Acid-Base and Tautomeric Equilibria in the Solid State: ^{15}N NMR Spectroscopy of Histidine and Imidazole

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Abstract: Lyophilized powders of ^{15}N-enriched histidine and imidazole, prepared from aqueous solutions with pH values ranging between 2 and 12.5, were examined using solid-state NMR techniques. Isotropic and anisotropic chemical shifts for the histidine ring nitrogens were determined for the various ionic forms. The ^{15}N shift anisotropy is strongly influenced by the protonation state of the nitrogen and is found to double upon the deprotonation of the π nitrogen at neutral pH. The "pH" dependence of the isotropic shifts closely parallels that seen in solution except that only one tautomer, the N^{τ}-H, is observed for the neutral and anionic species; this form is the predominant one in solution. Although the isotropic shift values for the various species in the solid do not differ appreciably from those measured in solution, both acid-base and tautomeric exchange processes are slow and each form is separately identified. These solid-state results therefore permit a more precise description of tautomeric structure (and possibly of hydrogen-bond interactions) than can be obtained from solution ^{15}N chemical shift data alone.

Introduction

High-resolution nuclear magnetic resonance spectroscopy has been widely employed to examine structural and dynamic properties of molecules in solution. However, in the past few years the development of dilute spin double resonance techniques[1] together with magic angle sample spinning (MASS)[2,3] has permitted similar high resolution spectra to be obtained for solid samples,[4,5] thereby making NMR one of relatively few techniques applicable to molecules in both liquid and solid phases. Comparative studies may hold particular significance for application to biologically important molecules such as amino acids and proteins, since, at present, most structural information comes from X-ray diffraction studies of crystals, while functional properties are determined almost exclusively in solution. In addition to providing interesting comparisons between isotropic chemical shifts in solutions and in solids, solid-state NMR experiments can supply information on the anisotropy of magnetic resonance interactions. For example, chemical shift and dipolar coupling tensors may be measured in solids, and these data can provide insight into local electronic structure as well as into the detailed nature of dynamical processes.[6-8]

Histidyl residues are often found as integral components of structurally or functionally important sites in proteins.[9,10] As we will see below, both isotropic and anisotropic ^{15}N chemical shifts of histidine are very sensitive to tautomeric structure, hydrogen-bond interactions, and the state of protonation of the imidazole ring system.[11] Moreover, despite inherently poor NMR sensitivity, ^{15}N is a relatively favorable nucleus for solid-state studies of biological molecules because such systems often contain relatively few different nitrogen nuclei and because ^{15}N chemical shifts are spread over a wide range. Problems of resolution are therefore significantly reduced compared to the situation for ^{13}C and ^{1}H NMR. Examination of histidyl residues in proteins by solid-state ^{15}N NMR would appear to be desirable, and, when isotopic labeling is possible,[12] quite feasible.

pH is a critical parameter in solution studies of enzymes, because most enzymes are active only over a relatively narrow range. In addition, pK_a's of enzyme functional groups often bear importantly on their catalytic mechanisms and consequently are of considerable interest. The meaning of these parameters for solid samples is not at all clear. These questions, of course, are relevant to any comparative study of solution and solid states, not just to the study of enzymes. In what follows we examine histidine and imidazole in the solid state and compare the results with those of the corresponding solution studies,[13] paying particular attention to the significance of pH and pK_a for solid-state studies.

Experimental Section

Histidine enriched in ^{15}N to 95 atom % at the π position only and at both the π and τ positions was purchased from Isotope Labelling Corp. and used without additional purification. Imidazole, similarly enriched at both ring nitrogen positions, was purchased from the same company and was recrystallized from benzene before use. Samples for NMR were prepared by lyophilizing aqueous solutions whose pH values were adjusted with HCl and NaOH. Typically 50 mg of powder was used for each spectrum.

Cross-polarization and MASS experiments were performed on a homebuilt spectrometer operating at a field of 6.9T. ^{15}N signals were observed at 29.82 MHz with a proton frequency of 294.2 MHz. ^{15}N and ^{1}H Hartmann-Hahn fields amounted to 100 and 10 G, respectively, and single contact cross-polarization was carried out for 3ms. The intensities of lines due to different chemical species depend on the relative efficiency of cross-polarization. For this reason, intensity measurements of the same signal in different spectra recorded under identical conditions may be safely compared; however, quantitative measurements of concentrations of different species in the same spectrum must be made with caution.

Signals from protonated and nonprotonated ^{15}N atoms were separated using a rotationally synchronized pulse sequence which has been described in detail elsewhere.[14] The essential feature is the incorporation

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(1) A. Pines, M. G. Gibby, and J. S. Waugh *J. Chem. Phys.*, **59**, 569 (1973).
(2) I. J. Lowe, *Phys. Rev. Lett.*, **2**, 285 (1959).
(3) E. R. Andrew, A. Bradbury, and R. G. Eades, *Nature (London)*, **182**, 1659 (1958).
(4) J. Schaefer and E. O. Stejskal, *J. Am. Chem. Soc.*, **98**, 1031 (1976).
(5) E. O. Stejskal, J. Schaefer, and R. A. McKay, *J. Magn. Reson.*, **25**, 569.
(6) M. Mehring, "NMR Basic Principles and Progress", Vol. 11, Springer Verlag, New York, 1976.
(7) U. Haeberlen, "High Resolution NMR in Solids", Academic Press, New York, 1976.
(8) H. W. Spiess in "NMR Basic Principles and Progress", Vol. 15, Springer Verlag, New York, 1978.
(9) J. L. Markley, *Acc. Chem. Res.*, *8*, 70 (1975).
(10) E. Reich, D. B. Rifkin, and E. Shaw, "Proteases and Biological Control", Cold Spring Harbor Conferences on Cell Proliferation, Vol. 2, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., 1975, pp 1-64.
(11) M. Witanowski, L. Stefaniak, H. Januszewski, Z. Grabowski, and G. A. Webb, *Tetrahedron*, 637 (1972).
(12) W. W. Bachovchin and J. D. Roberts, *J. Am. Chem. Soc.*, **100**, 8041 (1978).
(13) F. Blomberg, W. Maurer, and H. Rüterjans, *J. Am. Chem. Soc.*, **99**, 8149 (1977).

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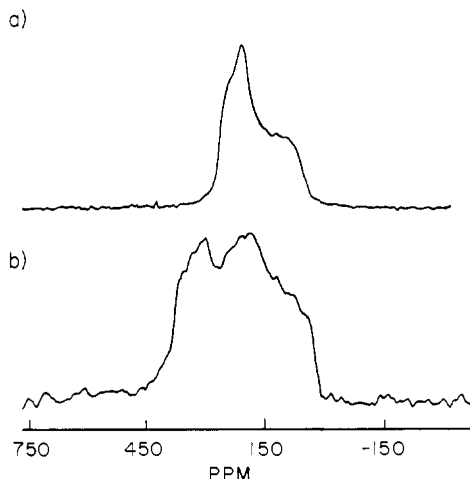


Figure 1. ^{15}N NMR spectra of histidine spectra isotopically labeled at the N$^{\pi}$ position. Powder samples were prepared from aqueous solutions at (a) pH 2 and (b) pH 7. The spectrum in (b) suggests the presence of more than one chemically distinct species.

of a variable length delay without proton decoupling prior to acquisition. This procedure allows the system to develop under the influence of the heteronuclear dipolar Hamiltonian before the FID is sampled. For a dilute ^{15}N–^{1}H spin pair, the magnetization undergoes damped dipolar oscillations during the coupled period, the extent of the evolution being transmitted to the first point of the FID in the form of a phase modulation. The length of the delay period may be set to null the signal from the protonated species.[14,15] Sampling is constrained to begin at the peak of a rotational echo so that no net phase angle due to chemical shift anisotropy is accumulated before the acquisition begins. In addition, 180° refocusing pulses are employed to remove phase shifts due to resonance offsets and isotropic chemical shifts.

Chemical shift tensors were calculated by numerical methods from the sideband intensities in MASS spectra, as previously described.[16] Spectra were obtained at slow spinning speeds to increase accuracy. The rotors used were Andrew–Beam models constructed of Delrin and were operated at rotation rates ranging from 1.5 to 4.0 kHz.[17] The tensors may be expressed in terms of an isotropic shift σ, an anisotropy δ, and an asymmetry parameter η. These quantities are obtained from the diagonal elements σ_{xx}, σ_{yy}, and σ_{zz} by taking $\sigma = (\sigma_{xx} + \sigma_{yy} + \sigma_{zz})/3$, $\delta = \sigma_{zz} - \sigma$, and $\eta = (\sigma_{yy} - \sigma_{xx})/\delta$. The Cartesian principal values are understood to be arranged so that $|\sigma_{zz} - \sigma| \geq |\sigma_{xx} - \sigma| \geq |\sigma_{yy} - \sigma|$.[7] Isotropic chemical shifts were measured in ppm relative to external powdered $(^{15}NH_4)_2SO_4$.

Results

^{15}N NMR Spectra of L-[^{15}N$^{\pi}$]Histidine. Figure 1a shows a proton-decoupled powder spectrum of L-histidine labeled specifically at the π position and lyophilized from a solution at pH 2.0. A MASS spectrum of a similar sample lyophilized from a solution of pH 5.5 (Figure 2) demonstrates that the spectrum of Figure 1a is due to a single chemical species. The principal values of the shift tensor may be obtained directly from the powder pattern to an accuracy of about ±3 ppm, and are in good agreement with those obtained from MASS (Table I) and with the results of a recent single-crystal study of natural abundance His·HCl·H_2O.[18]

Figure 1b illustrates the striking changes that occur in the spectrum when the pH of the solution from which the powder is prepared is raised to 7.0. The line shape reflects the presence of at least two overlapping powder patterns, indicating the presence of at least two distinct species. Figure 2 shows a series of MASS spectra taken of samples prepared from solutions with pH values ranging from 5.5 to 8.4. Relatively fast rotation rates of 3.5–4.0 kHz were chosen in order to minimize sideband intensities. These spectra demonstrate that the powder spectrum of Figure 1b is due

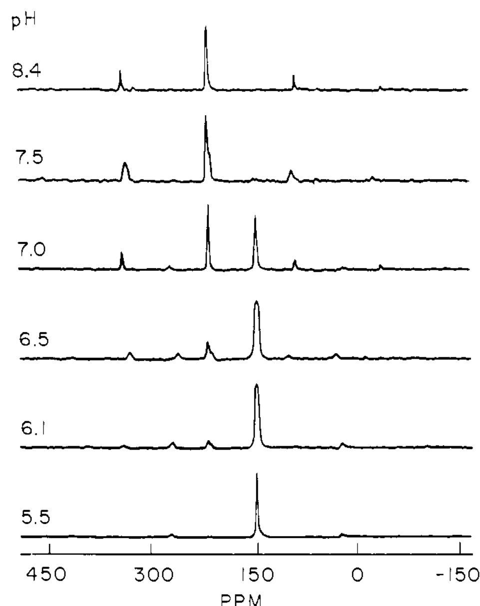


Figure 2. A series of MASS spectra obtained from solid samples lyophilized from solution with pH values ranging between 5.5 and 8.4. The spectra provide information on the isotropic chemical shifts of the cationic and neutral forms, and on the changing composition of the mixture. Intensities of the peaks are not to scale. Deviations from the magic angle are responsible for some additional line broadening evident in the spectra.

to only two species and that the relative amount of each species present in the powder depends on the pH of the solution from which it was prepared. At low pH the form with an isotropic shift of 155 ppm predominates. As the pH is increased this species disappears progressively and is replaced by a new one with an isotropic shift of 222 ppm. The midpoint of this transition occurs at pH 6.3 ± 0.2, after correction for different cross-polarization behavior is made.

The spectra of Figures 1 and 2 also show that the chemical shift tensors of these two forms are quite different. This is easily recognized by noting the presence of only two weak rotational sidebands in the pH 5.5 spectrum, while at pH 8.4 the first upper and lower sidebands are noticeably more intense and a second upper sideband is also observable. At pH 5.5 the π tensor is about 210 ppm in breadth with an asymmetry of 0.45. At pH 8.4 the breadth of the π tensor increases to 380 ppm with each element approximately doubling in size.

Increases in the pH beyond 8.4 result in the appearance of a third distinct form, which is observed alone in MASS spectra at pH 12.3. The midpoint of this transition occurs near pH 9.5. Although the isotropic shift of this species differs by only 6 ppm, the σ_{xx} and σ_{yy} principal values are different from those of the form which predominates at pH 8.4. As a result, the tensor is more nearly axially symmetric. Note that the σ_{zz}' element, which is probably roughly perpendicular to the plane of the ring, remains unchanged from its value at pH 8.4.

The principal values of the tensors for all three forms observed are compared in Table I, where they are identified with cationic, neutral, and anionic species (vide infra).

^{15}N NMR Spectra of DL-[^{15}N$^{\pi}$,^{15}N$^{\tau}$]Histidine. Changes in the spectrum of the τ nitrogen as a function of pH were observed by studying histidine enriched with ^{15}N at both nitrogens of the imidazole ring. Assignments were made by comparison with spectra of singly labeled histidine. A representative spectrum is shown in Figure 3a. In contrast to the behavior of the π nitrogen which undergoes a 67-ppm downfield shift with increasing pH, the τ nitrogen undergoes only a small upfield shift from 152 ppm at pH 5.5 to 147 ppm at pH 8.4. No further change in isotropic shift is observed for higher pH values (Table I).

Suppression of Signals from Protonated Nitrogens. Although there is abundant reason to assign the low-field signal in the above spectra (e.g., Figure 2, pH 7.0) to a nonprotonated nitrogen, this identity can be demonstrated conclusively with the rotationally

(14) M. G. Munowitz, R. G. Griffin, G. Bodenhausen, and T. H. Huang, *J. Am. Chem. Soc.*, **103**, 2529 (1981).
(15) S. J. Opella and M. H. Frey, *J. Am. Chem. Soc.*, **101** 5854 (1979).
(16) J. Herzfeld and A. E. Berger, *J. Chem. Phys.*, **73**, 6021 (1980).
(17) E. R. Andrew, *Prog. NMR Spectrosc.*, **8**, 1 (1971).
(18) G. Harbison, J. Herzfeld, and R. G. Griffin, *J. Am. Chem. Soc.*, **103**, 4752 (1981).

Table I. ^{15}N Chemical Shift Parameters for the Ring Nitrogens of Histidine in Various Ionic States

		solution	powder				
		σ(ppm)^a	σ(ppm)^b	σ_{xx}'(ppm)^c	σ_{yy}'(ppm)^c	σ_{zz}'(ppm)^c	η
π	cation	155	155 ± 1	88	33	−121	0.45
	neutral	211	222	160	60	−220	0.45
	anion	197	228	138 ± 10	82 ± 10	−220	0.25 ± 0.1
τ	cation	153	152	89	21	−110	0.62
	neutral	158	147	88	18	−106	0.66
	anion	173	147	97	15	−112	0.73

^{a} Relative to 4 M $^{15}NH_4NO_3$ in 2 M HNO_3 (from ref 13). ^{b} Relative to $(^{15}NH_4)_2SO_4$ powder. ^{c} Traceless tensor elements (indicated by primes), obtained by numerical analysis of the sideband intensities in MASS spectra (according to ref 16). Uncertainties ±5 ppm except where indicated.

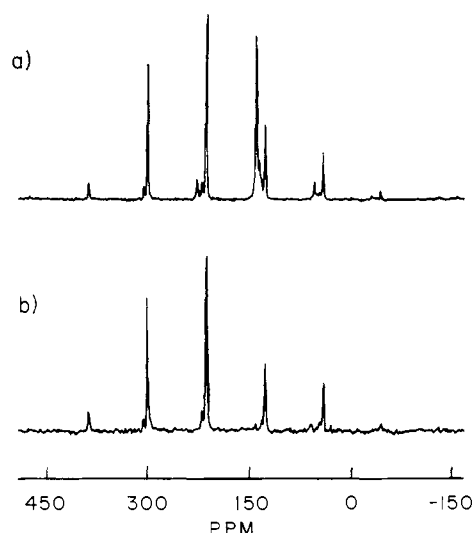


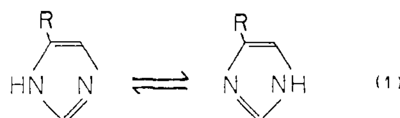
Figure 3. MASS spectra of histidine isotopically labeled at both the π and τ positions, and lyophilized from a pH 9 solution. (a) Normal spectrum. (b) Spectrum obtained with a rotationally synchronized pulse sequence allowing for heteronuclear dipolar coupling prior to acquisition. The lines which are missing in the latter spectrum arise from protonated ^{15}N atoms.

synchronized pulse sequence described above. The result of employing such a sequence on the doubly ^{15}N-labeled sample of histidine is shown in Figure 3b. In this case, an interval of 230 μs without proton decoupling was inserted prior to a detection period initiated after exactly one rotational period. Comparison of this spectrum with the one in Figure 3a shows that the centerband near 150 ppm and its associated sidebands are due to the protonated τ nitrogen. The lines remaining in the spectrum are identical with those obtained from the singly labeled material and are due to the nonprotonated imidazole ring nitrogen.

^{15}N NMR Spectra of Imidazole. The MASS spectrum of polycrystalline free base imidazole, doubly labeled with ^{15}N, was recorded. Two signals with isotropic shifts of 150 and 222 ppm were observed.

Discussion

Imidazoles characteristically exist as a mixture of tautomeric forms (eq 1) with the equilibrium constant, which is equal to 1



for imidazole itself, depending on the nature of R.[19] Because both nitrogens in imidazole are chemically equivalent, and because in aqueous solutions the tautomeric exchange is very rapid, imidazole in solution at high pH exhibits only a single ^{15}N NMR

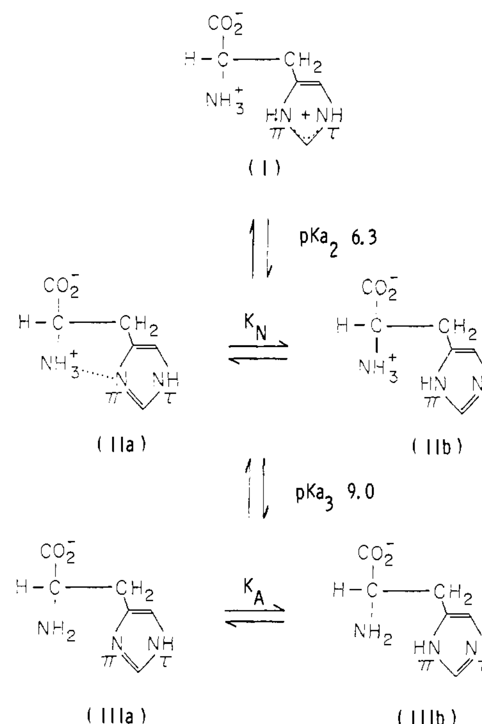


Figure 4. Possible ionic and tautomeric forms of histidine from pH 2 to 12.5.

signal with a chemical shift of 186 ppm.[12] Nevertheless, the ^{15}N chemical shifts of the protonated and nonprotonated nitrogens should be quite different, and in MASS spectra of polycrystalline free base imidazole we do observe two ^{15}N resonances separated by 72 ppm. Thus, the tautomeric exchange of eq 1 is slow or nonexistent in the solid. We assign the signal with an isotropic shift of 150 ppm to the >NH nitrogen of imidazole and the signal at 222 ppm to the ≥N nitrogen. The average of these isotropic shifts is equal to the shift of the single resonance observed for imidazole in solution. This is the expected result if both nitrogens have the same isotropic shift in solution as in the solid. As the pH of the solution is decreased, the ^{15}N NMR signal shifts progressively upfield from 186 to 155 ppm, delineating a smooth pH curve and demonstrating that the ionic equilibrium, like the tautomeric equilibrium, is fast on the NMR time scale for imidazole in solution.

By contrast, the ^{15}N NMR pH dependence for histidine in solution is considerably more complex. This is due to the inequivalence of the histidyl ring nitrogens and to the presence of an additional ionization (i.e., of the α-amino group) which strongly influences the tautomeric equilibrium. There are, therefore, five distinct species of histidine which must be considered in interpreting the solution and solid-state ^{15}N NMR data. These are the one cationic, two neutral, and two anionic forms, shown in Figure 4 and labeled I, IIa, IIb, IIIa, and IIIb, respectively.

In solutions at low pH only I is present and the π and τ nitrogens of this cationic species give rise to high-field signals separated by

(19) J. Elguero, C. Marzin, A. Katritzky, and P. Linda, "The Tautomerism of Heterocycles", Academic Press, New York, 1976.

2.4 ppm (Table I). Ionization of the carboxylate group with a pK_a of 1.8 has no discernible effect on the ^{15}N chemical shifts. As the pH is increased, the imidazole ring deprotonates first and the neutral forms IIa and IIb increase progressively relative to I. However, because the three forms are in rapid exchange, the ^{15}N NMR spectrum yields a chemical shift which represents a weighted average of the shifts of each species present.

Under conditions in which the imidazole ring is completely deprotonated (pH 8.0), both nitrogen resonances are found downfield of their positions from the cationic species. The π signal shifts considerably further downfield (56 ppm) than that of the τ (5 ppm), indicating that the deprotonation occurs largely at the π position. Nevertheless, analyses of coupling constants and of ^{13}C chemical shifts have demonstrated that significant concentrations of both species IIa and IIb are present so that the ^{15}N chemical shifts are averaged values. The mole fraction of species IIa was estimated to be 0.88 by both of the above methods. It should be noted that the ^{15}N chemical shifts could not be used to evaluate this equilibrium because of uncertainty in the limiting values of the shifts for the pure tautomers.

The reason that species IIa predominates to such an extent over IIb in neutral solutions is due, at least in part, to the stabilizing effect of the hydrogen-bond interaction possible for IIa, but not for IIb (Figure 4). The effect of this hydrogen bond on the equilibrium constant K_N is most readily discerned by the changes in the ^{15}N shifts upon ionization of the amino group at high pH. This ionization eliminates the possibility for the interaction and the π resonance is observed to shift 15 ppm upfield while the τ resonance shifts an equal amount downfield, reflecting a redistribution of tautomeric forms such that species IIIa is not so strongly favored over IIIb as IIa is over IIb. However, the mole fraction of IIIa was calculated to be 0.76, indicating that the N^{τ}–H tautomer still predominates even in the absence of the hydrogen bond.

Examination of histidine in solid samples prepared from solutions with pH values between 2.0 and 12.5 reveals both similarities to and differences from the solution studies. In the cationic form at "low pH" in the solid, the isotropic shifts of both nitrogens are very close to the corresponding chemical shifts in solution (Table I). This fact is interesting and may have significance for the interpretation of solution data.

As the "pH" of the solid is increased, increasing amounts of the neutral form of histidine can be detected. In the solid, however, the exchange between cationic and neutral forms is slow, and, consequently, separate resonances are observed for each species. A second important difference in the solid is that only a single tautomer corresponding to species IIa is observed. Thus the π resonance undergoes a larger downfield shift (67 vs. 56 ppm) upon ionization of the imidazole ring than in solution, while the τ signal undergoes a small upfield shift (5 ppm) in contrast to the small downfield shift observed in solution (Table I). This upfield shift of the τ signal is in accord with expectations for transition of species I to species IIa. The possibility that this single pair of signals might represent a weighted average of both tautomers can be ruled out because of the observation of two signals for imidazole. Species IIa then appears to be even more highly favored over IIb in the solid than in solution.

Further increases in the "pH" of the solid result in a small downfield shift of the π signal but no change in that of the τ signal. The midpoint of this transition occurs near pH 9.5 and is due to deprotonation of the α-amino group. This result demonstrates that the hydrogen bond between the protonated α-amino group and π nitrogen is present in the solid as in solution. However, unlike the situation in solution, removal of this hydrogen bond does not bring about a redistribution of tautomeric forms, as evidenced by the absence of any new signals in the high "pH" spectra. The lack of change in the position of the τ signal rules out the possibility that fast exchange may be responsible for the absence of new signals corresponding to species IIIb. Apparently, in the solid, the N^{τ}–H tautomer is overwhelmingly favored even without the stabilization provided by the hydrogen bond. The 6-ppm difference in the isotropic shift of the π nitrogen between neutral and anionic forms can be assigned directly to the effect of the hydrogen bond. However, this nonbonded interaction exerts a greater effect on the principal values of the shift tensor since these are somewhat different in the anionic as compared to the neutral form (Table I). The existence and behavior of the hydrogen bond in solid samples are also supported by X-ray and neutron crystallographic studies. For example, IIa is analogous to the zwitterion observed in crystals obtained from neutral solution, whereas molecules similar to IIIa have been detected in metal complexes of histidine.[20–24]

The midpoint for the transition between cationic and neutral forms of histidine in the solid occurs at a "pH" of 6.3, while that of the transition between neutral and anionic forms is near a "pH" of 9.5. These correspond very closely to the measured pK_a's in solution. Thus, pH and pK_a appear to have meaning for solid-state studies, at least in the sense that the distribution of titrating forms is not significantly altered between the solid and the solution from which it was prepared.

The ability to apply ^{15}N chemical shift data to a quantitative analysis of tautomeric structure and hydrogen-bond interactions of histidyl residues in proteins is very desirable for a number of reasons. However, such application requires knowledge of the limiting values of the chemical shifts of each nitrogen in each of the pure tautomers. The unavailability of these data from solution investigations has until now limited the usefulness of ^{15}N chemical shift data. For example, in the ^{15}N solution study of histidine, the tautomeric equilibrium was quantitatively evaluated using ^1J ^{13}C–^{15}N coupling constants rather than ^{15}N chemical shifts. This situation can now be remedied since slow exchange in the solid allows these extreme values to be measured directly. The requirement that the isotropic shifts do not differ significantly between solid and solution states seems fulfilled in view of the good agreement noted above. A more important drawback is the fact that we have so far observed only one of the tautomeric forms of histidine in the solid. However, if we assume that the averaged shift seen in solution is close to the shift that would be observed in the solid under conditions of fast exchange, then we can estimate that 0.86 ± 0.02 mol fraction of histidine exists as the N^{τ}–H tautomer at pH 8.0. This assumption is reasonable in view of the similarity between solution and solid isotropic shift for the protonated form. The result is in good agreement with the value of 0.88 calculated from the coupling constants and from the ^{13}C chemical shifts.[25] At high pH in solution we calculate that the anionic form of histidine is 0.66 ± 0.02 mol fraction N^{τ}–H tautomer. This does not agree as well with the value of 0.76 calculated in the ^{15}N solution study; however, this latter calculation very likely overestimates the difference in ^{15}N shifts between tautomers. Our value does agree well with a value of 0.70 calculated from ^{13}C NMR shifts for a number of histidine derivatives in which the α-amino function is blocked.[25]

Conclusions

The rates of both intermolecular and intramolecular proton exchange processes in histidine are slower in the solid than in solution, resulting in separate NMR signals for the various ionic and tautomeric states of the molecule. This allows chemical shift anisotropies to be determined for the different forms of the compound. The N^{π} tensor is extremely sensitive to the ionic state, nearly doubling in size in response to the deprotonation of the ring which occurs near neutral pH. The subsequent deprotonation of the amino group at high pH, accompanied by the loss of a hydrogen bond between the amino nitrogen and the π nitrogen, does

(20) M. S. Lehmann, T. F. Koetzle, and W. C. Hamilton, *Int. J. Peptide Protein Res.*, **4** 229 (1972).
(21) H. Fuess and H. Bartunik, *Acta Crystallogr., Sect. B* **32**, 2803 (1976).
(22) K. Oda and H. Koyama, *Acta Crystallogr., Sect. B* **28**, 639 (1972).
(23) H. Fuess, D. Hohlwein, and S. A. Mason, *Acta Crystallogr., Sect. B* **33**, 654 (1977).
(24) R. Candlin and M. M. Harding, *J. Chem. Soc. A*, 421 (1967).
(25) W. F. Reynolds, I. R. Peat, M. H. Freedman, and J. R. Lyerla, Jr., *J. Am. Chem. Soc.*, **95**, 328 (1973).

not further affect the anisotropy of the tensor, although it does reduce the asymmetry parameter. The τ tensor is largely unaffected by these events, in accord with a picture of poor communication between the ring nitrogens in the solid. In the solid state, only a single tautomeric species is detected for each of the protonation states, in contrast to the mixture of tautomers found in solution. The predominant tautomer found in solution, however, is the one also observed in the solid state. The isotropic chemical shift values can be used to interpret the solution chemical shifts in terms of the tautomeric equilibria, and the results agree reasonably well with the analysis based on coupling constants in solution. We anticipate that the methods used here will permit a similar analysis to be made of the state of histidine in a large system, such as a protein.

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Registry No. L-Histidine, 71-00-1; DL-histidine, 4998-57-6; imidazole, 288-32-4; L-histidine cation, 70805-60-6; L-histidine anion, 26302-81-8; DL-histidine cation, 80448-36-8; DL-histidine anion, 80448-37-9.

Origins of Inhomogeneous Broadening in the Vibronic Spectra of Visual Chromophores and Visual Pigments

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Abstract: The origins of inhomogeneous broadening in the vibronic spectra of visual chromophores and pigments are analyzed using the INDO-PSDCI molecular orbital theory and the expansion of selected coordinate potential functions in a generalized anharmonic basis set. Our analysis differentiates between two mechanisms of inhomogeneous broadening: "horizontal" and "vertical". Horizontal inhomogeneity is described in terms of a distributed coordinate effect, whereas vertical inhomogeneity is described in terms of a $\pi^* \leftarrow \pi$ vibronic effect. The primary source of inhomogeneous broadening in the ambient temperature electronic absorption spectra of the retinal isomers in solution is horizontal inhomogeneity in the C_6–C_7 torsional coordinate. Our results are therefore in general agreement with the previous literature on this subject. However, our calculations indicate that at 1.8 K, the C_6–C_7 torsional angle of *all-trans*-retinal is restricted to a relatively small range of dihedral angles ($\phi_{6,7} = 52.4 \pm 4°$). The primary source of broadening of the principal absorption bands of the visual chromophores at very low temperatures is shown to be vertical inhomogeneity associated with the C_6–C_7 torsional potential surface. The λ_{max} absorption bands of the pigments, rhodopsin and bacteriorhodopsin, are predicted to be broadened by vertical inhomogeneity associated with one or more barrierless excited state potential surface(s) for double-bond isomerization. In particular, the C_{11}=C_{12} torsional surface in the lowest $\pi\pi^*$ state of the 11-cis chromophore is sufficient unto itself for producing spectral diffuseness in the λ_{max} band of rhodopsin down to 1.8 K. The C_6–C_7 torsional potential surface is predicted to be a minor source of inhomogeneous broadening in rhodopsin and bacteriorhodopsin. We conclude that the mechanism of spectral broadening in the isolated chromophores is distinctly different from that responsible for spectral broadening in the visual and bacterial pigments.

I. Introduction

The low-energy electronic λ_{max} absorption bands of the visual chromophores (Figure 1) and the visual pigments rhodopsin and bacteriohodopsin (Figure 2) are characterized by a total absence of distinct vibronic structure.[2] The λ_{max} absorption bands of the retinal isomers are completely inhomogeneously broadened in low-temperature (77 K) solvent glasses although many of the higher-energy electronic bands display a degree of vibronic development (Figure 1). Furthermore, this inhomogeneous broadening of the retinal λ_{max} band persists at extremely low temperatures (1.8 K) where the Boltzmann population of upper vibrational levels of the ground state is negligible.[3]

Christensen and Kohler were the first investigators to study this phenomenon in detail, and these investigators experimentally demonstrated that torsional freedom around the 6–7 single bond is the essential element in producing spectral diffuseness in retinyl polyenes.[4] A salient experimental demonstration of this effect can be observed in the absorption spectrum of anhydrovitamin A, a molecule with a 6,7 double bond which restricts torsional distortion (Figure 3). Theoretical calculations provide general support for Christensen and Kohler's arguments by predicting that the ground state potential surface of the 6,7 dihedral torsional angle is shallow[5,6] whereas the excited state potential surface is much steeper.[6] Subsequent experimental and theoretical studies have provided additional evidence to support the above interpretation.[7-9] However, a number of important questions remain to be answered. For example, why does the principal absorption band of *all-trans*-retinal remain diffuse at liquid helium temperature?[3] Furthermore, why are the main absorption bands of the visual pigments also diffuse at low temperatures (4–77 K)?[10,11] The above two questions are interesting because they imply a

(1) UCAR graduate student fellow.
(2) Birge, R. R. *Annu. Rev. Biophys. Bioeng.* **1981**, *10*, 315.
(3) The absorption spectrum of *all-trans*-retinal in EPA at 1.8 K (liquid helium) exhibits no enhancement in the vibronic structure of the λ_{max} absorption band over that shown in Figure 1. Auerbach, R. A.; Birge, R. R.; Kohler, B. E.; Sullivan, M. J., unpublished results.
(4) Christensen, R. L.; Kohler, B. E. *Photochem. Photobiol.* **1973**, *18*, 293.
(5) Honig, B.; Hudson, B.; Sykes, B. D.; Karplus, M. *Proc. Natl. Acad. Sci. U.S.A.* **1971**, *68*, 1289.
(6) Warshel, A.; Karplus, M. *J. Am. Chem. Soc.* **1974**, *96*, 5677.
(7) (a) Hemley, R.; Kohler, B. E. *Biophys. J.* **1977**, *20*, 377. (b) Das, P. K.; Becker, R. S. *J. Phys. Chem.* **1978**, *82*, 2081, 2093.
(8) (a) Blatz, P. E.; Dewhurst, P. B.; Balasvbramaniyan, V.; Balasvbramaniyan, P.; Lin, M. *Photochem. Photobiol.* **1970**, *11*, 1. (b) Mao, B.; Govindjee, R.; Ebrey, T. G.; Arnaboldi, M.; Balogh-Nair, V.; Nakanishi, K.; Crouch, R. *Biochemistry* **1981**, *20*, 428.
(9) Honig, B.; Dinur, U.; Birge, R. R.; Ebrey, T. G. *J. Am. Chem. Soc.* **1980**, *102*, 488.
(10) Yoshizawa, T.; Wald, G. *Nature (London)* **1963**, *197*, 1279.
(11) Becher, B.; Tokunaga, F.; Ebrey, T. G. *Biochemistry* **1978**, *17*, 2293.

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