Conformational Stability of Phenylalanine in Aqueous Solution by Nuclear Magnetic Resonance[1]

J. R. Cavanaugh

Contribution from the Eastern Regional Research Laboratory,[2] Philadelphia, Pennsylvania 19118. *Received November 15, 1967*

Abstract: The nmr spectral parameters for the aliphatic protons of the phenylalanine anion and cation in aqueous solution have been obtained as a function of both temperature and concentration. The temperature variation of the vicinal coupling constants for the anion depends critically on the concentration: at low concentration, the coupling constants diverge with increasing temperature; at high concentrations, they converge. The results for the anion are inconsistent with the interpretation that the anomalous behavior may arise from nonstaggered conformations. It appears that deviations from the staggered conformations are small but that the anion rotamer energies vary with both temperature and concentration, the two less favorable conformations gaining enhanced stability at low temperatures and concentrations. This variation can be related to changes in the dielectric constant of the aqueous solution but may be indicative of specific solute–solvent interactions. The spectral parameters corresponding to the vicinal coupling constants for the cation exhibit little variation either with temperature or concentration. These results suggest that the rotational isomerism for the cation may be interpreted in terms of staggered conformations whose relative energies are independent of temperature and concentration.

In a previous investigation,[3] the temperature variations of the vicinal coupling constants for the phenylalanine anion and dipolar ion in aqueous solution were found to be anomalous. For the anion, the coupling constants diverge with increasing temperature; for the dipolar ion, the larger coupling is constant over the measured temperature range. Two interpretations are consistent with these data. If the potential energy curve for the internal rotation about the C_α–C_β bond is assumed to be temperature independent, the potential energy minima do not correspond to the staggered conformations. On the other hand, if the rotational isomers do correspond to the staggered conformations, then the potential energies of the rotamers must vary with temperature. It is the purpose of the present communication to establish the proper interpretation from additional data on the concentration dependence of the spectrum and to examine the implications thereof.

Experimental Section

The nmr spectra were recorded on a Varian Associates DA-60-IL spectrometer,[4] equipped with a variable-temperature probe and operated at 60 MHz in the internal-lock, frequency-sweep mode. The frequency sweep was calibrated in the vicinity of each resonance peak by counting the frequency difference between fixed and swept oscillators. The line positions were calculated as the averages of at least four recordings taken with alternating upfield and downfield sweep. Reproducibility of the measurements was better than 0.1 Hz for most resonances (see below). The variable-temperature apparatus was calibrated using the internal chemical shifts of ethylene glycol and methanol as standards for the high- and low-temperature scales, respectively. Accuracy of the settings was $\pm 1°$.

L-Phenylalanine was obtained from a commercial source and was of the highest purity. The anion was prepared by dissolving the amino acid in NaOH solution in D_2O, containing a slight excess of an equivalent amount of NaOH; the cation, in HCl solution in D_2O, containing a slight excess of an equivalent amount of HCl. Concentrations are reported in moles of phenylalanine/liter of solvent. *t*-Butyl alcohol and acetic acid (2%) were added as internal references for the anion and cation, respectively.

Results and Discussion

Analysis of the Spectra. The general features of the proton spectrum of phenylalanine in aqueous solution have been discussed previously.[5] The spectral analyses for the anion are straightforward; the chemical shifts

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(2) Eastern Utilization Research and Development Division, Agricultural Research Service, U. S. Department of Agriculture.

(3) J. R. Cavanaugh, *J. Am. Chem. Soc.*, **89**, 1558 (1967).

(4) Mention of commercial products does not constitute an endorsement by the U. S. Department of Agriculture over others of a similar nature not mentioned.

(5) *Cf.* ref 3 and references quoted therein. In addition, it is to be noted that the line widths (full width at half-height) for the anion proton resonances increase substantially with concentration at low sample temperatures, with a value of ~2.5 Hz for the most concentrated sample at 15°.

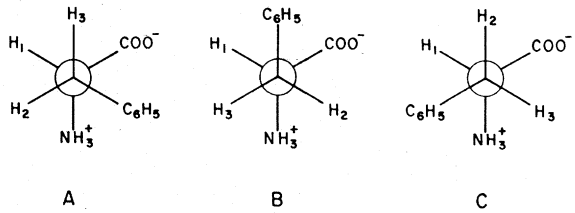

Figure 1. Newman projections of the staggered rotamers of the phenylalanine dipolar ion.

and coupling constants for the three aliphatic protons are presented in Table I. The reported coupling constants are estimated to be accurate to better than 0.1 Hz.

Table I. Chemical Shifts[a] and Coupling Constants[b] for the Phenylalanine Anion[c]

Concn[d]	Temp[e]	$J_{geminal}$	$J_{vicinal}$		$\Delta\nu_\alpha$	$\Delta\nu_\beta$	
0.3	15	−13.50	5.53	7.25	134.25	95.75	103.72
	33	−13.56	5.54	7.49	134.53	94.70	105.09
	50	−13.53	5.50	7.62	134.69	93.90	106.18
	70	−13.65	5.44	7.80	134.80	93.24	107.09
	95	−13.72	5.34	7.93	134.89	92.44	108.40
2	20	−13.40	4.91	8.22	136.43	91.77	110.86
	33	−13.46	4.91	8.28	137.13	92.17	111.59
	50	−13.53	4.92	8.40	138.25	92.63	112.77
	70	−13.58	4.96	8.40	138.79	92.89	113.62
	98	−13.67	4.93	8.41	139.09	92.94	114.17
3	15	−13.35	4.25	9.02	138.85	90.46	117.40
	33	−13.49	4.41	9.06	140.10	91.39	117.95
	50	−13.49	4.52	8.97	140.73	91.92	118.23
	70	−13.56	4.62	8.92	141.15	92.37	118.41
	95	−13.66	4.74	8.78	141.14	92.51	118.31

[a] In Hz at 60 MHz downfield from *t*-butyl alcohol (methyl resonance). [b] In Hz. [c] The smaller of the two vicinal coupling constants is associated with the β-proton resonance at lower field. [d] In moles/liter of solvent. [e] In °C.

The spectra of the cation, on the other hand, present several difficulties. The internal chemical shifts of the β protons at 60 MHz are quite small; the spectra contain several transitions of very low intensity. (The reproducibility of these measurements exceeds 0.1 Hz as quoted previously.) The resulting indefiniteness produces a corresponding uncertainty in the values for the spectral parameters. Moreover, the analysis of the spectrum is highly sensitive to very small variations in the input parameters. Therefore, data for the temperature and concentration dependence for the cation will not be reported in detail; certain tentative conclusions which can be reached will be discussed in a later section.

The Variation of the Anion Vicinal Coupling Constants. The rotational isomerism of amino acids in solution is usually represented in terms of an equilibrium mixture of the three staggered rotamers illustrated in Figure 1 for the phenylalanine dipolar ion. Since the internal rotation is sufficiently rapid, the observed coupling constants are weighted averages over those corresponding to the individual rotamers; that is

$$\begin{aligned} J_{12} &= aJ_{12}^A + bJ_{12}^B + cJ_{12}^C \\ J_{13} &= aJ_{13}^A + bJ_{13}^B + cJ_{13}^C \\ J_{23} &= aJ_{23}^A + bJ_{23}^B + cJ_{23}^C \end{aligned} \tag{1}$$

where J_{ij}^k is the coupling constant between nuclei i and j in the kth conformation and a, b, and c are the appropriate normalized populations. These conform to a Boltzmann distribution

$$a:b:c = e^{-E_A/RT}:e^{-E_B/RT}:e^{-E_C/RT} \tag{2}$$

where E_A, E_B, and E_C are the rotamer energies. It is assumed that the rotamer partition functions except those containing internal rotation coordinates are identical and that entropy differences between rotamers are small and may be neglected.[6]

It is usually further assumed that the vicinal coupling constant is a function of the dihedral angle (the Karplus equation[7]); J_{ij}^k may be replaced by J_g and J_t for protons *gauche* or *trans* to one another. Equations 1 become in part

$$\begin{aligned} J_{12} &= (1 - b)J_g + bJ_t \\ J_{13} &= (1 - c)J_g + cJ_t \end{aligned} \tag{3}$$

If J_g and J_t were known, the populations and consequently the rotamer energies could be evaluated from measurements at a single temperature, and the temperature dependence of the coupling constants could be predicted. The lack of agreement between the experimental and predicted temperature dependence for the phenylalanine anion and dipolar ion[3] shows that one or more of the assumptions underlying this point of view have broken down.[8] Two choices appear open: either the rotamer energies are not independent of temperature and the vicinal coupling constants do not vary simply in accordance with what would be predicted from the Boltzmann factors, or the rotational isomers are not represented by the classical staggered conformations and replacement of J_{ij}^k by J_g and J_t and the resulting eq 3 are no longer applicable.

According to the latter view, nonstaggered conformations of appropriate energy can be found that will yield satisfactory agreement with experiment. This has been accomplished for the 1 *M* sample[3] by means of a computer "direct-search" computation in which the molecular conformations and energies were independently varied in order to best fit the calculated with the experimental temperature dependence using eq 1 and 2. The vicinal coupling constants were obtained from the Karplus equation[7]

$$J_{\text{vicinal}} = \begin{cases} K_1 \cos^2 \varphi + C & 0 \leqslant \varphi \leqslant \pi/2 \\ K_2 \cos^2 \varphi + C & \pi/2 \leqslant \varphi \leqslant \pi \end{cases} \tag{4}$$

where φ is the dihedral angle and K_1, K_2, and C are constants.

With these conformations as a basis and with the assumption that they do not change with concentration, eq 1, 2, and 4 may be used to evaluate the populations and the rotamer energies for the other concentrations from spectral measurements at a given temperature.

(6) It must be remembered, however, that we are concerned with condensed systems and that, strictly speaking, the free energies of the rotamer–water aggregates determine the populations.

(7) M. Karplus, *J. Chem. Phys.*, **30**, 11 (1959).

(8) It does not appear that assigning more than one value to J_g (*cf.* G. M. Whitesides, J. P. Sevenair, and R. W. Goetz, *J. Am. Chem. Soc.*, **89**, 1135 (1967)) would yield any better agreement with experiment. For example, the temperature variation of the vicinal coupling constants (based on the experimental data for the ~1 *M* solution of the anion at 50°) calculated by eq 1 and 2 with two *gauche* coupling constants, 2 Hz apart, differs from that predicted by eq 2 and 3 with a single J_g by at most 0.02 Hz over the 100° temperature range. Therefore, for simplicity, we will assume that a single J_g is adequate to describe the system.

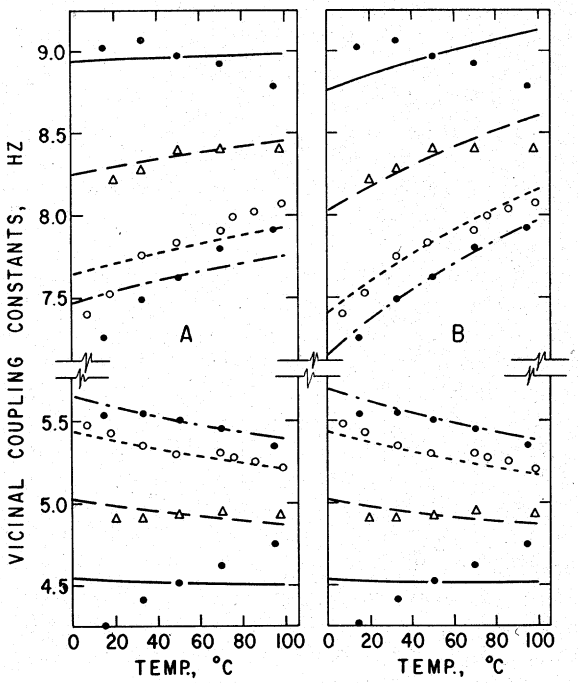

Figure 2. Observed and calculated temperature dependence of the vicinal coupling constants for the phenylalanine anion for nonstaggered conformations and the energies indicated in Table II. A and B refer to sets I and II, respectively: ● and — — —, concn = 0.3; ○ and - - -, concn = 1; △ and — — —, concn = 2; and ● and —, concn = 3 moles/l. of solvent.

The results of the calculations appropriate to 50° are shown in Table II.[9] From these values the temperature variations of the vicinal coupling constants for the several concentrations are readily determined. They are compared to the experimental points in Figure 2.

Table II. Rotamer Energies[a] Calculated from the Observed Vicinal Coupling Constants[b] Using Eq 1, 2, and 4 for the Conformations Given in Ref 3[c]

	Concn[d]	E_A	E_B	E_C
I	0.3	86	952	0
	2	38	382	0
	3	13	66	0
II	0.3	0	572	495
	2	0	281	420
	3	0	97	331

[a] Calories/mole. [b] At 50°; a value of 8.36 Hz was chosen for the larger coupling for the 2 moles/l. of solvent concentration as a closer approximation to a smooth curve. [c] Set I refers to the conformations given in ref 3, Table III; set II refers to those in ref 3, Table IV. [d] In moles/liter of solvent.

The over-all agreement is most unsatisfactory. Although in both cases the fit is good at some concentrations, it is very poor at others. The calculated curves are nearly parallel; the slopes of the experimental curves change sign as the concentration is increased. At a concentration of 3 moles/l. of solvent, the vicinal coupling constants vary nearly as originally expected; that is, they converge with increasing temperature.

(9) The results shown in Table II are for $J_{12} > J_{13}$. While the conformations and energies obtained with $J_{13} > J_{12}$ are considerably different, the final results secured (that is, the temperature dependence of the vicinal coupling constants based on these conformations) are essentially identical. Note, however, that if eq 3 hold, then steric considerations (that is, that $c > b$) imply that $J_{13} > J_{12}$, since $J_g < J_t$.

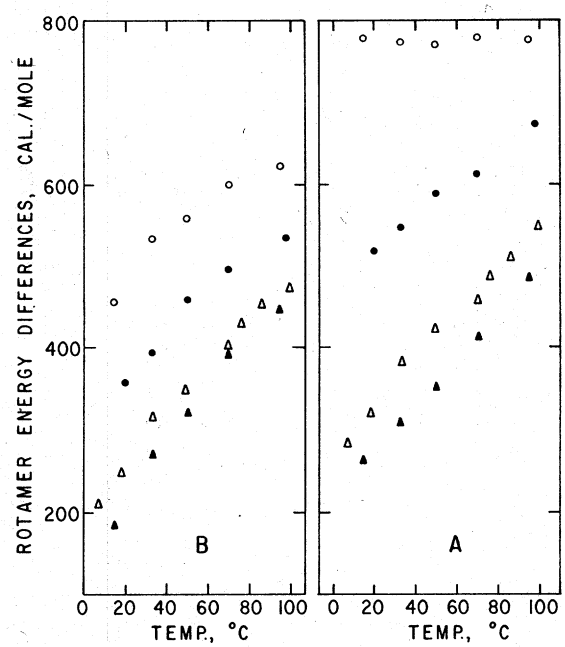

Figure 3. Anion rotamer energies: (A) $E_A - E_C$; (B), $E_B - E_C$. ▲, △, ●, and ○ refer to concentrations of 0.3, 1, 2, and 3 moles/l. of solvent, respectively.

The interpretation based on temperature-independent though concentration-dependent rotamer energies within the framework of nonclassical conformations appears to fail rather badly. In order to maintain this framework, conformational changes attendant on concentration would be required as well; that is, suitable though different conformations could probably be found for all concentrations that would fit the observed data. Such a viewpoint seems to be untenable. The variations of the phenylalanine anion vicinal coupling constants suggest that the approach involving nonstaggered conformations and temperature-independent rotamer energies is unsatisfactory. It appears that variations of the rotamer energies with temperature must be taken into account.

The Temperature and Concentration Dependence of the Anion Rotamer Energies. The variation of the conformer energies of the phenylalanine anion may be put on a quantitative basis by means of eq 2 and 3. With $J_g = 2.60$ and $J_t = 13.56$ Hz,[10] the relative rotamer energies are readily determined from the observed vicinal coupling constants as a function of temperature and concentration.[11] The energies of the A and B rotamers, relative to that of the most stable rotamer, C, are displayed in Figures 3A and 3B, respectively. Both increase with increasing concentration and temperature, the variation being approximately linear. Moreover, with increasing concentration (higher relative energies), the slopes of the curves diminish; at a concentration of 3, $E_A - E_C$ is approximately constant.

The rotamer energies correlate with the dielectric constants of the aqueous solutions. Figure 4 illustrates this relationship. The values for the dielectric con-

(10) K. G. R. Pachler, *Spectrochim. Acta*, **20**, 581 (1964).

(11) The values so computed depend on the values assigned to J_g and J_t (and on the assumption that a single value for J_g is adequate). However, the over-all dependence of the rotamer energies on temperature and concentration would be expected to be largely independent of small variations in the numeric values actually assigned to the coupling constants.

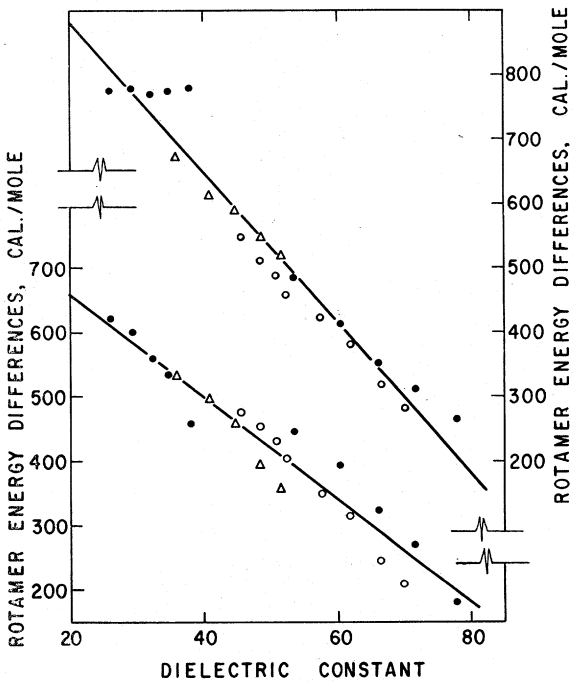

Figure 4. Anion rotamer energies *vs.* dielectric constant of the solution: upper portion, $E_A - E_C$; lower portion, $E_B - E_C$. Left-hand ●, concn = 3; △, concn = 2; ○, concn = 1; and right hand ●, concn = 0.3 moles/l. of solvent. Solid lines are linear least-squares curves.

stant of the solution, $\epsilon(C,t)$, are obtained from the equation

$$\epsilon(C,t) = \epsilon(t) - 14C\frac{\epsilon(t)}{78} \quad (5)$$

where $\epsilon(t)$ is the dielectric constant of water as a function of temperature.[12] The dielectric decrement for the sodium salt of phenylalanine is taken as 14.[13] It is also assumed that the decrement is linear in C and is proportional to the dielectric constant of water at the appropriate temperature. Equation 5 is quite approximate as neither assumption holds rigorously over the temperature and concentration ranges studied.[14] Moreover, the decrement is usually given in terms of molar concentrations whereas moles/liter of solvent have been used here. In spite of the approximate nature of eq 5, the correlation exhibited in Figure 4 is quite good and takes into account rotamer energy variations with temperature as well as with concentration.

However, the causal relationships between the dielectric constant and the conformational stability remain in doubt. The variations in rotamer energies can probably be ultimately traced to variations in solute–solvent interactions. Since the nature of such interactions in aqueous solution is at present poorly understood, only further investigation can determine the precise role they play. Whether the proper description of the variations in rotamer energies can be based on specific solute–solvent interactions or couched in terms of the classical electrostatic theory of dielectrics, which has been successfully applied to the study of rotational isomerism in substituted ethanes,[15] is an open question.

The Variation of the Cation Vicinal Coupling Constants. The repeated spacings corresponding to the cation vicinal coupling constants exhibit little variation with temperature or concentration. The larger spacings fall in a relatively narrow range about 7.2 Hz; the smaller spacings, about 6.0 Hz. The greatest variation occurs for the sample of concentration 0.3 mole/l. of solvent; the larger and smaller spacings change by −0.26 Hz and +0.29 Hz, respectively, over the (increasing) temperature scale. This small variation undoubtedly reflects a similar behavior of the actual vicinal coupling constants themselves. Since the spectral analysis magnifies the experimental error of the measurements, it is difficult to establish this behavior in a precise and straightforward manner.

The over-all range of these values, however, suggests that a limit may be placed on the possible variations of the vicinal coupling constants themselves. In view of the small spread of these limits (and the fact that the repeated spacings vary in the direction expected from the simple theory, that is, they converge with increasing temperature) it is probable that the rotational isomerism of the cation may be represented in terms of the classical staggered conformations whose relative energies are independent of temperature and concentration. The limitations of the experimental data and the spectral analysis preclude at this time quantitative statements regarding the extent of deviations from constant values.

Conclusion

The variation of the vicinal coupling constants for the phenylalanine anion suggests that the rotational isomers are the classical staggered conformations whose relative energies vary with temperature and concentration. These effects are most probably related to intermolecular interactions present in the phenylalanine–water solutions. Other amino acids also show anomalous behavior in the variation of the vicinal coupling constants: serine, for which the cation but not the anion is reported to have temperature-dependent conformational energies;[16] and tyrosine, for which preliminary experiments in this laboratory show unusual temperature but not concentration effects. It is possible that the elucidation of the nature and causes of the deviations from ideal behavior in these systems could lead to a better knowledge of solute–solvent interactions in aqueous media.

Acknowledgment. The technical assistance of Messrs. R. J. Szamborski and R. C. Beam is gratefully acknowledged.

(12) G. C. Akerlof and H. I. Oshry, *J. Am. Chem. Soc.*, **72**, 2844 (1950).

(13) This value was chosen from data on salts of carboxylic acids; *cf.* G. H. Haggis, J. B. Hasted, and T. J. Buchanan, *J. Chem. Phys.*, **20**, 1452 (1952).

(14) *Cf.* C. P. Smyth, "Dielectric Behavior and Structure," McGraw-Hill Book Co., Inc., New York, N. Y., 1955, Chapter 3. The assumption that the decrement is proportional to the dielectric constant of water at a particular temperature is equivalent to the assumption that the percentage decrease in the dielectric constant is temperature independent. For examples of actual aqueous systems, see J. B. Hasted and G. W. Roderick, *J. Chem. Phys.*, **29**, 17 (1958); J. B. Hasted, D. M. Ritson, and C. H. Collie, *ibid.*, **16**, 1 (1948).

(15) R. J. Abraham, L. Cavalli, and K. G. R. Pachler, *Mol. Phys.*, **11**, 471 (1966); R. J. Abraham and M. A. Cooper, *J. Chem. Soc., B*, 202 (1967).

(16) H. Ogura, Y. Arata, and S. Fujiwara, *J. Mol. Spectry.*, **23**, 76 (1967).