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Natural-Abundance Carbon-13 Nuclear Magnetic Resonance Studies in 20-mm Sample Tubes. Observation of Numerous Single-Carbon Resonances of Hen Egg-White Lysozyme†

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ABSTRACT: The development of a probe for sample tubes of 20-mm outside diameter has increased the sensitivity of natural abundance ^{13}C Fourier transform nuclear magnetic resonance to the point that single-carbon resonances of proteins can be studied. Numerous narrow single-carbon resonances are observed in the aromatic region of the ^{13}C spectrum of native hen egg-white lysozyme. Theoretical and experimental evidence is presented to show that these narrow resonances are those of the 28 nonprotonated aromatic carbons. The 59 protonated aromatic carbons give rise to a background of broad peaks. Partial assignments for the nonprotonated carbon resonances are presented. Significant chemical-shift variations occur upon folding of the protein into its native conformation. For example, the γ carbons of the six tryptophan residues resonate at 81.4, 82.1, 83.2, 83.8, and 85.2 ppm upfield from CS_2. The peak at 85.2 ppm is a two-carbon resonance. Upon denaturation with guanidinium chloride, all six carbons resonate at about 83.8 ppm. ^{13}C chemical shifts and assignments for aqueous tryptophan at pH 4 are also presented.

Nuclear magnetic resonance (nmr) is suitable for detailed studies of biopolymers in solution because it can, in principle, yield an individual signal for every atom with a nonzero nuclear spin. Proton nmr, which has been widely used for studies of proteins (Roberts and Jardetzky, 1970), is characterized by a small range of chemical shifts, and spectral splittings arising from homonuclear scalar coupling. As a result, proton spectra of proteins are relatively unresolved envelopes. Only a few resonances, such as those of $H^{\epsilon 1}$ of histidine residues, fall outside the main spectral region and thus can be resolved into single-proton resonances. It is known (Levy and Nelson, 1972) that the range of ^{13}C chemical shifts is much greater than that of protons. Moreover, because of the low natural abundance (1.1%) of ^{13}C, splittings from homonuclear coupling are normally not observed. Splittings caused by heteronuclear coupling to

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protons (or, less frequently, other nuclei) can be eliminated by strong irradiation at the resonance frequencies of the protons (or other nuclei). Thus, ^{13}C nmr spectra of large molecules are more resolved and simpler to interpret than their proton spectra. We have already shown (Allerhand *et al.*, 1970) that existing Fourier transform (FT) nmr equipment provides enough sensitivity for recording proton-decoupled natural-abundance ^{13}C nmr spectra of proteins. The potential of ^{13}C nmr for detailed studies of biopolymers in solution depends on the magnitude of ^{13}C chemical-shift nonequivalence induced by the folding of the molecule into its native conformation. Several natural-abundance ^{13}C FT nmr spectra of proteins have been reported, some recorded on "home-built" equipment operating at about 15 MHz (Allerhand *et al.*, 1970, 1971a; Glushko *et al.*, 1972), others recorded on commercial instruments operating above 20 MHz (Chien and Brandts, 1971; Conti and Paci, 1971; Moon and Richards, 1972). In all cases, however, the signal-to-noise ratio was not sufficient for observing *single-carbon* resonances.

It is pertinent to our discussion that the signal-to-noise ratio in a proton-decoupled ^{13}C nmr spectrum depends on the nuclear Overhauser effect (NOE) (Kuhlmann and Grant, 1968; Kuhlmann *et al.*, 1970), which produces an increase in intensity when strong irradiation at the proton resonance frequency is introduced during the ^{13}C nmr experiment. The NOE causes a maximum intensity increase of a factor of 2.988 when the ^{13}C nuclei are undergoing purely dipolar ^{13}C–^{1}H relaxation (Kuhlmann *et al.*, 1970) and eq 1 is satisfied

$$(\omega_H + \omega_C)\tau_R \ll 1 \qquad (1)$$

where τ_R is the correlation time for molecular rotation and ω_H and ω_C are the resonance frequencies (in radians per second) of ^{1}H and ^{13}C, respectively.

It is now generally accepted that, with currently available instrumentation, 0.01 M is the lowest practical concentration for observing *single-carbon resonances* in natural-abundance ^{13}C spectra, when the maximum NOE factor of 2.988 occurs (Doddrell and Allerhand, 1971; Levy and Nelson, 1972). Theoretical considerations (Doddrell *et al.*, 1972) and experimental results (Oldfield, E., and Allerhand, A., manuscript in preparation) indicate that the NOE may be nearly nonexistent for carbons on or near the backbone of a native protein, because τ_R is so large that eq 1 is not valid. Then the lowest concentration for observing single-carbon resonances in natural-abundance ^{13}C spectra is expected to be about 0.03 M (with 1 day or less of signal accumulation). This molarity represents a 45% solution for a protein of mol wt 15,000. Clearly, a practical approach to the detection of single-carbon resonances of proteins requires an increase in instrumental sensitivity.

While proton nmr studies are usually made in sample tubes with an outside diameter of 5 mm, most existing ^{13}C nmr instruments utilize tubes with a diameter of 12 or 13 mm. We have recently constructed a probe which uses spinning sample tubes with an outside diameter of 20 mm (18-mm inside diameter). This relatively inexpensive development (Allerhand *et al.*, 1972) produces an increase in sensitivity of about a factor of three with respect to commercial FT nmr equipment, in spite of the fact that we work at 15.18 MHz while most commercial FT nmr instruments operate at ^{13}C resonance frequencies above 20 MHz. Happily, the magnetic field inhomogeneity over our large sample volume (about 10 ml) is still sufficiently small (0.3 Hz) for high-resolution studies. With the new probe, systematic studies of single-carbon resonances of proteins become practical.

We report here the observation and partial assignment of numerous narrow single-carbon resonances in the aromatic region of the proton-decoupled natural-abundance ^{13}C spectrum of native lysozyme at 15.18 MHz. We show that all the narrow resonances arise from nonprotonated carbons, a fact that greatly simplifies the assignments. We also show that denaturation removes the large chemical-shift nonequivalence observed in native lysozyme.

Experimental Section

Materials. Hen egg-white lysozyme (EC 3.2.1.17) was obtained from Miles Laboratories (grade I, six times recrystallized, lyophilized, lot no. 7103). Tryptophan was purchased from Sigma Chemical Co. All other materials were reagent grade.

Methods. SAMPLE PREPARATION. Lysozyme (3 g) was dialyzed in Spectrapor tubing No. 132680 (Spectrum Medical Industries), three times for 8 hr against 2.5 l. of water at 4°, and then lyophilized. Lysozyme (2.05 g) was then dissolved in 8.5 ml of 0.1 M NaCl. For the undialyzed samples, 2.5 g of the commercial lysozyme was dissolved in 8.5 ml of 0.1 M NaCl (for spectra of native enzyme) or in 8.5 ml of a solution 0.1 M in NaCl and 6.2 M in guanidinium chloride. The pH was adjusted with hydrochloric acid. pH measurements were made before and after each spectral run with a Radiometer PHM 52 Digital pH meter equipped with a Radiometer GK2322C combined electrode. The sample was then passed through a 0.8-μ Millipore filter. Identical ^{13}C nmr spectra were obtained from dialyzed and undialyzed lysozyme, and also from a sample that had been passed through a column of Sephadex G-25 equilibrated with deionized water. It is safe to assume that no small molecule impurities contributed to the observed spectra of the undialyzed material.

NMR SPECTRA. ^{13}C spectra were recorded at 15.18 MHz on a "home-built" Fourier transform nmr apparatus, which consists of a Varian 12-in. high-resolution electromagnet (14.2 kG), radiofrequency circuits and probe of our own design, a Nicolet 1074 instrument computer (4096, 18-bit words) for data acquisition, and a PDP-8/E computer (Digital Equipment Corporation) for data processing. The probe uses spinning sample tubes with a 20-mm outside diameter (Allerhand *et al.*, 1972). A spectral width of 250 ppm (3795 Hz) was used for recording the complete ^{13}C spectrum of native lysozyme, with a digital resolution of 1.85 Hz, which was imposed by the limited memory size of the Nicolet 1074 unit. The resolution in the unsaturated carbon region was increased twofold by using 125-ppm spectral windows.

We would like to point out that after most of the research presented below was completed, we incorporated additional improvements into our equipment that have further reduced, by about a factor of three, the time required to obtain a given signal-to-noise ratio in ^{13}C spectra of proteins. To facilitate a comparison with commercial equipment, we have determined that about 10 min of accumulation time is now required on our instrument to get a spectrum of lysozyme comparable to that reported by Chien and Brandts (1971), which was obtained after about 13 hr of accumulation time. In Figure 1 we show a spectrum of lysozyme (20% w/v in H_2O) obtained in less than 10 min of signal accumulation time. The signal-to-noise ratio is not sufficient for unambiguous detection of single-carbon resonances. In Figure 2 we show spectra obtained with more than enough accumulations to

permit detection of single-carbon resonances, if any *resolved* ones should be present.

Results and Discussion

Figure 2A shows the proton-decoupled ^{13}C spectrum of native lysozyme (pH 4.08). Overall assignments can be made on the basis of reported ^{13}C chemical shifts of amino acids (Horsley *et al.*, 1970) and peptides (Glushko *et al.*, 1972; Christl and Roberts, 1972). The region below 90 ppm upfield from CS_2 contains the resonances of all unsaturated carbons. The saturated carbons resonate above 120 ppm. The aromatic region of the spectrum (about 37–85 ppm upfield from CS_2) showed the most promise for observing resolved single-carbon resonances. Figure 2B shows the region of unsaturated carbon resonances of native lysozyme, recorded with twice the digital resolution of that in Figure 2A. Peaks in the range 13–25 ppm upfield from CS_2 are carbonyl resonances. The strong resonance at 35.8 ppm in Figure 2B can be assigned to C^{ζ} of the 11 arginine residues of lysozyme. The rest of the unsaturated carbon region contains all the aromatic carbon resonances, *i.e.*, those of the three phenylalanine, three tyrosine, one histidine, and six tryptophan residues, a total of 87 carbons. This region consists of numerous narrow resonances (some with a line width of 2 Hz or less) labeled 1–22 in Figure 2B, superimposed on a background of overlapping broad peaks. We present below experimental proof that the narrow resonances are those of the 28 *nonprotonated* aromatic carbons (C^{γ} of Phe and His, C^{γ} and C^{ζ} of Tyr, C^{γ}, $C^{\delta 2}$, $C^{\epsilon 2}$ of Trp). However, it is instructive to first discuss the theory of line widths in ^{13}C spectra of proteins.

If we consider a proton-decoupled ^{13}C spectrum of a protein, the only important contribution to the natural line width of a carbon bonded to one or more hydrogens is from ^{13}C–^{1}H dipolar relaxation (Allerhand *et al.*, 1971b) given by (Solomon, 1955; Doddrell *et al.*, 1972)

$$W = (20\pi)^{-1}\hbar^2\gamma_C^2\gamma_H^2 N r_{CH}^{-6} f(\tau_R) \qquad (2)$$

where W is the line width in hertz, γ_C and γ_H are the gyromagnetic ratios of ^{13}C and ^{1}H, respectively, N is the number of directly attached hydrogens, r_{CH} is the C–H bond length, and $f(\tau_R)$ is defined by

$$f(\tau_R) = 4\tau_R + \frac{\tau_R}{1 + (\omega_H - \omega_C)^2\tau_R^2} + \frac{3\tau_R}{1 + \omega_C^2\tau_R^2} + \frac{6\tau_R}{(\omega_H + \omega_C)^2\tau_R^2} + \frac{6\tau_R}{1 + \omega_H^2\tau_R^2} \qquad (3)$$

We have assumed that the molecule is undergoing isotropic rotation with a correlation time τ_R. Equation 2 is a fairly good approximation for the α carbons of native lysozyme, but it applies to carbons on side chains only when internal rotations are much slower than the overall molecular reorientation. For native lysozyme, fluorescence polarization measurements yield a value of τ_R of 25 nsec (Irwin and Churchich, 1971; Rawitch, 1972) at room temperature, and ^{13}C spin–lattice relaxation studies yield 22 nsec at 40° (Allerhand, A., and Hailstone, R. K., unpublished results). If we choose the latter value, eq 2 predicts a line width of 36 Hz (2.4 ppm) at 15.18 MHz for the α carbons. The aromatic side chains are not expected to have fast internal rotations (Browne, D. T., Kenyon, G. L., Packer, E. L., Sternlicht, H., and Wilson, D. M., private communication) and thus a line width of this magnitude is also expected for protonated aromatic carbons. On the other hand, because of the inverse sixth-power dependence on carbon–hydrogen distances, the contribution to the line widths of *nonprotonated* carbons from ^{13}C–^{1}H dipolar relaxation will be reduced by at least an order of magnitude. Even when one includes other contributions, such as instrumental broadening and relaxation by chemical-shift anisotropy, the nonprotonated carbons are expected to have line widths of only a few hertz. This is indeed the case in the carbonyl region (Figure 2B). The resonance of the 11 arginine ζ carbons appears to be broadened by chemical-shift nonequivalence (a slight splitting is actually detected).

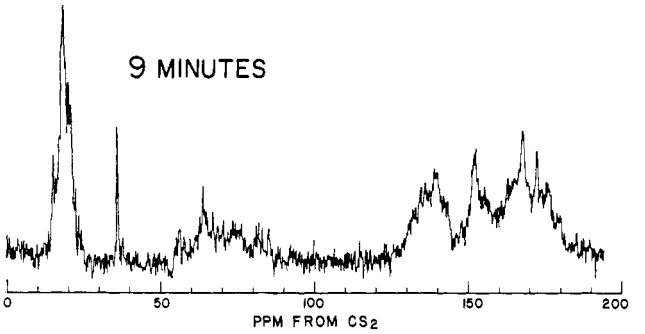

FIGURE 1: Proton-decoupled natural-abundance ^{13}C FT nmr spectrum of hen egg-white lysozyme (about 20% w/v in 0.1 M NaCl, pH 4.0, 43°) at 15.18 MHz in a 20-mm sample tube, recorded with a 250-ppm spectral window, 4096 points in the time domain, 1.09-sec recycle time, and 512 accumulations (9.3 min total time). The signal-to-noise ratio is comparable to that in a spectrum obtained by Chien and Brandts (1971) after 13.3 hr of signal accumulation time on a commercial FT nmr instrument operating at 25.2 MHz.

We tentatively ascribed the sharp peaks 1–22 (Figure 2B) to the 28 nonprotonated aromatic carbons, and the broad features to the 59 protonated ones. This assignment was confirmed experimentally by means of noise-modulated, off-resonance proton decoupling (Wenkert *et al.*, 1969), which selectively broadens protonated carbons. The residual broadening from incomplete proton decoupling is proportional to the square of the pertinent carbon–hydrogen coupling constant (Ernst, 1966). One-bond ^{13}C–^{1}H coupling constants are larger than 120 Hz (Levy and Nelson, 1972), while long-range carbon–hydrogen coupling constants are smaller than 15 Hz (Levy and Nelson, 1972). The effectiveness of noise-modulated off-resonance decoupling for identifying resonances of nonprotonated carbons is illustrated in the aromatic region of the spectrum of 0.05 M tryptophan (Figure 3). Only the resonances of C^{γ}, $C^{\delta 2}$, and $C^{\epsilon 2}$ are sharp peaks in the noise-modulated off-resonance decoupled spectrum (Figure 3A). The protonated carbons are barely discernible broad humps. The corresponding fully proton-decoupled spectrum shows the expected eight sharp resonances (Figure 3B).

The region of unsaturated carbons in the noise-modulated off-resonance proton-decoupled ^{13}C spectrum of native lysozyme is shown in Figure 1C. As expected, the region from 10 to 40 ppm upfield from CS_2 is superimposable with the corresponding one in the fully decoupled spectrum (Figure 2B). In the range 50–90 ppm, all the sharp resonances of Figure 2B are still sharp in Figure 2C, and can thus be assigned to nonprotonated carbons. The envelopes of protonated-carbon resonances in Figure 2B are broadened further in Figure 2C. It should be noted that only narrow

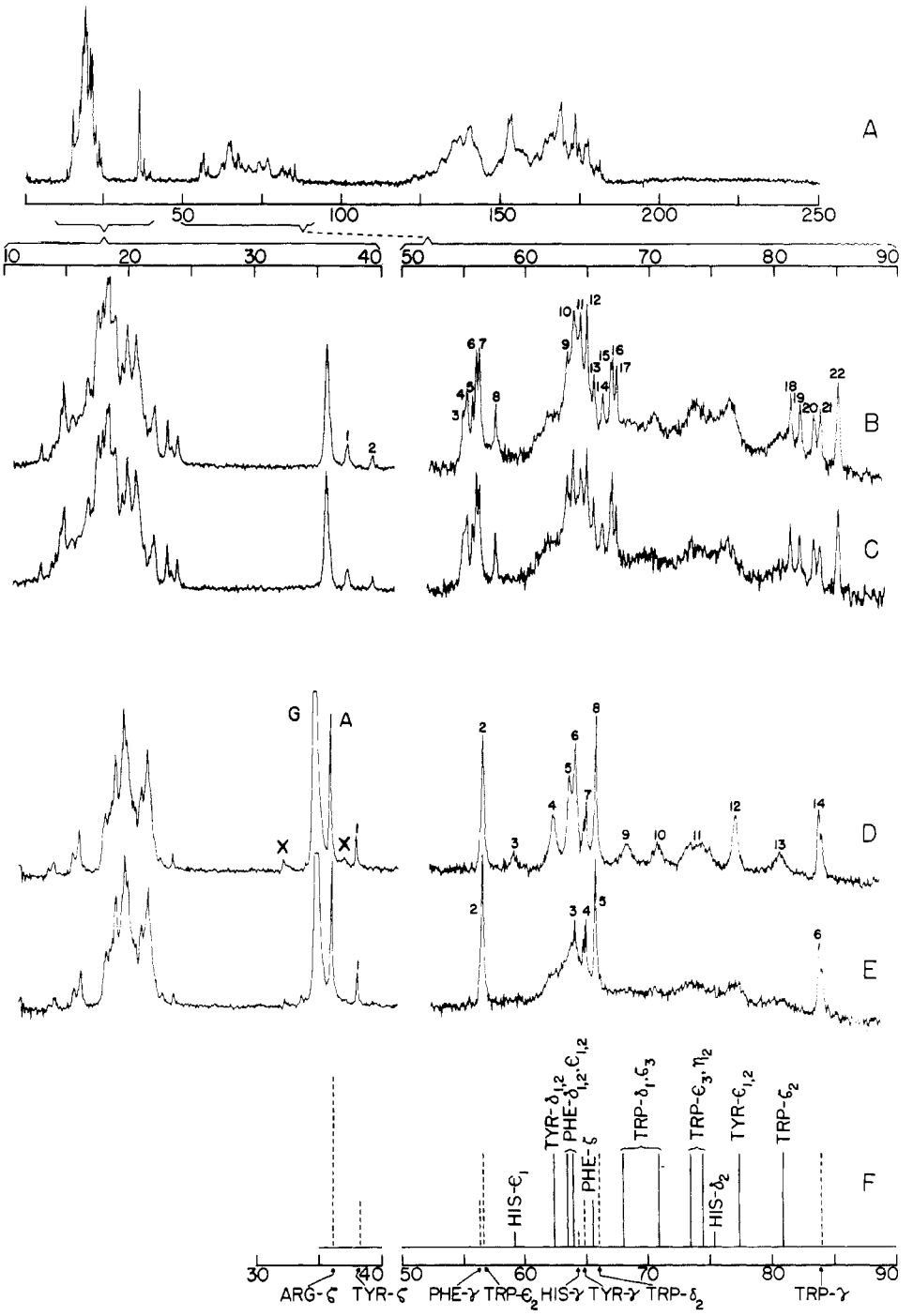

FIGURE 2: Proton-decoupled natural-abundance ^{13}C FT nmr spectra of hen egg-white lysozyme ($C_{613}H_{950}N_{192}O_{188}S_{10}$) at 15.18 MHz in a 20-mm sample tube, with 4096 points in the time domain. Horizontal scale is in parts per million upfield from CS_2. (A) Fully proton-decoupled total spectrum of native lysozyme (about 20% w/v in 0.1 M NaCl, pH 4.1, 45°), recorded with a 250-ppm spectral width, 1.38-sec recycle time, and 32,768 accumulations (12 hr total time; note comment below). (B) Fully proton-decoupled unsaturated carbon region of native lysozyme (about 25% w/v in 0.1 M NaCl, pH 4.0, 40°), recorded with a 125-ppm spectral width, 1.09-sec recycle time, 131,072 accumulations (40 hr total time; note comment below), 0.15-Hz digital broadening, and about 1-Hz resolution. Peaks 1–22 arise from the 28 nonprotonated aromatic carbons (see text). (C) Same as B, but with noise-modulated off-resonance proton decoupling. The decoupling frequency was centered at about 6 ppm upfield from tetramethylsilane. The random-noise modulation bandwidth was about 300 Hz. (D) Fully proton-decoupled unsaturated-carbon region of guanidine-denatured lysozyme (about 25% w/v in 0.1 M NaCl and 6.2 M guanidinium chloride, pH 4.3, 45°), recorded with a 125-ppm spectral width, 1.09-sec recycle time, 65,536 accumulations (20 hr total time; note comment below), 0.15-Hz digital broadening, and about 1-Hz resolution. The resonance of the 6.2 M guanidinium ion (denoted by G) is the only signal that is strong enough to yield detectable spinning side bands (denoted by X). Peak A is the resonance of the ζ carbons of the 11 arginine residues. Peaks 1–14 arise from the 87 aromatic carbons. (E) Same as D, but with noise-modulated off-resonance proton decoupling. Decoupling conditions were the same as for native lysozyme in C. Peaks 1–6 arise from the 28 nonprotonated aromatic carbons. (F) Simulated fully proton-decoupled ^{13}C spectrum of the unsaturated-carbon region (except carbonyls) of lysozyme, based on reported ^{13}C chemical shifts in pentapeptides (Glushko *et al.*, 1972; Glushko, V. G., Gurd, F. R. N., Keim, P., Lawson, P. J., Marshal, R. C., Nigen, A. M., and Vigna, R. A., private communication). Protonated and nonprotonated carbons are shown as solid and dashed lines, respectively. The chemical shifts of the histidine residue are those of the protonated form. Please note (Experimental Section) that the signal accumulation time required to obtain spectra A–E has been reduced to 4, 13, 13, 7, and 7 hr, respectively, by means of instrumental modifications introduced after these results were obtained.

TABLE I: Assignments of the ^{13}C Resonances of Tryptophan at pH 4.0.[a]

Chemical Shift[b]	Assignment[c]	Chemical Shift[b]	Assignment[c]
18.52	CO	73.39	C^{ϵ_3}, C^{η_2}
56.41	C^{ϵ_2}	74.43	
66.18	C^{δ_2}	80.87	C^{ζ_2}
67.68	C^{δ_1}, C^{ζ_3}	85.26	C^{γ}
70.76		137.6	C^{α}
		166.3	C^{β}

[a] 0.05 M aqueous solution at 38°, with dioxane as an internal standard. [b] Ppm upfield from CS_2. Chemical shift of dioxane taken as 126.3 ppm. Accurate to ±0.05 ppm for the unsaturated carbons, ±0.2 ppm for the saturated ones. [c] The assignments for C^{α}, C^{β}, and the carbonyl are based on known amino acid chemical shifts (Horsley *et al.*, 1970). The assignments for the indole resonances are based on those of 3-methylindole (Parker and Roberts, 1970).

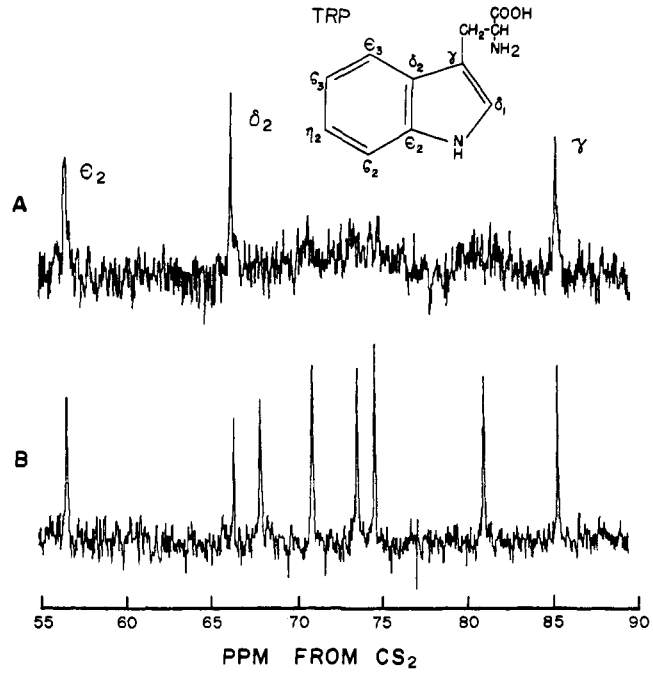

FIGURE 3: Proton-decoupled natural-abundance ^{13}C FT nmr spectra of the aromatic region of aqueous tryptophan (0.05 M, pH 4.0, 38°) at 15.18 MHz in a 20-mm sample tube, recorded with a 62.5-ppm spectral window, 4096 points in the time domain, and a 31.6-sec recycle time. (A) Noise-modulated off-resonance proton-decoupled spectrum, as in Figure 2C, recorded with 1024 accumulations (9 hr total time). (B) Fully proton-decoupled spectrum, recorded with 512 accumulations (4.5 hr total time). The very long recycle time of over 30 sec was dictated by the long spin–lattice relaxation times of the nonprotonated carbons of tryptophan, a relatively small molecule.

protonated-carbon resonances are dramatically broadened by noise-modulated off-resonance decoupling. If the line width of a fully decoupled ^{13}C resonance approaches the magnitude of the ^{13}C–^{1}H coupling constant, the effect of incomplete proton decoupling is small.

Knowledge of ^{13}C chemical shifts of model compounds is, of course, helpful in assigning ^{13}C resonances of proteins. Except for the relatively insoluble tryptophan, the ^{13}C chemical shifts of the aromatic side chains in the free amino acids (Horsley *et al.*, 1970) and small peptides (Gurd *et al.* 1971; Freedman *et al.*, 1971; Christl and Roberts, 1972; Glushko *et al.*, 1972) have been reported. We present in Table I ^{13}C chemical shifts and assignments for tryptophan. The assignments were easily made by comparison with those of 3-methylindole reported by Parker and Roberts (1970). The ^{13}C chemical shifts of the indole moiety of tryptophan differ by 0.4 ppm or less from those of the tryptophan residue in Gly-Gly-Trp-Gly-Gly (Glushko, V. G., Gurd, F. R. N., Keim, P., Lawson, P. J., Marshall, R. C., Nigen, A. M., and Vigna, R. A., private communication) with the exception of C^{γ}, which moves downfield by about 1.2 ppm in the pentapeptide.

Assignments in the Spectrum of Denatured Lysozyme. The region of unsaturated carbon resonances of lysozyme denatured with guanidinium chloride is shown in Figure 2D. The carbonyl region is appreciably less complex than that of native lysozyme, an indication that carbonyl chemical shifts are subject to major effects from protein folding. We have not yet attempted to make any assignments in the carbonyl region. The very strong resonance at about 35 ppm (peak G in Figure 2D) is that of guanidinium chloride. Its spinning side bands are also observed (X in Figure 2D). The spinning side bands of all other resonances are too weak to be observed. The ζ carbons of the 11 arginine residues resonate at 36.0 ppm (peak A in Figure 2D). The corresponding chemical shift of the free amino acid is 36.1 ppm (Horsley *et al.*, 1970). Peaks 1–14 in Figure 2D contain all the aromatic resonances. Rapid segmental motion of the denatured protein backbone (Allerhand *et al.*, 1971a) causes appreciable narrowing of the resonances of protonated aromatic carbons upon denaturation. The narrowing is greater for phenylalanine and tyrosine than for tryptophan, an effect that merits further study.

There is good agreement between the observed spectrum (Figure 2D) and a simulated one based on chemical shifts of small peptides (Figure 2F). C^{γ} of the phenylalanines and C^{ϵ_2} of the tryptophans yield an unresolved nine-carbon resonance (peak 2 in Figure 2D). C^{γ} of the lone histidine overlaps with $C^{\delta_{1,2}}$ or $C^{\epsilon_{1,2}}$ of the phenylalanines (peak 6 in Figure 2D), but it can be identified by means of noise-modulated off-resonance proton decoupling (peak 3 in Figure 2E). The narrow peaks 1–6 in Figure 2E are those of the nonprotonated aromatic carbons.

Residues in different locations along the protein chain show no chemical-shift nonequivalence, with the exception of C^{γ} of the three tyrosines and C^{γ} of the six tryptophans (peaks 7 and 14, respectively, in Figure 2D), which are partly resolved into two peaks each with a splitting of about 0.2 ppm.

Assignments in the Spectrum of Native Lysozyme. The chemical shifts of the nonprotonated aromatic carbons of native lysozyme are given in Table II, together with the corresponding values of denatured lysozyme and those of the pentapeptides (Gly-Gly-X-Gly-Gly) reported by Glushko *et al.* (1972) and Glushko, V. G., Gurd, F. R. N., Keim, P., Lawson, P. J., Marshall, R. C., Nigen, A. M., and Vigna, R. A. (private communication). Peaks 1 and 2 (Figure 2B) can be assigned to C^{ζ} of the three tyrosine residues. Clearly, these peaks are two-carbon and single-carbon resonances, respectively. They are shifted 0.6 ppm downfield and 1.4 ppm upfield, respectively, from their common position in denatured

TABLE II: ^{13}C Chemical Shifts of Nonprotonated Aromatic Carbons of Lysozyme.[a]

Native Lysozyme[b]	Denatured Lysozyme[c]	Small Peptide[d]	Assignment[e]
37.55 (1)	38.10 (1)	38.2	Tyr C^{ζ}
39.56 (2)			
55.01 (3)[f]			
55.25 (4)			
55.74 (5)	56.53 (2)	56.4	Phe C^{γ}
56.04 (6)		56.7	Trp C^{ϵ_2}
56.29 (7)			
57.63 (8)			
63.37 (9)			
63.92 (10)			
64.41 (11)			
64.96 (12)	64.04 (3)	64.3	His C^{γ}
65.50 (13)	64.96 (4)[g]	64.9	Tyr C^{γ}
66.24 (14)	65.75 (5)	66.0	Trp C^{δ_2}
66.85 (15)			
67.03 (16)			
67.34 (17)			
81.37 (18)			
82.11 (19)			
83.20 (20)	83.81 (6)[h]	84.1	Trp C^{γ}
83.75 (21)			
85.22 (22)			

[a] Chemical shifts in ppm upfield from CS_2. [b] Accurate to ± 0.1 ppm. Numbers in parentheses are peak designations in Figure 2B. Sample conditions are those of Figures 2B and 2C. [c] Accurate to ± 0.1 ppm. Numbers in parentheses are peak designations in Figure 2E. Sample conditions are those of Figures 2D and 2E. [d] Taken from Glushko *et al.* (1972), except for the tryptophan values, which are preliminary results of F. R. N. Gurd and coworkers for Gly-Gly-Trp-Gly-Gly at pH 2. The chemical shifts of the histidine residue are those of the protonated form. [e] IUPAC-IUB nomenclature. Carbon designations of Trp are shown in Figure 3. [f] Shoulder (see Figures 2B and 2C). [g] More intense resonance of a partly resolved doublet. The weaker component is at 64.8 ppm. [h] This resonance shows some splitting.

lysozyme. Peaks 3–8 contain the resonances of C^{γ} of the phenylalanines and C^{ϵ_2} of the tryptophans, a total of nine carbons. It is likely that peaks 5, 8, and the low-field shoulder (peak 3) are single-carbon resonances, while peak 4 (which is small but broad) and peaks 6 and 7 are two-carbon resonances. The maximum shifts in position with respect to denatured lysozyme are 1.5 ppm downfield (peak 3) and 1.1 ppm upfield (peak 8). Peaks 9–17 contain the γ carbons of the histidine and the tyrosines and C^{δ_2} of the tryptophans, a total of ten carbons for nine peaks. They overlap with a large number of broad protonated-carbon resonances. It is likely that the region of peaks 10–12 contains four nonprotonated carbons, while peaks 9 and 13–17 are all single-carbon resonances. The five peaks 18–22 can be assigned to the γ carbons of the six tryptophan residues. Peaks 18 and 19 overlap with a broad peak assignable to C^{ζ_2} of the tryptophan residues. Clearly, peaks 18–21 are single-carbon resonances and peak 22 is a two-carbon resonance. The changes in chemical shift with respect to their common position in denatured lysozyme are: 2.4 ppm downfield (peak 18), 1.7 ppm downfield (peak 19), 0.6 ppm downfield (peak 20), no measurable shift (peak 21), and 1.4 ppm upfield (peak 22). These values are subject to an error of up to ± 0.2 ppm, because of a slight splitting of C^{γ} in the spectrum of denatured lysozyme (see text above and peak 14 in Figure 2D).

Our results demonstrate that folding of a protein into its native conformation produces some large ^{13}C chemical-shift variations, which permits the detection of resonances of individual nonprotonated aromatic carbons. Research directed at the assignment of these resonances to specific residues is in progress. We believe that the results presented above prove the potential of ^{13}C nmr spectroscopy for studies of proteins in solution. It should be possible to study in great detail numerous interactions of small molecules with proteins. Particularly promising is the possibility of observing the effect of paramagnetic centers, both natural (as in heme proteins) and artificial (as in spin-labeled proteins). In the case of lysozyme, organic spin labels (Wien *et al.*, 1972) or inorganic paramagnetic ions such as Gd^{3+} (Butchard *et al.*, 1972) can be introduced. The broadening effect of electron spins on nuclear spin resonances can be used for determining interatomic distances in solution (Wien *et al.*, 1972, and references therein cited). One such recent study of spin-labeled lysozyme by means of proton nmr (Wien *et al.*, 1972) was severely limited by the small number of observable single-proton resonances.

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Oxidative Modification of Proteins in the Presence of Ferrous Ion and Air. Effect of Ionic Constituents of the Reaction Medium on the Nature of the Oxidation Products†

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ABSTRACT: Proteins—represented by chymotrypsinogen, cytochrome *c*, myoglobin, phosvitin, and ribonuclease—undergo a structural modification upon brief exposure to ferrous ion, in air. The proteins become reducible by sodium borohydride, indicating that the modification is an oxidative one. The reaction does not occur when oxygen or ferrous ion is excluded, or when another cation (Fe^{3+}, Mg^{2+}, or Cu^{2+}) is substituted for Fe^{2+}. The ion exchange chromatographic profiles of acid hydrolyzates of the several oxidized proteins, labeled in their oxidatively modified side chains with [^{3}H]sodium borohydride, show a far-reaching similarity of composition in terms of labeled products. The nature of the reaction products is strikingly dependent on the composition of the reaction medium: different products are obtained after reaction in phosphate or in tris(hydroxymethyl)aminomethane buffer. The principal product obtained in sodium phosphate buffer has properties expected of a derivative of threonine. The principal product in tris(hydroxymethyl)aminomethane hydrochloride buffer is probably a derivative of lysine. This lysine derivative and some additional, minor products appear to be formed not only in proteins but also in poly-L-lysine. The reaction shares significant features with the spontaneous generation of collagen cross-links and may provide a model for the reaction catalyzed by lysine oxidase. Cytochrome *c*, compared with the other proteins tested, shows a particularly enhanced susceptibility to the oxidative modification but only in phosphate buffer.

Certain aspects of an earlier study of the aerobic reduction of ferricytochrome *c* by ferrous ion implied that the reaction might involve not only the heme group but also the protein moiety of the cytochrome molecule (Taborsky, 1972). In this article, we give evidence that the protein undergoes an oxidative modification in the course of the reaction. It is likely that this oxidation is a general feature of the aerobic interaction between proteins and ferrous ion, but cytochrome *c* appears to be particularly susceptible. This study also revealed that the oxidative modification of proteins is markedly affected by the ionic constitution of the reaction medium.

Materials and Experimental Procedure

Cytochrome *c* (horse heart; type III, Sigma), ribonuclease A (bovine pancreas; type IIA, Sigma), chymotrypsinogen A (bovine pancreas; 3× crystallized, Worthington), myoglobin (horse heart; 2× crystallized, Mann), and the synthetic polypeptides, poly-L-glutamic acid (mol wt 58,300; Miles) and poly-L-lysine (mol wt 11,100; Miles), were used as supplied. Phosvitin (hen egg volk) was prepared according to Joubert and Cook (1958) and was rendered metal-free as described earlier (Taborsky, 1963).

Ion exchange resins (Beckman and Bio-Rad), [^{14}C]amino acids (Schwarz-Mann), [^{3}H]sodium borohydride (New England Nuclear), [^{2}H]sodium borohydride (Alfa Inorganics), and other chemicals of reagent grade quality were used as obtained.

The routine experimental procedure consisted of four stages: (1) reaction with ferrous ion, in the presence of air, resulting in the oxidative modification of the protein (or poly-

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