mass spectral data. In addition, elimination of manual interpretation and manual instrument control would permit all necessary experiments to be performed on a single aliquot of sample. This would allow complete structural analysis of considerably smaller sample quantities (potentially <1 μg total mixture) thus conserving precious samples.

Experimental Section

High resolution mass spectra were obtained utilizing a Varian-MAT 711 mass spectrometer operated at an ionizing voltage of 70 eV, an ionizing current of 1.6 mA, and a scan rate of 22 sec/decade at a nominal resolving power of 10,000. LEV spectra were recorded at an ionizing voltage of 13.5 eV (uncorrected). This value was determined (utilizing standard samples of estrone, estrone methyl ether, and estradiol) to yield only molecular ion peaks in the molecular ion region of estrogens (above $C_{18}H_{24}$[8]). First field-free region metastable ions were analyzed on the Varian-MAT 711, with additional metastable ion data collected utilizing a Varian-MAT 311 (second field-free region) (see Acknowledgment).

The computer times mentioned above are those for an IBM 360/50 at the Stanford Medical School's ACME facility, a computer which is four to five times slower for this program than the computer utilized in a previous study (IBM 360/67).[8]

Acknowledgment. We are indebted to Dr. A. L. Burlingame, Space Sciences Laboratory, University of California, Berkeley, for making available to us time on a Varian-MAT 311 mass spectrometer for determination of some of the metastable ion data obtained for mixtures A–C.

Rotamer Stability of Histidine and Histidine Derivatives[1]

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Abstract: The nuclear magnetic resonance vicinal coupling constants for histidine and *im*-benzyl-, *N*-acyl-, and *O*-methylhistidines have been obtained in deuterium oxide solutions at acidic, neutral, and basic pH. Equations used in calculating relative rotamer populations have been derived which include terms for the functions of dihedral angles, substituent electronegativities, and orientation in vicinal coupling. A model is presented in which the rotamer populations of histidine and its derivatives are determined by an electrostatic interaction between the carboxylate anion and the imidazole ring, which stabilizes the conformation in which these groups are in close proximity.

As a result of our interest in the solution structure of histidine analogs and peptides which contain these analogs,[2] we have reexamined the proton magnetic resonance data concerning histidine and histidine derivatives. An understanding of histidine conformations and the relative importance of the interactions which determine these orientations were found to be important in problems of peptide secondary and tertiary structure.[2] Proton magnetic resonance techniques are particularly well suited to an investigation of these questions. Previous papers have described the relative populations of rotamer conformations[3] of histidine and factors which affect the energy differences.[4] Others have noted the variations of pmr[5] and ^{13}C nmr[6] parameters with pH and have attempted to correlate these with electronic structure.[7] Pmr studies attempting to describe the structure of the low energy histidine rotamer require assignment of the frequency of the β-methylene hydrogens. The chemical shift differences of these protons are small and the reasons given for the assignments have not been convincing.[3b,8] As a result of this difficulty, the conformation of the preferred histidine rotamer has not been established.

Solid-state conformations as determined by X-ray crystallography[9] indicate that energy differences separating conformers are comparable to the hydrogen bonding and packing energies in the crystal. A unit cell of L-*N*-acetylhistidine contains both an open (imidazole and carboxylate groups are trans) and closed (adjacent) conformation. As the identification of the preferred amino acid conformer is necessary for application to problems of peptide solution structure, we have examined this aspect in the pmr studies in this paper.

Work with histidine analogs[2a,b] in which the 4-imidazolyl ring was replaced by the stable free radical 1,3-dioxy-4,4,5,5-tetramethyldihydro-2-imidazoloyl showed electron spin resonance line broadening associated with low rotation rates[10] when the carboxyl group was in the anionic form. On protonation esr

(1) Supported in part by the Public Health Service Research Grant AM 08066 from the National Institute of Arthritis and Metabolic Disease.

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Table I. Pmr Data for Histidine Derivatives at 23°, Coupling Constants (Hz)

Compd no.	Species in solution[a]			$\nu_\beta - \nu_{\beta'}$[b]	$J_{\beta\beta'}$	$J_{\alpha\beta'}$[c]	$J_{\alpha\beta}$	$J_{\alpha\beta} - J_{\alpha\beta'}$
1	NH_3^+	ImH^+	COOH	0		6.4	6.4	0
2	AcNH	ImH^+	COOH	10.8	15.0	5.4	7.8	2.3
3	NH_3^+	$BzImH^+$[d]	COOH	0		6.7	6.7	0
4	BocNH[e]	$BzImH^+$	COOH	0		6.6	6.6	0
5	NH_3^+	ImH^+	CO_2CH_3	0		6.7	6.7	0
6	NH_2	Im	CO_2CH_3	0		7.2	7.2	0
7	NH_3^+	ImH^+	COO^-	0		6.7	6.7	0
8	NH_3^+	$BzImH^+$	COO^-	14.2	14.6	4.7	8.1	3.4
9	NH_3^+	Im	COO^-	0		6.5	6.5	0
10	BocNH	$BzImH^+$	COO^-	23.4	15.0	3.9	9.1	5.2
11	AcNH	ImH^+	COO^-	17.3	15.0	4.4	9.0	4.6
12	NH_2	Im	COO^-	20.0	14.6	4.7	8.4	3.7
13	BocNH	BzIm	COO^-	33.0	15.0	2.8	10.0	7.2
14	NH_2	BzIm	COO^-	31.2	14.4	4.3	9.1	4.8
15	AcNH	Im	COO^-	16.0	15.0	4.1	9.7	5.6

[a] All solutions were prepared in D_2O at approximately 0.15 mmol/ml; the columns represent the functional substitution of the amino, imidazole, and carboxyl groups of histidine. [b] Hz at 100 MHz. [c] The low field methylene proton has the smaller coupling constant and is indicated here as the β' hydrogen. [d] $BzImH^+$ = monoprotonated *im*-benzyl-4-imidazolyl. [e] Boc = *tert*-butyloxycarbonyl.

spectra characteristic of rapidly interconverting isomers were observed. It was postulated[2a] that an intramolecular ion–dipole association between the carboxylate and nitronyl nitroxide functions forms a cyclic conformer which slows the rate of interconversion. The analogy between these esr spectral changes and the variation of the pmr $C_\alpha H-C_\beta H_2$ coupling constants discussed in this paper has been noted.[2a]

In general, the methine and methylene protons of histidine appear as an A_2X pattern at low pH and as a 12-line ABX pattern at high pH and the variation of the histidine anion pmr spectra with temperature and concentration has been correlated with the solvent dielectric constant.[4] While this indicates that hydrogen bonding and electrostatic interactions may be important factors influencing conformer equilibrium, they have not been included in assumptions used to identify the preferred conformation. Previous detailed analyses of the proton magnetic resonance solution spectra have relied on analogies with related compounds[3b,8] or assumptions concerning steric repulsions[4,5a] to make critical conclusions concerning rotamer identity. In this investigation, the pmr spectra of histidine and *im*-benzyl-, *N*-acyl-, and *O*-methylhistidines were obtained in deuterium oxide solution at different pD values. It was concluded from these data that the primary influence determining rotational stability of histidine derivatives is an electrostatic interaction between the carboxyl group and the imidazole ring.

Experimental Section

Acetyl-L-histidine, *tert*-butyloxycarbonyl-*im*-benzyl-L-histidine, and L-histidine methyl ester hydrochloride were obtained from commercial sources and were of the highest purity. Their melting points were in agreement with known values and they were homogeneous according to thin-layer chromatography, electrophoresis at pH 1.85, and pmr. The *im*-benzylhistidine was prepared from the *tert*-butyloxycarbonyl-*im*-benzyl derivative by dissolving in anhydrous trifluoroacetic acid. Evaporation yielded the dipositive *im*-benzylhistidine ion which appeared as a single spot on electrophoresis.

The pmr spectra were recorded on a JOEL JMN-4H-100 or a Varian Associates A60-A spectrometer. The coupling constants were calculated from spectra taken on the 100-MHz instrument at 23°. Amino acid concentrations were approximately 0.15 mmol/ml in D_2O. The methyl resonance of *tert*-butyl alcohol was used as an internal reference (2% v/v) with an internal lock. The pD of these solutions was adjusted using trifluoroacetic acid and sodium deuteroxide solutions in D_2O. The ionic species inferred from the solution acidity were consistent with the chemical shift values expected for the respective ionic states.[3b,5a,11] The values reported here are in agreement with previously reported values taken under similar conditions with 60-MHz instruments.[3b,5a]

Results

In general, the spectra showed sharp lines although those taken in the pH range of 7–10 were broadened.[5a] All spectra were considered to be second order and were analyzed by reported procedures.[12] The spectra of histidine dipositive (**1**) and zwitterions (**9**), the *tert*-butyloxy-*im*-benzyl positive ion (**4**), and the methyl ester at acid and basic pD's (**5** and **6**) show all of the characteristics of A_2X spectra. Those of the *im*-benzyl dipositive ion (**3**) and the monoprotonated histidine zwitterion (**7**) showed some broadening of the central peak in the X region and of the upfield peak in the A_2 region while showing seven resolved peaks so that no further analysis could be undertaken. The methylene chemical shift differences and coupling constants for the 15 structural and ionic histidine derivatives are presented in Table I. The pK_a values of histidine are 1.77, 6.10, and 9.18 in water at 25°.[13] The pmr spectra of **1–5** were taken at pD 0.5; **7**, **8**, and **10** at pD 4; **9** at pD 7.5; and **6** and **11–15** at pD 12. A correlation between the magnitude of the $J_{\alpha\beta} - J_{\alpha\beta'}$ coupling constant difference and the chemical shift difference of the β-methylene hydrogens, $\nu_\beta - \nu_{\beta'}$, was apparent from the data in Table I. This is plotted in Figure 1.

Discussion

The coupling constant differences between $J_{\alpha\beta}$ and $J_{\alpha\beta'}$ of a $C_\alpha HXY-C_\beta HH'Z$ unit are dependent on the coupling constants of the individual $C_\alpha HXY-C_\beta HH'Z$ rotamers and the relative populations of these rotamers. The rotamer coupling constants are functions of $C_\alpha H-C_\beta H$ and $C_\alpha H-C_\beta H'$ dihedral angles, the electronegativity of the XY and Z substituents, and the orientation of these substituents. These functions can be approximated so the relative rotamer populations may be calculated from coupling constant

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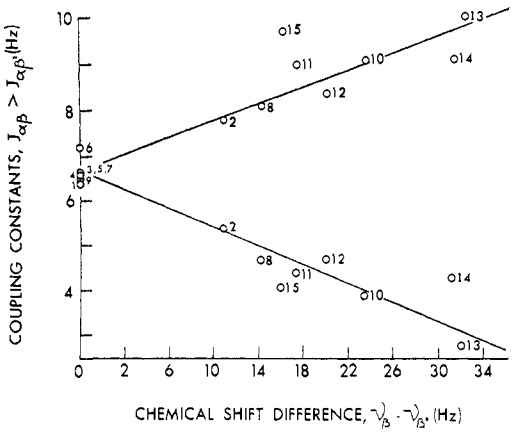

Figure 1. A plot of vicinal $\alpha\beta$-coupling constants against β-proton chemical shift difference of histidine derivatives.

data. As this work is directed toward identification of the stable histidine conformer, the relative populations are correlated with the known structural properties of the histidine derivatives to indicate which mechanism of conformational stabilization is operating.

In this series, the identity of the bound atoms on the $C_\alpha HXY$-$C_\beta HH'Z$ fragment was not changed so variation in the internal bond angles and interatomic distances was not considered. As there is no evidence that amino acids tend to exist in stable configurations in aqueous solutions,[3c] the pmr spectra are taken to be time averaged and the chemical shift and coupling constants are determined by the conformational equilibria of the asymmetric amino acids. Where the contributing equilibrium is between rotational states, the energy difference between the staggered and eclipsed conformations is sufficiently large so that only the sterically favored staggered rotamer contributes to the pmr spectrum.[14] The sum of the mole fractions of the rotamers indicated in Figure 2 is equal to one.

Application of these principles relates the observed vicinal coupling constants $J_{\alpha\beta}$ and $J_{\alpha\beta'}$ to the coupling constants of the individual gauche (G) and trans (T) rotamers of Figure 2 through eq 1 and 2. The difference in the mole fraction n_I, n_{II}, and n_{III} is a function of the free energy difference between the respective rotamers as shown in eq 3 and 4.

$$J_{\alpha\beta} = n_I J_{G_I} + n_{II} J_{T_{II}} + n_{III} J_{G_{III}} = n_I(J_{G_I} - J_{G_{III}}) + n_{II}(J_{T_{III}} - J_{G_{III}}) + J_{G_{III}} \quad (1)$$

$$J_{\alpha\beta'} = n_I J_{T'_I} + n_{II} J_{G'_{II}} + n_{III} J_{G'_{III}} = n_I(J_{T'_I} - J_{G'_{III}}) + n_{II}(J_{G'_{II}} - J_{G'_{III}}) + J_{G'_{III}} \quad (2)$$

$$n_{III}/n_I = e^{(-\Delta F_{I-III}/RT)} \quad (3)$$

$$n_{III}/n_{II} = e^{(-\Delta F_{II-III}/RT)} \quad (4)$$

The chemical shift difference between the β-methylene hydrogens may also be dependent on the relative rotamer populations. As shown in Figure 1, a fairly good correlation exists between these chemical shift differences ($\nu_\beta - \nu_{\beta'}$) and the vicinal coupling constant difference $J_{\alpha\beta} - J_{\alpha\beta'}$. It appears, then, that the β chemical shift difference for histidine derivatives is approximately related to the rotamer populations as

(14) S. Mizushima, "Structure of Molecules and Internal Rotation," Academic Press, New York, N. Y., 1954.

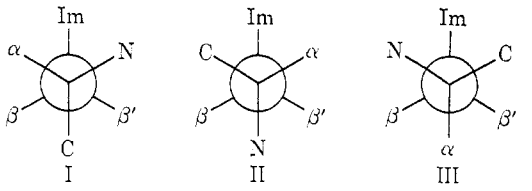

Figure 2. Newman projections of the staggered conformations of histidine where the α-amino, carboxyl, and proton substituents are indicated by N, C, and α and the β carbon imidazole and geminal protons by Im, β and β'.

shown in eq 5 and 6 and the chemical shift difference in eq 7. The substituent effects are denoted H, N, and C for the proton, amino, and carboxyl substituents of the α carbon and the orientations gauche and trans to the relevant β protons are shown by the subscripts G and T. The substituent effects for protons are not expected to change significantly with small variations in dihedral angles[15] so that functions gauche to both C_β protons may be taken as equal even though the respective dihedral angles may differ somewhat. That is, C_G-$C_{G'}$, N_G-$N_{G'}$, and H_G-$H_{G'}$ are taken as zero.

$$\nu_\beta = \nu_0 + n_I(H_G + N_T + C_G) + n_{II}(H_T + N_G + C_G) + n_{III}(H_G + N_G + C_T) \quad (5)$$

$$\nu_{\beta'} = \nu_0 + n_I(H_{T'} + N_{G'} + C_{G'}) + n_{II}(H_{G'} + N_{G'} + C_{T'}) + n_{III}(H_{G'} + N_{T'} + C_{G'}) \quad (6)$$

$$\nu_\beta - \nu_{\beta'} = n_I(H_G\text{-}H_{T'} + N_T\text{-}N_{G'}) + n_{II}(H_T\text{-}H_{G'} + C_G\text{-}C_{T'}) + n_{III}(N_G\text{-}N_{T'} + C_T\text{-}C_{G'}) \quad (7)$$

It has been shown[3a] that if $J_{\alpha\beta} = J_{\alpha\beta'}$ when $n_I = n_{II}$, then $\nu_\beta - \nu_{\beta'} = 0$, when $n_I = {}^1/_3$, so that the coupling constants observed at $\nu_\beta - \nu_{\beta'} = 0$ are J_{av} values. However, if the substituent effects for the amino and carboxyl groups were the same [N_G-N_T = C_G-C_T], then $J_\beta = J_{\beta'}$ in rotamers I and II and the relation will not hold. The chemical shifts of geminal methylene protons adjacent to an asymmetric center are not necessarily equal even when rotamer populations are equal ($\nu_0 = \nu_0'$). This asymmetrically induced difference does not appear to be significant in aqueous solutions of histidine derivatives as a number of spectra appear as A_2X patterns.

The effects of substituents on the chemical shifts are dependent upon electric dipole moments, magnetic susceptibility, anisotropy, and, to a lesser extent, van der Waal's interactions. Specific solvational interactions are also important in rigid systems.[15] The manner in which these substituent and solvational effects vary with their orientation to a vicinal proton is not well defined. Orientational variations of substituent effects on coupling constants, however, are significantly less complicated and may be calculated with reasonable accuracy using known relationships.[16] Calculation of the gauche and trans coupling constants (J_G and J_T of eq 1) for each of the contributing conformations leads to a determination of the relative rotamer populations. Three factors have been identified

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(16) G. M. Whitesides, J. P. Sevenair, and R. W. Goetz, *J. Amer. Chem. Soc.*, 89, 1135 (1967).

as influencing the vicinal coupling constants, the dihedral angle between the coupled vicinal protons,[17] the electronegativity of substituents on the HCCH fragment,[18] and the orientation of the electronegative substituent with respect to the coupled protons.[19]

The angular dependence of coupling for a $C_\alpha HXY-C_\beta HH'Z$ fragment is described by the Karplus equation[17] (eq 8). The values for the constants A, B_1,

$$J_{HH'} = A + B_1 \cos^2 \phi \quad 0° \geq \phi \geq 90°$$
$$= A + B_2 \cos^2 \phi \quad 90° \geq \phi \geq 180° \tag{8a}$$

$$J_{HH'} = (A^u + B^u \cos^2 \phi)(1 + D\Delta\chi) \tag{8b}$$

and B_2 were calculated for the $NH_2{}^+RCH(CHR)COO^-$ fragment by Abraham and McLauchlan[20] where $B_1 = 10.5$ and $B_2 = 13.7$; A was assumed to equal zero. The ratio of the constants was determined by fitting the shape of the $\cos^2 \phi$ function to the observed data, and the magnitude of the constants was a function of the XY and Z substituent electronegativity as shown in the generalized form (8b),[17] where A^u and B^u are the A and B values of Abraham and McLauchlan uncorrected for electronegativity effects and D weighs the influence of the substituent electronegativity $\Delta\chi$. Any error in estimating the values of these constants is minimized as the solution of eq 8 is most sensitive to changes in the angle ϕ.

The effect of substituent electronegativity on vicinal coupling constants has been estimated by eq 9 for substituted ethanes.[21] The group electronegativity E_i is calculated from the chemical shift difference between the methyl and methylene hydrogens δ_{int} for an i substituted ethane.[21] It is difficult to apply these equations to amino acids as the ethane fragment is polysubstituted with ionic functions for which group electronegativity values are not reported. We have therefore utilized the calculated amino acid charge density distributions of Del Re, Pullman, and Yonezawa[7] in lieu of group electronegativities. These workers related the calculated carbon and hydrogen charge densities, Q_C and Q_H, to chemical shift by eq 11 where

$$J_{av} = 18.0 - 0.80\sum_{i=1}^{6} E_i \tag{9}$$

$$E_i = 0.684\delta_{int} + 1.78 \tag{10}$$

$$\delta_H = -9.92Q_C + 133.93Q_H + K \tag{11}$$

K is determined by the reference frequency used. One can therefore calculate a set of terms $\delta_{CH_3} - \delta_{C_\alpha HXY}$ and $\delta_{CH_3} - \delta_{C_\beta H_2 Z}$ which is equal to the sum of δ_{int} terms in eq 9. Using the same parameters of eq 9, 10, and 11, the calculated average coupling constant values may be obtained from eq 12.

$$J_{av} = 13.31 - 73.26(Q_H{}^\alpha + Q_H{}^\beta) - 5.43(Q_C{}^\alpha + Q_C{}^\beta) \tag{12}$$

(17) M. Karplus, *J. Chem. Phys.*, **30**, 11 (1959).
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(20) R. J. Abraham and K. A. McLauchlan, *Mol. Phys.*, **5**, 513 (1962).
(21) J. R. Cavanaugh and B. P. Dailey, *J. Chem. Phys.*, **34**, 1099 (1961).

The average of the calculated values for the seven histidine derivatives of Table I which show no rotational orientation is 6.71 Hz with an average difference of 0.17 Hz. The seven observed values average to 6.68 Hz. The calculated J_{av} values for the oriented derivatives are significantly higher (0.2–0.9 Hz) than the observed $^1/_2(J_{\alpha\beta} + J_{\alpha\beta'})$ constants.

The electronegativity of the substituents increases as the substituents are protonated so that some decrease in the average coupling constants is expected. The calculated J_{av} values for histidine anion and its dipositive ion are 7.3 and 6.5 Hz, respectively, indicating that a decrease of less than 1 Hz is produced on protonation. The small electronegativity effect in amino acids has been suggested by a number of observations. Both ^{13}C and proton chemical shift data for the α position[6,11] indicate that there is little charge density variation on ionization of the amino acid. This may result from the fact that substitution of a number of electronegative groups on an HCCH fragment appears to attenuate the electronegativity effect on proton–proton splitting.[22]

In the individual rotamer, the magnitude of the substituent effect on the J_G and J_T values is dependent on the orientation of the coupled protons. It has been reported that the influence of a substituent on a gauche coupling constant is significant only if the substituent is trans and coplanar to one of the coupled protons.[23] This was described more quantitatively in Pachler's calculations[24] of coupling constant variations with rotation of the carbon–carbon bond in fluoroethane. The gauche and trans vicinal coupling constants J_{Gt} (F trans) and J_{Gg} (F gauche) differ from their ethane counterparts by -1.0 Hz and $+0.6$ Hz, respectively. The calculated fluoroethane J_{Tg} differs from ethane by -1.4 Hz. If it is assumed that these substituent electronegativity to coupling constant ratios are linear as in eq 9, then the electronegativity effect on an averaged coupling constant may be calculated using the generalized Karplus equation, eq 13, where $\Delta\chi$ is the

$$J_{av} = J_{av}{}^u(1 + D\sum\Delta\chi) = (A^u + B^u \cos^2 \phi)(1 + D\sum\Delta\chi) \qquad D = -0.09 \tag{13}$$

calculated electronegativity difference shown in Table II. The gauche and trans coupling constants may be

Table II. Substituent Electronegativity Values

Substituent	Group electronegativity	$\Delta\chi$
H	2.15[a]	0
NH_2	2.91[a]	0.76
$NH_3{}^+$	3.66[b]	1.51
COOH	2.60[a]	0.45
COO^-	2.48[b]	0.33
Im	2.55[b]	0.40
ImH^+	2.73[c]	0.58

[a] From J. R. Cavanaugh and B. P. Dailey, *J. Chem. Phys.*, **34**, 1099 (1961). [b] Calculated from the charge density values of Del Re, *et al.*, ref 7, using eq 9 and 12. [c] Estimated by authors.

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(23) (a) H. Booth, *Tetrahedron Lett.*, 411 (1965); (b) R. J. Abraham, L. Cavalli, and K. G. R. Pachler, *Mol. Phys.*, **11**, 471 (1966).
(24) K. G. R. Pachler, *Tetrahedron Lett.*, 1955 (1970).

Table III. Calculated Coupling Constants and Rotamer Populations of Histidines

Compd no.	Species in solution			Obsd[a] vicinal coupling, Hz		Calcd[b] J_{av}, Hz	Series 1[c] $J_{\alpha\beta'} > J_{\alpha\beta}$			Series 2[d] $J_{\alpha\beta} > J_{\alpha\beta'}$		
				$J_{low\ field}$	$J_{high\ field}$	J_{av}		n_I	n_{II}	n_{III}	n_I	n_{II}	n_{III}
1	NH_3^+	ImH^+	CO_2H			6.4	6.5	0.35	0.35	0.30	0.35	0.35	0.30
2	AcNH	ImH^+	CO_2H	5.4	7.8	7.0	7.0	0.43	0.24	0.33	0.24	0.43	0.33
3	NH_3^+	$BzImH^+$	CO_2H			6.7	6.5	0.37	0.36	0.27	0.36	0.37	0.27
4	BocNH	$BzImH^+$	CO_2H			6.6	7.0	0.32	0.32	0.36	0.32	0.32	0.36
5	NH_3^+	ImH^+	CO_2CH_3			6.7	6.5	0.37	0.36	0.27	0.36	0.37	0.27
6	NH_2	Im	CO_2CH_3			7.2	7.2	0.36	0.36	0.28	0.36	0.36	0.28
7	NH_3^+	ImH^+	CO_2^-			6.7	6.6	0.37	0.37	0.26	0.33	0.36	0.31
8	NH_3^+	$BzImH^+$	CO_2^-	4.7	8.1	6.4	6.6	0.49	0.21	0.30	0.18	0.45	0.37
9	NH_3^+	Im	CO_2^-			6.5	6.7	0.35	0.32	0.33	0.32	0.32	0.36
10	BocNH	$BzImH^+$	CO_2^-	3.9	9.1	6.5	7.1	0.51	0.12	0.37	0.09	0.50	0.41
11	AcNH	ImH^+	CO_2^-	4.4	9.0	6.7	7.1	0.50	0.15	0.35	0.13	0.49	0.38
12	NH_2	Im	CO_2^-	4.7	8.4	6.6	7.3	0.44	0.17	0.39	0.14	0.43	0.43
13	BocNH	BzIm	CO_2^-	2.8	10.0	6.4	7.3	0.56	0.04	0.40	0.00	0.55	0.45
14	NH_2	BzIm	CO_2^-	4.3	9.1	6.7	7.3	0.51	0.15	0.34	0.11	0.48	0.41
15	AcNH	Im	CO_2^-	4.1	9.7	6.9	7.3	0.54	0.13	0.33	0.09	0.53	0.38

[a] In D_2O. [b] Calculated using eq 12 or 13. [c] n_I, n_{II}, and n_{III} calculated from eq 16 and 17 with $\phi_{\alpha\beta}^{I} = 295°$, $\phi_{\alpha\beta}^{II} = 185°$, $\phi_{\alpha\beta}^{III} = 60°$, $\phi_{\alpha\beta'}^{I} = 175°$, $\phi_{\alpha\beta'}^{II} = 65°$, $\phi_{\alpha\beta'}^{III} = 300°$. [d] n_I, n_{II}, and n_{III} calculated from eq 16 and 17 with $\phi_{\alpha\beta}^{I} = 300°$, $\phi_{\alpha\beta}^{II} = 180°$, $\phi_{\alpha\beta}^{III} = 60°$, $\phi_{\alpha\beta'}^{I} = 180°$, $\phi_{\alpha\beta'}^{II} = 60°$, $\phi_{\alpha\beta'}^{III} = 300°$ for compounds with anionic carboxyl functions.

Figure 3. Newman projections showing gauche coupling where the substituents are oriented gauche (χ_i) and trans (χ_j) to the coupled protons and trans coupling where the substituents must be oriented gauche (χ_k) to the coupled protons.

calculated from eq 14 and 15. The equations of Pachler[24] were used to calculate the parameters a, b, and c which weigh the effect of the electronegativity difference of substituents gauche to a gauche HCCH unit ($\Delta\chi_i$), trans to a gauche HCCH unit ($\Delta\chi_j$), and gauche to a trans HCCH unit ($\Delta\chi_k$), respectively (Figure 3). The generalized eq 13 is in fair agreement with that calculated by Karplus,[17] where the value of $D = {}^1/_3(a + b + c)$ was given as -0.07.

$$J_G = J_G^u(1 + a\sum_i \Delta\chi_i + b\sum_j \Delta\chi_j) \quad a = 0.13 \quad b = -0.35 \quad (14)$$

$$J_T = J_T^u(1 + c\sum_k \Delta\chi_k) \quad c = -0.06 \quad (15)$$

The equations presented thus far may be combined to give eq 16 and 17 for $\alpha\beta$ and $\alpha\beta'$ vicinal coupling

$$J_{\alpha\beta} = n_I(1 + a\Delta\chi_i^I + b\Delta\chi_j^I)(B_1^u \cos^2\phi_{\alpha\beta}^I) + n_{II}(1 + c\Delta\chi_k^{II})(B_2^u \cos^2\phi_{\alpha\beta}^{II}) + n_{III}(1 + a\Delta\chi_i^{III} + b\Delta\chi_j^{III})(B_1^u \cos^2\phi_{\alpha\beta}^{III}) \quad (16)$$

$$J_{\alpha\beta'} = n_I(1 + c\Delta\chi_k^{I'})(B_2^u \cos^2\phi_{\alpha\beta'}^{I'}) + n_{II}(1 + a\Delta\chi_i^{II'} + b\Delta\chi_j^{II'})(B_1^u \cos^2\phi_{\alpha\beta'}^{II'}) + n_{III}(1 + a\Delta\chi_i^{III'} + b\Delta\chi_j^{III'})(B_1^u \cos^2\phi_{\alpha\beta'}^{III'}) \quad (17)$$

constants which contain terms for the dihedral angle, substituent electronegativity, and substituent orientation. The term $\Delta\chi_i^I$ refers to those substituents gauche to the gauche vicinal α and β protons and $\Delta\chi_i^{I'}$ to those gauche to the gauche vicinal α and β' protons in rotamer I. The other $\Delta\chi$ terms are defined analogously. The B_1^u and B_2^u symbols are the B_1 and B_2 values of eq 8 not corrected for electronegativity effects; $B_1^u = 13.2$ Hz and $B_2^u = 17.4$ Hz.

The dihedral angle may be approximated from assumptions about the factors influencing rotamer orientations. Nonbonded repulsion between bulky groups appears to increase the dihedral angle by about 5°;[16,25] electrostatic attractions are assumed to decrease the angle by the same amount. The calculation of the relative rotamer populations is most sensitive to changes in the dihedral angle ϕ, and less dependent on the estimated values of B_1^u, B_2^u, and electronegativity terms. A three-degree variation in the dihedral angle ϕ^I produces a change up to 1% in the relative rotamer populations. The same population change would be calculated from a 2.3 Hz (20%) change in B_1^u, or a 20% change in the substituent electronegativity factor $(1 + a\Delta\chi_i^I + b\Delta\chi_j^I)$ in eq 16.

The relative rotamer populations n_I, n_{II}, and n_{III} were calculated from eq 16 and 17 as shown in Table III. The determination of the predominant histidine rotamer depends on the assignment of the high and low field β-methylene hydrogens to the β and β' protons of Figure 2. Assignment of the high field methylene proton which has the larger vicinal coupling to the β' proton results in n_I having the larger relative population. Reversing this assignment gives a predominant n_{II} rotamer. In previous discussions of histidine rotamer isomerization, the former assignment has been made on the basis that the carboxyl function has a larger steric requirement than the amino group.[3] It follows that the rotamer with the carboxyl group trans to the imidazole ring would predominate. The rotamer populations of series 1, Table III, were made using this assignment with ϕ angles estimated from probable nonbonded interactions.

The rotamer populations of series 2, Table III, were based on the assignment of the larger vicinal coupling to the $\alpha\beta$ protons. The resulting predominant rotamer, n_{II}, has the amino group trans and the carboxyl function gauche to the imidazole ring for compounds that show rotational isomerism. This assignment is rationalized on the basis of an electrostatic

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attraction between the polar imidazole ring and the carboxyl anion when these are in a gauche orientation.

Several approaches have been made to distinguish between these two assignments. Assumptions have been made concerning the steric requirements of the substituents,[4,5a] and analogies between the assignments of histidine and malic acid have been made[3b] and questioned.[8] The ambiguity of these arguments has been acknowledged.[3b] In general, these arguments have urged the assignments used in the series 1 calculations. The approach utilized here is to examine a sufficiently diverse series of derivatives so that one of the assignments is rendered untenable.

The $\phi_{\alpha\beta}$ and $\phi_{\alpha\beta'}$ values for the series 1 calculations were estimated by assuming that steric and ion–ion repulsions were the dominant influences on rotational isomerism. Exclusive consideration of steric interactions would give the relative rotamer populations $n_I > n_{II} > n_{III}$ as the steric requirement of the carboxyl group is greater than the amine. The n_{III} rotamer, with both of these substituents gauche to the imidazole, would be least favored. However, it is difficult to rationalize the calculated rotamer population for the extended series on the basis of these assumptions. For example, the ester derivatives **5** and **6** appear to be sterically similar to the histidine ions **1** and **7**; if so, the ester **6** should be similar to **12**, but the relative rotamer populations of **12** differ markedly. Phenylalanine has steric requirements which are similar to those of histidine. In contrast to histidine, however, little difference in rotamer population is observed on conversion of the dipolar ion to the anion.[26]

The significantly increased steric factors present in *tert*-butyloxycarbonyl-*im*-benzylhistidine should decrease the population of the most sterically hindered n_{III} rotamer. The calculated n_{III} population of 0.40 for the anion **13** does not agree with this estimate. In general, the n_{III} populations for compounds in series 1 which show rotational isomerism tend to be significantly greater than the n_{II} (trans amine–imidazole) rotamers although they are sterically more hindered. This indicates that the system is relatively insensitive to steric effects and that some other cause must be provided for the conformational stability of the n_{III} conformation.

Electrostatic repulsion also appears to be a minor factor in determining residence times of histidines. Compounds **1**, **3**, and **5** are dipositive ions and should favor a conformation where the ammonium ion and the protonated imidazole are separated. None of these compounds show rotational stability. This lack of preferred conformations is in contrast to the cysteine dinegative ion which has $\delta\nu_{\beta\beta'} = 22.3$ Hz in aqueous solutions.[3b] Here both steric and electronic factors are complimentary.

The common features of the structures in Table III suggest that an attractive interaction between the carboxylate anion and the imidazole ring may be the primary determining factor in rotamer stability. The $\phi_{\alpha\beta}$ and $\phi_{\alpha\delta'}$ values of series 2 calculations were made using this assumption. It follows from this assumption that the favored conformer would have the carboxyl group and the imidazole ring in closest proximity consistent with the minimum nonbonded interactions so that the relative rotamer populations should be $n_{II} > n_{III} > n_I$. This order of population is obtained in series 2 by assigning the upfield resonance to the β hydrogen and, consequently, that the $J_{\alpha\beta}$ coupling constant be greater than $J_{\alpha\beta'}$.

Ion–ion interactions are possible in acylated amino acid zwitterions **10** and **11**, as well as ion–dipole interactions in anions **12–15**. All of these compounds show a high population of the n_{II} isomer and a low population of the n_I isomer. The lack of high rotamer stability of the zwitterion derivatives **7**, **8**, and **9** indicates that the interaction between the anion and the imidazole ring may be superseded by the attractive interaction between the carboxylate and ammonium ions. The carboxylate anion may interact with either the ammonium ion or the protonated imidazole ring in compounds **7** and **8**. The spectrum of **7** shows some distortion of the A_2X pattern but was not sufficiently resolved to be analyzed. Compound **8** shows a preference for the n_{II} conformer. The difference may be attributed to an increased steric bulk of the *im*-benzylimidazole ion in **8**.

Intramolecular hydrogen bonds in aqueous solutions have been observed in a number of systems. Changes in molecular conformations of allohydroxyproline anion[27] and serine[28] have been explained in this way. The preferred conformation of serine has the β-hydroxyl group gauche to both the amino and carboxyl functions as indicated by pmr[28] and X-ray diffraction studies.[29] Other systems which show intramolecular hydrogen bonds in aqueous solutions are 5-hydroxy-1,3-dioxanes[30] and 2-methoxyethanol.[31] It appears that water is not very efficient at interrupting intramolecular bonding perhaps because its own solvent–solvent interactions are stronger.[31]

The ion–dipole interaction between the carboxylate anion and the imidazole may be rationalized on the basis of the highly polar nature of the imidazole ring. The dipole moment of 1-methylimidazole in dioxane of 3.8 D[26] is similar to 1-nitropropane, for example, where $\delta = 3.7$ D. The strong bonding interaction of the *im*-benzyl derivatives **13** and **14** indicates that the interaction is not necessarily hydrogen bonding, but may be the different ion–dipole bond between the carboxylate anion and the electron deficient N-alkylated imidazole ring.

Conclusion

The combination of an amine, a carboxylate ion, and an amphoteric imidazole ring in a single molecule introduces a large number of factors which influence the conformational stability of histidine in aqueous solution. Successful analogies between histidine and other amino acids are difficult to make as the relative importance of these factors are not obvious. A number of substituted histidine derivatives have been observed at different pH levels in order to determine the importance of steric, electronic, and hydrogen bonding

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interactions. The relative rotamer populations of these derivatives were calculated using equations containing terms approximating the dihedral angle, substituent electronegativity, and orientation effects on vicinal coupling constants. As a result of this study, it appears that the dominant factor in this system is the ion–ion or ion–dipole interaction between the carboxylate anion and the imidazolium ion or the polar imidazole ring itself. The coupling constant and chemical shift data have been found to be consistent with a preferred conformation for histidine in basic solution where the carboxyl and imidazole functions are in close proximity. In acidic and isoelectric solutions the ions show equally populated conformations.

Conformation of Cyclic Peptides. VII. Cyclic Hexapeptides Containing the D-Phe-L-Pro Sequence[1]

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Abstract: The cyclic peptides *cyclo*-(L-xxx-D-Phe-L-Pro)$_2$, where xxx is Ala, Orn, or His, were prepared and their conformations were studied by proton magnetic resonance and model building. The effects on the peptide proton resonances of solvent variation, addition of a stable nitroxyl, and, in one case, dilution in chloroform show that the peptide proton of the D-Phe residue is exposed to solvent and that of the L-xxx residue is sequestered, although not completely hidden from small hydrogen bond accepting molecules. Rationalizable differences in peptide solvation by the isomeric butyl alcohols were demonstrated. These observations, together with the observed vicinal coupling constants, indicate no major differences among the backbones of the three peptides. The proposed conformation has the L-xxx residues extended between hairpin turns like those formed by the D-Phe-L-Pro sequence of gramicidin S. The D-Phe-L-Pro peptide bond is trans. χ_1 for both the phenylalanine and histidine side chains is near 180°.

Rational design of peptides with structures suited to particular functions requires predictive power for both backbone and side-chain conformation. To obtain experimental data on which to base predictions about side chains, peptides with well-defined backbones are necessary. Cyclic hexapeptides with C_2 symmetry containing the sequence D-xxx-L-yyy, or its enantiomer, seem likely to possess the desired rigidity.

Empirical estimates of conformational energies indicate that the sequence D-xxx-L-yyy (or its enantiomer) is a particularly favorable one for a sharp reversal of peptide chain direction (a hairpin bend). In making the turn, the first residue can lie in the major region of stability (for a D residue) around $\phi = +100°$, $\psi = -100°$, while the second residue lies in the right-handed helix region of stability (for an L residue) near $\phi = -60°$, $\psi = -60°$.[2-4] Two such turns, connected by two more residues, make up a cyclic peptide that can be expected to have a stable backbone conformation. We have investigated this system, using the sequence D-Phe-L-Pro to form the hairpin bend. The use of proline as the second residue introduces a further backbone constraint, in that ϕ for the proline residue is restricted by the ring structure to a narrow range near $-60°$. The sequence D-Phe-L-Pro is considered to form hairpin turns in the gramicidin S structure inferred from proton magnetic resonance data.[5,6]

In this work we describe the conformations, obtained by model building in the light of pmr studies, of the cyclic peptides *cyclo*-(L-Ala-D-Phe-L-Pro)$_2$ (**1**), *cyclo*-(L-His-D-Phe-L-Pro)$_2$ (**2**), and *cyclo*-(L-Orn-D-Phe-L-Pro)$_2$ (**3**). A study of the analog, *cyclo*-(Gly-D-Phe-L-Pro)$_2$, has been completed by Blout, Deber, and Pease.[7]

The high solubility of **1** in organic solvents permitted an inquiry into its self-association in chloroform and an examination of the solvation of its peptide protons by alcohols of varying steric requirements, in addition to studies using the more usual solvents.

Experimental Section

Proton magnetic resonance spectra were obtained using the 250-MHz spectrometer of the NMR Facility for Biomedical Research at Carnegie-Mellon University.[1] Resonances were assigned to amino acid residues in the usual manner, using spin decoupling in frequency sweep operation.

Materials. Except for hexafluoro-2-propanol-d_2, solvents for the pmr work were commercial products used without further purification. The dimethyl-d_6 sulfoxide was the nominal 100.0% material of Diaprep Inc. Hexafluoro-2-propanol-d_2 was prepared by high-pressure deuteration of hexafluoroacetone over Adams

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