THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 250, No. 16, Issue of August 25, pp. 6381–6402, 1975
Printed in U.S.A.

Studies of Individual Carbon Sites of Proteins in Solution by Natural Abundance Carbon 13 Nuclear Magnetic Resonance Spectroscopy

STRATEGIES FOR ASSIGNMENTS*

(Received for publication, December 26, 1974)

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Natural abundance ^{13}C Fourier transform NMR spectra (at 15.18 MHz, in 20-mm sample tubes) of aqueous native proteins yield numerous narrow single carbon resonances of nonprotonated aromatic carbons. Techniques for the assignment of these resonances are presented. Each technique is applied to one or more of the following proteins: ferricytochrome *c* from horse heart and *Candida krusei*, ferrocytochrome *c* and cyanoferricytochrome *c* from horse heart, lysozyme from hen egg white, cyanoferrimyoglobins from horse and sperm whale skeletal muscle, and carbon monoxide myoglobin from horse. In all of the protein spectra we have examined, methine aromatic carbons give rise to broad bands. Studies of the narrow resonances of nonprotonated aromatic carbons of proteins are facilitated by removal of these broad bands by means of the convolution-difference method, preferably from spectra recorded under conditions of noise-modulated off-resonance proton decoupling.

We present a summary of the chemical shift ranges for the various types of nonprotonated aromatic carbons of amino acid resiuues and hemes of diamagnetic proteins, based on our results for hen egg white lysozyme, horse heart ferrocytochrome *c*, horse carbon monoxide myoglobin, and carbon monoxide hemoglobins from various species. Heme carbon resonances disappear from their positions in the spectra of ferrocytochrome *c* and carbon monoxide myoglobin upon conversion of these proteins to paramagnetic states. The resonance of C^{γ} of the coordinated His-18 is not detected in the spectrum of horse heart ferricytochrome *c*. Horse heart cyanoferricytochrome *c* yields a resolved single carbon resonance for every one of the 18 nonprotonated aromatic carbons of amino acid residues, including C^{γ} of His-18. The temperature dependence of the ^{13}C chemical shifts of ferricytochrome *c* and cyanoferricytochrome *c* yields information about paramagnetic contributions to these chemical shifts.

Spectra recorded under conditions of low power selective proton decoupling are used to distinguish C^{ζ} resonances of tyrosine residues from those of arginine residues. The resonances of $C^{\delta 2}$ and $C^{\epsilon 2}$ of tryptophan residues are identified by means of the partially relaxed Fourier transform (PRFT) method. The PRFT method yields the specific assignments of $C^{\epsilon 2}$ and $C^{\delta 2}$ of the lone Trp-59 residue of horse heart ferrocytochrome *c*, ferricytochrome *c*, and cyanoferricytochrome *c*. The PRFT method also yields the identification of the resonances of $C^{\epsilon 2}$ and $C^{\delta 2}$ of the 6 tryptophan residues of hen egg white lysozyme in D_2O. The resonances of some nonprotonated aromatic carbons and C of arginine residues of hen egg white lysozyme and horse heart cytochrome *c* undergo upfield shifts when labile hydrogens are replaced by deuterium.

The pH dependence of chemical shifts yields specific assignments for C^{γ} of His-15 of hen egg white lysozyme, C^{γ} of His-26 and His-33 of horse heart ferrocytochrome *c*, and C^{γ} of His-32 of *C. krusei* ferricytochrome *c*. The resonances of C^{γ} of His-39 and His-45 of *C. krusei* ferricytochrome *c* are assigned, but not on a 1:1 basis. The effect of pH is also used for assigning the carbonyl resonance of Gly-1 of horse carbon monoxide myoglobin.

Gd^{3+} bound to hen egg white lysozyme in the region of Glu-35 and Asp-52 is used as a line-broadening probe for specifically assigning the resonances of C^{γ} and $C^{\delta 2}$ of Trp-108. Conversion of Trp-62 of hen egg white lysozyme to oxindolealanine-62 affects the chemical shifts of C^{γ}, $C^{\delta 2}$, and $C^{\epsilon 2}$ of 2 tryptophan residues, but has no detectable effect on the chemical shifts of all other nonprotonated aromatic carbons. The affected resonances are assigned to Trp-62 and Trp-63, but not on a 1:1 basis.

* This work was supported by the National Science Foundation (Grant GP-40688X), the United States Public Health Service (Grant NS-10977-02), and by Eli Lilly and Company.

‡ Supported by a Fellowship from the European Molecular Biology Organization.

The aromatic regions of the ^{13}C NMR spectra of cyanoferrimyoglobins from sperm whale and horse are compared. The pH dependence of the chemical shifts of C^{γ} of many histidine residues complicates the use of this comparison for making specific assignments of the resonances of variant aromatic residues. A comparison of the ^{13}C NMR spectra of ferricytochromes *c* from horse heart and *C. krusei* yields the specific assignment for C^{γ} of Tyr-52 of the latter.

Fast electron transfer between horse heart ferrocytochrome *c* and ferricytochrome *c* produces chemical exchange effects in spectra of mixtures of the two species. Narrow exchange-averaged resonances are assigned to 16 carbons situated far from the iron atom. Their fast exchange behavior is used to establish a 1:1 correspondence between resonances in spectra of the two oxidation-reduction states. The resonances of the remaining 18 nonprotonated aromatic carbons are narrow in the spectrum of ferrocytochrome *c* but become broad upon addition of ferricytochrome *c*. These resonances are assigned to C^{γ} of His-18, C^{ζ} of Tyr-67, and the 16 nonprotonated aromatic carbons of the heme. Specific assignments are presented for C^{γ} of His-18 and C^{ζ} of Tyr-67 of ferrocytochrome *c*. Temperature dependence of chemical shifts is used to assign the C^{ζ} resonance of Tyr-67 of ferricytochrome *c*, and the resonances of C^{ϵ} of Tyr-67 and C^{γ} of His-18 of cyanoferricytochrome *c*.

We have shown that the natural abundance ^{13}C Fourier transform NMR spectra of small native proteins yield numerous narrow single carbon resonances of nonprotonated aromatic carbons (1–3). The detection of these resonances is facilitated by the use of a 20-mm probe (4). In the preceding paper (5) we presented theoretical and experimental values of linewidths, spin-lattice relaxation times, and nuclear Overhauser enhancements for nonprotonated aromatic carbon resonances of various native proteins. In this report we discuss techniques for assigning these resonances to specific residues. We apply each technique to one or more of the following proteins: cytochrome *c* from horse heart and *Candida krusei*, lysozyme from hen egg white, and myoglobin from horse and sperm whale. Some specific assignments of individual carbon resonances of hemoglobins are presented in the following paper (6).

Cytochromes *c*, lysozymes, and myoglobins from some species are attractive proteins for initial ^{13}C NMR investigations, because the large quantities ($\gtrsim$0.5 g) that are needed for our 20-mm sample tubes (4) are relatively easy to obtain. Proton NMR spectroscopy has been used to study solutions of hen egg white lysozyme (7–19), cytochrome *c* from various species (20–31), and myoglobins from various species (23, 32–39). In each case, only a few single proton resonances have been observed (even at magnetic field strengths above 50 kG), most notably those arising from $H^{\epsilon 1}$ of histidine residues (13–15, 24–27, 38), $H^{\epsilon 1}$ of tryptophan residues (16, 17), and some hydrogens situated near the iron of a heme (23, 28–31, 39). Natural abundance ^{13}C NMR spectra of horse heart cytochrome *c* (2), hen egg white lysozyme (1, 3, 40–43) and myoglobins (44–47) have been reported. However, most of the spectra (40–47) were recorded under conditions of insufficient sensitivity for the detection of single carbon resonances.

This report is concerned mainly with the narrow resonances of nonprotonated aromatic carbons: C^{γ} of phenylalanine and histidine residues, C^{γ} and C^{ζ} of tyrosine residues, and C^{γ}, $C^{\delta 2}$, and $C^{\epsilon 2}$ of tryptophan residues (Fig. 1*A*). The 16 nonprotonated aromatic carbons of the heme of diamagnetic heme proteins also yield narrow resonances (2). In addition, we must consider ζ carbons of arginine residues, because their resonances fall at the downfield edge of the aromatic region, close to those of C^{ζ} of tyrosine residues (1, 2, 5). Hen egg white lysozyme has 3 phenylalanine residues, 3 tyrosine residues, 1 histidine residue, 6 tryptophan residues, and 11 arginine residues (48). The aromatic residues contribute a total of 28 nonprotonated aromatic carbons. The lone tryptophan, 4 phenylalanine, 4 tyrosine, and 3 histidine residues of horse heart cytochrome *c* (48) contribute a total of 18 nonprotonated aromatic carbons. If we include contributions from the 16 nonprotonated aromatic carbons of the heme and C^{ζ} of the 2 arginine residues, we expect up to 36 narrow resonances in the aromatic region of the ^{13}C NMR spectrum of horse heart cytochrome *c*. In the case of horse myoglobin, the 2 tyrosine, 7 phenylalanine, 11 histidine, and 2 tryptophan residues contain a total of 28 nonprotonated aromatic carbons (48). The replacement of Phe-151 by Tyr-151 and of Asn-12 by His-12 when going from horse to sperm whale increases this number to 30. If we include C^{ζ} of arginine residues and the 16 nonprotonated aromatic carbons of the heme, we expect up to 46 and 50 narrow resonances in the aromatic regions of the ^{13}C NMR spectra of horse and sperm whale myoglobins, respectively.

EXPERIMENTAL PROCEDURE

Horse heart cytochrome *c* was purchased from Sigma Chemical Co., St. Louis, Mo. (type III and VI) and from Calbiochem, La Jolla, Calif. (A grade). *Candida krusei* cytochrome *c* (type VII), hen egg white lysozyme (grade I), horse skeletal muscle myoglobin, sperm whale skeletal muscle myoglobin, Gly-His-Gly, Gly-Phe amide acetate, Gly-Tyr amide hydrochloride, L-arginine hydrochloride, *N*-bromosuccinimide, and gadolinium oxide (99.9%) were purchased from Sigma Chemical Co. L-Tryptophan was obtained from Matheson, Coleman & Bell, Norwood, Ohio. Oxindole was purchased from Aldrich Chemical Co., Milwaukee, Wis. Lanthanum oxide (ultrapure grade) was obtained from Alfa Products, Beverly, Mass. ^{13}CO (28.5% ^{13}C) was purchased from Monsanto Research Corp., Miamisburg, Ohio. K^{13}CN (85 to 90% ^{13}C) was obtained from Mallinckrodt Chemical Works, St. Louis, Mo.

Unless otherwise stated, protein solutions were concentrated in a model 52 or 402 stirred ultrafiltration cell (Amicon Corp., Lexington, Mass.) equipped with a suitable Diaflo membrane. Samples of ferrocytochrome *c* and ferricytochrome *c* in H_2O were prepared as described previously (2). Samples of cyanoferricytochrome *c* were prepared by addition of KCN in phosphate buffer to a buffered solution of ferricytochrome *c*. Solutions of horse heart ferri- and ferrocytochrome *c* in D_2O were prepared by concentrating and diluting 4 g of protein five times over a 6-hour period, each time using 50 ml of a 0.05 M phosphate/0.1 M NaCl solution in D_2O (pH meter reading 6.7), at 25°. A solution of horse heart ferrocytochrome *c* in D_2O was prepared by adding solid sodium dithionite to a D_2O solution of ferricytochrome *c* under argon.

Hen egg white lysozyme was purified on a column of DEAE-Sephadex.[1] Samples in H_2O were prepared by dilution of a 20 mM stock solution of protein. pH was adjusted with 5 M HCl or NaOH. A

[1] R. S. Norton, R. F. Childers, and A. Allerhand, manuscript in preparation.

lysozyme solution in D_2O was prepared as follows. Thirteen milliliters of stock solution were diluted to 55 ml with D_2O, and then concentrated to the original volume. The above process was carried out five times (total time 19 hours, at 25°). The lysozyme solution (pH meter reading 5.5) was then kept at 37° for 24 hours, concentrated to 10 ml, diluted to 55 ml with D_2O, and concentrated to 11 ml.

For the preparation of carbon monoxide myoglobin, ferrimyoglobin was reduced anaerobically with sodium dithionite, in a 0.05 M phosphate/0.1 M NaCl buffer, pH 7.0, and then carbonylated. Small molecules were removed on a Sephadex G-25 (medium) column. pH values in the range 6.4 to 8.1 were obtained by equilibration and elution with the appropriate buffer. NaOH (1 M) was used to raise the pH to 10.1. For the preparation of cyanoferrimyoglobin, first an equimolar quantity of potassium ferricyanide was added to a solution of commercial myoglobin, in order to ensure full oxidation to ferrimyoglobin. Then small molecule impurities were removed and pH was adjusted on a Sephadex G-25 (medium) column, and KCN in phosphate buffer (pH 7.0) was added.

pH measurements were carried out as described previously (1). The pH was measured at room temperature, except for the pH titration experiments on horse heart ferrocytochrome *c*. In this case the pH was measured at 36°, the temperature used for the NMR titration experiments. Most protein solutions were passed through a membrane filter (8 μm pore size) from Millipore Corp., Bedford, Mass. or from Nuclepore Corp., Pleasanton, Calif. Protein purity was monitored by cellulose acetate electrophoresis in a Microzone cell (Beckman Instruments Inc., Fullerton, Calif.), in some cases both before and after the NMR experiments. Except in the case of sperm whale myoglobin, all protein samples were at least 90% electrophoretically homogeneous, as estimated with the use of a Beckman Microzone R110 densitometer. The main component (~65%) of the sperm whale myoglobin migrated with an authentic sample of Fraction IV of sperm whale myoglobin kindly provided by Professor F. R. N. Gurd, Department of Chemistry, Indiana University. Protein concentrations were determined on a Cary 14 spectrophotometer, using published extinction coefficients for lysozyme (49), cytochromes *c* (50), and myoglobins (51).

Aqueous solutions of $GdCl_3$ and $LaCl_3$ were prepared by dissolving Gd_2O_3 and La_2O_3 in dilute HCl. The indole ring (Fig. 1*A*) of Trp-62 of hen egg white lysozyme was oxidized to oxindole (Fig. 1*B*) by reaction with *N*-bromosuccinimide using the procedure of Hayashi *et al.* (52), except that after dialysis the protein solution was concentrated by ultrafiltration and not lyophilized. Our product was electrophoretically homogeneous and had an ultraviolet absorption spectrum in agreement with that reported by Hayashi *et al.* (52) for oxindolealanine-62-lysozyme.

Unless otherwise indicated, ^{13}C NMR spectra were obtained as described in the preceding paper (5). For single frequency selective proton-decoupling experiments, the ^1H radiofrequency peak field strength was about 0.02 G. Chemical shifts were measured digitally, and are reported in parts per million downfield from Me_4Si. Except for the samples listed below, internal dioxane (at 67.86 ppm downfield from external Me_4Si) was used as an internal standard. External ethylene glycol (at 64.3 ppm downfield from Me_4Si) was used as a reference for the chemical shifts of hen egg white lysozyme at pH 5 (in the absence and presence of La^{3+}), oxindolealanine-62-lysozyme, and the ferricytochromes *c* from *C. krusei* and horse heart at pH 5.5.

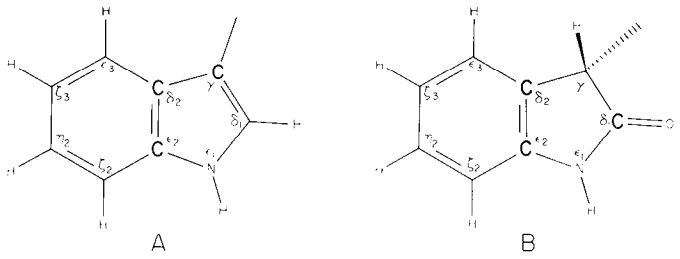


FIG. 1. Structures. *A*, indole ring of a tryptophan residue. *B*, oxindole ring of an oxindolealanine residue.

NOISE-MODULATED OFF-RESONANCE PROTON DECOUPLING

In the aromatic regions of ^{13}C NMR spectra of native proteins we observe narrow resonances and broad features (1–3, 5). If the effect of internal rotation about C^α—C^β and C^β—C^γ bonds of aromatic amino acid residues could be neglected, then we could confidently assume that all of the narrow resonances arise from nonprotonated carbons (5). Such an assumption is not necessary, because an unambiguous experimental method exists for distinguishing nonprotonated carbons from methine carbons, namely noise-modulated off-resonance proton decoupling (53). Incomplete proton decoupling produces a residual broadening of a ^{13}C resonance which is proportional to the square of the pertinent ^{13}C-^1H scalar coupling constant. One-bond ^{13}C-^1H scalar coupling constants are larger than 100 Hz, while long range carbon-hydrogen scalar coupling constants are typically smaller than 10 Hz (54). In noise-modulated off-resonance proton-decoupling experiments, one adjusts the average frequency and the power of the ^1H irradiation to produce negligible broadening of nonprotonated carbon resonances but large broadening of any narrow methine carbon resonances. In Fig. 2*A* we show the fully proton-decou-

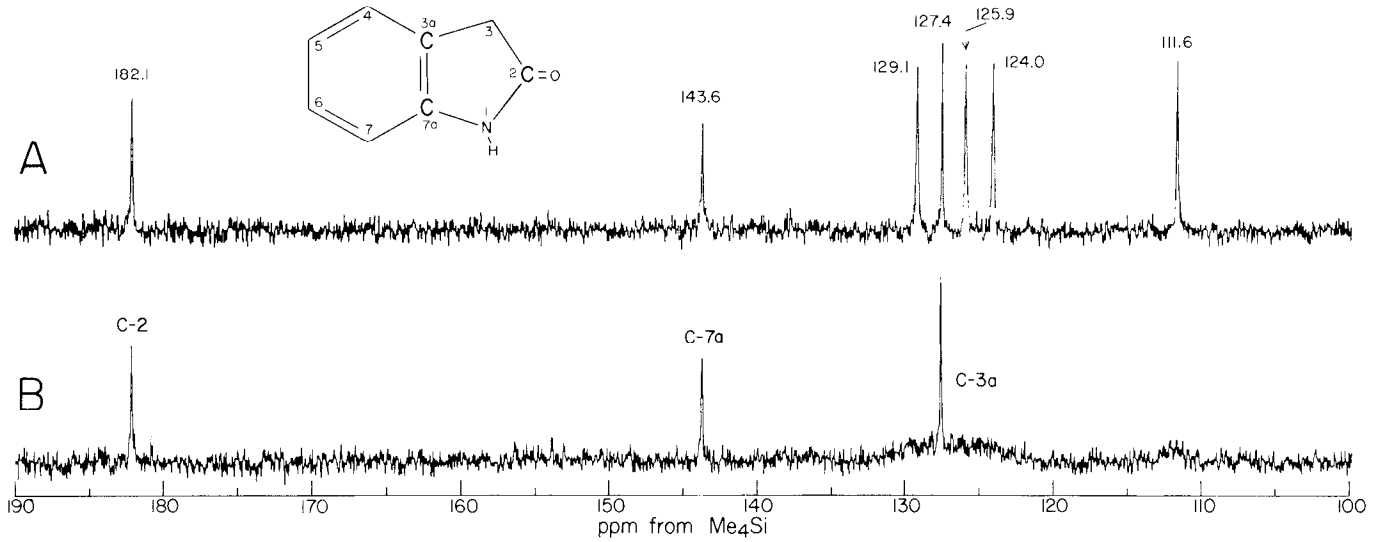


FIG. 2. Unsaturated carbon regions in natural abundance ^{13}C Fourier transform NMR spectra of 56 mM oxindole in H_2O (0.1 M NaCl, pH 3.1, 44°), recorded at 15.18 MHz using 16,384 time domain addresses, a spectral width of 3,787.9 Hz, a recycle time of 60 s, 512 accumulations, and a digital broadening of 0.29 Hz. *A*, fully proton-decoupled. ^1H irradiation (0.6 G peak field strength) was centered about 3.5 ppm downfield from Me_4Si, and had a noise modulation bandwidth of about 7 ppm. *Numbers* above the peaks are chemical shifts in parts per million downfield from Me_4Si. *B*, noise-modulated off-resonance proton-decoupled (see "Experimental Procedure" of Ref. 5).

pled ^{13}C NMR spectrum of oxindole, which is of interest in connection with our studies of chemically modified hen egg white lysozyme (see below). Chemical shift considerations (54) are sufficient for assigning the resonance at 182.1 ppm to C-2 (carbonyl) and the one at 143.6 ppm to C-7a (Fig. 2*A*). The third nonprotonated carbon (C-3a) was identified by recording the ^{13}C NMR spectrum under conditions of noise-modulated off-resonance proton decoupling (Fig. 2*B*).

Noise-modulated off-resonance proton-decoupling experiments have shown that all of the narrow resonances in the aromatic region of the ^{13}C NMR spectra of all of the native proteins that we have examined (1–3, 5, 6) arise from nonprotonated carbons. Nonprotonated carbon resonances of proteins that fall within the range of the strong methine carbon bands are often better resolved in spectra recorded under conditions of noise-modulated off-resonance proton decoupling than in fully proton-decoupled spectra, because noise-modulated off-resonance proton decoupling produces broader bands of methine carbon resonances than those obtained with full proton decoupling. For example, in the case of sperm whale cyanoferrimyoglobin, several narrow resonances in the range 128 to 138 ppm are better resolved under conditions of noise-modulated off-resonance proton decoupling (Fig. 3*B*), than when efficient proton decoupling is used (Fig. 3*A*). We routinely use noise-modulated off-resonance proton decoupling when studying only the resonances of nonprotonated carbons.

CONVOLUTION-DIFFERENCE SPECTRA

The convolution-difference method has been used to improve the resolution in proton NMR spectra of proteins (18, 19). We find it useful for improving the resolution of nonprotonated aromatic carbon resonances and for eliminating the broad methine carbon features from our ^{13}C NMR spectra of proteins.

Multiplication of a time domain signal by e^{-t/τ_1} produces a broadening of $1/\pi\tau_1$ Hz after Fourier transformation (55, 56). In order to improve the signal to noise ratio without considerable loss of resolution, the constant τ_1 is normally chosen so that $1/\pi\tau_1$ is equal to or slightly smaller than the linewidth (57). For example, the spectrum of Fig. 3*B* was obtained when the accumulated time domain signal from sperm whale cyanoferrimyoglobin was processed with $1/\pi\tau_1 = 0.662$ Hz. When the same time domain signal (retrieved from a disk) was Fourier transformed after applying a digital broadening $1/\pi\tau_2 = 9.33$ Hz, the spectrum of Fig. 3*C* resulted. When the spectrum of Fig. 3*C* (multiplied by a constant $K = 0.9$) was digitally subtracted from Fig. 3*B*, the result was a convolution-difference spectrum (Fig. 3*D*). In order to facilitate the discussion of the relatively complex patterns of narrow aromatic carbon resonances of myoglobins, we find it convenient to divide the aromatic portion of the ^{13}C NMR spectra of these proteins into regions a to f, shown in Figs. 3 and 4. Clearly, Fig. 3*D* is more suitable than Fig. 3*B* for studying the narrow peaks in regions c to e. The choice of values of τ_1, τ_2, and K has been discussed by Campbell *et al.* (19). In Fig. 3*E* we show the final form of our convolution-difference spectrum of sperm whale cyanoferrimyoglobin. This spectrum was derived from Fig. 3*D* by means of a digital base-line adjustment, in which the residual broad components of Fig. 3*D* were treated as a base-line imperfection.

CHEMICAL SHIFTS

General Considerations—In Fig. 5 we show the chemical shift ranges for the various types of nonprotonated aromatic carbons of diamagnetic proteins, based on our assignments for hen egg white lysozyme, horse heart ferrocytochrome *c*, carbon monoxide myoglobins (see below), and carbon monoxide hemoglobins (6). Also shown in Fig. 5 are the relatively invariant positions (*thick vertical lines*) of the corresponding resonances in a denatured protein (1) and in small peptides (58–60). It follows from Fig. 5 that γ-carbons of tryptophan residues yield resonances that do not overlap with any other nonprotonated carbon resonances. Resonances of C^{ζ} of tyrosine residues overlap only with those of C^{ζ} of arginine residues. The resonances of C^{γ} of phenylalanine, $C^{\epsilon 2}$ of tryptophan, and C^{γ} of histidine residues have chemical shift ranges that overlap. The resonances of C^{γ} of tyrosine, $C^{\delta 2}$ of tryptophan, and C^{γ} of histidine residues also have overlapping ranges of chemical shifts. Fig. 5 also summarizes the large chemical shift variations that a particular type of amino acid residue can experience when situated in different portions of a folded diamagnetic protein. The results of Fig. 5 are based on our limited observations and assignments. It is likely that as more proteins are studied, the range of chemical shifts for each type of nonprotonated aromatic carbon will extend beyond the limits shown in Fig. 5. For example, even though Fig. 5 indicates no overlap between the ranges of chemical shifts of C^{γ} of phenylalanine and C^{γ} of tyrosine, a more extensive survey of ^{13}C NMR spectra of native proteins may reveal such an overlap. The chemical shift ranges of Fig. 5 must be used with caution.

Hen Egg White Lysozyme—The aromatic regions of convolution-difference ^{13}C NMR spectra of hen egg white lysozyme in H_2O (pH 3) and in D_2O (pH meter reading 3) are shown in Fig. 6. Chemical shifts are given in Table I. In the section under "Selective Proton Decoupling" we show that Peaks 1 to 3 arise from C^{ζ} of the 11 arginine residues, and that Peaks 4 to 6 are the resonances of C^{ζ} of the 3 tyrosine residues. It follows from Fig. 5 that the resonances of C^{γ} of the 6 tryptophan residues of lysozyme are Peaks 22 to 26 of Fig. 6. Integrated intensities indicate that Peaks 22 to 25 are single carbon resonances and that Peak 26 is a 2-carbon resonance (5). The group of resonances in the range 137 to 139 ppm (*Peaks 7* to *13* of Fig. 6) arise from C^{γ} of the 3 phenylalanine and $C^{\epsilon 2}$ of the 6 tryptophan residues. C^{γ} of the lone histidine residue should not contribute to this region at pH 3, because the apparent pK_a of His-15 is about 5.5 (13, 14, 61, 62). Integrated intensities (5) indicate that Peaks 10 and 13 are single carbon resonances, Peaks 11 and 12 are 2-carbon resonances, and Peaks 7 to 9 are the resonances of the remaining 3 carbons. A comparison of the chemical shifts of Peaks 7 to 13 of Fig. 6, *A* and *B* (Table I) indicates that there are significant deuterium isotope effects on the chemical shifts of some of these resonances (see below). In the range 126 to 131 ppm of the lysozyme spectra (Fig. 6), there are six single carbon resonances (Peaks 14 to 16, 18, 19, and 21) and two 2-carbon resonances (Peaks 17 and 20), for a total of 10 carbons (5). Fig. 5 indicates that C^{γ} of the 3 tyrosine and $C^{\delta 2}$ of the 6 tryptophan residues contribute to this region. C^{γ} of His-15 is the 10th contributor (see "Effect of pH").

Heme Proteins (General Considerations)—The application of the chemical shift information of Fig. 5 to the interpretation of ^{13}C NMR spectra of heme proteins is not as straightforward as in the case of lysozyme. The presence of a heme can have large effects on the ^{13}C NMR spectrum of a protein. The 16 nonprotonated aromatic carbons of the heme contribute narrow resonances if the heme is in a diamagnetic state, as in ferrocytochrome *c* (2), carbon monoxide myoglobin (see below), and carbon monoxide hemoglobin (6). Some of these

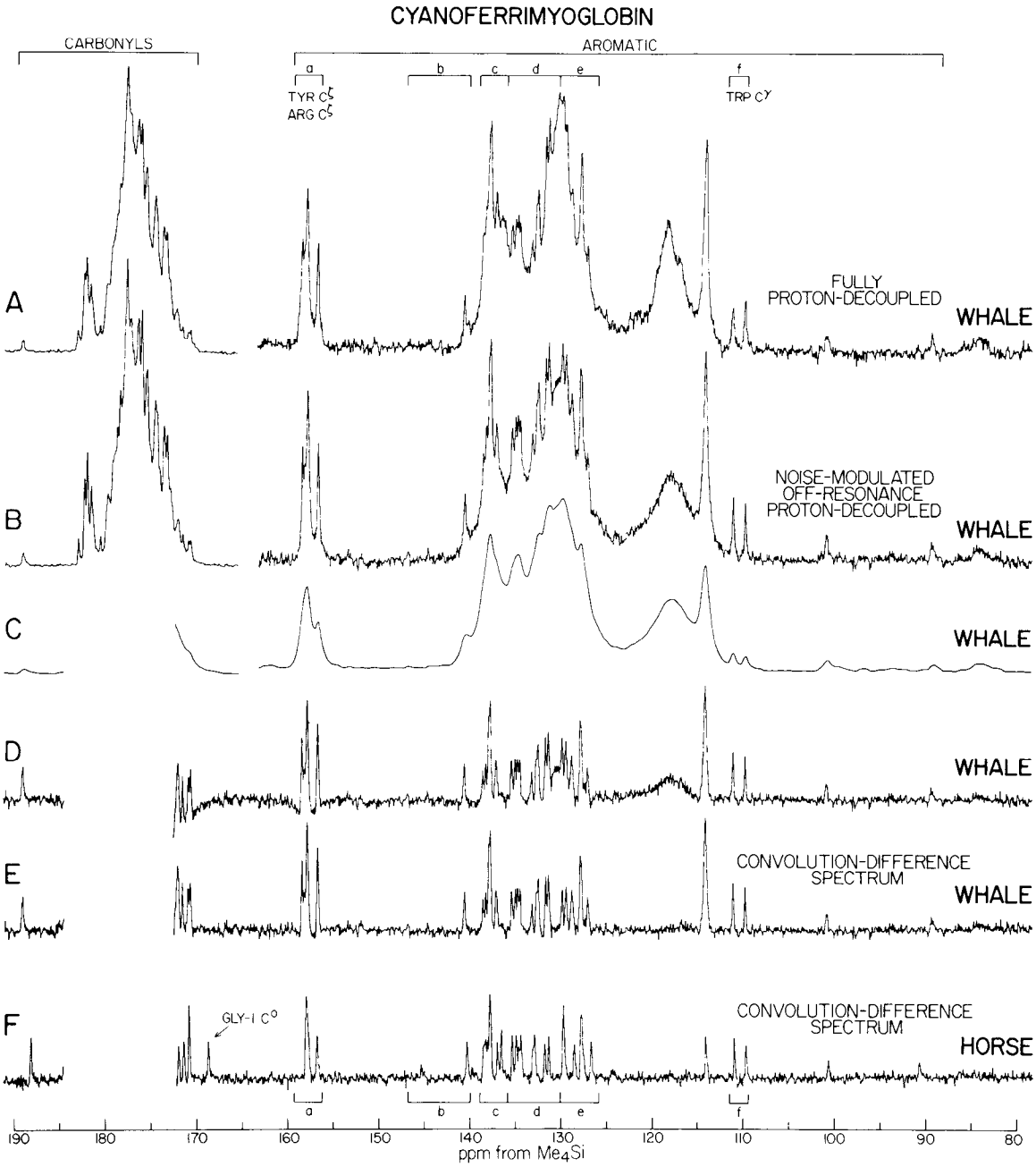
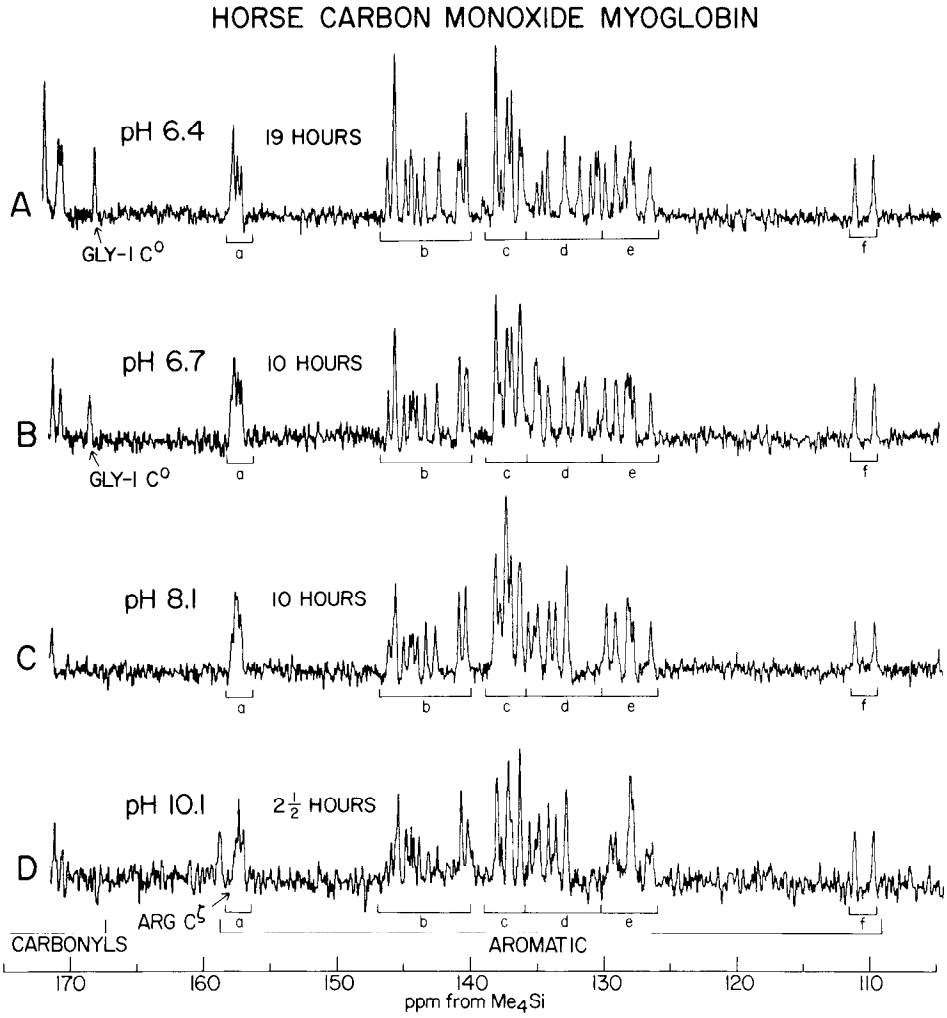


FIG. 3. *A*, unsaturated carbon regions in the fully proton-decoupled natural abundance ^{13}C Fourier transform NMR spectrum of 8.8 mM sperm whale cyanoferrimyoglobin in H_2O (0.1 M NaCl/0.05 M phosphate buffer, pH 6.8, 36°), prepared with the use of $K^{13}CN$ (85 to 90% ^{13}C) in an unsuccessful attempt to detect the ^{13}C resonance of the bound cyanide.[4] The spectrum was recorded at 15.18 MHz using 8,192 time domain addresses, a 4,000-Hz spectral width, 65,536 accumulations, a recycle time of 1.105 s (20 hours total time), and a digital broadening of 0.62 Hz (τ_1 = 0.51 s). The peak at about 114 ppm arises from excess free $H^{13}CN$ which is in fast exchange with about 0.5% free $^{13}CN^-$ (54). The carbonyl region is presented with 0.3 times the vertical gain of the aromatic region. *B*, as spectrum *A*, but recorded under conditions of noise-modulated off-resonance proton decoupling. *C*, as spectrum *B*, but with digital broadening of 9.33 Hz (τ_2 = 0.034 s). *D*, convolution-difference spectrum, obtained by digitally subtracting nine-tenths (K = 0.9) of spectrum *C* from spectrum *B*. Carbonyl resonances have the same vertical gain as the rest of the spectrum. *E*, final convolution-difference spectrum, obtained from spectrum *D* by a digital base-line adjustment. *F*, same as *E*, but 8.7 mM horse cyanoferrimyoglobin. Sample conditions were the same as for the sperm whale protein, except that KCN of natural isotopic composition was used.

heme carbon resonances are readily identified, while others overlap with resonances of aromatic amino acid residues (Fig. 5). The histidine residue coordinated to the iron of the diamagnetic horse heart ferrocytochrome *c* yields a C^γ resonance shifted considerably upfield of C^γ resonances of uncoordinated histidine residues (2). Part of this upfield shift must arise from the strong ring current effect of the porphyrin ring (23, 39). Ring current effects may also be important for other amino acid carbons near the heme, such as C^ζ of Tyr-67 of cytochrome *c* (63).

The chemical shift ranges of Fig. 5 may not apply to carbons near the iron of a heme protein in a paramagnetic state, as a result of large contact or pseudocontact contributions (or both) to the chemical shifts of these carbons (23, 64). Furthermore, the resonances of carbons near the paramagnetic center are subject to paramagnetic broadening (64).



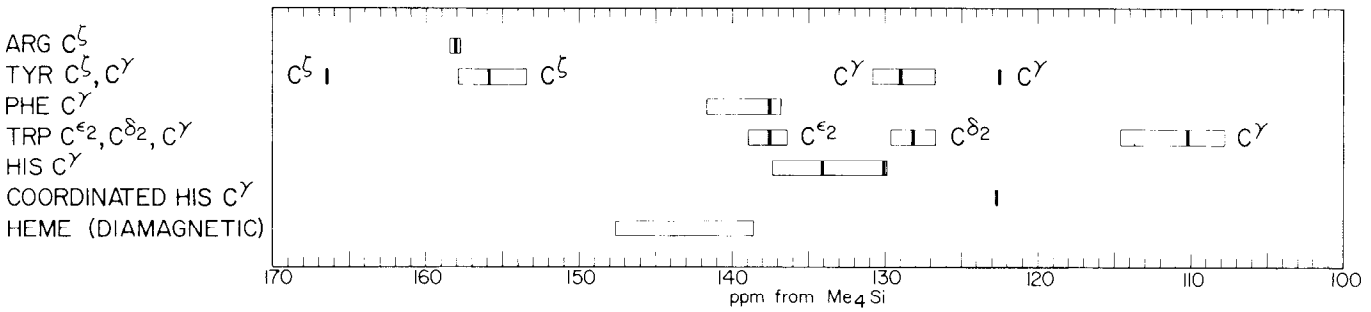


FIG. 4. Effect of pH on the chemical shifts of nonprotonated aromatic carbons, Cζ of arginine residues, and some carbonyls in the natural abundance ^{13}C Fourier transform NMR spectra of horse carbon monoxide myoglobin in H_2O (0.1 M NaCl/0.05 M phosphate buffer, 36°). The protein at pH 6.4 was prepared with the use of 28.5% ^{13}C-enriched carbon monoxide, in connection with other experiments (5). Spectra were recorded at 15.18 MHz using 8,192 time domain addresses and a 4,000-Hz spectral width. Full proton decoupling was used, except for the spectrum at pH 10.1, which was recorded with noise-modulated off-resonance proton decoupling. The convolution-difference method was applied with $\tau_2 = 0.034$ s and $K = 0.9$. The value of τ_1 was 0.34 s (pH 6.4 and 10.1) or 0.51 s (pH 6.7 and 8.1). *A*, pH 6.4, 8.6 mM protein, 32,768 accumulations, 2.105-s recycle time (19.2 hours total time). *B*, pH 6.7, 9.7 mM protein, 32,768 accumulations, 1,105-s recycle time (10.1 hours total time). *C*, pH 8.1, 9.9 mM protein, other conditions as in *B*. *D*, pH 10.1, 9.9 mM protein, 8,192 accumulations, 1.105-s recycle time (2.5 hours total time).

FIG. 5. Observed ranges of chemical shifts (*boxes*) of nonprotonated aromatic carbons and Cζ of arginine residues of some native diamagnetic proteins in H_2O. The thick lines are the relatively invariant chemical shifts of these carbons in a denatured protein (1) and in small peptides (58–60), except for the chemical shift of Cγ of coordinated histidine, which is the value observed for His-18 of horse heart ferrocytochrome *c* (Table II). The histidine lines at 130 ppm and 134 ppm refer to Cγ of the imidazolium and imidazole forms, respectively. The tyrosine lines at 129 ppm and 122.5 ppm refer to Cγ of the phenolic and phenolate forms, respectively. The tyrosine lines at 156 ppm and 166.5 ppm refer to Cζ of the phenolic and phenolate forms, respectively.

Horse Heart Ferrocytochrome c—Fig. 7*A* shows the aromatic region of the convolution-difference ^{13}C NMR spectrum of this diamagnetic protein. Chemical shifts are given in Table II. Peak intensities indicate that all 34 nonprotonated aromatic carbons (including those of the heme) and the ζ carbons of the 2 arginine residues yield narrow resonances. We show in the section under "Chemical Exchange" that one set of resonances (Peaks 1 to 3, 15, 19 to 28, and 30) arises from 18 carbons located relatively far from the iron, and that another set (Peaks 4 to 14, 16 to 18, and 29) arises from 18 carbons near the iron. The latter set of peaks must be assigned to the 16 nonprotonated aromatic carbons of the heme and 2 amino acid carbons near the heme. On the basis of crystallographic information (63), these 2 amino acid carbons are Cγ of the coordinated His-18 and Cζ of Tyr-67. Using crystal coordinates (of tuna ferrocytochrome *c*) kindly supplied by Professor R. E. Dickerson, Department of Chemistry, California Institute of Technology, Pasadena, Calif., and the ring current data of Shulman *et al.* (39), we calculate upfield ring current shifts of about 5 ppm and about 2 ppm for Cγ of His-18 and Cζ of Tyr-67, respectively. Peak 4 (Fig. 7*A*) is about 3 to 4 ppm upfield from Peaks 2 and 3, which are assigned below to Cζ

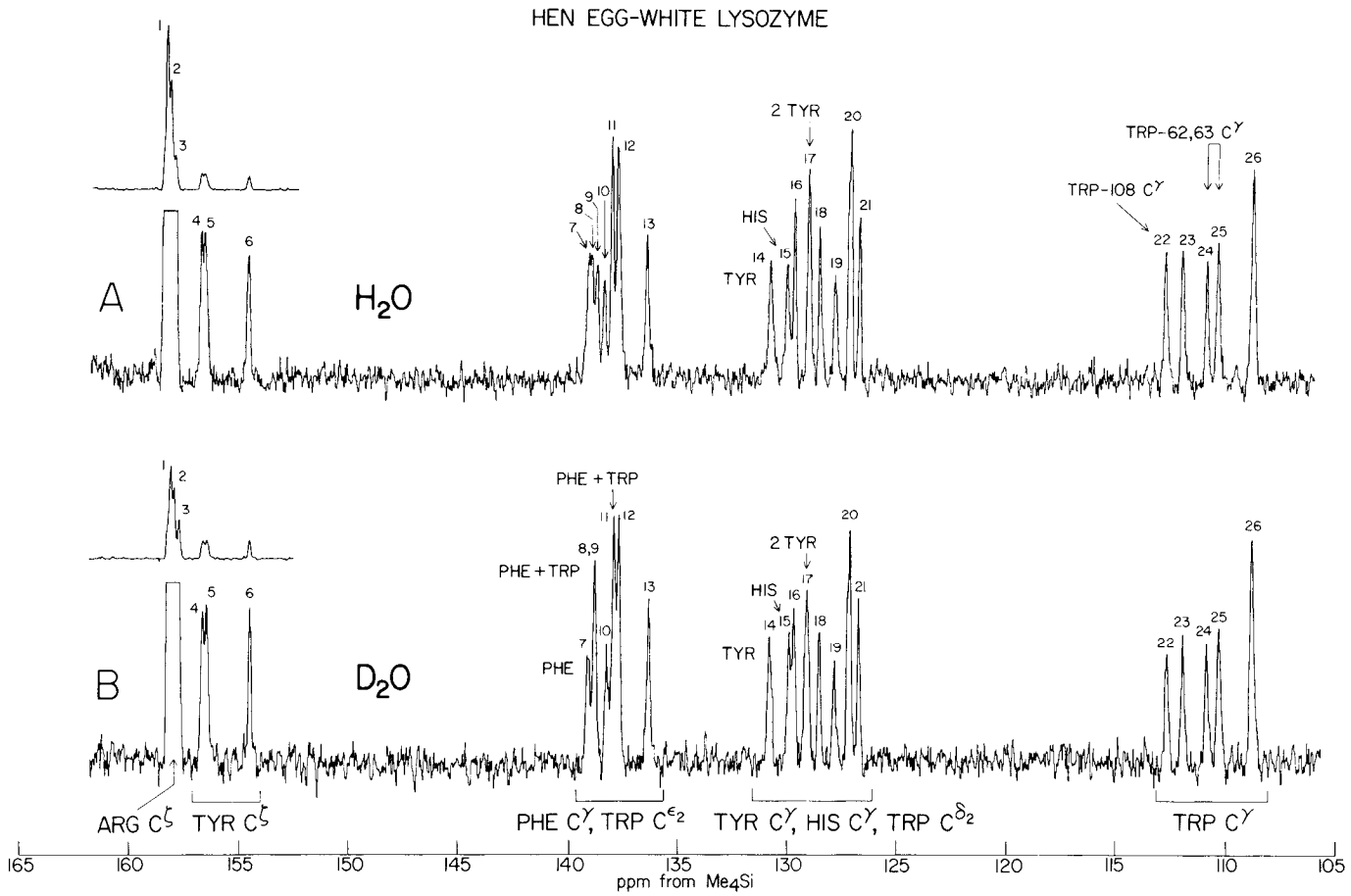


FIG. 6. Regions of aromatic carbons and C^{ζ} of arginine residues in the convolution-difference natural abundance ^{13}C Fourier transform NMR spectra of hen egg white lysozyme. Each spectrum was recorded at 15.18 MHz under conditions of noise-modulated off-resonance proton decoupling, using 8,192 time domain addresses, a spectral width of 3,787.9 Hz, 49,152 accumulations, a recycle time of 2.205 s (30 hours total time). The convolution-difference procedure was used with $\tau_1 = 0.72$ s, $\tau_2 = 0.036$ s, and $K = 1.0$. *Peak numbers* are those of Table I. The *insets* (*Peaks 1 to 6*) are shown with one-eighth the vertical gain of the main spectrum. Assignments are given in the text. *A*, 14.6 mM protein in H_2O, pH 3.05, 0.1 M NaCl, 44°. *B*, 13.8 mM protein in D_2O, pH meter reading 3.08, 0.1 M NaCl, 42°.

of Tyr-48, Tyr-74, and Tyr-97. Furthermore, Peaks 5 to 14, 16 to 18, and 29 are at least 8 ppm upfield of Peaks 2 and 3. We assign Peak 4 to C^{ζ} of Tyr-67. Peak 29 is about 7 ppm upfield from the normal range of chemical shifts of C^{γ} of histidine residues (Fig. 5). Furthermore, all other peaks identified above as arising from carbons near the heme are actually downfield from the range of resonances of C^{γ} of histidine residues (Fig. 5). We assign peak 29 to C^{γ} of His-18. By elimination, Peaks 5 to 14 and 16 to 18, which arise from 16 carbons (2), must be assigned to the nonprotonated aromatic carbons of the heme. Peaks 5 to 14 and 16 to 18 have chemical shifts in the range 138.6 to 147.6 ppm (Table II). Reported chemical shifts of analogous carbons of some diamagnetic metalloporphyrins are in the range 135.9 to 147.5 ppm (65).

On the basis of Fig. 5, Peaks 1 to 3 (which arise from 5 carbons) must be the resonances of C^{ζ} of Tyr-48, Tyr-74, Tyr-97, and the 2 arginine residues. We show in the section under "Selective Proton Decoupling" that Peak 1 (a 2-carbon resonance) arises from C^{ζ} of the arginine residues. Therefore, Peaks 2 and 3 (a 2-carbon resonance) must be assigned to C^{ζ} of the 3 tyrosine residues under consideration. Peaks 15 and 19 to 23 arise from 6 carbons (2). On the basis of Fig. 5, 5 of these carbons are $C^{\epsilon 2}$ of Trp-59 and C^{γ} of the 4 phenylalanine residues. The sixth contributor must be C^{γ} of His-26 or His-33, because C^{γ} of His-18 has already been assigned. Peaks 24 to 28 arise from 6 carbons (2). Fig. 5 indicates that these are the resonances of $C^{\delta 2}$ of Trp-59, C^{γ} of the 4 tyrosine residues, and C^{γ} of 1 of the 2 uncoordinated histidine residues.

Horse Heart Ferricytochrome c—In the section under "Chemical Exchange" we present the connections between Peaks 2 to 16 in the spectrum of ferricytochrome *c* (Fig. 7*B*) and the corresponding resonances of ferrocytochrome *c* (Fig. 7*A*), but not the connection between Peak 4 of ferrocytochrome *c* (already assigned to C^{ζ} of Tyr-67) and Peak 1 of ferricytochrome *c*. The chemical shift of Peak 1 of ferricytochrome *c* exhibits a large temperature dependence (Table III). As a first approximation, the contact and pseudocontact contributions to the ^{13}C chemical shifts of ferricytochrome *c* should be proportional to the reciprocal of the absolute temperature (64). The chemical shift of Peak 1 extrapolated to $1/T = 0$ is about 150 ppm, which is close to the chemical shift of Peak 4 of the diamagnetic ferrocytochrome *c* (Table II). A similar result has been obtained by Patel.[2] The large paramagnetic contribution to the chemical shift of C^{ζ} of Tyr-67 in the spectrum of ferricytochrome *c* is consistent with the proximity of this carbon to the iron in the crystalline protein (66).

The relatively small temperature dependence of the chemical shifts of Peaks 2 to 10 and 12 to 16 of ferricytochrome *c* (Table III) is consistent with the assignment of these peaks to

[2] D. J. Patel, private communication.

TABLE I

Chemical shifts of nonprotonated aromatic carbons and C^{ζ} of arginine residues of hen egg white lysozyme in H_2O and D_2O

$\delta_A{}^B$ designates a chemical shift, in parts per million downfield from Me_4Si, for the protein in Solvent *A* at temperature *B*. Estimated accuracy is ±0.05 ppm. Peak numbers and sample conditions are those of Fig. 6, *A* and *B* for the protein in H_2O and D_2O, respectively, except that dioxane was added and the spectra were recorded with full proton decoupling, a recycle time of 1.105 s, and 32,768 accumulations for the H_2O solution and 16,384 accumulations for the D_2O solution.

ASSIGNMENT	PEAK	$\delta^{42}_{H_2O}$	$\delta^{42}_{H_2O}-\delta^{42}_{D_2O}$
11 Arg C^{ζ}	1	158.1_9	0.2_2[a]
	2	158.0_7	0.2_4[a]
	3	157.8_6	0.2_7[a]
Tyr C^{ζ}	4	156.6_1	0.0_9
Tyr C^{ζ}	5	156.4_9	0.1_5
Tyr C^{ζ}	6	154.5_4	0.1_2
Phe C^{γ}	7	139.0_1	-0.0_3
Trp-62 or 63 $C^{\epsilon 2}$	8	138.8_6	0.1_6
Phe C^{γ}	9	138.7_0	0.0_0
Trp $C^{\epsilon 2}$	10	138.3_1	0.1_6
Phe C^{γ} + 3 Trp $C^{\epsilon 2}$	11[b,c]	137.9_6	d
	12[b,c]	137.7_3	d
Trp $C^{\epsilon 2}$	13	136.3_9	0.1_5
Tyr C^{γ}	14	130.6_9	0.0_0
His-15 C^{γ}	15	129.9_8	0.1_4
Trp-108 $C^{\delta 2}$	16	129.6_3	0.0_0
2 Tyr C^{γ}	17[b]	129.0_2	-0.0_3
Trp $C^{\delta 2}$	18	128.5_0	0.0_6
Trp-62 or 63 $C^{\delta 2}$	19	127.7_8	0.0_4
2 Trp $C^{\delta 2}$	20[b,e]	127.1_3	0.0_3
Trp $C^{\delta 2}$	21	126.6_9	0.0_2
Trp-108 C^{γ}	22	112.6_9	0.1_2
Trp C^{γ}	23	111.9_0	-0.0_3
Trp-62 or 63 C^{γ}	24	110.8_4	0.0_7
Trp-63 or 62 C^{γ}	25	110.3_2	0.0_9
2 Trp C^{γ}	26[b]	108.7_6	0.0_3

[a] Because of the poor resolution of the resonances of C^{ζ} of the 11 arginine residues, these values do not necessarily yield the isotope effects for every single carbon resonance.

[b] Two-carbon resonance.

[c] Peak 12 of the protein in H_2O contains the $C^{\epsilon 2}$ resonance of Trp-63 or Trp-62. The other contributor to this peak is probably C^{γ} of a phenylalanine residue.

[d] Unambiguous connections between Peaks 11 and 12 of the protein in H_2O and Peaks 11 (137.7_9 ppm) and 12 (137.5_8 ppm) of the protein in D_2O have not been made (see text).

[e] Contains the resonance of $C^{\delta 2}$ of Trp-63 or Trp-62.

carbons relatively far from the iron (see "Chemical Exchange"). Even in the absence of the connections between the resonances of ferrocytochrome *c* and ferricytochrome *c*, the small temperature dependence of the chemical shifts of the above resonances would be a sufficient criterion for using Fig. 5 in assigning them. The 16 nonprotonated aromatic carbons of the heme and C^{γ} of His-18 do not yield detectable resonances in our spectrum of ferricytochrome *c* (Fig. 7*B*).

Horse Heart Cyanoferricytochrome c—As in the case of ferricytochrome *c*, we can assign Peak 1 (Fig. 7*C*) to C^{ζ} of

TABLE II

Chemical shifts of nonprotonated aromatic carbons and C^{ζ} of arginine residues of horse heart ferrocytochrome c in H_2O and D_2O

$\delta_A{}^B$ designates a chemical shift, in parts per million downfield from Me_4Si, for the protein in Solvent *A* at temperature *B*. Estimated accuracy is ±0.05 ppm. Peak numbers are those of Figs. 7*A* and 12. Unless otherwise noted, each chemical shift of the protein in H_2O is the arithmetic average of pH invariant values shown in Fig. 12. Chemical shifts in D_2O were obtained using about 20 mM ferrocytochrome *c* (in 0.1 M dithionite/0.1 M NaCl/0.05 M phosphate buffer, pH 6.7), 8,192 accumulations, a recycle time of 2.105 s, and other spectral conditions as in Fig. 7*A*.

ASSIGNMENT	PEAK	$\delta^{36}_{H_2O}$	$\delta^{36}_{H_2O}-\delta^{36}_{D_2O}$
2 Arg C^{ζ}	1	158.2_1	0.1_0
Tyr C^{ζ}	2	157.8_9	0.0_1
2 Tyr C^{ζ}	3	156.8_1[a]	0.0_2
Tyr-67 C^{ζ}	4	153.4_9	0.0_5
2 heme carbons	5,6	147.5_5	b
2 heme carbons	7	147.0_3	-0.0_5
heme	8	146.6_3	-0.0_3
heme	9	146.3_2	-0.0_5
heme	10	145.6_8	-0.0_5
2 heme carbons	11	144.5_1	-0.0_6
heme	12	144.2_4	-0.0_4
heme	13	143.8_8	-0.0_4
2 heme carbons	14	143.1_0	-0.0_5
Phe C^{γ}	15	141.5_6	-0.0_8
heme	16	140.6_2	-0.0_6
heme	17	139.1_2	-0.0_4
heme	18	138.6_3	-0.0_5
Trp-59 $C^{\epsilon 2}$	19	137.8_3	0.0_8
Phe C^{γ}	20	137.3_4	-0.0_6
2 Phe C^{γ}	21,22	136.7_7	b
His-26 C^{γ}	23	135.5_3[c]	-0.0_3
His-33 C^{γ}	24	132.2_7[d]	e
Tyr C^{γ}	25	130.7_6	-0.0_4
Tyr C^{γ}	26	128.2_8	-0.0_8
Tyr C^{γ} + Trp-59 $C^{\delta 2}$	27	127.8_1	-0.0_7
Tyr C^{γ}	28	126.7_1	-0.0_4
His-18 C^{γ}	29	122.7_0	0.1_0
Trp-59 C^{γ}	30	110.3_4	-0.0_1

[a] One contributor to this 2-carbon resonance begins to move downfield at about pH 9.

[b] Could not be determined accurately, because of partial resolution of the components of this 2-carbon resonance.

[c] Arithmetic average of chemical shifts in the pH range 5.5 to 9.4 (Fig. 12).

[d] At pH 6.68.

[e] Not determined, because of the pH dependence of this chemical shift.

Tyr-67 on the basis of the temperature dependence of the chemical shift (Table IV). Again, extrapolation to $1/T = 0$ yields a chemical shift of about 150 ppm. The relatively broad resonance at 123.6 ppm (*Peak 18* of Fig. 7*C*) is assigned to C^{γ} of the coordinated His-18. The chemical shift of this peak shows a strong temperature dependence (Table IV). Extrapolation to

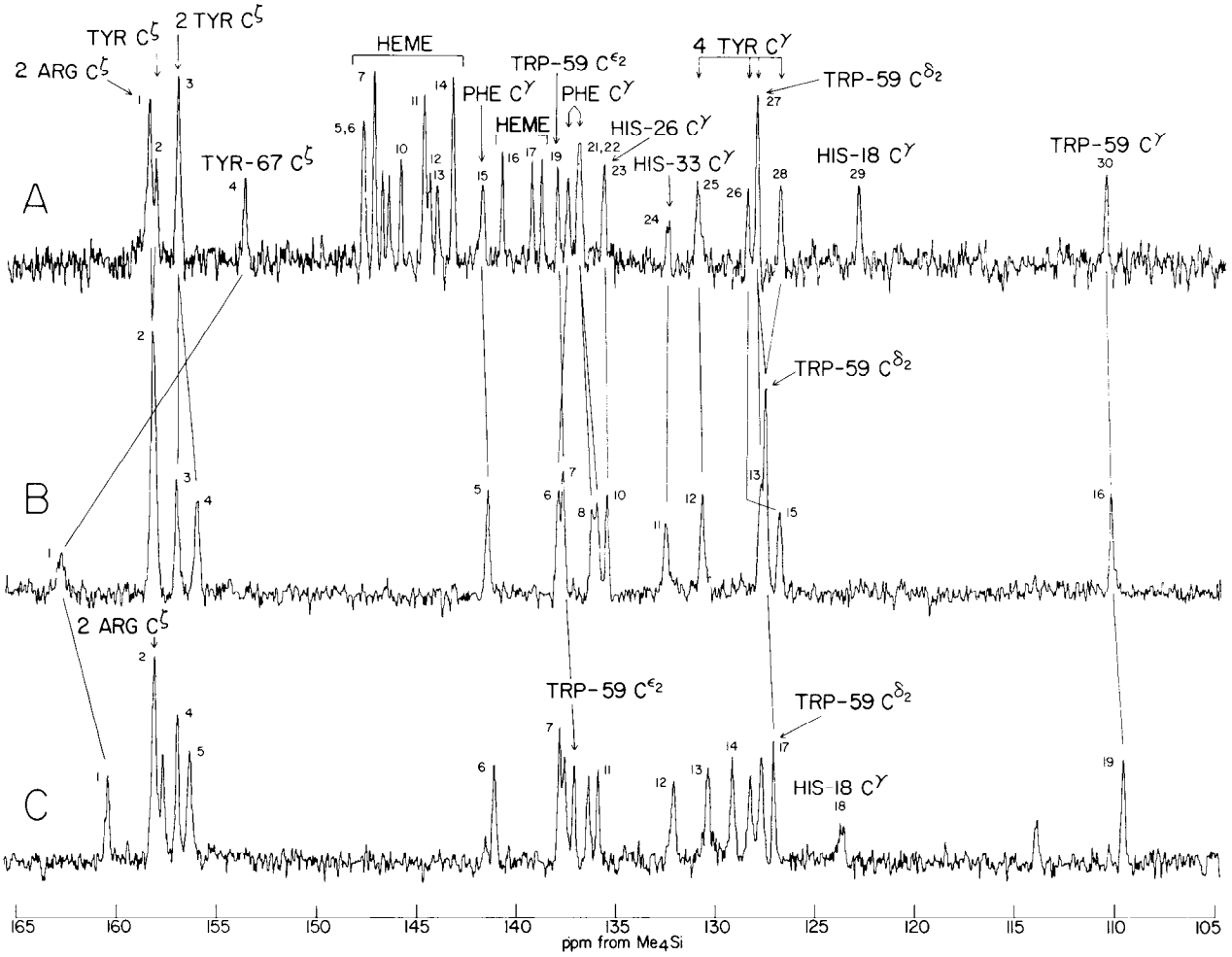


FIG. 7. Regions of aromatic carbons and Cζ of arginine residues in the convolution-difference natural abundance ^{13}C Fourier transform NMR spectra of horse heart cytochrome *c* in H_2O (0.1 M NaCl/0.05 M phosphate buffer). Each spectrum was recorded at 15.18 MHz, with noise-modulated off-resonance proton decoupling, 8,192 time domain addresses, a 4,000-Hz spectral width, and a recycle time of 1.105 s. The convolution-difference procedure was carried out with $\tau_1 = 0.51$ s, $\tau_2 = 0.034$ s, $K = 0.9$. Assignments are given in the text. *A*, 11.5 mM ferrocytochrome *c*, pH 6.7, 40°, after 16,384 accumulations (5 hours total time). *Peak numbers* are those of Table II. *B*, 19.4 mM ferricytochrome *c*, pH 6.9, 36°, after 32,768 accumulations (10 hours total time). Peak numbers are those of Table III. *C*, 19.4 mM cyanoferricytochrome *c*, pH 6.9, 36°, after 32,768 accumulations (10 hours total time). Peak numbers are those of Table IV. The peak at about 114 ppm arises from excess free HCN which is in fast exchange with about 0.5% free CN^- (54).

$1/T = 0$ yields a chemical shift of 113 ppm, which eliminates the possibility that this is a heme carbon resonance. Supporting evidence for our assignment is presented in the section under "Deuterium Isotope Effects on Chemical Shifts." The possibility that Peak 18 arises from the coordinated cyanide was eliminated by an examination of the ^{13}C NMR spectrum of a sample of cyanoferricytochrome *c* prepared with $K^{13}CN$ (85 to 90% ^{13}C enriched).

We do not have many connections between the resonances of cyanoferricytochrome *c* and those of ferricytochrome *c* and ferrocytochrome *c*. Nevertheless, the very small temperature dependence of the chemical shifts of Peaks 2 to 11, 13, 14, 16, 17, and 19 of cyanoferricytochrome *c* (Table IV) validates the use of Fig. 5 in assigning these resonances. In the section under "PRFT[3] Method" we assign Peak 2 of cyanoferricytochrome *c* (Fig. 7*C*) to Cζ of the 2 arginine residues. By elimination, Peaks 3 to 5 must arise from Cζ of Tyr-48, Tyr-74, and Tyr-97. Peaks 6 to 11 (six single carbon resonances) are assigned to Cε2 of Trp-59, Cγ of the 4 phenylalanine, and Cγ of 1 of the 2 uncoordinated histidine residues. Peak 19 is assigned to Cγ of Trp-59. Peaks 12 to 17 (six single carbon resonances) are assigned to the remaining 6 nonprotonated aromatic carbons of amino acid residues: Cγ of the 4 tyrosine residues, Cδ2 of Trp-59, and Cγ of 1 of the 2 uncoordinated histidine residues.

Myoglobin—Below pH 9, region a in spectra of the diamagnetic horse carbon monoxide myoglobin (Fig. 4) contains the resonances of Cζ of the 2 arginine and 2 tyrosine residues. The effect of high pH is discussed in another section. The two resonances in region f (at 109.7 ppm and 111.1 ppm) must arise from Cγ of Trp-7 and Trp-14. On the basis of comparisons with ^{13}C NMR spectra of hemoglobins from various species (6), we have tentatively assigned the peak at 111.1 ppm to Trp-14. The remaining 40 nonprotonated aromatic carbons (including those of the heme) of carbon monoxide myoglobin must yield resonances in regions b to e.

Region b in the spectrum of carbon monoxide myoglobin (Fig. 4) covers the range 140 to 147 ppm. Peak intensities suggest that 14 carbons contribute here. No resonances of hen egg white lysozyme fall in this range (Table I). Only one resonance of an amino acid residue of horse heart ferrocytochrome *c* occurs in this range (Fig. 7*A*). However, the 16

[3] The abbreviation used is: PRFT, partially relaxed Fourier transform.

TABLE III

Chemical shifts of nonprotonated aromatic carbons and C^{ζ} of arginine residues of horse heart ferricytochrome c in H_2O (at 36° and 11°) and D_2O (at 36°)

$\delta_A{}^B$ designates a chemical shift, in parts per million downfield from Me_4Si, for the protein in Solvent A at temperature B. Estimated accuracy is ±0.05 ppm. Peak numbers, samples, and spectral conditions are those of Fig. 7*B* (H_2O) and Fig. 10*A* (D_2O), except that for the spectrum at 11° the protein concentration was 12 mM, the pH was 6.7, and the spectrum was recorded under conditions of full proton decoupling.

ASSIGNMENT	PEAK	$\delta^{36}_{H_2O}$	$\delta^{36}_{H_2O}-\delta^{36}_{D_2O}$	$\delta^{36}_{H_2O}-\delta^{11}_{H_2O}$
Tyr-67 C^{ζ}	1	162.6$_7$	−0.0$_3$	−1.1$_3$
Tyr C^{ζ}	2	158.1$_4$	0.0$_6$	a
2 Arg C^{ζ}			0.2$_6$[b]	
Tyr C^{ζ}	3	156.9$_5$	0.1$_0$	−0.1$_0$
Tyr C^{ζ}	4	155.9$_5$	0.1$_3$	0.2$_3$
Phe C^{γ}	5	141.3$_8$	−0.0$_3$	−0.1$_3$
Phe C^{γ}	6	137.8$_5$	−0.0$_6$	−0.1$_3$
Trp-59 $C^{\epsilon 2}$	7	137.6$_2$	0.1$_0$	0.0$_6$
Phe C^{γ}	8	136.1$_7$	0.0$_0$	0.1$_9$
Phe C^{γ}	9	135.9$_2$	0.0$_0$	0.1$_3$
His-26 C^{γ}	10	135.4$_0$	−0.0$_6$	−0.0$_3$
His-33 C^{γ}	11	132.4$_7$	[c]	[d]
Tyr C^{γ}	12	130.6$_1$	−0.0$_6$	0.0$_0$
Tyr C^{γ}	13	127.6$_5$	[e]	a
Tyr C^{γ} + Trp-59 $C^{\delta 2}$	14	127.4$_6$	[e]	a
Tyr C^{γ}	15	126.7$_2$	−0.0$_6$	0.1$_6$
Trp-59 C^{γ}	16	110.1$_2$	0.0$_3$	0.1$_3$

[a] Could not be measured accurately because of partial splitting. The chemical shifts change by 0.1 ppm or less when going from 36° to 11°.

[b] Two-carbon resonance.

[c] Not measured, because the chemical shift is pH-dependent.

[d] This resonance was not detected at 11°.

[e] Could not be measured accurately because of partial splitting. The chemical shifts involved change by 0.1 ppm or less when going from H_2O to D_2O.

TABLE IV

Chemical shifts of nonprotonated aromatic carbons and C^{ζ} of arginine residues of horse heart cyanoferricytochrome c in H_2O (at 36° and 11°) and D_2O (at 36°)

$\delta_A{}^B$ designates a chemical shift, in parts per million downfield from Me_4Si, for the protein in Solvent A at temperature B. Estimated accuracy is ±0.05 ppm. Peak numbers are those of Figs. 7*C* and 9*A*. The chemical shifts of the protein in H_2O at 36° were obtained from the spectrum in Fig. 9*A*. The chemical shifts of the protein in D_2O at 36° were obtained using the same sample and spectral conditions as in Fig. 9*C*, except that a normal spectrum was recorded, using 28,894 accumulations and a recycle time of 2.105 s. For the spectrum at 11°, conditions were as for ferricytochrome *c* at 11° (Table III) except that the protein concentration was 9.6 mM, and 8,192 accumulations were used.

ASSIGNMENT	PEAK	$\delta^{36}_{H_2O}$	$\delta^{36}_{H_2O}-\delta^{36}_{D_2O}$	$\delta^{36}_{H_2O}-\delta^{11}_{H_2O}$
Tyr-67 C^{ζ}	1	160.3$_9$	0.0$_6$	−0.8$_4$
2 Arg C^{ζ}	2	158.0$_8$	0.1$_9$	0.1$_6$
Tyr C^{ζ}	3	157.6$_3$	0.0$_6$	−0.2$_9$
Tyr C^{ζ}	4	156.9$_2$	0.1$_0$	−0.0$_3$
Tyr C^{ζ}	5	156.2$_7$	0.1$_0$	0.1$_6$
a	6	141.0$_6$	−0.0$_3$	−0.1$_0$
a	7	137.7$_8$	b	−0.0$_6$
a	8	137.5$_9$	b	0.3$_2$
Trp-59 $C^{\epsilon 2}$	9	137.0$_4$	0.1$_6$	0.1$_9$
a	10	136.3$_3$	0.0$_0$	0.1$_3$
a	11	135.8$_8$	−0.0$_3$	−0.0$_3$
c	12	131.9$_6$	0.1$_6$	d
c	13	130.3$_5$	−0.0$_6$	0.0$_0$
c	14	129.1$_3$	−0.0$_3$	−0.1$_6$
c	15	128.2$_0$	0.0$_0$	0.6$_1$
c	16	127.6$_2$	−0.0$_6$	0.0$_3$
Trp-59 $C^{\delta 2}$	17	127.0$_4$	0.0$_3$	0.1$_0$
His-18 C^{γ}	18	123.6$_0$	0.2$_3$	−0.9$_3$
Trp-59 C^{γ}	19	109.5$_1$	0.0$_0$	0.0$_6$

[a] Peaks 6 to 8, 10, and 11 are the resonances of C^{γ} of 1 histidine residue (probably His-26) and the 4 phenylalanine residues.

[b] Could not be measured accurately because of partial resolution. The chemical shifts involved change by 0.15 ppm or less when going from H_2O to D_2O.

[c] Peak 12 is tentatively assigned[4] to C^{γ} of His-33. If this assignment is correct, Peaks 13 to 16 must all be C^{γ} resonances of tyrosine residues.

[d] Not detected at 11°.

nonprotonated aromatic carbons of the heme of horse heart ferrocytochrome *c* yield resonances in the range 138.6 to 147.6 ppm (Table II). Therefore, region b in spectra of horse carbon monoxide myoglobin (Fig. 4) should contain the resonances of most of the nonprotonated aromatic carbons of the heme. This expectation can be verified by comparing the spectra of the diamagnetic carbon monoxide myoglobin and the paramagnetic ferrimyoglobin or cyanoferrimyoglobin. We have seen above that there are no narrow heme carbon resonances in the range 138 to 148 ppm in spectra of ferricytochrome *c* (Fig. 7*B*) and cyanoferricytochrome *c* (Fig. 7*C*). A similar behavior is observed in the ^{13}C NMR spectra of paramagnetic myoglobins: region b in the spectra of horse ferrimyoglobin[4] and cyanoferrimyoglobin (Fig. 3*F*) contains one narrow single carbon resonance at 140.3 ppm. Region b in the spectrum of horse carbon monoxide myoglobin probably contains the resonances of 1 amino acid carbon and 13 of the 16 nonprotonated aromatic carbons of the heme.

On the basis of Fig. 5, the nonprotonated aromatic carbons of the heme that do not yield resonances in region b of Fig. 4 are expected to show up in region c. The remaining resonances of region c, and those in regions d and e, must arise from $C^{\epsilon 2}$ and $C^{\delta 2}$ of the 2 tryptophan residues, and C^{γ} of the 7 phenylalanine, 11 histidine, and 2 tyrosine residues (a total of 24 carbons).

Two resonances upfield of region f in spectra of cyanoferrimyoglobins (at about 91 ppm and 101 ppm in Fig. 3*F*) may arise from paramagnetically shifted resonances of 2 of the 16 nonprotonated aromatic carbons of the heme. This extremely tentative assignment is based on ^{13}C chemical shifts of dicyanoferriporphyrins (67).

SELECTIVE PROTON DECOUPLING

In order to determine whether selective proton decoupling can be used to distinguish C^{ζ} resonances of arginine from those of tyrosine, we first examined the ^{13}C NMR spectra of L-arginine and *p*-cresol (both in D_2O) in the absence of proton decoupling. These spectra yielded information about the values of long range scalar couplings of C^{ζ} of arginine to aliphatic hydrogens, and of C^{ζ} of tyrosine to aromatic hydro-

[4] E. Oldfield and A. Allerhand, unpublished results.

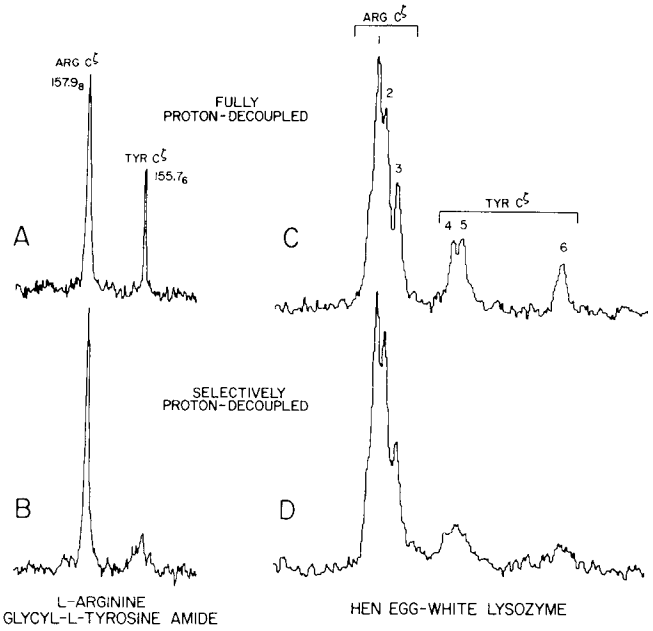


FIG. 8. *A*, resonances of C^{ζ} of arginine and tyrosine in the fully proton-decoupled natural abundance ^{13}C Fourier transform NMR spectrum of 0.29 M glycyl-L-tyrosine amide hydrochloride and 1.13 M L-arginine hydrochloride in D_2O (pH meter reading 3.0) at 37°, recorded at 15.18 MHz, using 16,384 time domain addresses, a 4,000-Hz spectral width, 16 accumulations, a recycle time of 30 s (8 min total time), and a digital broadening of 0.47 Hz. *Numbers* are chemical shifts in parts per million downfield from Me_4Si. Estimated accuracy is ±0.05 ppm. *B*, same as *A*, but selectively proton-decoupled spectrum, at 30°. Low power single frequency ^1H irradiation was set about 3.2 ppm downfield from Me_4Si (see "Experimental Procedure"). *C*, same as *A*, but 14.7 mM hen egg white lysozyme in D_2O (0.1 M NaCl, pH meter reading 3.0), at 44°, recorded with 8,192 time domain addresses, a spectral width of 3,787.9 Hz, 16,384 accumulations, a recycle time of 2.205 s (10 hours total time), and a digital broadening of 0.44 Hz. *Peak numbers* are those of Fig. 6*B*. Chemical shifts are given in Table I. *D*, same sample and conditions as spectrum *C*, but selectively proton-decoupled (as spectrum *B*), at 31°.

gens. Without proton decoupling, the resonance of C^{ζ} of L-arginine in D_2O is a poorly resolved multiplet with an over-all "width at half-height" of about 8 Hz. The largest coupling is probably to H^{δ} (54). In the absence of proton decoupling, the phenolic carbon of *p*-cresol yields a partly resolved 1:2:1 triplet that must result from scalar coupling to 2 equivalent aromatic hydrogens, with a coupling constant of about 8 Hz, and much weaker scalar coupling to the other 2 aromatic hydrogens. The stronger coupling is probably to the hydrogens three bonds removed and not to the ortho hydrogens (54).

If we apply coherent irradiation at the resonance frequency of H^{δ} of arginine residues (about 3.2 ppm downfield from Me_4Si), of low enough power to prevent decoupling of the aromatic protons of tyrosine residues (which have chemical shifts in the range 6.8 to 7.2 ppm), then the ^{13}C NMR spectrum of a protein should yield a partly split feature (with an over-all width of at least 16 Hz) for each C^{ζ} of tyrosine, but a sharp resonance for each C^{ζ} of arginine. In order to avoid the complicating effect of observable scalar couplings of C^{ζ} of arginine to slowly exchanging NH protons, a distinct possibility at low pH (68), D_2O is used as solvent for our selective proton decoupling experiments. We first applied the procedure to a test sample of aqueous glycyl-L-tyrosine amide and L-arginine. In Fig. 8*A* we show the resonances of C^{ζ} of arginine and tyrosine in the fully proton-decoupled ^{13}C NMR spectrum of this sample. In Fig. 8*B* we show the same resonances, but obtained with low power (0.02 G) single frequency proton irradiation, set 3.2 ppm downfield from Me_4Si. At our static magnetic field strength of 14.2 kG, these conditions yield essentially full proton decoupling for C^{ζ} of arginine, but practically no decoupling for C^{ζ} of tyrosine. Although in this experiment the proton irradiation power must be kept low enough to prevent excitation of aromatic proton resonances, it must be sufficiently high to cover possible variations in chemical shifts of arginine protons in proteins. With the proton irradiation power used for Fig. 8*B*, an upfield shift of the proton irradiation frequency from 3.2 ppm to 2.2 ppm caused no increase in the linewidth of the resonance of C^{ζ} of L-arginine.

In Fig. 8*C* we show the region of C^{ζ} of arginine and tyrosine residues in the fully proton-decoupled ^{13}C NMR spectrum of hen egg white lysozyme in D_2O (*Peaks 1* to *6* of Fig. 6*B*). When the selective proton-decoupling conditions of Fig. 8*B* were applied to the protein, the spectrum of Fig. 8*D* was obtained. Clearly, Peaks 4 to 6 of Fig. 8*C* are the resonances of C^{ζ} of the 3 tyrosine residues.

We have also used selective proton decoupling to distinguish the resonances of C^{ζ} of the 2 arginine residues from those of C^{ζ} of the 4 tyrosine residues of horse heart ferrocytochrome *c* (spectrum not shown). Our results indicate that Peak 1 of Fig. 7*A* (a 2-carbon resonance) must be assigned to C^{ζ} of the 2 arginine residues. It follows from the connections presented in the section under "Chemical Exchange" that Peak 2 of ferricytochrome *c* (Fig. 7*B*), which is a 3-carbon resonance, contains contributions from C^{ζ} of 1 tyrosine and C^{ζ} of the 2 arginine residues.

PRFT METHOD

Theoretical and experimental results presented in the preceding paper (5) suggest that resonances of $C^{\delta 2}$ and $C^{\epsilon 2}$ of tryptophan residues can be identified on the basis of their T_1 values. Our use of T_1 values for making assignments depends on the dominance of the ^{13}C-^1H dipolar relaxation mechanism (5). The most important contributions to the ^{13}C-^1H dipolar relaxation of nonprotonated aromatic carbons are those from hydrogens two bonds away (5). The γ carbon of a tyrosine or phenylalanine residue has 4 such hydrogens, C^{γ} of a histidine has 3 when the residue is in the imidazole form (69) and 4 when in the imidazolium form, and $C^{\delta 2}$ of a tryptophan residue has only 1 such hydrogen (Fig. 1*A*). As a result, the T_1 of $C^{\delta 2}$ of a tryptophan residue is much longer than that of C^{γ} of a tyrosine or histidine residue (5). Whenever two classes of carbons have measurably different T_1 values, PRFT spectra (70) can be used to distinguish their resonances (71–73). The PRFT method can also be used for distinguishing resonances of $C^{\epsilon 2}$ of tryptophan residues from those of C^{γ} of phenylalanine and histidine residues, if D_2O is used as solvent (73). In H_2O, there are 2 hydrogens two bonds removed from $C^{\epsilon 2}$ of a tryptophan residue (Fig. 1*A*). In D_2O solution, there will be only 1 hydrogen two bonds removed from $C^{\epsilon 2}$ after the hydrogen attached to $N^{\epsilon 1}$ has been exchanged with deuterium. Because of the relatively small gyromagnetic ratio of ^2H, ^{13}C-^2H dipolar relaxation can be neglected here. Therefore, the identification of resonances of $C^{\epsilon 2}$ of tryptophan residues by the PRFT method is easier if D_2O is the solvent (73).

Fig. 9*A* shows the aromatic region of the "normal" convolution-difference ^{13}C NMR spectrum of horse heart cyanoferricytochrome *c* in H_2O. Here we define a "normal" spectrum as one obtained with the use of 90° radiofrequency pulse excitation, and with the interval between successive 90° pulses

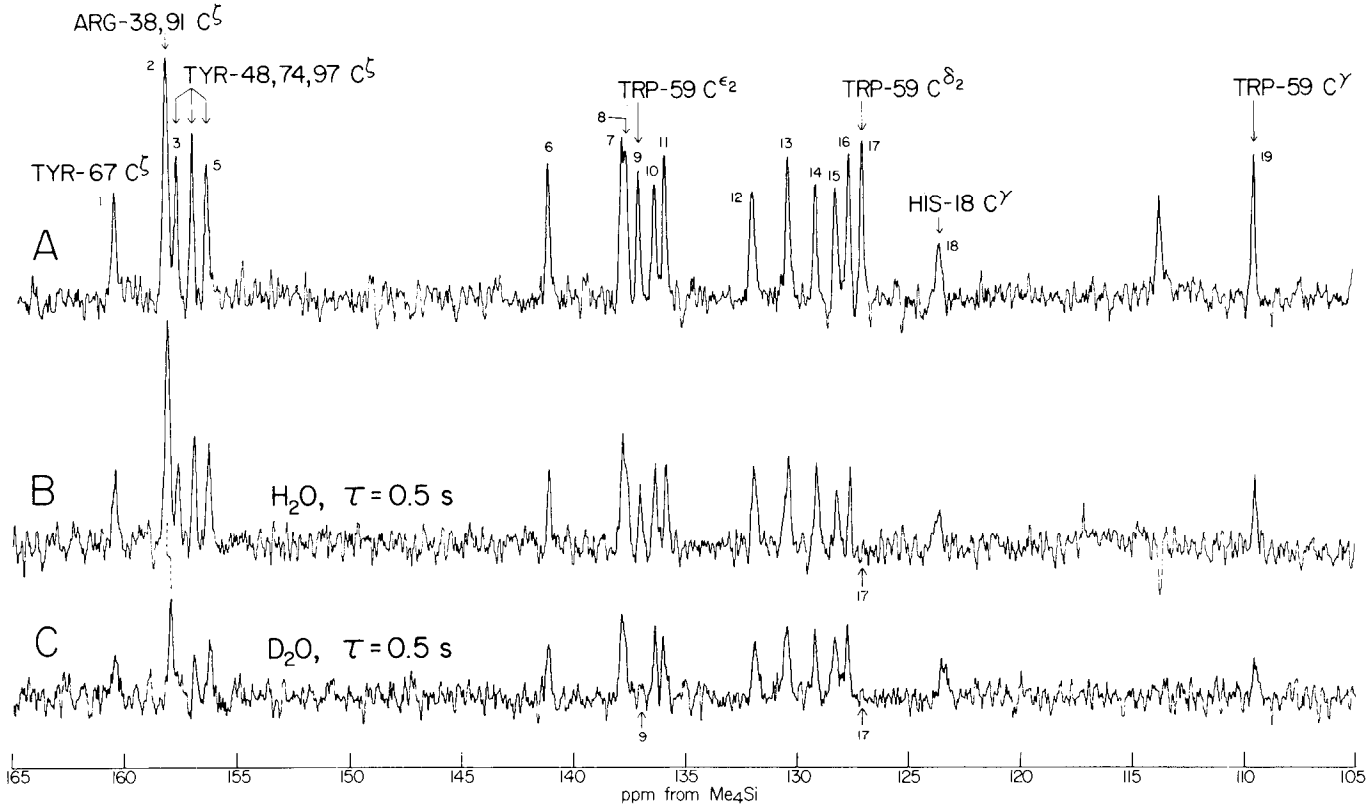


FIG. 9. Region of aromatic carbons and C^{γ} of arginine residues in convolution-difference natural abundance ^{13}C Fourier transform NMR spectra of 14.7 mM horse heart cyanoferricytochrome *c* (in 0.1 M NaCl/0.05 M phosphate buffer), at 36°. Each spectrum was recorded at 15.18 MHz, under conditions of noise-modulated off-resonance proton decoupling, using 8,192 time domain addresses, a spectral width of 4,000 Hz, and 32,768 accumulations. The convolution-difference procedure was carried out with τ_1 = 0.34 s, τ_2 = 0.034 s, K = 0.9. *Peak numbers* are those of Fig. 7*C*. The peak at about 114 ppm arises from excess free HCN which is in fast exchange with about 0.5% free CN^- (54). *A*, normal spectrum of protein in H_2O, pH 6.7. Recycle time was 2.105 s (19.2 hours total time). *B*, PRFT spectrum of same sample as in *A*, with τ = 0.5 s. Recycle time (interval between successive 90° pulses) was 2.605 s (24 hours total time). *C*, as spectrum *B*, but after deuterium exchange, in D_2O, pH meter reading 6.7.

sufficiently long (relative to the pertinent T_1 values) that the peak intensities do not differ measurably from the equilibrium intensities (56, 57). We have already established (see above) that the resonance of $C^{\epsilon 2}$ of Trp-59 must be one of Peaks 6 to 11, and that the resonance of $C^{\delta 2}$ of Trp-59 must be one of Peaks 12 to 17. In Fig. 9*B* we show a PRFT spectrum of the same sample used in Fig. 9*A*, recorded with an interval (τ) of 0.5 s between each 180° radiofrequency pulse and the following 90° pulse. In a PRFT NMR spectrum, a resonance will appear negative (relative to the normal spectrum) if $\tau < T_1 \ln 2$, nulled if $\tau = T_1 \ln 2$, and positive if $\tau > T_1 \ln 2$ (70). Peaks 12 to 16 are positive in the PRFT spectrum of Fig. 9*B*, while Peak 17 is nulled. We conclude that Peak 17 has a considerably longer T_1 value than Peaks 12 to 16. On this basis, it is assigned to $C^{\delta 2}$ of Trp-59. The PRFT spectrum of Fig. 9*B* (protein in H_2O) does not clearly identify the resonance of $C^{\epsilon 2}$ of Trp-59 (one of Peaks 6 to 11). In Fig. 9*C* we show a PRFT spectrum of horse heart cyanoferricytochrome *c* in D_2O, recorded using the same τ value as for the sample in H_2O. Now Peak 9 is nulled, while Peaks 6 to 8, 10, and 11 are positive. We assign Peak 9 to $C^{\epsilon 2}$ of Trp-59.

We have also used the PRFT method to identify the resonances of $C^{\delta 2}$ and $C^{\epsilon 2}$ of Trp-59 of horse heart ferricytochrome *c*, using a sample in D_2O (Fig. 10). It follows from Fig. 10 that Peak 7 is the resonance of $C^{\epsilon 2}$ of Trp-59, and that $C^{\delta 2}$ of Trp-59 contributes to Peak 14 (a 2-carbon resonance). We can now use the connections between Fig. 7, *A* and *B* (discussed in the section under "Chemical Exchange") to conclude that Peak 19 in the spectrum of ferrocytochrome *c* (Fig. 7*A*) is the resonance of $C^{\epsilon 2}$ of Trp-59, and that either Peak 28 or one-half of Peak 27 is the resonance of $C^{\delta 2}$ of Trp-59. A PRFT spectrum of horse heart ferrocytochrome *c* in D_2O (not shown) confirms that Peak 19 is the resonance of $C^{\epsilon 2}$, and indicates that $C^{\delta 2}$ of Trp-59 contributes to Peak 27 (a 2-carbon resonance).

In Fig. 11*A* we show the aromatic region of the normal convolution-difference ^{13}C NMR spectrum of hen egg white lysozyme in D_2O. The PRFT spectra of Fig. 11*B* (τ = 0.6 s) and Fig. 11*C* (τ = 0.5 s) clearly identify the resonances of $C^{\delta 2}$ of the tryptophan residues as Peaks 16 and 18 to 21. Peak 20 is a 2-carbon resonance. Peaks 14, 15, and 17 must be assigned to C^{γ} of the lone histidine and the 3 tyrosine residues. The specific assignment of C^{γ} of His-15 to Peak 15 is presented in the section under "Effect of pH." Because of the poor resolution of most of the resonances of $C^{\epsilon 2}$ of tryptophan and C^{γ} of phenylalanine residues (Peaks 7 to 13), it is somewhat more difficult to identify the resonances of $C^{\epsilon 2}$ of tryptophan residues of lysozyme by the PRFT method. The PRFT spectrum with τ = 0.5 s (Fig. 11*C*) indicates that the 3 phenylalanine residues give rise to Peak 7, one-half of Peak 8-9, and one-half of Peak 11. The resonances of $C^{\epsilon 2}$ of the 6 tryptophan residues are the remaining components in the range 136 to 139 ppm. In this case, the PRFT spectrum with τ = 0.6 s (Fig. 11*B*) is less favorable than the one with τ = 0.5 s for distinguishing the resonances of C^{γ} of phenylalanine from those of $C^{\epsilon 2}$ of tryptophan residues.

In the case of horse heart cytochrome *c* (Fig. 7), the peaks are

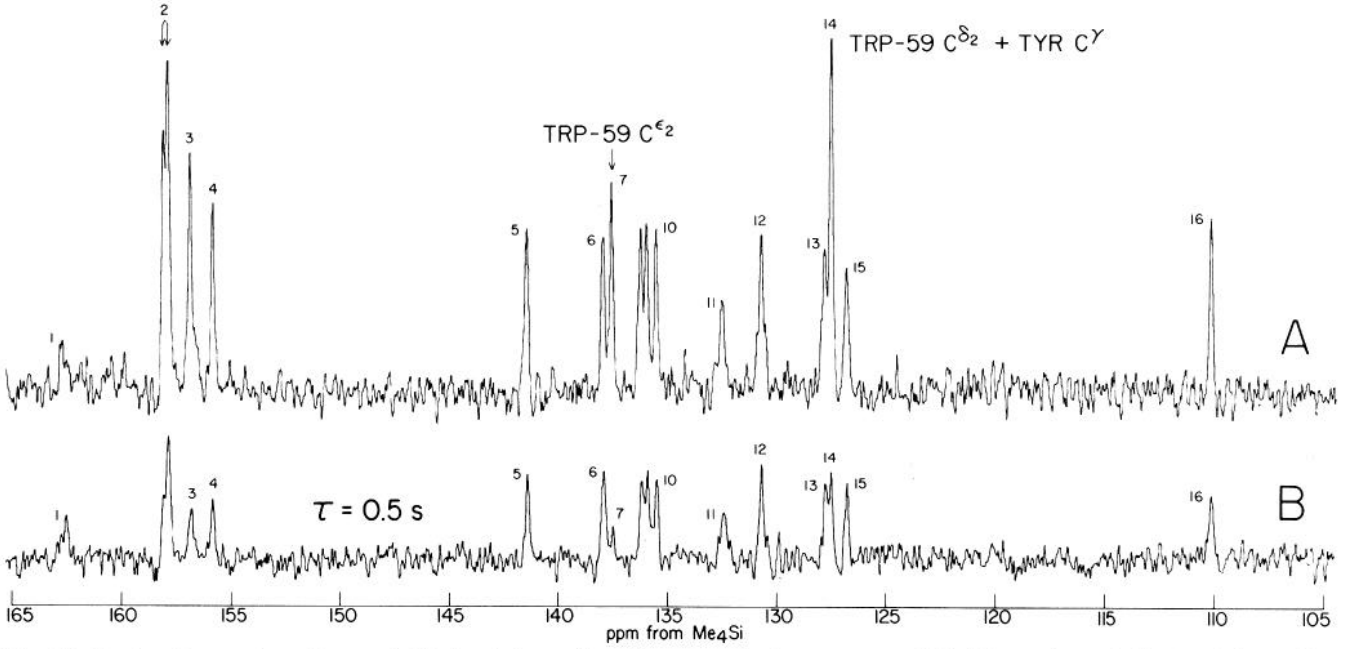


FIG. 10. Region of aromatic carbons and C^{γ} of arginine residues in convolution-difference natural abundance ^{13}C Fourier transform NMR spectra of about 20 mM horse heart ferricytochrome *c* in D_2O (0.1 M NaCl/0.05 M phosphate buffer, pH meter reading 6.7) at 36°. Each spectrum was recorded at 15.18 MHz, under conditions of noise-modulated off-resonance proton decoupling, using 8,192 time domain addresses, and a spectral width of 4,000 Hz. The convolution-difference procedure was carried out as in Fig. 9. *Peak numbers* are those of Fig. 7*B*. Chemical shifts are given in Table III. *Peak 2*, which contains the unresolved resonances of C^{ζ} of 1 tyrosine and the 2 arginine residues when H_2O is the solvent (Fig. 7*B* and Table III), becomes partly split when going to a D_2O solution, as a result of the relatively large upfield shift of the C^{ζ} resonances of the arginine residues (see text). *A*, normal spectrum, after 12,288 accumulations with a recycle time of 2.105 s (7.2 hours total time). *B*, PRFT spectrum with τ = 0.5 s, after 24,576 accumulations with a recycle time of 2.605 s (17.8 hours total time). The vertical gain in spectrum *B* is one-half that of spectrum *A*, to compensate for the 2-fold increase in the number of accumulations.

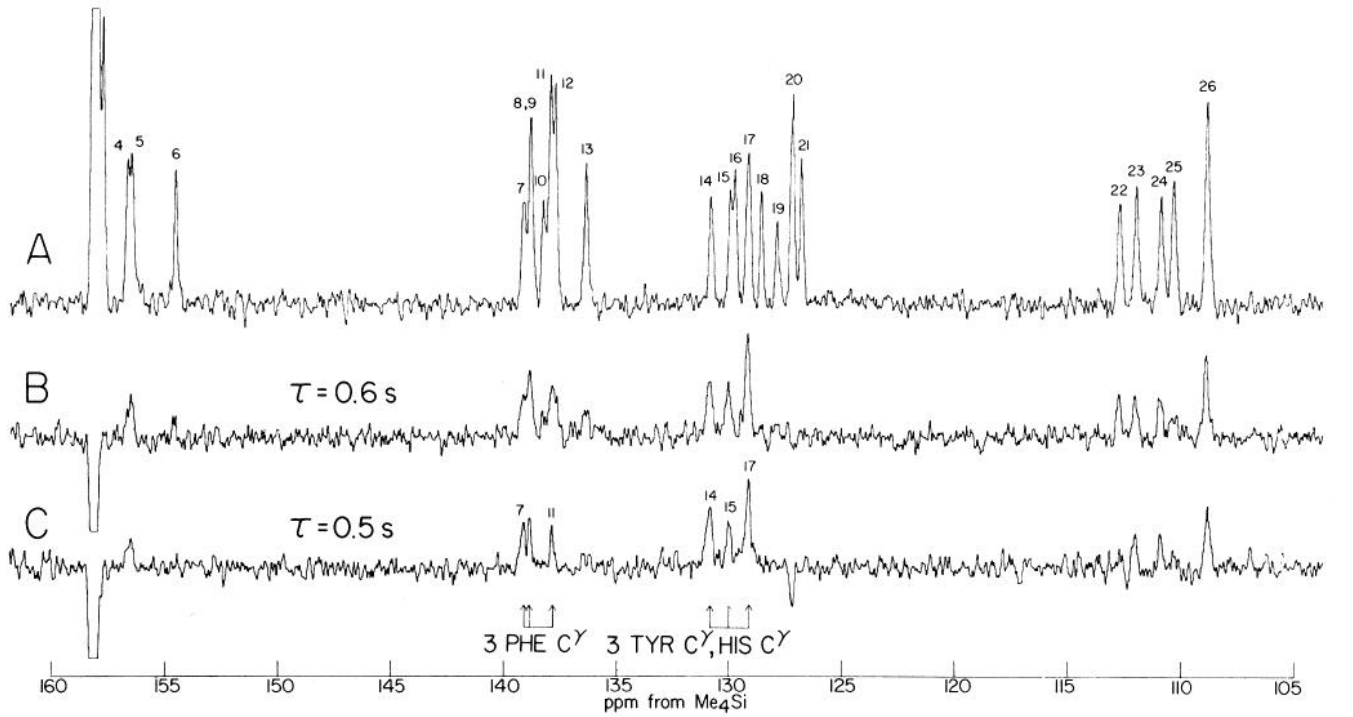


FIG. 11. Region of aromatic carbons and C^{ζ} of arginine residues in convolution-difference natural abundance ^{13}C Fourier transform NMR spectra of 13.8 mM hen egg white lysozyme in D_2O (0.1 M NaCl, pH meter reading 3.1) at 42°. Each spectrum was recorded at 15.18 MHz under conditions of noise-modulated off-resonance proton decoupling, using 8,192 time domain addresses, a spectral width of 3,787.9 Hz, and 49,152 accumulations. The convolution-difference procedure was applied with τ_1 = 0.36 s, τ_2 = 0.033 s, and K = 1.0. *Peak numbers* are those of Fig. 6*B* and Table I. *A*, normal spectrum, recorded using a recycle time of 2.205 s (30 hours total time). *B*, PRFT spectrum with τ = 0.6 s and a recycle time of 2.805 s (38 hours total time). *C*, PRFT spectrum with τ = 0.5 s and a recycle time of 2.705 s (37 hours total time).

relatively well resolved and it is easy to establish by inspection a 1:1 correspondence between peaks in spectra of H_2O and D_2O solutions, even for resonances that undergo significant chemical shift changes when going from H_2O to D_2O (Tables II to IV). However, it is not trivial to establish such a 1:1 correspondence in the region of resonances of C^{γ} of phenylalanine and $C^{\epsilon 2}$ of tryptophan residues of hen egg white lysozyme. Details are given in the next section. Furthermore, as explained above, the PRFT method does not yield a clear identification of the resonances of $C^{\epsilon 2}$ of tryptophan residues of lysozyme in H_2O (73).

The possibility of wrong assignments by the PRFT method arises if some histidine, phenylalanine, or tyrosine residues undergo fast internal rotation about C^{α}—C^{β} or C^{β}—C^{γ}, or both. In the preceding paper (5) we discussed this possibility in detail. As an example, fast internal rotation could lengthen the T_1 value of C^{γ} of a phenylalanine residue to the point that its resonance would be incorrectly assigned to $C^{\epsilon 2}$ of a tryptophan residue. Relaxation data presented in the preceding paper (5) indicate that, in general, aromatic amino acid side chains of native proteins do not have sufficiently fast internal rotation to produce a significant effect on ^{13}C T_1 values. A possible exception is 1 tryptophan residue of hen egg white lysozyme (5). Peak 25 in the spectrum of lysozyme (Fig. 6) has a slightly longer T_1 value than the other resonances of C^{γ} of tryptophan residues (5). The difference is small but reproducible. It can be seen in the PRFT spectrum of Fig. 11*B*. Various experimental results presented below and elsewhere[1] indicate that Peak 25 (Fig. 6) arises either from Trp-62 or from Trp-63. It would be tempting to use the long T_1 value to assign this resonance to Trp-62, because x-ray studies suggest the possibility of internal rotation about the C^{β}—C^{γ} bond of this residue, at least in the crystalline enzyme (49). However, we do not believe that such a specific assignment is justified at this time. Small differences in ^{13}C T_1 values may be caused by a number of factors other than internal rotation (5).

The PRFT spectra of Fig. 9 identify the resonances of C^{ζ} of the 2 arginine residues of horse heart cyanoferricytochrome *c*. When H_2O is the solvent, the T_1 value of Peak 2 is about one-half that of Peaks 1 and 3 to 5 (Fig. 9*B*). In D_2O (Fig. 9*C*), the T_1 value of Peak 2 is about twice as long as in H_2O. The T_1 values of Peaks 1 and 3 to 5 increase by only about 50% when going from H_2O to D_2O. On the basis of arguments presented in the preceding paper (5), these results indicate that Peak 2 (a 2-carbon resonance) should be assigned to C^{ζ} of the 2 arginine residues.

The PRFT method may fail to distinguish between C^{ζ} resonances of tyrosine and arginine residues if the latter are undergoing fast segmental motions (5), or if paramagnetic species of the aqueous environment contribute significantly to the relaxation of solvent-exposed ζ carbons. The PRFT method is particularly ineffective when there are numerous arginine residues which yield overlapping C^{ζ} resonances with different T_1 values, as in the case of lysozyme (5). Selective proton decoupling is a convenient method of general applicability for distinguishing C^{ζ} resonances of arginine from those of tyrosine residues.

DEUTERIUM ISOTOPE EFFECTS ON CHEMICAL SHIFTS

We have examined the effect of deuterium substitution of labile hydrogens on the chemical shifts of nonprotonated aromatic carbons and C^{ζ} of arginine residues of native proteins (Tables I to IV) and some model compounds (Table V). Our results indicate that the resonances of carbons bonded to hydrogen-bearing nitrogens or oxygens undergo a measurable upfield shift when the labile hydrogens are replaced by deuterium. The effect is greatest for C^{ζ} of an arginine residue, which is bonded to 3 hydrogen-bearing nitrogens (Tables I to V). Note that in the case of a titratable residue we report a deuterium isotope effect only if the pH is sufficiently far away from the pK_a to ensure that the residue is fully in one form (Tables I to V). We did not measure the isotope effect for C^{γ} of a histidine residue that was not fully in the imidazole or imidazolium form. Therefore, the isotope effects we do present for C^{γ} of histidine residues (Tables I to V) are not affected by significant displacements in the acid-base equilibria when going from H_2O to D_2O solutions at constant pH meter reading.

Many of the observed deuterium isotope effects on chemical shifts are barely outside our experimental error (Tables I to V). Furthermore, we believe that more proteins must be studied in order to establish the generality of our results. Therefore, at this time deuterium isotope effects alone should not be used for making assignments, but only as supporting evidence for results obtained by other procedures, such as the PRFT method and selective proton decoupling (see above).

We have already assigned C^{ζ} of the 11 arginine residues of hen egg white lysozyme to Peaks 1 to 3 of Fig. 6. When going from H_2O to D_2O solution, this group of peaks undergoes an

TABLE V

Deuterium isotope effects on some ^{13}C chemical shifts of model compounds

Chemical shifts were measured with respect to internal dioxane. δ_A is a chemical shift, in parts per million downfield from Me_4Si, in Solvent A. Estimated accuracy is ±0.05 ppm. Each spectrum was recorded with full proton decoupling and 16,384 time domain addresses. A spectral width of 4,000 Hz and 128 scans were used, except for Gly-His-Gly (see Footnote *e*).

COMPOUND	CARBON	δ_{H_2O}	$\delta_{H_2O}-\delta_{D_2O}$
L-Arginine[a]	C^{ζ}	158.27	0.19
Gly-Tyr amide[b]	Tyr C^{ζ}	155.89	0.13
	Tyr C^{γ}	129.42	0.00
Gly-Phe amide[c]	Phe C^{γ}	137.72	-0.03
L-Tryptophan[d]	$C^{\epsilon 2}$	137.75	0.13
	$C^{\delta 2}$	127.97	0.00
	C^{γ}	108.71	-0.06
Gly-His-Gly[e] pH 3.0	His C^{γ}	129.38	0.09
Gly-His-Gly[e] pH 9.6	His C^{γ}	133.46	-0.03

[a] L-Arginine hydrochloride (0.18 M) at about 36°, pH 6.78 for the H_2O solution, and pH meter reading 6.79 for the D_2O solution. The recycle time was 10 s for the H_2O solution and 60 s for the D_2O solution.

[b] Glycyl-L-tyrosine amide hydrochloride (0.14 M) at about 36°, pH 6.78 for the H_2O solution, and pH meter reading 6.76 for the D_2O solution. The recycle time was 10 s.

[c] Glycyl-L-phenylalanine amide acetate (0.15 M) at 42°, pH 6.74 for the H_2O solution, and pH meter reading 6.77 for the D_2O solution. The recycle time was 15 s.

[d] L-Tryptophan (0.05 M) at about 36°, pH 4.00 for the H_2O solution, and pH meter reading 4.00 for the D_2O solution. The recycle time was 20 s.

[e] Gly-His-Gly (0.05 M) at about 35°. For the D_2O solution, the indicated pH is a pH meter reading. Each spectrum was recorded using a spectral width of 3,787.9 Hz, a recycle time of 15 s, and 256 scans (pH 3) or 512 scans (pH 9.6).

over-all upfield shift of about 0.2 ppm (Table I). An upfield isotope shift of about 0.2 ppm is also observed for C^{ζ} of L-arginine (Table V) and the arginine residues of horse heart ferricytochrome *c* (Table III) and cyanoferricytochrome *c* (Table IV). The upfield shift is about 0.1 ppm in the case of ferrocytochrome *c* (Table II). The resonances of C^{ζ} of tyrosine residues undergo smaller upfield shifts than those of C^{ζ} of arginine residues (Tables I to V).

The resonances of $C^{\epsilon 2}$ of tryptophan residues show upfield isotope shifts of about 0.1 ppm, while those of C^{γ} of phenylalanine residues do not undergo upfield shifts (Tables I to V). The PRFT spectra of hen egg white lysozyme in D_2O (Fig. 11) indicate that Peak 7, one-half of Peak 8-9, and one-half of Peak 11 (Figs. 6*B* and 11*A*) are the resonances of C^{γ} of the 3 phenylalanine residues. The chemical shifts of Peaks 7 and 9 do not change when going from H_2O to D_2O solution, but Peak 8 moves upfield by 0.1_6 ppm (Table I). These results are consistent with our PRFT assignments of Peaks 7, 8, and 9 (in D_2O) to C^{γ} of a phenylalanine, $C^{\epsilon 2}$ of a tryptophan, and C^{γ} of a phenylalanine residue, respectively. Peaks 10 and 13 show upfield deuterium isotope shifts of about 0.1_5 ppm (Table I), consistent with their assignment to $C^{\epsilon 2}$ of tryptophan residues. Peaks 11 and 12 of lysozyme in H_2O (Fig. 6*A*) must arise from the same 4 carbons as Peaks 11 and 12 of lysozyme in D_2O (Fig. 6*B*). Peak 12 of the H_2O solution has about the same chemical shift as Peak 11 of the D_2O solution (Table I). If there is no deuterium isotope effect on the chemical shift of C^{γ} of the phenylalanine residue that contributes to Peaks 11 and 12, then Peak 12 of the H_2O solution (Fig. 6*A*) contains the resonance of this carbon. However, independent evidence for this identification is needed.

Our results (Tables I to V) suggest that C^{γ} of a histidine residue undergoes an upfield isotope shift ($\gtrsim$ 0.1 ppm) only when there is a hydrogen attached to $N^{\delta 1}$. The histidine coordinated to a heme has a hydrogen on $N^{\delta 1}$, and so does an uncoordinated histidine residue when it is in the imidazolium form, but not when it is in the imidazole form (69). For example, His-15 of hen egg white lysozyme at pH 3 is fully in the imidazolium form (13, 14, 61, 62), and its C^{γ} resonance (*Peak 15* of Fig. 6) moves upfield by 0.1_4 ppm when going from H_2O to D_2O, at pH meter reading 3 (Table I). In the next section we assign Peak 23 in the spectrum of horse heart ferrocytochrome *c* (Fig. 7*A*) and Peak 10 in the spectrum of ferricytochrome *c* (Fig. 7*B*) to C^{γ} of His-26. In both the ferrous and ferric states of the protein, the chemical shift of the C^{γ} resonance of His-26 does not undergo a measurable deuterium isotope shift at pH 6.7 (Tables II and III). At this pH, His-26 is fully in the imidazole form (see "Effect of pH"). In contrast, Peak 29 of ferrocytochrome *c* (Fig. 7*A*) and Peak 18 of cyanoferricytochrome *c* (Fig. 7*C*). which have been assigned to C^{γ} of the coordinated His-18 residue (see "Chemical Shifts"), move upfield by 0.1_0 ppm and 0.2_3 ppm, respectively, when going from H_2O to D_2O (Tables II and IV).

Note that the C^{γ} resonances of some of the 6 tryptophan residues of hen egg white lysozyme undergo small upfield shifts when going from H_2O to D_2O (Table I).

EFFECT OF PH

In Fig. 12 we show the effect of pH on the chemical shifts of the nonprotonated aromatic carbons and C^{ζ} of arginine residues of horse heart ferrocytochrome *c*, in the pH range 4.4 to 9.4. The chemical shifts of nonprotonated aromatic carbons of nontitrating amino acid residues and of the heme exhibit a remarkable pH invariance. This is strong evidence for a lack of major conformational variations in horse heart ferrocytochrome *c* in the pH range 4.4 to 9.4. One of the two contributors to Peak 3 (C^{ζ} of a tyrosine) begins to move slightly downfield as the pH is raised to 9.4, a behavior consistent with the onset of deprotonation of a tyrosine hydroxyl (59, 60). However, only Peaks 23 and 24 show major chemical shift variations in the pH range we have studied (Fig. 12). The downfield motion of these two single carbon resonances as the pH is raised from 4.4 is consistent only with assignments to C^{γ} of histidine residues. His-18 does not titrate in the pH range under consideration (74), and in any case we have already assigned Peak 29 to C^{γ} of this residue. Consequently, Peaks 23 and 24 must arise from C^{γ} of His-26 and His-33. We were not able to detect Peak 24 at pH values above 6.7, possiby as a result of broadening caused by traces of paramagnetic ions (75). However, the pH dependence of the chemical shift of this resonance in the pH range 4.4 to 6.7 indicates a histidine with an apparent pK_a of 6 or higher. His-33 of horse heart ferrocytochrome *c* has an apparent pK_a of about 6.5 (25, 27). Therefore, we assign Peak 24 to C^{γ} of His-33. By elimination, Peak 23 must be assigned to C^{γ} of His-26.

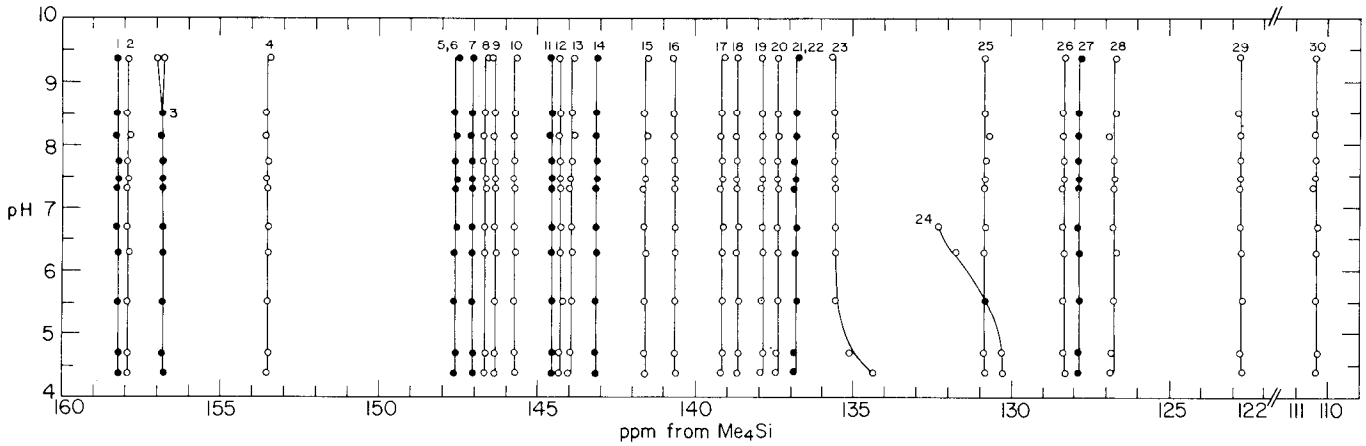


FIG. 12. Effect of pH on the chemical shifts of the nonprotonated aromatic carbons and C^{ζ} of arginine residues of horse heart ferrocytochrome *c* in H_2O (0.1 M KCl/0.05 M phosphate buffer) at 36°. Peak numbers are those of Fig. 7*A*. Single carbon and 2-carbon resonances are indicated by *open* and *solid circles*, respectively. Protein concentrations were in the range 8.7 to 13.4 mM. The number of accumulations per spectrum was 8,192, 16,384, or 32,768. A digital broadening of 0.6 to 0.9 Hz was applied. Other spectral conditions were as in Fig. 7*A*, except that the convolution-difference procedure was not applied. Full proton decoupling was used at pH 6.28.

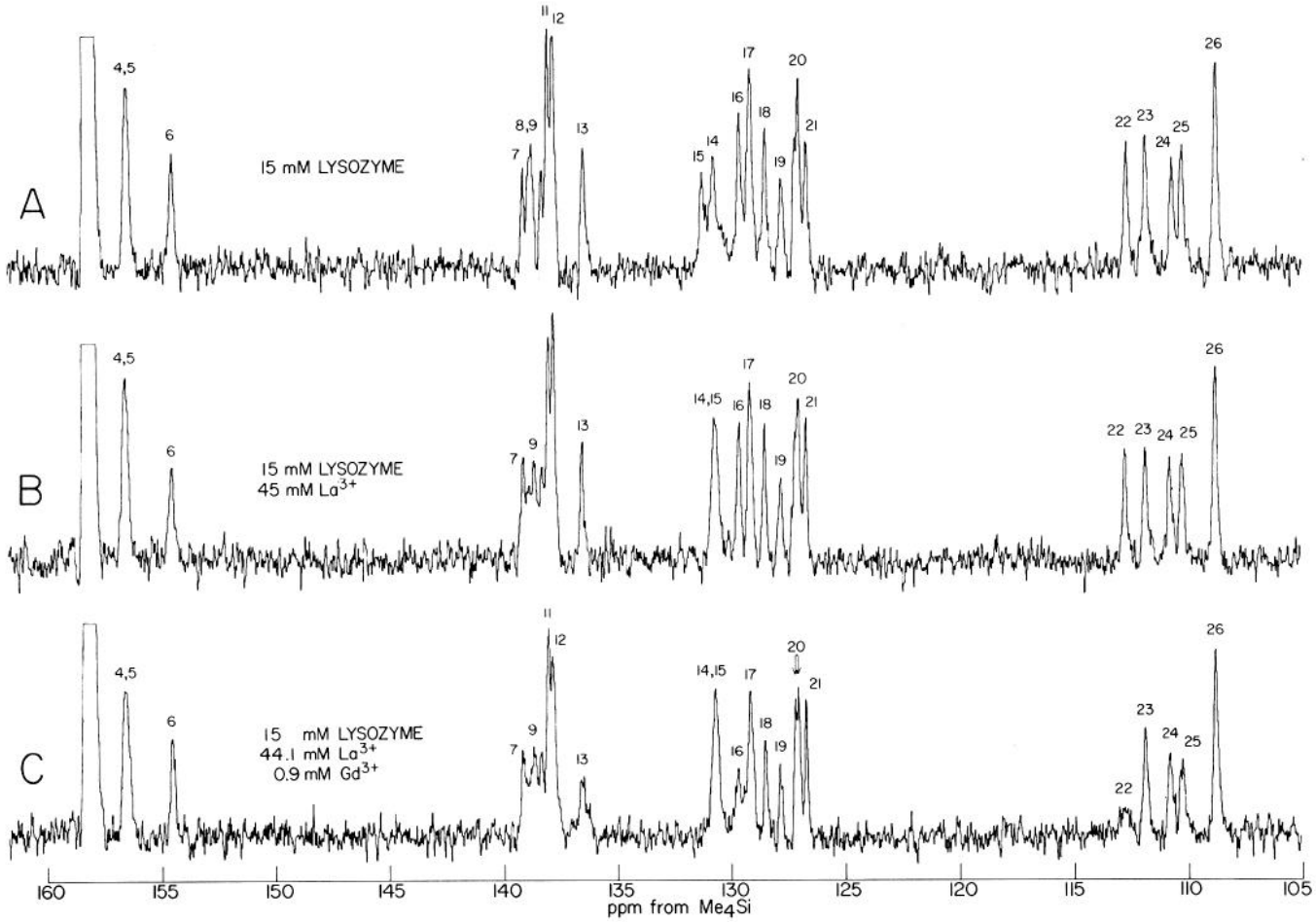


FIG. 13. Effect of lanthanide ions on the region of aromatic carbons and C^{ζ} of arginine residues in the convolution-difference natural abundance ^{13}C Fourier transform NMR spectrum of 15 mM hen egg white lysozyme in H_2O (pH 5.0, about 40°). Each spectrum was recorded at 15.18 MHz, under conditions of noise-modulated off-resonance proton decoupling, using 16,384 time domain addresses, a spectral width of 3,787.9 Hz, 32,768 accumulations, and a recycle time of 2.205 s (20 hours total time). The convolution-difference method was applied with $\tau_1 = 0.72$ s, $\tau_2 = 0.036$ s, and $K = 0.82$. *Peak numbers* are those of Fig. 6*A*. *A*, without lanthanide ions. The C^{γ} resonance of His-15 (*Peak 15*) is upfield of Peak 14 at pH 3 (Fig. 6*A*), but moves downfield when the pH is raised to 5. *B*, with 45 mM $LaCl_3$. *C*, with 44.1 mM $LaCl_3$ and 0.9 mM $GdCl_3$.

Stellwagen and Shulman (25) and Cohen and Hayes (27) identified the resonance of $H^{\epsilon 1}$ of His-33 in the proton NMR spectrum of horse heart ferrocytochrome *c*, and obtained an apparent pK_a of about 6.5 for this residue. These authors did not observe a resolved resonance for $H^{\epsilon 1}$ of His-26. Stellwagen and Shulman (25) identified the resonance of the imidazole NH hydrogen (presumably $H^{\epsilon 2}$) of His-26 in the proton NMR spectrum of horse heart ferrocytochrome *c* in H_2O. The chemical shift of this resonance was invariant in the pH range 6 to 9, but the signal could not be detected outside this pH range (25). We believe that our ^{13}C NMR spectra provide the first evidence for the pK_a of His-26. It follows from the behavior of Peak 23 of Fig. 12 that the pK_a of His-26 is lower than 4.5.

In the case of hen egg white lysozyme, as the pH is raised from 3 (Fig. 6*A*) to 4 (not shown), only Peak 15 moves significantly, in the downfield direction. At pH 5, this peak is already downfield of Peak 14 (Fig. 13*A*). On this basis, Peak 15 of Fig. 6 is assigned to C^{γ} of the lone His-15 residue.

Horse and sperm whale myoglobins have 11 and 12 histidine residues, respectively (48). Many of these histidine residues have apparent pK_a values in the range 5.5 to 7.5 (38, 76, 77). Fig. 4 shows the effect of pH on the aromatic region (and the upfield edge of the carbonyl region, discussed below) in convolution-difference ^{13}C NMR spectra of horse carbon monoxide myoglobin. The chemical shift ranges of Fig. 5 indicate that the C^{γ} resonances of many histidine residues should appear in region d of Fig. 4. Indeed, region d shows a complex pH dependence, with a downfield migration of many resonances as the pH is raised. A detailed study of the pH dependence of the chemical shifts of the nonprotonated aromatic carbons of horse myoglobin is in progress.[5]

Region a in the spectra of horse carbon monoxide myoglobin below pH 9 (Fig. 4, *A* to *C*) contains the resonances of C^{ζ} of the 2 tyrosine and 2 arginine residues. As the pH is raised to 10.1 (Fig. 4*D*) the resonance of 1 carbon moves about 1 ppm downfield of region a, a behavior expected for C^{ζ} of a tyrosine residue which begins to deprotonate significantly (59, 60), presumably the relatively exposed Tyr-103 (78).

The upfield edge of the carbonyl region is also shown in Fig. 4, in order to illustrate the identification of the carbonyl resonance of the NH_2-terminal glycine residue of horse myoglobin. A peak at 168.2 ppm at pH 6.4 (Fig. 4*A*) moves to 168.5 ppm when the pH is raised to 6.7 (Fig. 4*B*) and merges with the main carbonyl band when the pH is raised to 8.1 (Fig. 4*C*). The chemical shift of this resonance (at low pH) is practically the same as that of the carbonyl of NH_2-terminal glycine residues

[5] D. J. Wilbur and A. Allerhand, unpublished results.

(in the $-NH_3^+$ form) in small peptides (79). The pH dependence of the chemical shift is also consistent with that observed for the carbonyl of NH_2-terminal glycine residues of small peptides (79). Furthermore, ferrimyoglobins from pilot whale, Dall porpoise, and harbor seal, all of which have a glycine as the NH_2-terminal residue, have apparent pK_a values for this residue of 7.43, 7.22, and 7.66, respectively (80). We assign the resonance at 168.2 ppm in Fig. 4*A* to the carbonyl of the NH_2-terminal glycine residue of horse carbon monoxide myoglobin. A comparison of ^{13}C NMR spectra of horse and sperm whale myoglobin supports this assignment (see "Proteins from Different Species").

EFFECTS OF PARAMAGNETIC SPECIES

A paramagnetic center can change the chemical shift of a nuclear magnetic resonance. It may also provide an effective relaxation mechanism, thereby causing a line broadening and a decrease in the nuclear spin-lattice relaxation time (64). The paramagnetic shift of a nuclear magnetic resonance can have contributions from a contact shift and a pseudocontact (or dipolar) shift. The latter is proportional to r^{-3}, where r is the distance between the nucleus and the paramagnetic center (64). The paramagnetic contribution to the linewidth and to $1/T_1$ of the nuclear spin is proportional to r^{-6} (64). There are some paramagnetic species (those with relatively long electron relaxation times) that produce negligible shifts but large effects on T_1 and the linewidths of nuclear resonances. These species are called relaxation probes (64). At the other extreme, there are some paramagnetic species (with relatively short electron relaxation times) that cause large shifts but small effects on T_1 and on the linewidths of nuclear resonances. They are called shift probes (64).

The effects of paramagnetic sites on NMR signals have been used extensively in studies of protein solutions (12, 15, 18–23, 32–37, 39, 64, 81–88). For example, there is strong evidence that hen egg white lysozyme has a site in the vicinity of Glu-35 and Asp-52 that strongly binds lanthanide ions (18, 83, 89), Co^{2+} (12), Mn^{2+} (84), and Cu^{2+} ions (90). Proton NMR studies have been reported on the lysozyme complexes of Co^{2+} (12), Mn^{2+} (84, 85), Eu^{2+} (83), Gd^{3+} (18, 19, 83, 85–87), and Pr^{3+} (18). Some assignments of proton NMR resonances of lysozyme have been made with the use of Co^{2+} (12) and Pr^{3+} (18) as shift probes. The work of Williams and co-workers (18, 19) on the effect of Gd^{3+} on the proton NMR spectrum of hen egg white lysozyme suggests the use of Gd^{3+} as a relaxation probe for assigning some ^{13}C resonances of the protein. In this study, we report some initial assignments that have resulted from the use of low concentrations of Gd^{3+}. The specific sample conditions chosen here closely parallel those used by Williams and co-workers (18, 19) in their proton NMR studies. In Fig. 13*A* we show the aromatic region of the convolution-difference ^{13}C NMR spectrum of 15 mM hen egg white lysozyme at pH 5. In Fig. 13*B* we show the effect of a 45 mM concentration of the diamagnetic La^{3+} ion on the spectrum. On the basis of published values of the association constant (83, 86, 89), we estimate that under the conditions of Fig. 13*B* only about 15 to 25% of the protein is not binding lanthanide ions. A comparison of the chemical shifts of Fig. 13*B* with those of Fig. 13*A* indicates that La^{3+} binding causes a relatively large change in the chemical shift of C^γ of His-15 (Peak 15), a minor change in the chemical shift of Peak 8, and no other measurable changes. We conclude that binding of La^{3+} (and by inference binding of Gd^{3+}) causes no major changes in the conformation of lysozyme. In Fig. 13*C* we show the effect of replacing about 2% of the La^{3+} ions by Gd^{3+} without changing the total lanthanide concentration.

We have used the published crystal coordinates of hen egg white lysozyme (49) and the coordinates of Gd^{3+} in the crystalline Gd^{3+}-lysozyme complex (kindly supplied by Dr. C. C. F. Blake and Dr. L. N. Johnson, Department of Zoology, Oxford University, Oxford, England) to compute the distances of the gadolinium to all of the nonprotonated aromatic carbons. We first consider the γ carbons of the 6 tryptophan residues (*Peaks 22* to *26* of Fig. 13). Only Peak 22 is significantly broadened upon addition of a small concentration of Gd^{3+} (Fig. 13*C*). C^γ of Trp-108 is much closer to the bound Gd^{3+} ions than the γ carbons of the other tryptophan residues. The values of r^6 are about 5, 6, 10, 50, and 100 times greater for the γ carbons of Trp-63, Trp-62, Trp-111, Trp-28, and Trp 123, respectively, than for C^γ of Trp-108. We assign Peak 22 to C^γ of Trp-108. We are now in the process of verifying this assignment by other methods.

We have shown in the section under "PRFT Method" that Peaks 16 and 18 to 21 of Figs. 6 and 13 arise from $C^{\delta 2}$ of the 6 tryptophan residues of lysozyme. Clearly, Peak 16 is more affected by Gd^{3+} than Peaks 18 to 21 (Fig. 13*C*). On the basis of r^6 values,[1] Peak 16 is assigned to $C^{\delta 2}$ of Trp-108. As we increase the Gd^{3+}:La^{3+} ratio above the value of Fig. 13*C*, additional peaks begin to broaden. A series of spectra at different Gd^{3+}:La^{3+} ratios and constant lanthanide concentration yields numerous additional assignments.[1]

SPECIFIC CHEMICAL MODIFICATIONS

A comparison of the ^{13}C NMR spectrum of an unmodified protein with that of a chemically modified version of the same protein may yield specific assignments, if the chemical modification does not produce a significant conformational reorganization. The example presented below satisfies this criterion.

The indole ring (Fig. 1*A*) of Trp-62 of hen egg white lysozyme can be quantitatively oxidized to oxindole (Fig. 1*B*) by treating the protein with a stoichiometric amount of *N*-bromosuccinimide (52). Under the mild reaction conditions given by Hayashi *et al.* (52), the other 5 tryptophan residues are not attacked (52, 91). The results of Takahashi *et al.* (92) and our ^{13}C NMR spectra (see below) indicate that the conformation of oxindolealanine-62-lysozyme does not differ significantly from that of unmodified lysozyme. Oxidation of a tryptophan to an oxindolealanine residue converts C^γ from a nonprotonated aromatic carbon to a methine aliphatic carbon (Fig. 1). The effect on the aromatic region of the spectrum should be the disappearance of the C^γ resonance of the oxidized tryptophan residue. On the basis of the ^{13}C chemical shifts of aqueous oxindole (Fig. 2), we expect that $C^{\delta 2}$ of an oxindolealanine residue (Fig. 1*B*) will not be shifted outside the range of $C^{\delta 2}$ resonances of tryptophan residues (*Peaks 16* and *18* to *21* of Fig. 6), while $C^{\epsilon 2}$ will move from the range 136 to 139 ppm to about 144 ppm. Finally, $C^{\delta 1}$ of a tryptophan residue (Fig. 1*A*) is a methine aromatic carbon which therefore yields a broad resonance not present in our convolution-difference spectra, while $C^{\delta 1}$ of an oxindolealanine residue (Fig. 1*B*) should yield a narrow resonance in the carbonyl region. On the basis of the spectrum of oxindole (Fig. 2), this resonance should have a chemical shift of about 182 ppm, which is well downfield of any carbonyl resonances of unmodified lysozyme at low pH (see below).

In Fig. 14 we compare the aromatic regions in the convolu-

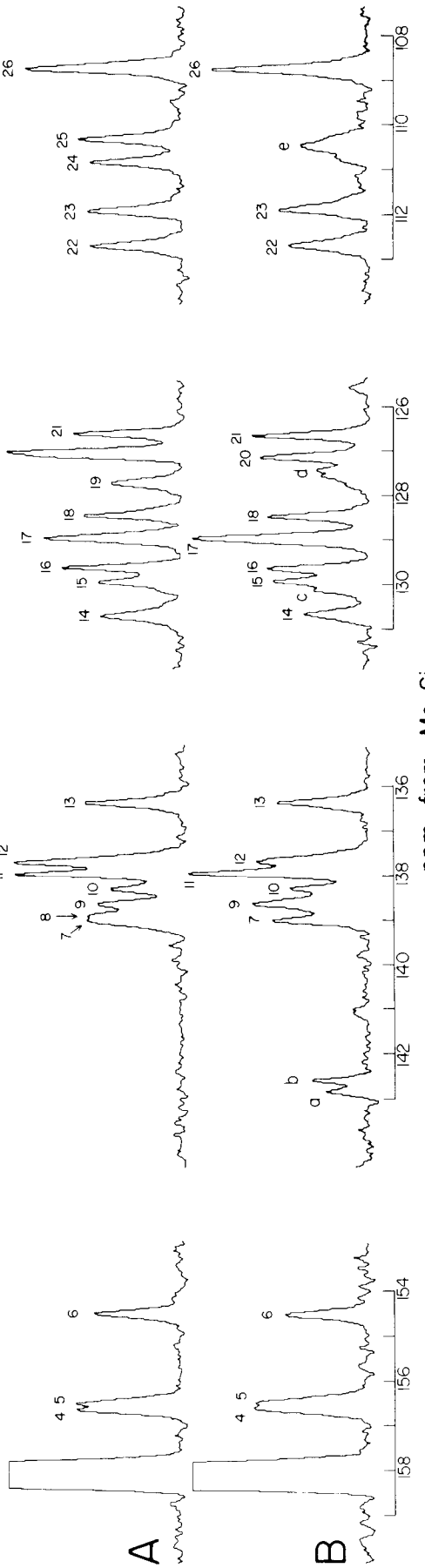


FIG. 14. Region of aromatic carbons and C^{ζ} of arginine residues in the convolution-difference natural abundance ^{13}C Fourier transform NMR spectra of hen egg white lysozyme and oxindolealanine-62-lysozyme. Each spectrum was recorded at 15.18 MHz under conditions of noise-modulated off-resonance proton decoupling, using 8,192 time domain addresses, a spectral width of 3,787.9 Hz, 65,536 accumulations, and a recycle time of 2.205 s (40 hours total time). The convolution-difference method was applied with $\tau_1 = 0.36$ s, $\tau_2 = 0.033$ s, $K = 0.9$. *A*, 14.6 mM lysozyme in H_2O (0.1 M NaCl, pH 3.1, 44°). *Peak numbers* are those of Fig. 6*A* and Table I. *B*, 14.1 mM oxindolealanine-62-lysozyme in H_2O (0.1 M NaCl, pH 3.1, 42°). Peak designations are discussed in the text.

tion-difference ^{13}C NMR spectra of unmodified hen egg white lysozyme (Fig. 14*A*) and oxindolealanine-62-lysozyme (Fig. 14*B*). The carbonyl regions (without applying the convolution-difference method) are shown in Fig. 15. The peak at 181.9 ppm in the spectrum of oxindolealanine-62-lysozyme (Fig. 15*B*) has the intensity of a 1-carbon resonance and no counterpart in the spectrum of the unmodified protein (Fig. 15*A*). This peak is assigned to $C^{\delta 1}$ of the oxindolealanine-62 residue. Consider now the region of C^{γ} resonances of tryptophan residues (*Peaks 22* to *26* of Fig. 14*A*). Peaks 22, 23, and 26, which arise from 4 carbons, are completely unaffected by the conversion to the oxindolealanine-62 form. However, instead of Peaks 24 and 25 of the intact protein, the oxindole derivative yields Peak e (Fig. 14*B*). The new resonance has an integrated intensity of about 1.1 to 1.5 carbons and a chemical shift of 110.4 ppm, a value between those of Peaks 24 and 25 of intact lysozyme (Table I). Trp-62 gives rise to Peak 24 or 25 of intact lysozyme (Fig. 14*A*). The second contributor to Peaks 24 and 25 (which yields *Peak e* in the spectrum of oxindolealanine-62 lysozyme, Fig. 14*B*), is a tryptophan residue whose chemical shifts are influenced by the modification of Trp-62. On the basis of the proximity of the side chains of residues 62 and 63 (49), we tentatively identify Trp-63 as the second contributor to Peaks 24 and 25. Strong independent evidence for this assignment is presented elsewhere.[1] Note that Peak e of Fig. 14*B* is relatively broad. We may have here unresolved chemical shift nonequivalence at Trp-63, as an indirect result of the stereoisomerism (or other nonequivalence) at oxindolealanine-62 (see below).

In the region of resonances of C^{γ} of phenylalanine and $C^{\epsilon 2}$ of tryptophan residues (*Peaks 7* to *13* of Fig. 14*A*), Peak 8 and one of the two contributors to Peak 12 of unmodified lysozyme (Fig. 14*A*) are replaced by Peaks a, b, and a contribution to Peak 9 when going to oxindolealanine-62-lysozyme (Fig. 14*B*). The sum of the intensities of Peaks a and b corresponds to 1 carbon. On the basis of their chemical shifts and total intensity, these peaks must both be assigned to $C^{\epsilon 2}$ of the oxindolealanine-62 residue. There are two nonequivalent forms of oxindoleala-

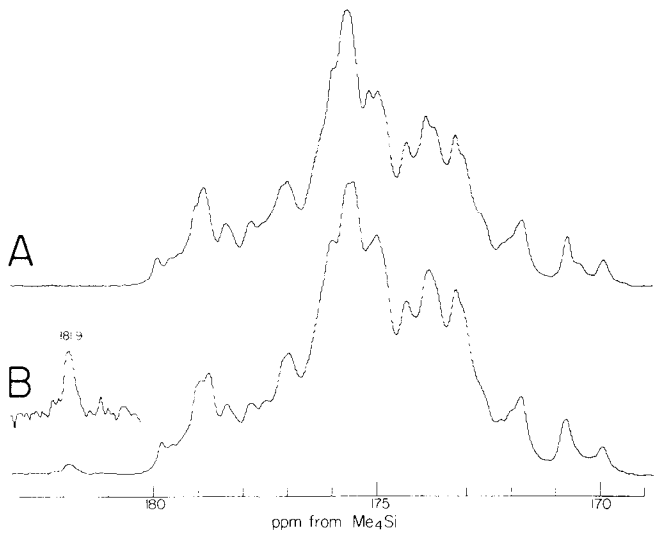


FIG. 15. Carbonyl region in the natural abundance ^{13}C Fourier transform NMR spectra of hen egg white lysozyme (*A*) and oxindolealanine-62-lysozyme (*B*). Samples and time domain NMR data are those of Fig. 14. Each spectrum was processed with 0.88-Hz digital broadening, and without applying the convolution-difference procedure. The *inset* at 181.9 ppm in spectrum *B* was obtained from a convolution-difference spectrum (whose aromatic region is shown in Fig. 14*B*).

nine-62-lysozyme, possibly as a result of stereoisomerism at C^{γ} of the oxindole ring (Fig. 1*B*). By elimination, $C^{\epsilon 2}$ of Trp-63 of the modified protein contributes to Peak 9 (Fig. 14*B*). Peak 8 and one-half of Peak 12 (a 2-carbon resonance) of intact lysozyme (Fig. 14*A*) are assigned to $C^{\epsilon 2}$ of Trp-62 and Trp-63, but not on a 1:1 basis.

Consider now the region of resonances of C^{γ} of histidine and tyrosine and $C^{\delta 2}$ of tryptophan residues (*Peaks 14* to *21* of Fig. 14*A*). Oxidation of Trp-62 does not affect the resonances of lysozyme that contribute to Peaks 14 to 18 and 21, but Peak 19 and one-half of Peak 20 (Fig. 14*A*) are replaced by Peaks c, d, and a contribution to Peak 17 (Fig. 14*B*). These effects are quite reproducible. The broad Peak d has the intensity of a 1-carbon resonance. Its chemical shift of 127.5 ppm is between those of Peaks 19 and 20 of intact lysozyme (Table I). The arguments used above to assign Peaks 24 and 25 of lysozyme to C^{γ} of Trp-62 and Trp-63 (but not on a 1:1 basis) can now be invoked to assign Peak 19 and one-half of Peak 20 to $C^{\delta 2}$ of these tryptophan residues, again not on a 1:1 basis.

The chemical shifts of all of the nonprotonated aromatic carbons of lysozyme, except for those of Trp-62 and Trp-63, remain unchanged when Trp-62 is converted to oxindolealanine. This result indicates that the conformation of lysozyme is not affected significantly by the oxidation of Trp-62.

PROTEINS FROM DIFFERENT SPECIES

Some assignments of proton resonances of proteins have been made from comparisons of spectra of analogous proteins from different species (25, 27). This approach can also be used for assigning ^{13}C resonances.

The aromatic regions of the convolution-difference ^{13}C NMR spectra of sperm whale and horse cyanoferrimyoglobins are shown in Fig. 3, *E* and *F*, respectively. The upfield edge of the carbonyl regions is also shown. The peak most upfield in the carbonyl region (at 168.7 ppm) in the spectrum of horse cyanoferrimyoglobin at pH 6.8 (Fig. 3*F*) is also present in the spectra of horse ferrimyoglobin (not shown) and horse carbon monoxide myoglobin (Fig. 4*B*). We have already assigned this resonance to the carbonyl of the NH_2-terminal glycine residue (see "Effect of pH"). Supporting evidence for this assignment comes from a comparison of the spectra of sperm whale and horse myoglobins. On the basis of ^{13}C chemical shifts of small peptides (58, 93), Val-1 (in the $-NH_3^+$ form) of sperm whale myoglobins is expected to yield a carbonyl resonance about 1 to 2 ppm downfield from that of a Gly-1 (in the $-NH_3^+$ form), and therefore right at the upfield edge of the main carbonyl band. Indeed, the resonance at 168.7 ppm in the spectrum of horse cyanoferrimyoglobin (Fig. 3*F*) has no counterpart in the spectrum of sperm whale cyanoferrimyoglobin (Fig. 3*E*).

There are only two changes in the aromatic amino acid residues when going from sperm whale to horse myoglobin (48): the loss of His-12 (which becomes Asn-12) and the replacement of Tyr-151 by Phe-151. We must also consider, however, the loss of 2 of the 4 arginine residues (48). In region a (Fig. 3), there should be a decrease from 7 carbons (C^{ζ} of 3 tyrosine and 4 arginine residues) to 4 carbons (C^{ζ} of 2 tyrosine and 2 arginine residues) when going from sperm whale (Fig. 3*E*) to horse (Fig. 3*F*). Indeed, there is a change from two 2-carbon resonances and one 3-carbon resonance (Fig. 3*E*) to one 3-carbon resonance and one single-carbon resonance (Fig. 3*F*). We do not have any specific assignments for region a at this time. A comparison of Fig. 3, *E* and *F* does not automatically yield the assignments of the resonances of variant aromatic residues, even though the number of differences between the aromatic residues of horse and sperm whale myoglobin is very small. The resonances of C^{γ} of some invariant histidine residues may have different chemical shifts in spectra of the two species at pH 6.8 (Fig. 3, *E* and *F*), as a result of differences in pK_a values of analogous histidine residues in sperm whale and horse myoglobins. A complete study of the pH dependence of the ^{13}C NMR spectra of both myoglobins may yield the assignments of C^{γ} and C^{ζ} of Tyr-151 and C^{γ} of His-12 of the sperm whale protein, and also the assignment of C^{γ} of Phe-151 of the horse protein.

In Fig. 16 we compare the aromatic region of the convolution-difference ^{13}C NMR spectra of cytochrome *c* from *Candida krusei* and from horse heart, both at pH 5.5. Chemical shifts are given in Table VI. These spectra illustrate the sensitivity of our NMR instrument, both in concentration-limited and sample-limited studies. A sample with about 2.5 g of horse heart cytochrome *c* yielded single carbon resonances in 1 hour of signal averaging (Fig. 16*B*). When using about 0.5 g of *C. krusei* cytochrome *c*, single carbon resonances could be observed after 20 hours of signal accumulation (Fig. 16*A*).

When going from horse to *C. krusei* cytochrome *c*, Phe-46 becomes Tyr-52, there are 2 new aromatic residues (Phe-4 and His-45), and the number of arginine residues increases from 2 to 4 (48). On the basis of our results for horse heat ferricytochrome *c*, Peaks 1 to 21 of *C. krusei* ferricytochrome *c* (Fig. 16*A*) should arise from C^{ζ} of the 4 arginine residues and 20 of the 21 nonprotonated aromatic carbons of amino acid residues. The exception is C^{γ} of the coordinated His-24 residue. By analogy with horse heart ferricytochrome *c*, Peak 1 (Fig. 16*A*) is assigned to C^{ζ} of Tyr-73 (analogous to Tyr-67 of the horse protein). Peaks 2 to 6 must arise from C^{ζ} of the 4 arginine residues and the remaining 4 tyrosine residues. The signal to noise ratio of Fig. 16*A* is inadequate for a definite carbon count in this region. On the basis of a comparison with the spectrum of horse heart ferricytochrome *c*, we tentatively assign Peak 6 of *C. krusei* ferricytochrome *c* (Fig. 16*A*) to C^{ζ} of Tyr-52. In order to make this assignment we have assumed that the ζ carbons of the invariant 3 tyrosine and 2 arginine residues have

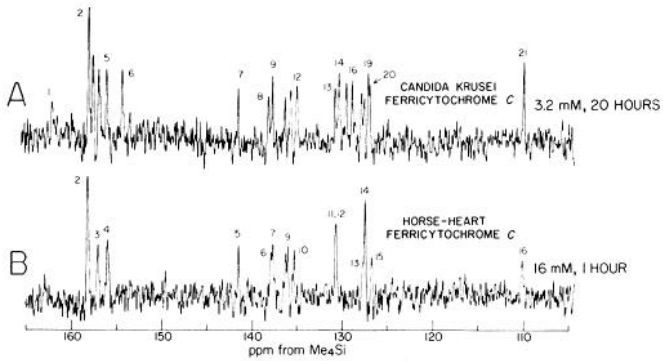


FIG. 16. Region of aromatic carbons and C^{ζ} of arginine residues in the convolution-difference natural abundance $^{13}C^{\zeta}$ Fourier transform NMR spectra of ferricytochromes *c* from *Candida krusei* and horse heart, in H_2O (0.1 M NaCl/0.05 M phosphate buffer), at pH 5.5, 36°. Each spectrum was recorded at 15.18 MHz, under conditions of noise-modulated off-resonance proton decoupling, using 8,192 time domain addresses, a 4,000-Hz spectral width, and a recycle time of 1.105 s. The convolution-difference method was applied as in Fig. 9. Chemical shifts and assignments are given in the text and in Table VI. *A*, 3.2 mM *C. krusei* ferricytochrome *c*, after 65,536 accumulations (20.1 hours total time). *B*, 16.2 mM horse heart ferricytochrome *c*, after 3,258 accumulations (60 min total time).

TABLE VI

Chemical shifts of nonprotonated aromatic carbons and C^{ζ} of arginine residues of ferricytochromes c from Candida krusei and horse heart at pH 5.5

Peak numbers are those of Fig. 16, *A* (*C. krusei*) and *B* (horse heart). The chemical shifts were obtained from the spectra in Fig. 16 and are given in parts per million downfield from Me_4Si. Estimated accuracy is ±0.1 ppm. Assignments for *C. krusei* are given in the text. Assignments for horse heart ferricytochrome *c* at pH 5.5 are based on Table III (pH 6.9).

CANDIDA KRUSEI			HORSE-HEART		
Assignment	Peak	Chemical Shift	Assignment	Peak	Chemical Shift
Tyr-73 C^{ζ}	1	162.2	Tyr-67 C^{ζ}	1	162.7[a]
	2	158.1	Tyr C^{ζ} + 2 Arg C^{ζ}	2	158.1
4 Arg C^{ζ}	3	157.7	Tyr C^{ζ}	3	156.9
4 Tyr C^{ζ}	4	157.0	Tyr C^{ζ}	4	155.9
	5	156.2			
	6	154.3			
	7	141.4	Phe C^{γ}	5	141.3
4 Phe C^{γ}	8	138.1	Phe C^{γ}	6	137.8
Trp-65 $C^{\varepsilon 2}$	9	137.6	Trp-59 $C^{\varepsilon 2}$	7	137.6
	10	136.3	Phe C^{γ}	8	136.1
	11	135.6	Phe C^{γ}	9	135.9
His-32 C^{γ}	12	134.9[b]	His-26 C^{γ}	10	135.1[c]
His-39 or 45 C^{γ}	13	130.7[d]	His-33 C^{γ} + Tyr C^{γ}	11,12	130.6[e]
Tyr C^{γ}	14	130.3			
His-45 or 39 C^{γ}	15	129.5[d]	Tyr C^{γ}	13	127.7
Tyr-52 C^{γ}	16	128.9			
	17	127.8	Tyr C^{γ} + Trp-59 C^{γ}	14	127.4
3 Tyr C^{γ}	18	127.5			
Trp-65 $C^{\delta 2}$	19	127.1	Tyr C^{γ}	15	126.7
	20	126.9			
Trp-65 C^{γ}	21	109.9	Trp-59 C^{γ}	16	110.1

[a] Barely detectable in Fig. 16*B* because of poor signal to noise ratio. At pH 6.9 (Peak 1 in Fig. 7*B*), the chemical shift of this resonance is 162.67 ppm (Table III).

[b] One hundred thirty five and two-tenths parts per million at pH 6.9.

[c] pH-dependent chemical shift (see Peak 10 of Table III).

[d] At pH 6.9 there are no resonances at 130.7 ppm and 129.5 ppm. Instead, there is a resonance at 133.6 ppm and perhaps one at about 131.4 ppm. A higher signal to noise ratio then that of Fig. 16*A* is needed to determine the pH dependence of the chemical shifts of C^{γ} of His-39 and His-45 of *C. krusei* cytochrome *c*.

[e] The C^{γ} resonance of His-33 (Peak 11) has a pH-dependent chemical shift (see Peak 11 of Table III).

about the same chemical shifts in the two proteins, and that the ζ carbons of the 2 additional arginine residues have chemical shifts comparable to those of the invariant arginine residues. Additional experiments, such as selective proton decoupling, are necessary to confirm our assignment.

Peaks 7 to 12 in the spectrum of *C. krusei* ferricytochrome *c* (Fig. 16*A*) must arise from $C^{\varepsilon 2}$ of Trp-65, C^{γ} of the 4 phenylalanine residues, and C^{γ} of 1 histidine residue (Fig. 5), a total of 6 carbons for six peaks. The chemical shifts of Peaks 7 to 11 (Fig. 16*A*) do not change when the pH is raised from 5.5 to 6.8, but Peak 12 moves downfield by 0.2$_4$ ppm, a behavior similar to that of the analogous His-26 of the horse protein. On this basis, Peak 12 of Fig. 16*A* is assigned to C^{γ} of His-32 of *C. krusei* ferricytochrome *c*. The chemical shift of this resonance is nearly the same as that of C^{γ} of His-26 of the horse protein (Table VI). Fig. 16 does not yield the specific assignment of C^{γ} of Phe-4 of *C. krusei* ferricytochrome *c* (missing from the sequence of the horse protein) nor the specific assignment of C^{γ} of Phe-46 of the horse protein (replaced by Tyr-52 when going to *C. krusei*), because Peaks 8 to 11 of Fig. 16*A* and Peaks 6 to 9 of Fig. 16*B* are close together.

Peaks 13 to 20 in the spectrum of *C. krusei* ferricytochrome *c* (Fig. 16*A*) must arise from $C^{\delta 2}$ of Trp-65, C^{γ} of His-39, C^{γ} of His-45, and C^{γ} of the 5 tyrosine residues, a total of 8 carbons for eight peaks. From the effect of pH, we assign Peaks 13 and 15 to C^{γ} of His-39 and His-45, but not on a 1:1 basis. The comparison of the spectra of horse and *C. krusei* ferricytochromes *c* strongly suggests that Peak 16 in the spectrum of the latter (Fig. 16*A*) arises from C^{γ} of Tyr-52.

CHEMICAL EXCHANGE

If a solution contains two forms of a protein which interchange sufficiently fast (see below), then it may be possible to establish a 1:1 correspondence between the resonances of the two forms. As a result, spectral assignments for one state will automatically yield assignments for the other. As an illustration, we shall consider the ferrous and ferric states of horse heart cytochrome *c*.

Ferrocytochrome *c* and ferricytochrome *c* undergo fast electron transfer in solution, with a rate that is dependent on ionic strength (30). The results of Gupta *et al.* (30) can be used to estimate that under our sample conditions (about 40°, ionic strength of 0.2) the rate constant (k) for the electron transfer process is about $7 \times 10^3\ M^{-1}\ s^{-1}$. The electron transfer produces

a fluctuation in the chemical shifts of the ^{13}C resonances between the values in the two oxidation-reduction states. It is useful to consider the cases of fast and slow exchange, defined by $2\pi|\nu_0 - \nu_r| \ll kC_t$ and $2\pi|\nu_0 - \nu_r| \gg kC_t$, respectively (94). Here ν_0 and ν_r are the resonance frequencies (in hertz) for 1 carbon of the oxidized and reduced species, respectively, and C_t is the total molar concentration of cytochrome *c*.

Peaks 1 to 3, 15, 19 to 28, and 30 of ferrocytochrome *c* (Fig. 7*A*), which arise from 18 nonprotonated carbons, remain narrow upon addition of ferricytochrome *c*, and undergo a change in position that is proportional to the mole fraction of the oxidized species (2). This fast exchange behavior (94) results from the small values of $|\nu_0 - \nu_r|$, relative to kC_t, for the above 18 carbons. In a spectrum of a mixture of ferrocytochrome *c* and ferricytochrome *c*, these 18 carbons do not yield distinct resonances for the ferrous and ferric proteins. Instead, each carbon yields an exchange-averaged resonance with a chemical shift equal to $x_o\nu_0 + x_r\nu_r$, where x_o and x_r are the mole fractions of the oxidized and reduced forms of the protein, respectively. A plot of chemical shifts as a function of x_0, from $x_0 = 0$ to $x_0 = 1$, yields the connections between Peaks 1 to 3, 15, 19 to 28, and 30 of the ferrous protein (Fig. 7*A*) and Peaks 2 to 16 of the ferric protein (Fig. 7*B*). The plot has been presented elsewhere (2). The connections are shown in Fig. 7.

The small values of $|\nu_0 - \nu_r|$ exhibited by the above 18 carbons, and the lack of detectable paramagnetic broadening of their resonances in the spectrum of ferricytochrome *c*, indicate that we are dealing here with carbons that are far from the iron atom, namely C^ζ of the 2 arginine residues and 16 of the 18 nonprotonated carbons of aromatic residues. The 2 excluded amino acid carbons are C^γ of His-18 and C^ζ of Tyr-67, which are relatively close to the iron atom (see "Chemical Shifts").

In contrast to the above behavior, some narrow resonances in the spectrum of fully reduced cytochrome *c* (*Peaks 4* to *14*, *16* to *18*, and *29* of Fig. 7*A*), which also arise from a total of 18 nonprotonated carbons, undergo considerable broadening when going to mixtures of ferrocytochrome *c* and ferricytochrome *c* (2). Only the resonance of one of these 18 carbons "reappears" in the spectrum of fully oxidized cytochrome *c* (*Peak 1* of Fig. 7*B*). The broadening of the resonance of this carbon in the spectrum of a mixture of the two oxidation-reduction states results from $2\pi|\nu_0 - \nu_r| \gtrsim kC_t$. In the case of the 17 nonprotonated aromatic carbons which did not yield detectable resonances in the spectrum of ferricytochrome *c*, the broadening in the spectra of mixed species is probably a consequence of large values of $|\nu_0 - \nu_r|$ (23, 67). However, there may be a contribution to the broadening from large linewidths of the corresponding resonances of ferricytochrome *c*. In any case, all of the resonances that become broad in the spectra of mixtures of the two oxidation-reduction states arise from carbons that undergo large paramagnetic shifts or broadenings, or both, when going to ferricytochrome *c*. We assign these resonances to carbons near the iron atom: C^ζ of Tyr-67, C^γ of His-18, and the 16 nonprotonated aromatic carbons of the heme (see "Chemical Shifts").

Acknowledgments—We thank Dr. Jona Allerhand for densitometer measurements on electrophoresis data, Professor C. C. F. Blake and Dr. L. N. Johnson for supplying crystal coordinates of Gd^{3+} bound to hen egg white lysozyme, Professor R. E. Dickerson for making available the crystal coordinates of tuna heart ferrocytochrome *c*, Professor F. R. N. Gurd for supplying electrophoresis standards, Dr. D. J. Patel for unpublished information, and Mrs. Carol Williams for her help.

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