Natural-Abundance Carbon-13 Nuclear Magnetic Resonance Spectroscopy of Proteins. Observation and Uses of Nonprotonated Aromatic Carbon Resonances

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The significant role that NMR can have for studies of proteins in solution depends heavily on the ability to resolve signals from individual atomic sites. The first ^{13}C NMR spectra of proteins, reported in 1970,[1] did not have adequate signal-to-noise ratios for detecting single-carbon resonances. A few years later, with improved instrumentation in the author's laboratory[2,3] it became practical to observe single-carbon resonances in natural-abundance ^{13}C NMR spectra of small proteins.[2,4-10] This account deals with the observation and uses of the numerous resolved single-carbon resonances (of nonprotonated carbons) found in the aromatic regions of proton-decoupled natural-abundance ^{13}C NMR spectra of small native proteins in solution. The coverage is restricted mainly to developments in the author's laboratory in the period 1972–1977. Other approaches for studying proteins by means of ^{13}C NMR have been reported.[11] Carbon-13 relaxation data have been used for studying motions of proteins in solution. The ^{13}C spectra of ligands or covalently attached reporter groups have been widely used. The incorporation of ^{13}C-enriched amino acid residues into an otherwise intact (or nearly intact) protein is being vigorously developed. These important topics have been reviewed recently by Howarth and Lilley.[11] The reader is referred to their article and the references cited therein.

General Features of Proton-Decoupled Natural-Abundance Carbon-13 NMR Spectra of Proteins

A carbon-13 NMR spectrum is normally obtained on a Fourier transform NMR spectrometer, with the use of many repetitive scans which are added together in the memory of a digital computer. Because of the low natural abundance of the ^{13}C isotope, splittings from ^{13}C–^{13}C scalar coupling are usually not observed in ^{13}C NMR spectra of molecules of natural isotopic composition. Therefore, under conditions of proton decoupling, each magnetically nonequivalent carbon normally yields one resonance. However, many spectrometers do not have enough sensitivity for detection of single-carbon resonances of a protein (with the use of a "reasonable" signal accumulation time per spectrum, such as 10 h or less). Unless otherwise indicated, all spectra presented in this account were recorded at 14.2 kG (15.18 MHz) on a "home-built" instrument equipped with a 20-mm probe.[3] Even with the best spectrometers, the observation of single-carbon resonances of a native protein is now practical only for proteins of low molecular weight which are very soluble and available in large quantities (typically a minimum of 0.5 to 2 g).[4-10,12] Furthermore, even in such favorable cases, the difference between success and failure often depends on careful choice of instrument conditions, with consideration of the pertinent relaxation parameters.[5,12,13]

We need to be concerned with three aspects of ^{13}C relaxation, i.e., the natural line width (W), the spin–lattice relaxation time (T_1) and the nuclear Overhauser effect (NOE). Differences in the line widths of resonances from different types of carbons (see below) and the magnetic field dependence of the line widths influence the choice of magnetic field strength for optimum resolution.[5,12,13] The values of T_1 influence the choice of recycle time (interval between successive scans in signal accumulation). The NOE is the redistribution of the populations of the ^{13}C spin energy levels as a result of ^{1}H irradiation (when ^{13}C–^{1}H dipolar relaxation is significant).[14] Here I define the NOE as the ratio of intensities with and without proton decoupling. The maximum value of the NOE for a ^{13}C resonance is 2.988.[14] Clearly, the value of the NOE has an important effect on the signal-to-noise ratio.

In order to compute the values of relaxation parameters, we need to know the relative importance of various relaxation mechanisms.[14] When dealing with ^{13}C NMR spectra of proteins, the significant relaxation mechanisms are ^{13}C–^{1}H dipole–dipole interactions,[5,12] chemical shift anisotropy (CSA),[12] ^{13}C–^{14}N dipole–dipole

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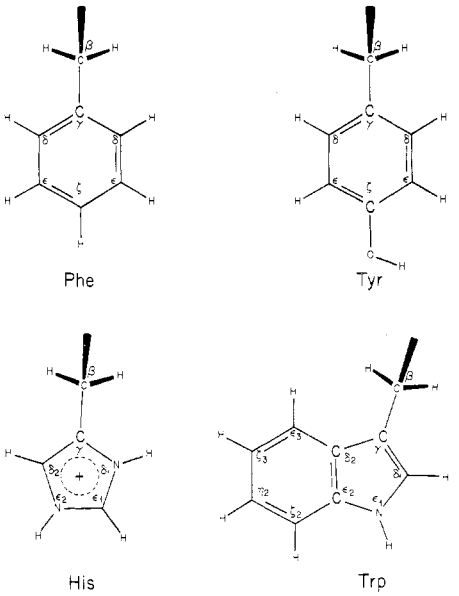

Figure 1. Structures of side chains of aromatic amino acid residues.

interactions,[5] and paramagnetic relaxation (if the protein contains a paramagnetic center).[4,6,9,10] The relative importance of these relaxation mechanisms depends on the type of carbon[5,12] and on the magnetic field strength.[12] We must also know the values of the "effective" rotational correlation times. The ^{13}C NMR spectrum of a protein is divided into the aliphatic region (typically about 10 to 75 ppm downfield from the ^{13}C resonance of Me_4Si), the aromatic region (about 105 to 160 ppm), and the carbonyl region (about 170 to 185 ppm).[2,5] The downfield edge of the aromatic region contains the resonances of C^{ζ} of arginine residues (at about 158 ppm).[6] For many aliphatic side-chain carbons, fast internal rotations (relative to the rate of overall molecular rotation) strongly affect the values of the relaxation parameters.[15] In contrast, the reported ^{13}C relaxation parameters of aromatic amino acid side chains can be interpreted by assuming that the "effective" correlation time is essentially equal to the overall rotational correlation time of the protein. Calculated and measured values of T_1, W, and the NOE for aromatic carbons of native proteins (at 14.2 and 63.4 kG) are given in ref 5, 12, and 13. One important result is that the NOE is generally in the range 1.0 to 1.4 instead of the value of about 3.0 often observed for small molecules.[14]

Observation of Nonprotonated Aromatic Carbon Resonances

The aromatic carbons of a protein (Figure 1) yield resonances which cover a large range of chemical shifts.[2,4-10] Typical chemical shift values observed for small peptides (and expected for random-coil proteins) are shown in Figure 2. It has been established that ^{13}C–^{1}H dipole–dipole relaxation is dominant for methine aromatic carbons (at all magnetic field strengths available today) *and* for nonprotonated aromatic carbons (at magnetic field strengths below about 40 kG).[5,12] Each ^{13}C–^{1}H dipolar contribution to the line width is proportional to the inverse sixth power of the distance (r) between the interacting nuclei.[14] Hydrogens directly bonded to a carbon ($r \approx 1.1$ Å) produce much larger contributions to the line width than nonbonded hydrogens ($r \gtrsim 2$ Å).[5] Therefore, the aromatic region of the ^{13}C NMR spectrum of a native protein at low magnetic field strengths should contain broad bands of methine carbons and narrow resonances of nonprotonated carbons.[5] This expectation can be verified experimentally: It is possible to set up "inefficient" proton decoupling conditions which produce a "smearing out" of any narrow methine carbon resonances but practically complete proton decoupling of nonprotonated carbon resonances. We routinely use noise-modulated off-resonance proton irradiation for this purpose (see Figures 2 and 3 of ref 6). Spectra recorded with noise-modulated off-resonance proton decoupling have shown that *all* the narrow resonances in the aromatic regions of the fully proton-decoupled ^{13}C NMR spectra of the proteins which have been studied in my laboratory (see below) arise from nonprotonated carbons.[2,4,6-10]

When studying nonprotonated aromatic carbon resonances, it is desirable to eliminate the broad bands of methine aromatic carbons by mathematical manipulation of the digitized spectrum. One simple procedure for this purpose is the convolution-difference method.[16] The spectra of hen egg-white lysozyme shown in Figure 3 were recorded under conditions of noise-modulated off-resonance proton decoupling and were subjected to the convolution-difference method, as described elsewhere.[6]

A molecule of hen egg-white lysozyme contains 28 nonprotonated aromatic carbons (from the three tyrosines, three phenylalanines, one histidine, and six tryptophans[17]). These carbons give rise to peaks 4–26 in the spectrum of native lysozyme (Figure 3B).[6] Under the conditions of Figure 3B it is legitimate to use relative intensities to determine how many carbons contribute to each peak.[5] Peaks 11, 12, 17, 20, and 26 are two-carbon resonances, and the other 18 numbered peaks in Figure 3B are single-carbon resonances.[5] Clearly, most of the 28 nonprotonated aromatic carbons give rise to single-carbon resonances in the spectrum of the native protein. However, in the spectrum of the denatured protein (Figure 3A), each *type* of residue yields chemical shifts essentially independent of its position in the amino acid sequence.[2] Figure 3 indicates that folding of lysozyme into its native conformation produces changes in chemical shifts that are quite different for amino acid residues (of the same type) at different positions in the sequence. This fortunate phenomenon is quite general for globular proteins.[6-10] When dealing with a small protein, it is realistic to expect that most of the nonprotonated aromatic carbons will yield resolved single-carbon resonances.[6-10]

Assignments to Specific Residues in the Sequence

There are seven types of nonprotonated aromatic carbons of amino acid residues: C^{γ} of phenylalanines, C^{γ} and C^{ζ} of tyrosines, C^{γ} of histidines, and C^{γ}, $C^{\delta 2}$, and

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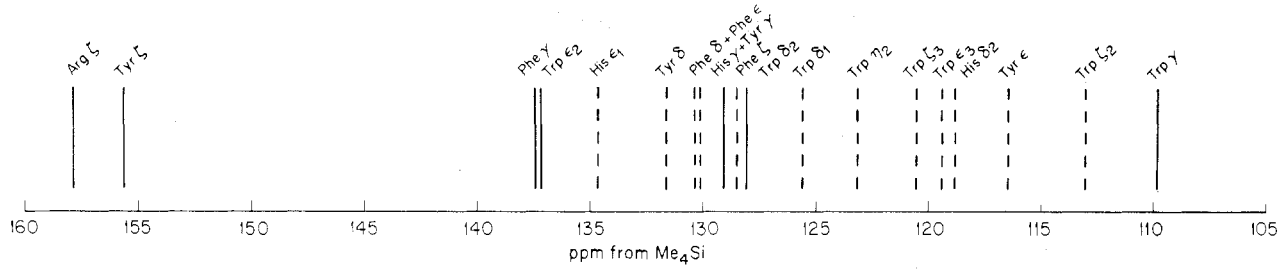

Figure 2. Chemical shifts of nonprotonated carbons (solid lines) and methine carbons (dashed lines) of aromatic amino acid side chains (and C^{ζ} of arginines) of small peptides. The values for histidine and tyrosine refer to the imidazolium and phenolic states, respectively. See the legend of Figure 2A of ref 13 for other details.

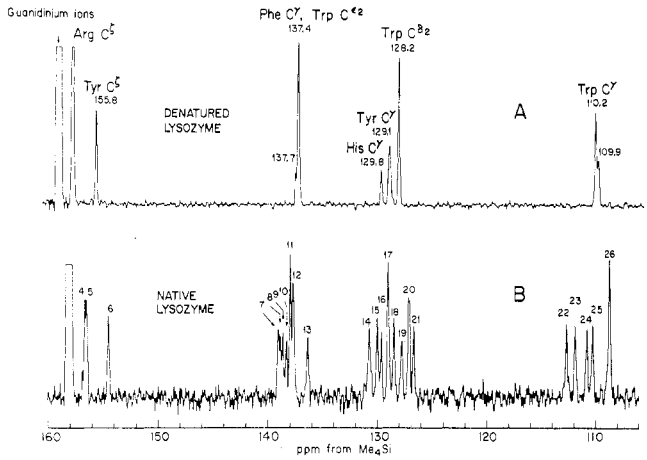

Figure 3. Region of aromatic carbons (and C^{ζ} of arginine residues) in the natural-abundance ^{13}C NMR spectra (at 14.2 kG, with a 20-mm probe) of 9 mM hen egg-white lysozyme in H_2O (0.1 M NaCl). Spectra were recorded under conditions of noise-modulated off-resonance proton decoupling,[6] and the convolution-difference method was applied (with 0.9 and 10 Hz digital broadening[6]) in order to remove the broad bands of methine aromatic carbons. (A) Denatured protein, 6 M guanidinium chloride, pH 3.0, 50 °C, after 40-h signal accumulation with a recycle time of 2.2 s. Assignments are those of ref 3. This spectrum is taken from ref 20. Numbers above peaks are chemical shifts. (B) Native protein, pH 3.4, 38 °C, after 10-h signal accumulation with a recycle time of 1.1 s. Peak numbers are those of ref 5, 6, and 18. This spectrum is taken from ref 27.

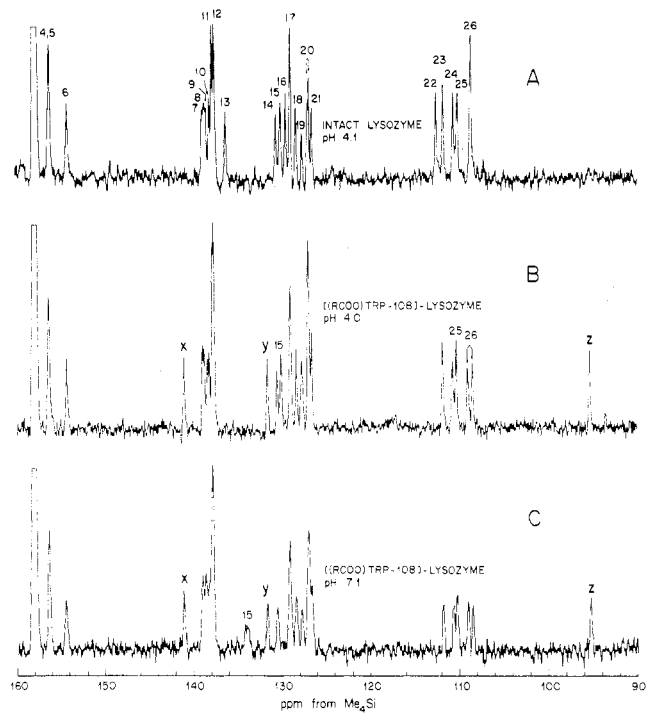

Figure 4. Region of aromatic carbons (and C^{ζ} of arginine residues) in convolution-difference natural-abundance ^{13}C NMR spectra at 14.2 kG, with a 20-mm probe and a recycle time of 1.1 s. (A) Intact hen egg-white lysozyme (11 mM), pH 4.1, 33 °C, 65 536 scans. (B) [(RCOO)Trp-108]lysozyme (8 mM), pH 4.0, 37 °C, 96 566 scans. (C) [(RCOO)Trp-108]lysozyme (8 mM), pH 7.1, 36 °C, 65 536 scans. See ref 24 for other details.

$C^{\epsilon 2}$ of tryptophans (Figure 1). When dealing with a diamagnetic heme protein, the 16 nonprotonated aromatic carbons of the heme also yield narrow resonances.[4,6] It is also necessary to consider the resonances of C^{ζ} of arginines, because they may overlap with those of C^{ζ} of tyrosines.[6,8] Before one tries to assign the resonances to specific residues in the amino acid sequence, it is often desirable to determine which type of carbon gives rise to each resonance. The resonances of C^{γ} of tryptophans can be identified by inspection (see Figures 2 and 3). The resonances of C^{ζ} of tyrosines and arginines can be distinguished from each other by means of selective proton decoupling[6] or (when feasible) by titration of the tyrosines.[8] The resonances of $C^{\delta 2}$ and $C^{\epsilon 2}$ of tryptophans can be identified with the use of partially relaxed Fourier transform NMR spectra.[6] The resonances of C^{γ} and C^{ζ} of titratable tyrosines and those of C^{γ} of titratable histidines can be identified from the pH dependence of their chemical shifts.[6,8-10,18]

One of the most challenging aspects of ^{13}C NMR of proteins is the task of assigning resonances to specific residues in the amino acid sequence. The main approaches have been relaxation probes, chemical modifications, and comparisons of spectra of analogous proteins from different species.[6-8,18-20] I will present one example of the use of each technique.

The broadening of a ^{13}C resonance by a paramagnetic species which behaves as a relaxation probe[21] is proportional to the inverse sixth power of the distance from the carbon to the relaxation probe.[21] Hen egg-white lysozyme has a site, in the vicinity of the carboxylate groups of Glu-35 and Asp-52, that strongly binds lanthanide ions.[22] In solution, the interchange between free lysozyme and the lysozyme–lanthanide complex is fast enough to yield exchange-averaged resonances. Therefore, gradual increases in the concentration of

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Table I
Assignments of the Nonprotonated Aromatic Carbon Resonances of Hen Egg-White Lysozyme to Specific Residues in the Sequence

assignment[a]	peak[b]	chemical shift,[c] ppm
Tyr-20 or -53 C^{ζ}	4	156.6
Tyr-53 or -20 C^{ζ}	5	156.4
Tyr-23 C^{ζ}	6	154.4
Phe-3 or -38 C^{γ}	7	139.0
Trp-63 C^{ϵ_2}	8	138.8
Phe-38 or 3 C^{γ}	9	138.6
Trp-28 or -111 C^{ϵ_2}	10	138.2
Trp-111 or -28 C^{ϵ_2} Trp-123 C^{ϵ_2}	11	137.9
Phe-34 C^{γ} Trp-62 C^{ϵ_2}	12	137.7
Trp-108 C^{ϵ_2}	13	136.3
Tyr-23 C^{γ}	14	130.7
His-15 C^{γ}	15	129.9
Trp-108 C^{δ_2}	16	129.6
Tyr-20 C^{γ} Tyr-53 C^{γ}	17	128.9
Trp-111 C^{δ_2}	18	128.4
Trp-63 C^{δ_2}	19	127.7
Trp-28 C^{δ_2} Trp-62 C^{δ_2}	20	127.1 127.0
Trp-123 C^{δ_2}	21	126.6
Trp-108 C^{γ}	22	112.6
Trp-123 C^{γ}	23	111.8
Trp-63 C^{γ}	24	110.7
Trp-62 C^{γ}	25	110.2
Trp-28 C^{γ} Trp-111 C^{γ}	26	108.7

[a] Taken from ref 18. [b] Peak designations are shown in Figure 3B. [c] From the spectrum of Figure 3B (protein in H_2O, pH 3.4, 0.1 M NaCl, 38 °C).

Gd^{3+} (a relaxation probe) produce a progressively greater number of detectably broadened ^{13}C resonances (see Figure 3 of ref 18). These broadening effects yield various specific assignments.[18]

A comparison of the ^{13}C NMR spectrum of an intact protein with that of a chemically modified version (at a specific residue) of the protein may yield specific assignments, if the chemical modification does not produce a significant conformational reorganization. Figure 4A shows the nonprotonated aromatic carbon resonances of intact hen egg-white lysozyme. Figure 4B is the corresponding spectrum of the same protein, but with Trp-108 converted into δ_1-hydroxytryptophan (esterified internally with the carboxylate group of Glu-35) (Figure 5B).[18,23,24] I call this protein [(RCOO)Trp-108]lysozyme. Peaks 13, 16, and 22 of the intact protein (Figure 4A) are missing from the spectrum of [(RCOO)Trp-108]lysozyme (Figure 4B) and are therefore assigned to Trp-108.[18,19] These assignments are identical with those based on the effects of Gd^{3+}. Table I lists all the known assignments for the nonprotonated aromatic carbons of hen egg-white lysozyme.[18]

Figure 6 shows the C^{γ} resonances of the tryptophan residues of various hemoglobins. Human adult hemoglobin has tryptophan residues at position 14 of the α chains and positions 15 and 37 of the β chains.[17] The α chains of human fetal hemoglobin are identical with the α chains of the adult hemoglobin. The sequence of the γ chains of human fetal hemoglobin differs considerably from that of the β chains of the adult protein.[17] However, the only change which involves a

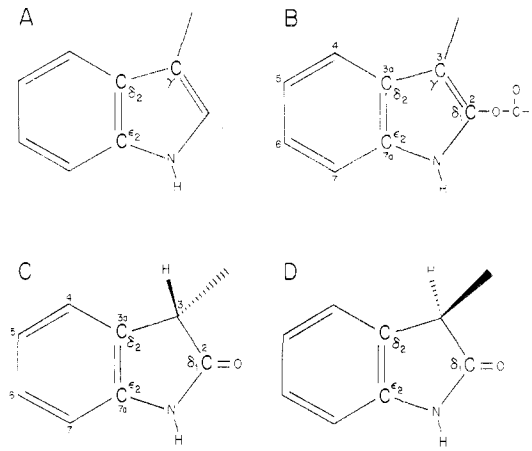

Figure 5. Structures. (A) Indolyl group of a tryptophan residue. (B) Modified indolyl group of a δ_1-hydroxytryptophan residue which has the hydroxyl group esterified. (C and D) Oxindolyl groups of the two diastereoisomers of an oxindolealanine residue.

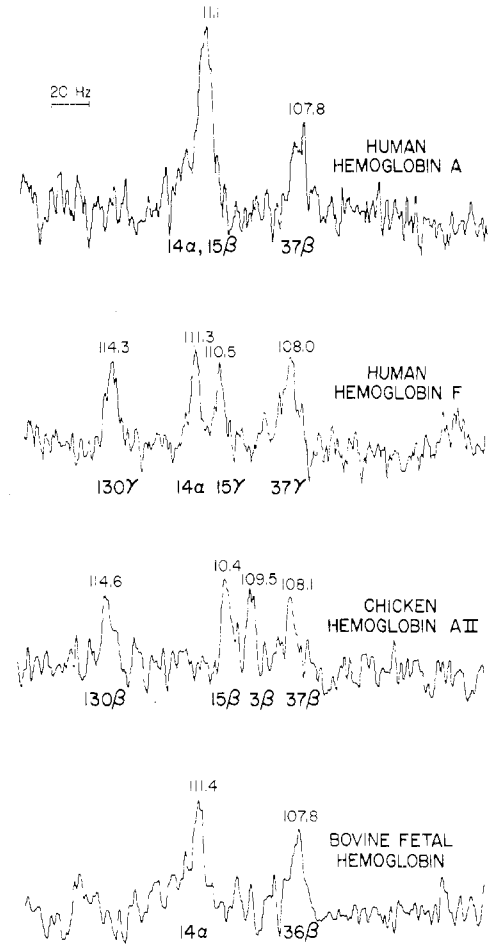

Figure 6. Resonances of C^{γ} of tryptophan residues in proton-decoupled natural-abundance ^{13}C NMR spectra (at 14.2 kG, with a 20-mm probe and a recycle time of 1.1 s) of some carbon monoxide hemoglobins in H_2O (0.1 M NaCl, 0.05 M phosphate buffer, pH 7.0, about 35 °C). Numbers above peaks are chemical shifts in parts per million downfield from Me_4Si. (A) Human adult hemoglobin (3.3 mM in tetramer) after 40-h signal accumulation. (B) Human fetal hemoglobin (2.3 mM in tetramer) after 77 h signal accumulation. (C) Chicken AII hemoglobin (2.6 mM in tetramer) after 40-h signal accumulation. (D) Bovine fetal hemoglobin (3.3 mM in tetramer) after 30-h signal accumulation. Taken from ref 7.

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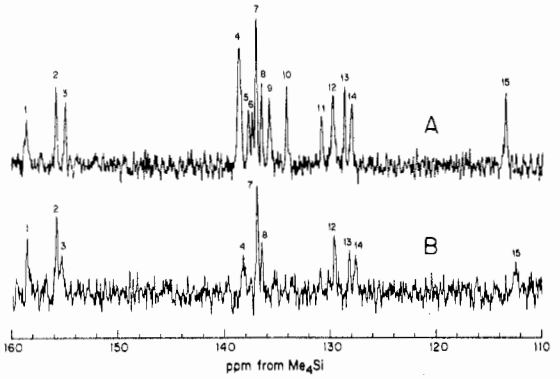

Figure 7. Region of aromatic carbons (and C^{ζ} of arginines) in convolution-difference natural-abundance ^{13}C NMR spectra of *P. aeruginosa* azurin (in H_2O, 0.05 M ammonium acetate, 31 °C). Each spectrum was recorded at 14.2 kG, with a 20-mm probe, noise-modulated off-resonance proton decoupling, and a recycle time of 1.1 s. (A) Cu(I) azurin (7.4 mM), pH 5.2, 65 536 scans. (B) Cu(II) azurin (5.8 mM), pH 5.3, 131 072 scans. Taken from ref 10.

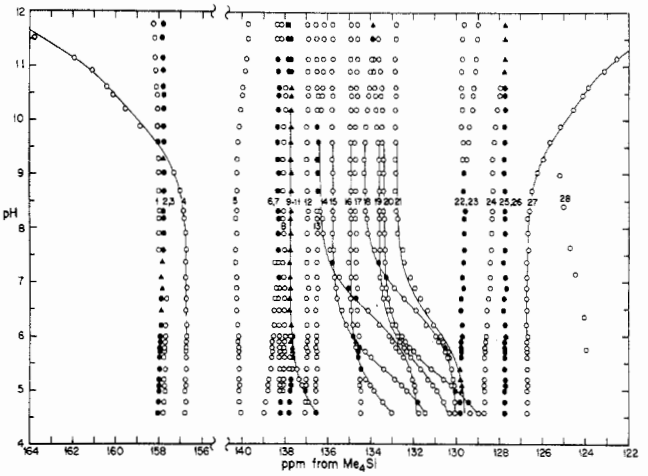

Figure 8. Effect of pH on the chemical shifts of the nonprotonated carbons of aromatic amino acid side chains (except C^{γ} of tryptophans) and the ζ carbons of arginine residues of horse cyanoferrimyoglobin at 38 °C. Because of the complex pH dependence of many chemical shifts, the peak numbering system (shown above the data at pH 8.3) assigns one number to each carbon and not to each peak, consecutively from left to right at pH ≈8. Open circles, closed circles, triangles, and squares indicate peaks that arise from one, two, three, and four carbons, respectively. The solid lines are best-fit theoretical titration curves, with one pK for each histidine and two pK values for Tyr-103 (peaks 4 and 27). Taken from ref 8 and 9.

tryptophan residue is the presence of Trp-130γ instead of Tyr-130β.[17] Chicken AII hemoglobin has no tryptophan residues in the α chains[17] and four in the β chains, three of which occupy the same positions as in the γ chains of human fetal hemoglobin (the fourth is Trp-3β).[25] Bovine fetal hemoglobin has Trp-14α and Trp-36β (the latter is analogous to Trp-37β of the other hemoglobins).[17] The pattern of chemical shifts of Figure 6 is remarkably consistent with the assumption that the chemical shift of C^{γ} of a tryptophan residue at a particular position in the amino acid sequence is unaffected (or only slightly affected) by substitutions elsewhere in the sequence.[7] The only exception is Trp-15β of human adult hemoglobin, which has a value significantly different from the ones observed for the corresponding residues of human fetal and chicken AII hemoglobins (Figure 6).[7] This difference does not create problems (see assignments under the peaks in Figure 6).[7] However, in less favorable situations, the method of species comparisons may not yield the desired assignments.[6]

Applications

The presence of a "built-in" or liganded paramagnetic metal ion which can act as a relaxation probe[21] can be used to estimate relative distances from some carbons to the metal ion, by measuring the paramagnetic contributions to the line widths and/or the spin–lattice relaxation times of the ^{13}C resonances. For example, the environments of the aromatic amino acid residues of azurin (a "blue" copper-containing protein) from *Pseudomonas aeruginosa* were investigated by observing the spectra of the copper(I) and copper(II) proteins and of mixtures of the two redox states.[10] All 17 nonprotonated aromatic carbons (and C^{ζ} of the single arginine residue) yield narrow resonances in the spectrum of the diamagnetic copper(I) protein (Figure 7A).[10] However, only 11 of these 18 carbons yield detectable resonances in the spectrum of the paramagnetic copper(II) protein (Figure 7B).[10] Furthermore, some of the detectable resonances of copper(II) azurin are considerably broader than the corresponding ones of the reduced protein. Clearly, many aromatic residues are close enough to the copper ion to undergo paramagnetic broadening. With the use of the assignments presented in Table I of ref 10, Figure 7 yields more detailed information. For example, the orientation of the indole ring of the single tryptophan residue places C^{γ} (peak 15) closer to the copper than C^{δ_2} (peak 13). Spectra of mixtures of the two redox states of azurin suggest that the two nontitrating histidine residues are coordinated to the copper.[10] In the future, better signal-to-noise ratios may permit more quantitative determinations of carbon–metal distances.

A comparison of the spectra of monomeric and self-associated hen egg-white lysozyme indicates that Trp-62 is located in a region of the intermolecular contact (as has also been determined by other methods[26]) and that self-association of lysozyme is not accompanied by a general conformational change.[27] A comparison of the spectra of intact hen egg-white lysozyme (Figure 4A) and [(RCOO)Trp-108]lysozyme (Figure 4B) reveals that the conversion of Trp-108 into the internal Glu-35 ester of δ_1-hydroxytryptophan-108 causes changes in the chemical shifts of the γ carbons of Trp-28 and Trp-111 (peak 26 in Figure 4).[24] It appears that the chemical modification of Trp-108 causes a minor conformational reorganization near Trp-28 and Trp-111. These two examples illustrate the utility of ^{13}C NMR for monitoring the effects of various perturbations on the conformation of a protein.

The effect of pH on the resonances of C^{ζ} and C^{γ} of each titrating tyrosine residue yields the pK value for that residue.[8] Figure 8 shows the effect of pH on the chemical shifts of the nonprotonated aromatic carbons (except C^{γ} of the two tryptophans) of horse cyanoferrimyoglobin. The two arginines, two tyrosines

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(Tyr-103 and Tyr-146), 7 phenylalanines, 11 histidines, and 2 tryptophans of this protein[17] contain 30 nonprotonated side-chain carbons, all of which yield observable resonances in spectra of the cyanoferrimyoglobin.[8,9] Clearly, peaks 4 and 27 arise from C^{ζ} and C^{γ}, respectively, of a tyrosine residue.[8] We can infer that the titratable tyrosine is Tyr-103 and not Tyr-146 from the fact that spectra of kangaroo myoglobin (a protein with a single tyrosine at position 146[17]) do not yield any titratable tyrosine residue.[8]

Proton NMR spectroscopy has been the favorite method for measuring the ionization behavior of histidine residues of proteins, because the resonances of H^{ϵ_1} (and sometimes H^{δ_2}) can often be observed as resolved single-hydrogen peaks.[28] Carbon-13 NMR is an attractive alternative, especially when dealing with a protein which contains many histidines.[9] The effect of pH on the chemical shift of C^{γ} of a histidine residue not only yields the pK value but also indicates which is the predominant tautomeric state of the imidazole form of the residue: If the imidazolium form of a histidine (Figure 1) deprotonates at N^{δ_1} to yield the "common" N^{ϵ_2}–H tautomer,[9,29] then the C^{γ} resonance should move about 6 ppm downfield.[29] If deprotonation yields the "uncommon" N^{δ_1}–H tautomer,[10] the C^{γ} resonance should move about 2 ppm upfield.[29] The C^{γ} resonances of 8 of the 11 histidines of horse cyanoferrimyoglobin exhibit titration behavior (peaks 11, 14–16, and 18–21 of Figure 8).[9] The direction of the titration shifts indicates that the imidazole form of each titrating histidine is predominantly in the N^{ϵ_2}–H tautomeric state. The effect of pH on the chemical shifts of the nonprotonated aromatic carbons of *P. aeruginosa* azurin (see Figure 2 of ref 10) indicates that only two of the four histidine residues titrate (peaks 11 and 14 of Figure 7A).[10] Peak 14 (Figure 7A) moves about 2 ppm *upfield* when going to high pH (with a pK of 7.5) and must therefore arise from a histidine residue whose imidazole form exists mainly in the "uncommon" N^{δ_1}–H tautomeric state.[10]

In favorable cases, ^{13}C NMR can be used to determine nondestructively the positions in the sequence of chemically modified aromatic amino acid residues, the amount of unreacted protein, and the nature of the chemical modifications.[19,20] For example, a comparison of the spectrum of intact hen egg-white lysozyme (Figure 4A) with the spectrum of one fraction obtained from the reaction mixture after treatment with iodine[24] (Figure 4B) indicates that Trp-108 is the only aromatic residue which has been altered (because peaks 13, 16, and 22 of the intact protein are missing from Figure 4B) and that the altered residue is not oxindolealanine but δ_1-hydroxytryptophan or an ester thereof (based on the chemical shifts of peaks x, y, and z of Figure 4B).[19] Figure 4B is a spectrum of chromatographically fractionated [(RCOO)Trp-108]lysozyme (see preceding section).[24] Actually, more detailed conclusions were obtained from spectra of *unfractionated* reaction mixtures of lysozyme with iodine.[19]

Application of ^{13}C NMR spectroscopy to the study of the reaction of equimolar amounts of *N*-bromosuccinimide and hen egg-white lysozyme (a known procedure for the specific conversion of Trp-62 into oxindolealanine[30]) revealed the complexities of a chemical modification which was previously thought to be very simple.[20]

Carbon-13 NMR can be used to study changes in the properties of a protein upon chemical modification of a specific residue. For example, ^{13}C NMR was used to study the effects of chemical modifications at Trp-108 of lysozyme on the self-association and lanthanide ion binding properties of the protein.[24] Both [(RCOO)-Trp-108]lysozyme and [oxindolealanine-108]lysozyme were studied.[24] Consider the spectra of [(RCOO)-Trp-108]lysozyme shown in Figure 4. The chemical shift of C^{γ} of Trp-62 (peak 25) does not change appreciably when going from pH 4 to pH 7. This fact, taken together with information presented in ref 27, indicates that [(RCOO)Trp-108]lysozyme does not self-associate significantly at neutral pH[24] (as has also been determined by other methods[26]), in contrast to the behavior of the intact protein under similar sample conditions.[27]

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Additions and Corrections

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Page 371. Figure 3 is inaccurate as presented. The correct sequence, reading from top to bottom, should be Photosynthetic Membrane, Vesicle, Micelle, and Sensitized Semiconductor.