rotamers I, II, and III correspond to $\chi^1$ values of −60, ±180, and +60°, respectively. The stereochemical assignment of I and II in half-cystyls 1 and 6 and tyrosyl 2 is arbitrary. [c] There is only a single $H^\alpha$-$H^\beta$ coupling constant. [d] Only the sum of $H^\alpha$-$H^{\beta2}$ and $H^\alpha$-$H^{\beta3}$ coupling constants is measurable at 270 MHz.

$[^3J(H^\alpha\text{-}H^{\beta2}), {}^3J(H^\alpha\text{-}H^{\beta3})]$ are approximately equal. On the other hand, two possible values are found for half-cystyl 6 (approximately 0 and +120°), corresponding to the two possible choices of assignment. Figure 3 illustrates these conformations and shows the values of $\chi^1$ which result from a precise interpretation of Figure 2. The upper conformations shown result from assignment of the upfield signal for each residue to $H^{\beta2}$; the lower conformations result from assignment of these signals to $H^{\beta3}$ in each case. Although the presence of coincident intersections in Figure 2 supports the assumption of fixed angles $\chi_1{}^1$ and $\chi_6{}^1$, the values derived imply virtually eclipsed conformers (Figure 2). Each of these conformers is expected to require an energy of internal rotation approximately 4 kcal/mol greater than that of the staggered conformers.[15]

Alternatively, the $\alpha$-$\beta$ proton vicinal coupling constants for half-cystyl 1 and half-cystyl 6 may be time-averaged resulting from interconversion of substantially populated rotameric isomers (rotamers). Standard analysis[18] of the data for the two half-cystyls with the assumption of three staggered rotamers would indicate that the fractional population of each rotamer (Figure 4) would be as shown in Table II. While it is reasonable to assume that side chains attached to the ring solely by the $C^\alpha$-$C^\beta$ bond show conventional rotational isomerism, the $C^\alpha$-$C^\beta$ bonds of the half-cystyls are a part of the 20-membered ring which may impose substantial structural limitations on the values of $\chi_1{}^1$ and $\chi_6{}^1$. The problem arises, therefore, of distinguishing these possibilities.

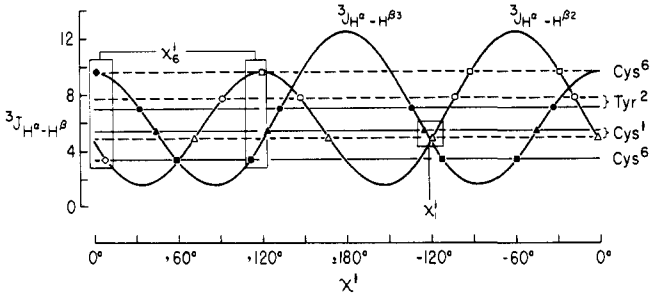



**Figure 2.** Graphical derivation of possible fixed values of the torsional angle $\chi^1$ for half-cystyls 1 and 6 of oxytocin in $D_2O$ at pD 3.8 and 25 °C. Experimentally observed coupling constants are indicated by horizontal lines that intersect the two Karplus curves.[17] The coupling constants indicated by the dashed and solid lines are arbitrarily assigned to the $\beta2$ and $\beta3$ protons, respectively. The intersections between the horizontal lines and Karplus curves for residues 1, 2, and 6 are indicated by triangles, circles, and squares, respectively; open and closed symbols represent intersections for $\beta2$ and $\beta3$ protons, respectively. Reversal of the assignments of the $\beta2$ and $\beta3$ protons for residue 6 leads to the compatible intersections denoted by the diamonds. Data for tyrosyl 2 are shown solely for comparison; the absence of coincident intersections is consistent with rotational averaging about the $C^\alpha$-$C^\beta$ bond. See text.

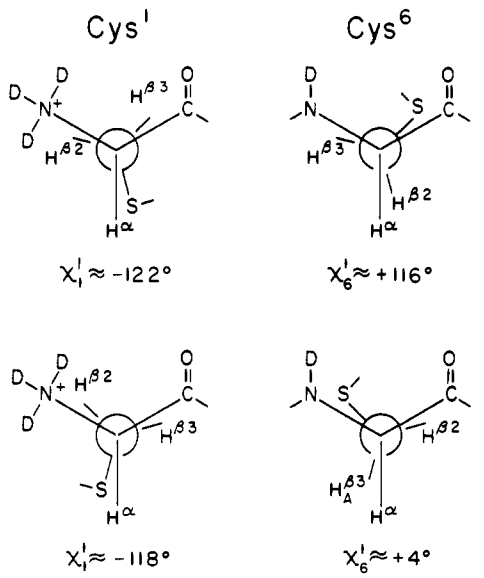



**Figure 3.** Possible fixed conformations about the $C^\alpha$-$C^\beta$ bonds in half-cystyls 1 and 6 of oxytocin in $D_2O$ at pD 3.8 and 25 °C. The deviations of these conformations from the eclipsed conformation are exaggerated for the sake of clarity. These torsional angles have estimated errors of approximately ±10°. See text.

One method of distinguishing the case in which a fixed conformation pertains from that in which rotational isomerism is manifest is based on the value of temperature dependencies of vicinal coupling constants. It will be seen in Table I that the only statistically significant temperature coefficients of coupling constants are associated with half-cystyl 6 and prolyl 7. It has been generally agreed that temperature coefficients >2 × $10^{-3}$ Hz/°C indicate that some rotational averaging is occurring.[19] The coincidence of solutions in Figure 2 is suggestive of both $\chi_1^1$ and $\chi_6^1$ having fixed conformations; on the other hand, the temperature dependence of $\chi_6^1$ makes rotational isomerism seem more likely. Additional experimental evidence is needed before either analysis is acceptable. Alternatively, it is possible that neither analysis describes the physical situation, and that either substantial libration is occurring about a single angle or that averaging is occurring among rotamers having average torsional angles well away from the normal staggered positions.

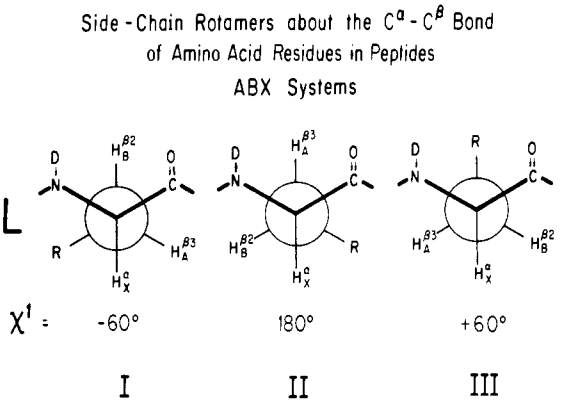



**Figure 4.** The classical, staggered rotamers about the $C^\alpha$-$C^\beta$ bond of an amino acid of the L configuration with two $\beta$ protons. R denoted the side chain starting with the $\gamma$ position. In an L-isoleucyl residue, which possesses only a single $\beta$ proton, R is the methyl group and $H^{\beta2}$ is replaced by an ethyl group. In this illustration the backbone amide proton is shown exchanged for a deuteron. In a peptide with a free amino terminus, the $ND_3^+$- group replaces the -ND- group in residue 1.

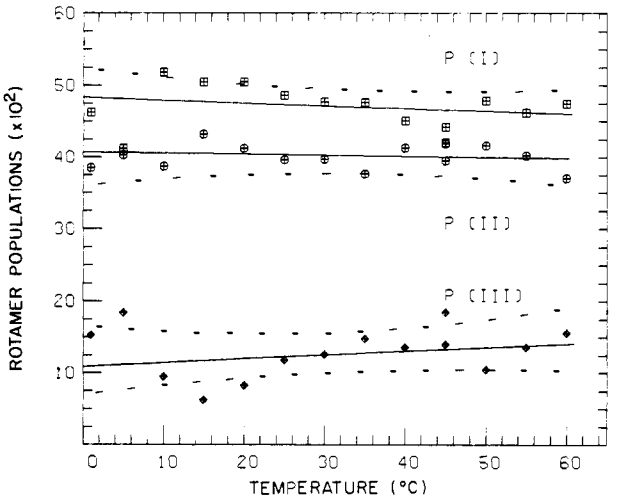



**Figure 5.** The dependencies of populations ($p$) of classical, staggered rotamers on temperature for tyrosyl 2 of oxytocin in $D_2O$. pD at 25 °C is 3.8. Calculation of $p$ is described in footnote $a$ of Table II. Solid lines represent regression of the data against temperature; dashed lines represent 95% confidence limits. Some confidence limits have been omitted for the sake of clarity.

The NMR and CD spectra of retro-D-tocinamide have been interpreted to indicate that the region of the ring near the disulfide occurs in at least two different conformations.[20] The differences in $^3J(H^\alpha$-$H^\beta)$ of the comparable half-cystyls of that compound and of oxytocin indicate, however, that it is most likely that the conformations of the two molecules are markedly different.

Table II also presents populations of rotamers for four other side chains, which were calculated from the data in Table I using standard assumptions.[18] The distributions of rotamers in all the pendent side chains is similar to those described for side chains in other small acyclic peptides.[8] Figure 5 shows the dependencies of the populations on temperature for tyrosyl 2.

Such information is directly useful in considering possible biologically active conformers of oxytocin. For example, rotamer III ($\chi_2^1$ = +60°) in tyrosyl 2 has a much lower population and higher energy than rotamers I and II. It has previously been suggested that, in the hypothetical model of the biologically active conformer of oxytocin, the tyrosyl side chain folds over the 20-membered ring.[21] The value of $\chi_2^1$ that would be most energetically favorable with regard to the $C^\alpha$-$C^\beta$ rotation in order to achieve such a folding is +60°. The results

in Table I indicate that <1 kcal/mol is required to transform the equilibrium mixture of conformers to this proposed conformer of tyrosyl 2.[22] It is probable that this amount of energy is small compared to the energy of binding of the peptide to its natural receptors.

The observed temperature dependencies of the chemical shifts range from 11 to −4 ppm downfield/°C × $10^4$. Some attempts have been made to use temperature dependencies of chemical shifts in conformational analysis.[23] In general, it seems unlikely that such analyses can be successfully applied to a molecule of the size of oxytocin, in which the number of interconverting equilibria affecting chemical shifts is large. The linearity of changes of chemical shifts and coupling constants with temperature indicates that the interconversions between conformations of oxytocin in water are continuous over the temperature range studied in this report.

This study illustrates how a substantial amount of conformational detail can be obtained from the $\alpha$ and $\beta$ proton NMR regions of a peptide such as oxytocin and how suggested biologically active conformations can be compared to the observed solution conformations.

**Note Added in Proof.** A recent publication [C. A. Boicelli, A. F. Bradbury, and J. Feeney, *J. Chem. Soc., Perkin Trans. 2*, 477 (1977)] reports comparable data for oxytocin and draws similar conclusions concerning the $\chi^1$'s of the half-cystyl residues.

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## References and Notes

(1) (a) The Vrije Universiteit, Brussels; (b) Mount Sinai Medical and Graduate Schools; (c) The Rockefeller University.
(2) V. J. Hruby, *Chem. Biochem. Amino Acids, Peptides, Proteins*, **3**, 1 (1974); J. Feeney, G. C. K. Roberts, J. H. Rockey, and A. S. V. Burgen, *Nature (London), New Biol.*, **232**, 108 (1971); J. D. Glickson in "Peptides, Chemistry, Structure and Biology", R. Walter and J. Meienhofer, Ed., Ann Arbor Science, Ann Arbor, Mich., 1975, pp 787–802; R. Deslauriers, I. C. P. Smith, and R. Walter, *J. Am. Chem. Soc.*, **96**, 2289 (1974).
(3) D. W. Urry and R. Walter, *Proc. Natl. Acad. Sci. U.S.A.*, **68**, 956 (1971).
(4) A. I. R. Brewster, V. J. Hruby, J. A. Glasel, and A. E. Tonelli, *Biochemistry*, **12**, 5295 (1973); D. Kotelchuck, H. A. Scheraga, and R. Walter, *Proc. Natl. Acad. Sci. U.S.A.*, **69**, 3629 (1972); B. Honig, E. A. Kabat, L. Katz, C. Levinthal, and T. T. Wu, *J. Mol. Biol.*, **80**, 277 (1973).
(5) J.-P. Meraldi, D. Yamamoto, V. J. Hruby, and A. I. R. Brewster in "Peptides, Chemistry, Structure and Biology", R. Walter and J. Meienhofer, Ed., Ann Arbor Science, Ann Arbor, Mich., 1975, pp 803–814.
(6) J. A. Glasel, V. J. Hruby, J. F. McKelvy, and A. F. Spatola, *J. Mol. Biol.*, **79**, 555 (1973); R. Deslauriers and I. C. P. Smith, *Top. Carbon-13 NMR Spectrosc.*, **2**, 1 (1976).
(7) L. C. Craig, E. J. Harfenist, and A. C. Paladini, *Biochemistry*, **3**, 764 (1964).
(8) J. Feeney, *Proc. R. Soc. London, Ser. A*, **345**, 61 (1975).
(9) M. Paabo and R. G. Bates, *Anal. Chem.*, **41**, 283 (1969).
(10) A. A. Bothner-By and S. M. Castellano, "Computer Programs for Chemistry", Vol. 1, D. F. DeTar, Ed., W. A. Banjamin, New York, N.Y., 1968, pp 10–53; "Itrcal", Nicolet Instrument Corp., Madison, Wis., 1973.
(11) J. A. Pople, W. G. Schneider, and H. J. Bernstein, "High-Resolution Nuclear Magnetic Resonance," McGraw-Hill, New York, N.Y., 1959, pp 130–138.
(12) W. A. Gibbons, C. F. Beyer, J. Dadok, R. F. Sprecher, and H. R. Wyssbrod, *Biochemistry*, **14**, 420 (1975).
(13) S. Hase and R. Walter, *Int. J. Pept. Protein Res.*, **5**, 283 (1973).
(14) R. A. Munsick, *Endocrinology*, **66**, 451 (1960).
(15) For a review see "Internal Rotation in Molecules", W. J. Orville-Thomas, Ed., Wiley, London, 1974.
(16) We have used torsional angle to denote the principal molecular angle defined by the modified Ingold–Prelog rules for amino acids and peptides [IUPAC–IUB Commission on Biochemical Nomenclature, *J. Mol. Biol.*, **52**, 1 (1970)].
(17) K. D. Kopple, G. R. Wiley, and R. Tauke, *Biopolymers*, **12**, 627 (1973).
(18) J. A. Pople, *Mol. Phys.*, **1**, 3 (1958).
(19) We have assumed that only the three classical, staggered rotamers are present when averaging is manifest. In the actual situation, however, there would be a continuum of conformations populated about these basic rotamers, and the population distribution of these conformations would depend upon the temperature and the form of the rotational energy as a function of $\chi^1$. This temperature-dependent distribution results in apparent temperature dependencies of $^3J_g$ and $^3J_t$—i.e., our approach remains valid if temperature-dependent corrections are made to $^3J_g$ and $^3J_t$ (in our present study, however, these small corrections are ignored for the sake of simplicity). In a theoretical study of a substituted ethane fragment Schug et al. [*J. Chem. Phys.*, **33**, 843 (1960)] estimated that $^3J_g$ and $^3J_t$ would be of the order of $1 \times 10^{-3}$ and $-1 \times 10^{-3}$ Hz/°C, respectively. We believe that these values are also reasonable for the $C^\alpha$–$C^\beta$ fragments in peptides. Therefore, any temperature dependency of $^3J(H^\alpha$–$H^\beta)$ outside this range would suggest conformational averaging, provided that the two temperature-dependent values of $^3J(H^\alpha$–$H^\beta)$ are not compatible with a single fixed conformation that changes with temperature. On the other hand, the absence of temperature dependency does not rule out conformational averaging (e.g., see ref 8), particularly when the values of $^3J(H^\alpha$–$H^\beta)$ are not compatible with a fixed conformation.
(20) K. D. Kopple, H. R. Dickinson, S. H. Nakagawa, and G. Flouret, *Biochemistry*, **15**, 2945 (1976).
(21) R. Walter, I. L. Schwartz, J. H. Darnell, and D. W. Urry, *Proc. Natl. Acad. Sci. U.S.A.*, **68**, 1355 (1971).
(22) At the physiological temperature of 37 °C, $p_I = 0.47 \pm 0.025$, $p_{II} = 0.40 \pm 0.025$, and $p_{III} = 0.13 \pm 0.025$ from Figure 5. The difference in Gibbs free energy between rotameric states III and II is calculated as follows: $G_{III} - G_{II} = RT \ln (p_{II}/p_{III}) = 700 \pm 170$ cal/mol; similarly, $G_{III} - G_I = 800 \pm 160$ cal/mol. (One-half of the estimated 95% confidence interval is given by the ± value.)
(23) E. W. Garbisch, Jr., B. L. Hawkins, and K. D. MacKay, "Conformational Analysis" G. Chiurdoglu, Ed., Academic Press, London, 1971, pp 93–109, and references cited therein.