Studies of Macromolecular Structure by ^{13}C Nuclear Magnetic Resonance. II. A Specific Labeling Approach to the Study of Histidine Residues in Proteins

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Abstract: Tryptophan synthetase α subunit specifically enriched in ^{13}C and labeled with deuterium at the C_2 (ring) position of the four histidine residues was prepared by *in vivo* incorporation of labeled histidine into *Escherichia coli* protein. The ^{13}C nmr spectrum of the labeled enzyme was determined as a function of magnetic field strength, concentration, temperature, solvent, and mode of decoupling. The ^{13}C nmr signal from the histidine C_2 carbons at 24 kG and pD 7 is an unresolved singlet of 50 $\pm$ 5 Hz width with a longitudinal relaxation time (T_1) of 0.5 sec. The correlation time for rotational reorientation, τ_c, for the C_2 carbons is calculated to be 2.7×10^{-8} sec, indicating that the histidine side chains are all highly immobilized within the enzyme. Quantitative estimates of the mechanisms determining the transverse relaxation times (T_2's) and therefore the line widths of signals from single C_2 carbons suggest that the individual resonances should be *ca.* 20 Hz wide. A major contribution (*ca.* 15 Hz) to C_2 carbon line width is made by scalar relaxation of the second kind with the directly bonded deuterium. It is suggested that ^{13}C nmr signals from deuterated macromolecules can be made much narrower by employing a strong deuterium decoupling rf field.

Carbon-13 nuclear magnetic resonance has been shown to have substantial value in the study of structures of biological macromolecules such as peptides and proteins[2–10] and nucleic acids.[11] Natural abundance ^{13}C nmr spectra of such species are generally better resolved than the corresponding proton nmr spectra because of the greater chemical shift range of the ^{13}C nucleus. An additional potential advantage of ^{13}C over ^{1}H nmr in the study of macromolecular structure is associated with the fact that the magnetogyric ratio, γ, of ^{13}C is approximately one-quarter that of ^{1}H.[12–14] The transverse relaxation times, T_2's, and hence the line widths of nmr signals from macromolecules, are often determined primarily by dipole–dipole interactions which have a quadratic dependence on the magnetogyric ratio of each of the interacting nuclei and an inverse sixth power dependence on the distance between the interacting nuclei. Thus, if all other factors (such as the distance between the interacting nuclei) are equal, ^{13}C nmr signals from macromolecules will be much narrower than ^{1}H signals, which are often broad enough to overlap, causing loss of resolution.[15] However, in many cases, this potential advantage is not realized because signals from ^{13}C nuclei directly bonded to hydrogen will typically have line widths comparable to the widths of proton signals.[16] This is due to the fact that typical C–H bond lengths are shorter than common hydrogen–hydrogen interatomic distances. However, the dipolar contribution can be reduced by substituting deuterium ($\gamma_D = 4.13 \times 10^3$ radians sec^{-1} G^{-1}) for directly bonded hydrogen ($\gamma_H = 2.69 \times 10^4$ radians sec^{-1} G^{-1}).[17]

Another very important advantage of ^{13}C over proton nmr in the study of macromolecules is a result of the fact that ^{13}C occurs at low natural abundance. It is thus possible to enrich particular locations in macromolecules with ^{13}C by as much as a factor of about 90 relative to natural abundance. This means that the signals from the labeled carbon atoms can be easily distinguished from signals arising from other unlabeled positions, even if overlap of signals from labeled and unlabeled positions occurs.

We have applied this specific labeling approach, which up to this time has not been used to study unmodified macromolecules,[18,19] to examination by ^{13}C nmr of the histidine residues in a native enzyme, tryptophan synthetase α subunit. Tryptophan synthetase α subunit contains four histidine residues,[20] one or more

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(18) A peptide corresponding to the 1–15 sequence of ribonuclease in which the phenylalanine was specifically enriched with ^{13}C has been studied by ^{13}C nmr.[19] Nigen, *et al.*,[9] have prepared carboxymethylated myoglobins and ribonuclease A using enriched [2-^{13}C]bromoacetic acid.
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of which appear to be located at or near the active site.[21,22] It has a molecular weight of 29,000, which corresponds roughly to the upper limit of molecular weight of enzymes which give proton nmr signals narrow enough to allow observation of a reasonable number of individual resonances.

This study was undertaken in order to evaluate the factors which determine ^{13}C nmr line widths in deuterated macromolecules and to determine the dynamic properties of the histidine side chains. Our results show the usefulness of the selective ^{13}C enrichment technique for study of particular regions of macromolecules in solution and illustrate the potential of deuteration of macromolecules as a method for narrowing the line widths in their ^{13}C nmr spectra. A preliminary account of this work has appeared.[7]

Experimental Section

Materials and Methods. Proton nmr spectra were obtained on Varian T-60 or HR-220 nmr spectrometers. Ultraviolet spectra were measured using a Cary Model 14 recording spectrophotometer or a Unicam SP500 spectrophotometer equipped with a Gilford Model 220 optical density converter. Radioactive samples were counted using a Nuclear-Chicago Mark I liquid scintillation counter. Amino acid analyses were performed by Mr. Vazken Tashinian of the Microanalytical Laboratory, Department of Chemistry, University of California, Berkeley, using a Beckman Model 120C amino acid analyzer.

Carbon-13 nmr spectra were determined using either a Varian XL-100 spectrometer modified for Fourier transform operation[23] or the 15-MHz spectrometer previously described.[14,17,24] The 15-MHz spectrometer was modified to permit simultaneous proton noise decoupling and coherent deuterium decoupling by double-tuning the normal ^{13}C transmitter coil of a Varian V4331A nmr probe for ^{13}C pulses (15 MHz) and ^{2}H decoupling (9 MHz) and by adding a ^{1}H decoupling coil. A ^{7}Li capillary lock (23 MHz) of the same x and y coordinates as the sample permitted long-term resolution of <1.5 Hz.[24] The T_1 relaxation measurements were done using the standard 180°, τ, 90° pulse sequence.[25]

[2-^{14}C]-L-Histidine was supplied by Calbiochem Corp. [^{13}C]Potassium cyanide (61% ^{13}C) was purchased from Prochem Limited. [2-^{13}C]-L-2-Deuteriohistidine was prepared as previously described.[11] *Escherichia coli* strains B8[26] and A2/F'A2[27] were obtained from Professor C. Yanofsky. Bacteria were grown in single strength Vogel and Bonner[28] minimal medium supplemented with 2.5 mg of indole per l. and 0.5% glucose. Cells were disrupted using a 10-kc Raytheon sonic oscillator. A crude sonic extract of strain A2/F'A2 was used as a source of tryptophan synthetase β_2 subunit.[22] Protein concentration was determined by the method of Lowry, *et al.*[29] Dialysis tubing was boiled in three changes of 5×10^{-3} *M* (ethylenedinitrilo)tetraacetic acid sodium salt, pH 7.0, before use in order to remove possible metal ion contaminants.

Samples of 1,4- and 1,5-dimethylimidazole were obtained from Professor D. S. Noyce and Dr. G. Stowe.[30] Natural abundance ^{13}C nmr spectra were determined for 20% (by volume) solutions of these materials in $CDCl_3$. Dioxane was used as an internal standard. The signals from the C_2 carbons of 1,4- and 1,5-dimethylimidazole occur at −69.9 and −70.1 ppm relative to dioxane, respectively, under these conditions.

Incorporation of [2-^{14}*C*]-L-Histidine into *E. coli* Strain B8. A series of growth media containing concentrations of [2-^{14}*C*]-L-histidine ranging from 1.14×10^{-5} to 4.56×10^{-4} *M* were inoculated with B8, and the resulting cultures were incubated with shaking at 37° until maximum bacterial density (as judged turbidimetrically using a Klett-Summerson colorimeter) had been achieved. Aliquots were removed from each culture, and the cells were removed by filtration using HAWP 025 00 filters (0.45 μm pore size) obtained from Millipore Corp. Incorporation of radioactivity into the cells was estimated by first drying the filters at 60° and then placing them in scintillation vials for counting (see Figure 1).

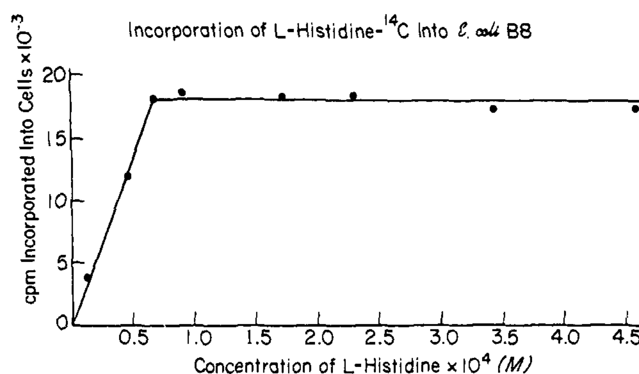

Figure 1. Incorporation of [2-^{14}*C*]-L-histidine into *E. coli* strain B8.

The specificity of labeling was determined by amino acid analysis. A crude sonic extract of labeled cells was centrifuged for 30 min at 144,000*g*. The resulting supernatant was dialyzed exhaustively, and a portion of the dialyzed solution was subjected to acidic hydrolysis (6 *N* HCl, 24 hr at 110°) and automatic amino acid analysis. Histidine contained >99% of the ^{14}C contained in the free amino acids present in the hydrolysate.

Incorporation of [2-^{13}*C*]-L-2-Deuteriohistidine into *E. coli* Strain B8. To approximately 12-l. portions of the growth medium previously described were added sufficient [2-^{13}*C*]-L-2-deuteriohistidine (as filter-sterilized solutions in D_2O) to give a final concentration of 9×10^{-5} *M* of the labeled histidine. The individual portions of media were inoculated with strain B8 and incubated in a New Brunswick Model FS314 fermentor assembly at 37° with stirring and aeration until maximum bacterial density had been achieved (approximately 30 hr after inoculation). The cells were harvested by centrifugation using a Sharples centrifuge operating at about 25°. A 500-g (wet weight) quantity of cells was isolated from a total of 108 l. of growth medium.

Isolation of ^{13}C-Labeled Tryptophan Synthetase α Subunit. Labeled α subunit was isolated using the Creighton and Yanofsky modification[31] of the method of Henning, *et al.*,[26] and was assayed in the conversion of indole to tryptophan in the presence of excess β_2 subunits.[32] Several additional minor modifications were employed. Sonic extracts were centrifuged at 144,000*g* for 30 min in order to remove cellular debris and ribosomes before addition of manganous chloride. In the ion exchange chromatography step, diethylaminoethyl (DEAE) Sephadex A-50 (Pharmacia Fine Chemicals Inc.) was substituted for DEAE-Selectacel. DEAE Sephadex A-50, equilibrated with 0.05 *M* potassium phosphate buffer, pH 7.0, was poured into each of two columns (2.5 × 100 cm) to a height of 90 cm after settling. Half of the protein sample was applied to each column and eluted with a linear gradient of potassium phosphate buffer, pH 7.0. The mixing flask contained 900 ml of 0.05 *M* buffer, and the other flask contained 900 ml of 0.30 *M* buffer. A flow rate of 12–18 ml/hr was employed, and fractions of 10 ml were collected. The α subunit was eluted at about 700 ml. The peak tubes from the DEAE Sephadex chromatography contained 300 mg of protein with a specific activity of 5000 units/mg. This protein was essentially homogeneous (<5% contaminants) as judged by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate.[33] Coomasie blue dye was used to stain the gels. Protein from the peak tubes was used for nmr studies without further purification.

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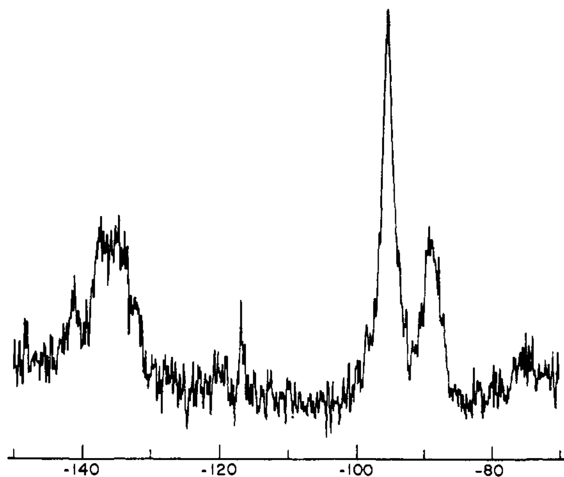

Figure 2. Carbon-13 nmr spectrum (102,000 accumulations) of labeled α subunit (2.9×10^{-3} M) in D_2O at 10° determined at 24 kG under conditions of proton noise decoupling. Scale: ppm relative to glycine α carbon. The 90° rf pulses were spaced at 0.51-sec intervals. Signals are artificially broadened 2.5 Hz by pretransform exponential filtering.

Preparation of Samples for Nmr Studies. Fractions of high specific activity from the DEAE Sephadex chromatography were combined, and the protein was precipitated by addition of ammonium sulfate (0.44 g/ml).[3] The precipitate was collected by centrifugation at 20,000*g* and was then suspended in a minimum amount of the appropriate buffer (0.1 M potassium phosphate in H_2O or D_2O, pH or pD 7.0). The suspension was transferred to dialysis tubing and exhaustively dialyzed against the appropriate buffer. When necessary, samples were further concentrated after dialysis by rolling the dialysis tubing in dry Sephadex G-50. If precipitate was present, it was removed by centrifugation at 20,000*g*.

Results

Incorporation of Exogenous Histidine into *E. coli* Protein. The amount of exogenous [2-^{14}C]-L-histidine incorporated into cells of *E. coli* strain B8 is proportional to the amount supplied at concentrations less than 8×10^{-5} M and is independent of the amount supplied at higher concentrations (Figure 1). Amino acid analysis of protein hydrolysates from B8 cells grown in the presence of [2-^{14}C]-L-histidine demonstrates that histidine is the only amino acid in the hydrolysate which is enriched in ^{14}C relative to natural abundance.

Isolation of Tryptophan Synthetase α Subunit Containing [2-^{13}C]-L-2-Deuteriohistidine. Tryptophan synthetase α subunit was isolated from the protein of *E. coli* strain B8 grown in the presence of [2-^{13}C]-L-2-deuteriohistidine by a procedure similar to that previously reported.[26,31] The α subunit used for nmr studies was at least 95% homogeneous as judged by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate and had a specific activity of 5000 units/mg, which corresponds to the highest specific activity previously reported for the enzyme.[31]

Carbon-13 and ^1H Nmr Studies. Carbon-13 nmr spectra of the labeled α subunit were obtained at 15 and 25 MHz and proton spectra at 220 MHz. All measurements were made on solutions in 0.1 M phosphate buffer, pH or pD 7.0.

The 25-MHz ^{13}C nmr spectra were obtained at 10° on a 2.9×10^{-3} M solution of the labeled α subunit in D_2O. The spectrum shown in Figure 2 was obtained under conditions of proton noise decoupling. The signals can be assigned by comparison with previously reported ^{13}C nmr spectra of amino acids and proteins.[1-7,12-14] The intense peak at −95 ppm relative to the glycine α carbon is attributable to the four labeled histidine C_2 carbons. Its width at half-height is 50 ± 5 Hz. Other signals in the spectrum arise from ^{13}C nuclei present at natural abundance. Signals from the α carbons and the carbons in aliphatic side chains appear as broad, unresolved peaks centered at *ca.* −15 and +12 ppm (not shown). The signals from the carbonyl carbons appear as a broad resonance centered at −135 ppm (290 amide carbonyl carbons) with a shoulder at −141 ppm (28 carboxylate carbonyl carbons). The band at −89 ppm consists of signals from 82 carbons in aromatic amino acid side chains. The narrow resonance at −116.5 ppm occurs in the region where signals from the 11 ϵ carbons of arginine and the six C_4 (ring) carbons of tyrosine are expected to occur. The signals from the 12 quaternary (ring) carbons of phenylalanine are obscured by the −95 ppm peak.

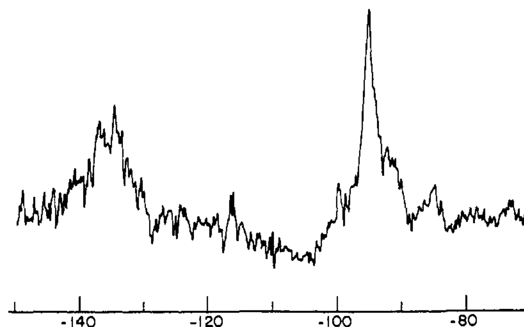

Figure 3. Carbon-13 nmr spectrum (80,000 accumulations) of labeled α subunit determined under conditions of full coupling; the filter broadening was 5 Hz. Other experimental details were identical with those of Figure 2.

The proton-coupled spectrum of the labeled α subunit taken under conditions similar to those used for the proton-decoupled spectrum is shown in Figure 3. The signal at −89 ppm in Figure 2 is attributable primarily to carbons directly bonded to hydrogen and is thus split into an apparent doublet in Figure 3. The signal at −95 ppm remains unsplit, demonstrating that a substantial fraction of the C_2 carbons of histidine are bonded to deuterium (rather than hydrogen) atoms. However, the appearance of a broad shoulder on the low-field side of the signal suggests that some of the deuterium label was lost during the *in vivo* incorporation, which is reasonable in view of the rate at which exchange occurs at the histidine C_2 position.[34]

The spectrum in Figure 4 was taken under conditions identical with those used in Figure 2 except that the pulse repeat time was 0.061 sec (rather than 0.51 sec). In Figure 4, the intensity of the −95-ppm peak is reduced relative to the −89-ppm signal, and the resonances from the carbonyl carbons are not observed.

The longitudinal relaxation time (T_1) of the −95-ppm signal was obtained under conditions of proton coupling similar to those used in Figure 3 and using the standard 180°, τ, 90° pulse sequence.[19] The signal could be characterized by a single T_1 value of 0.5 sec.

The labeled α subunit was also studied by ^{13}C nmr

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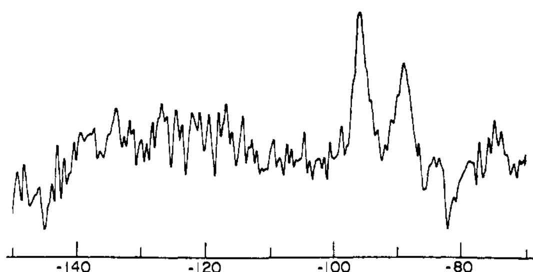

Figure 4. Carbon-13 nmr spectrum (81,000 accumulations) of labeled α subunit determined under conditions identical with those of Figure 2 except that the interval between rf pulses was 0.061 sec and the filter broadening was 8 Hz.

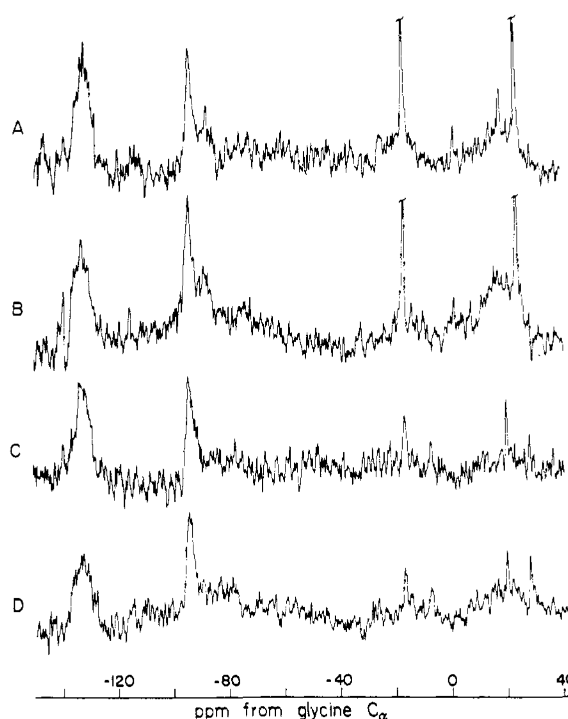

Figure 5. Carbon-13 nmr spectra (*ca.* 100,000 accumulations) of labeled α subunit (2.6×10^{-3} *M*) in D_2O at 2° determined at 14 kG. Ethyl alcohol was present as an internal standard. The 60° rf pulses were spaced at 0.5-sec intervals, the filter broadening was 5 Hz. Spectrum A is ^1H noise decoupled and ^2H coherently decoupled at the frequency of the histidine C_2 deuterium. Spectra B, C, and D are identical with spectrum A except that in spectrum B there is no ^2H decoupling, in spectrum C there is no ^1H decoupling, and in spectrum D there is neither ^1H nor ^2H decoupling.

at 15 MHz. Spectra were obtained using either H_2O or D_2O as a solvent at concentrations ranging from 2.6×10^{-3} to 4.3×10^{-3} *M* and temperatures from 2 to 15°. Spectra were obtained under conditions of full coupling, proton noise decoupling, coherent deuterium decoupling, and simultaneous proton noise decoupling and coherent deuterium decoupling (see Figure 5). The observed 35 ± 5 Hz width of the -95-ppm signal was essentially independent of the variations in temperature, concentration, and mode of decoupling. Furthermore, it was only slightly (30% or less) narrower than the corresponding signal in spectra obtained at 25 MHz.

Proton nmr spectra of the labeled α subunit were obtained at 220 MHz in D_2O. Spectra run at concentrations of 2.9×10^{-3} and 3.0×10^{-4} *M* were very similar in shape.

Discussion and Conclusions

Our experiments on the incorporation of [2-^{14}C]-L-histidine into *E. coli* protein show that it is possible to prepare *E. coli* enzymes specifically labeled at histidine residues by *in vivo* incorporation of exogenous, labeled amino acids. This result was expected by analogy to the results of extensive studies of histidine biosynthesis and transport in *Salmonella*.[35] Tryptophan synthetase α subunit isolated from *E. coli* strain B8 grown in the presence of [2-^{13}C]-L-2-deuteriohistidine contained histidine residues labeled with ^{13}C and deuterium and was fully active in comparison to unlabeled preparations of the enzyme. This result was also expected, since it seems very unlikely that substitution of ^{13}C for ^{12}C or ^2H for ^1H at histidine C_2 positions should have an appreciable effect on enzyme structure or mechanism.

Figures 2–5 convincingly demonstrate the value of the selective labeling technique in permitting unambiguous assignment and study of resonances which in natural abundance spectra would be weak and would overlap with other nearby signals. Because of the relatively wide range of ^{13}C chemical shifts, it is possible to assign all of the signals in the low-field portion of the spectrum by analogy to previously published spectra of amino acids.

Proton-decoupled ^{13}C nmr spectra of small molecules show a nuclear Overhauser enhancement (NOE) in signal intensity. This enhancement is as much as a factor of 3 if the carbon relaxation is dominated by a dipolar interaction with one or more protons.[36–38] In the case of proteins, where carbon atoms can have long rotational correlation times, τ_c, characteristic of the overall rate of rotation of macromolecules ($\tau_c \gtrsim 10^{-8}$ sec), a small NOE of *ca.* 1 can be observed even if the carbon atoms are directly bonded to hydrogen.[38–40] Quaternary carbons may or may not show a NOE depending on the factors determining their relaxation rates.[37] The deuterium-labeled C_2 carbons in the α subunit show essentially no NOE upon proton decoupling. This is reasonable since their spin–lattice relaxation rates would presumably be determined by the dipolar interaction with the directly bonded deuterium. The signal at *ca.* -89 ppm, which arises primarily from unlabeled carbons directly bonded to hydrogen, also shows essentially no NOE. This observation suggests that these carbons have long τ_c values.

In the α subunit, different classes of carbon atoms show different spin–lattice relaxation times depending on whether the carbon is directly bonded to deuterium, to hydrogen, or to neither. This is illustrated in Figures 2 and 4, where a shortening of the pulse repeti-

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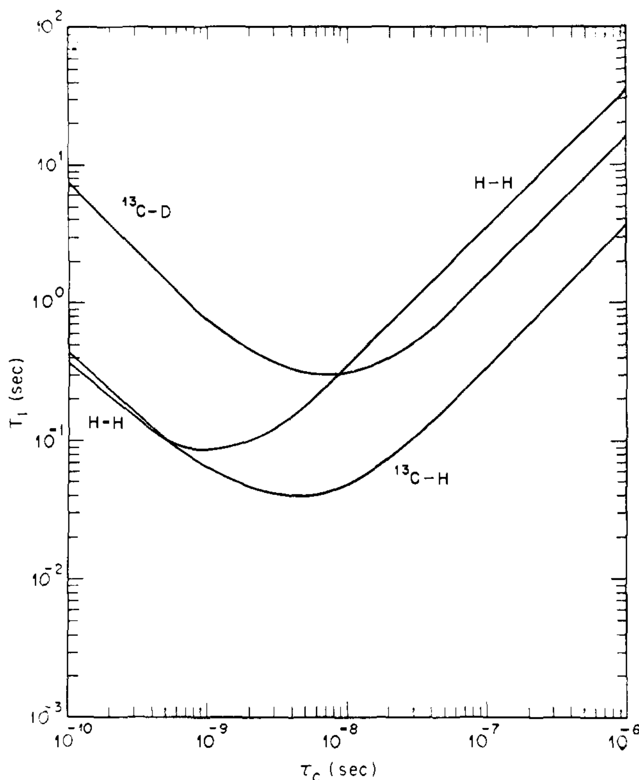

Figure 6. Contribution of dipole–dipole relaxation to longitudinal relaxation time (T_1) at 24 kG as a function of τ_c for ^{13}C at a distance of 1.09 Å from ^1H or ^2H or for ^1H at a distance of 1.78 Å from a second proton.

tion time from 0.51 to 0.061 sec causes large distortions in signal intensities. Those carbon spins having the longest T_1's have the most attenuated signal intensities.[41] Deuterium-labeled histidine C_2 carbons have T_1 values beween those of the aromatic carbons at −89 ppm (which have relatively short T_1's presumably dominated by dipole–dipole relaxation involving the directly bonded hydrogen) and those of the carbonyl carbons (which have relatively long T_1's because they have no directly bonded hydrogen or deuterium). T_1 of the deuterium-labeled histidine C_2 carbons was determined accurately using the standard 180°, τ, 90° pulse sequence[25] and has a value of 0.5 sec at 10° for all four of the C_2 carbons. If one makes the reasonable assumptions that the rotational motion of the α subunit is approximately isotropic and can be characterized by a single correlation time for rotational reorientation, τ_c,[42] and that the T_1 of carbon is dominated by the dipolar interaction with the directly bonded deuterium, one can calculate from eq 1 that τ_c for the C_2 carbons is approximately 2.7×10^{-8} sec.[6,45,46] In this equation,

$$\frac{1}{T_1^{\mathrm{C}}} = \frac{4}{15}\frac{\gamma_{\mathrm{C}}^2\gamma_{\mathrm{S}}^2\hbar^2 S(S+1)\tau_c}{r_{\mathrm{CD}}^6} \times \left[\frac{0.5}{1+(\omega_{\mathrm{C}}-\omega_{\mathrm{D}})^2\tau_c^2} + \frac{1.5}{1+\omega_{\mathrm{C}}^2\tau_c^2} + \frac{3}{1+(\omega_{\mathrm{C}}+\omega_{\mathrm{D}})^2\tau_c^2}\right] \quad (1)$$

γ_C and γ_S are the magnetogyric ratios of ^{13}C and ^2H, r_{CD} is the C–D distance, S is the spin of deuterium, and ω_C and ω_D are resonance frequencies expressed in units of radians per second of carbon and deuterium, respectively. Figure 6 illustrates how the contribution of dipole–dipole relaxation to T_1 varies as a function of τ_c for histidine C_2 carbons bonded to hydrogen and deuterium and for a hydrogen atom in a typical methylene group.

The value of 2.7×10^{-8} sec for τ_c is similar in magnitude to values of τ_c determined by nanosecond polarization spectroscopy for *overall* rotational reorientation of globular proteins having molecular weights comparable to that of α subunit.[47] The α carbons in native ribonuclease A also have a similar value of τ_c as determined by natural abundance ^{13}C nmr.[6] In contrast, carbons on some of the side chains on ribonuclease A have much shorter values of τ_c. For example, a significant number of the lysine ϵ carbons of ribonuclease A have a τ_c value of 7×10^{-11} sec.[6]

The fact that the four histidine C_2 carbons of α subunit have τ_c values of approximately 3×10^{-8} sec suggests that they are not free to rotate at a rate greater than the rate for overall rotation of the protein molecule. Thus, all of the imidazole side chains, including the one or more assumed to be at or near the active site, are "locked" rather tightly in the protein matrix. It is clear that knowledge about the environment and dynamic properties of microscopic regions of biological macromolecules must be available if a detailed understanding of the function of these molecules is to be obtained. The selective enrichment approach to ^{13}C nmr studies of these species seems particularly well suited to obtaining this type of information.

A major reason for undertaking these studies was to evaluate at least semiquantitatively the factors which determine ^{13}C nmr line widths in macromolecules, particularly deuterated ones, so that the potential advantages of ^{13}C nmr in study of macromolecular structure could be more clearly defined. We have considered the following as possible explanations of line broadening of the signals from the labeled histidine C_2 carbons: aggregation of protein molecules, spin–spin coupling (J coupling), chemical shift differences between signals from individual histidine residues, relaxation *via* chemical shift anisotropy, dipole–dipole relaxation, scalar relaxation of the second kind,[45] and relaxation mechanisms of the $\Delta\omega$ type attributable to tautomerism or partial protonation of the imidazole ring. Our measurements allow estimates of the importance of most of these factors, and we conclude that scalar relaxation makes a very significant contribution (*ca.* 15 Hz) to the width of signals from individual C_2 carbons. Most

(41) J. S. Waugh, *J. Mol. Spectrosc.*, **35**, 298 (1970).

(42) A value of the frictional coefficient, f, of α subunit may be calculated[43] from the observed sedimentation coefficient[26,44] and molecular weight. This value is only slightly higher than f_{min}, the frictional coefficient calculated for an unhydrated, spherical protein with the same molecular weight as α subunit. This implies that α subunit is approximately spherical.

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(46) Equation 1 gives rise to two real solutions for τ_c corresponding to a given value of T_1 (Figure 6). We choose the larger of these values because we observed no significant NOE for the carbon atoms giving rise to the peak at −89 ppm and narrow signals only from certain quaternary carbons and lysine ϵ carbons (*cf.* ref 6).

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other line broadening mechanisms are individually relatively unimportant.

The contribution of dipolar relaxation by the directly bonded deuterium to the observed line width, $(\pi T_2^{C})^{-1}$, can be calculated from eq 2 to be approxi-

$$\frac{1}{T_2^{C}} = \frac{4}{15}\frac{\gamma_C^2\gamma_S^2\hbar^2 S(S+1)\tau_c}{r_{CD}^6} \times \left[1 + \frac{0.25}{1+(\omega_C-\omega_D)^2\tau_c^2} + \frac{0.75}{1+\omega_C^2\tau_c^2} + \frac{1.5}{1+\omega_D^2\tau_c^2} + \frac{1.5}{1+(\omega_C+\omega_D)^2\tau_c^2}\right] \quad (2)$$

mately 3 Hz. For labeled C_2 carbons attached directly to hydrogen, this contribution is considerably greater, *i.e.*, approximately 40 Hz. Figure 7 shows the dipolar contribution to T_2 for histidine C_2 carbons and for a methylene hydrogen as a function of τ_c. Similar calculations[48] show that dipolar interaction with the two directly bonded nitrogens and the proton at C_4 contribute a total of only about 1 Hz. Dipolar relaxation involving solvent protons or exchangeable protons on the enzyme molecule is obviously not an important factor in determining line width because of the similarity of spectra run in H_2O and D_2O. The importance of dipolar interactions involving nonexchangeable protons on amino acid side chains which are close to C_2 carbons because of folding of the polypeptide chain cannot be quantitatively estimated until more is known about the tertiary structure of α subunit, but it is unlikely to be large in view of the inverse r^6 dependence of such contributions and the small magnetogyric ratio of the ^{13}C nucleus.

The only slight dependence of line width on magnetic field strength (14 *vs.* 24 kG) demonstrates that relaxation *via* chemical shift anisotropy, a process which can occur when the chemical shift tensor is anisotropic, is not important in determining line width, since this effect has a quadratic dependence on field strength.[45] Chemical shift differences between signals from individual histidine residues presumably exist because they have different environments by virtue of the structure of the polypeptide chain and also because they probably have different degrees of protonation at pD 7. However, since chemical shift differences are proportional to field strength and the width of the observed signal varies only slightly with field strength, these chemical shift differences are relatively small (*ca.* 1.5 ppm or less).

The observations that the width of the signal from the C_2 carbons does not depend on temperature within the limits of experimental error in the range of 2 to 15° or on concentration in the range of 2.6×10^{-3} to 4.3×10^{-3} *M* suggest that aggregation is not a significant source of line broadening. Line widths would be expected to increase with decreasing temperature if aggregations were occurring under the experimental conditions employed. This conclusion is further strengthened by the fact that the shapes of proton nmr spectra of the labeled protein determined at 2.9×10^{-3} and 3.4×10^{-4} *M* were very similar. Tryptophan synthetase α subunit is known to exist primarily as a monomer at 3.4×10^{-4} *M* in buffers similar to the ones we used.[26,44]

(48) D. J. Patel, C. K. Woodward, and F. A. Bovey, *Proc. Nat. Acad. Sci. U. S.*, **69**, 599 (1972).

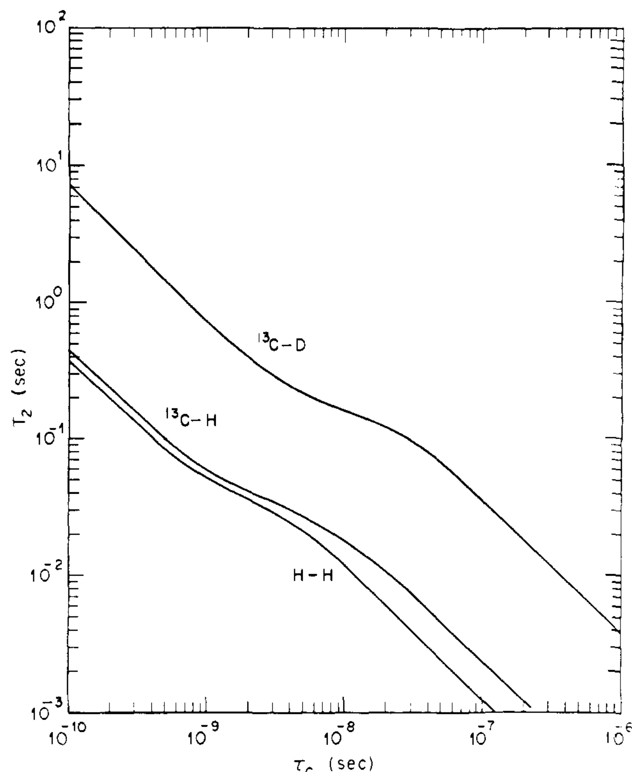

Figure 7. Contribution of dipole–dipole relaxation to transverse relaxation time (T_2) at 24 kG as a function of τ_c (*cf.* Figure 5).

Scalar relaxation of the second kind is a process in which a fluctuating magnetic field causing relaxation is produced by spin–spin coupling of the nucleus under observation to a second nucleus which is relaxing rapidly. It is sometimes an important source of line broadening in ^{13}C nmr spectra when the carbon in question is coupled to a nucleus, such as ^{35}Cl, ^{37}Cl, ^{79}Br, or ^{81}Br, which has a quadrupole moment,[49–51] since such nuclei often have rapid relaxation rates determined primarily by the magnitude of the quadrupole coupling constant. The spin–spin coupling constant for deuterium bonded to the C_2 carbon of histidine is $35 \times 2\pi$ radians sec^{-1}.[17] The quadrupolar coupling constant for the deuterium is estimated to be approximately 190 kHz.[52,53] Using these constants, one may calculate the spin–lattice relaxation time, T_1^{S}, and the transverse relaxation time, T_2^{S}, of the deuterium as a function of τ_c[45] (Figure 8). For a τ_c of 3×10^{-8} sec, $T_2^{S} \simeq 1.6 \times 10^{-4}$ sec and $T_1^{S} \simeq 1.3 \times 10^{-3}$ sec.

The contribution of scalar relaxation of the second kind to the spin–spin relaxation time of the C_2 carbon, T_2^{SC}, may be calculated from eq 3 if $(T_1^{S})^{-1} > J$. In eq

$$\frac{1}{T_2^{SC}} = \frac{J^2 S(S+1)T_1^{S}}{3} \quad (3)$$

3, S is the spin of deuterium. The calculated value of $(T_2^{SC})^{-1}$ for the C_2 carbons is approximately 45 sec^{-1},

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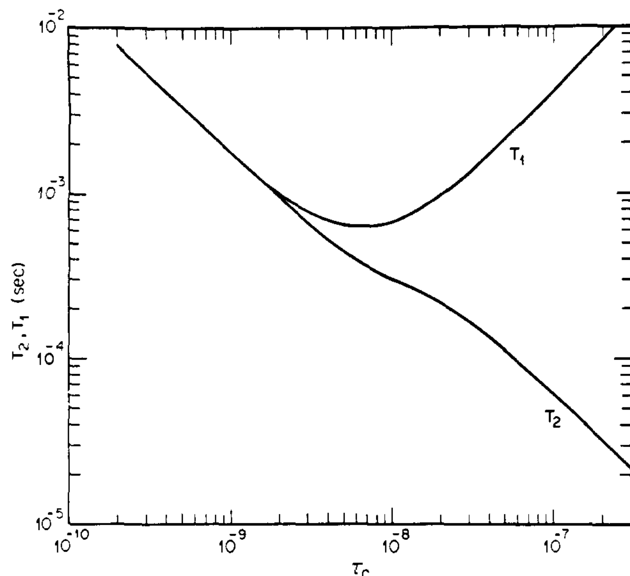


Figure 8. Contribution of quadrupole relaxation to T_1 and T_2 at 24 kG for a deuterium nucleus as a function of τ_c assuming a quadrupolar coupling constant of 190 kHz.

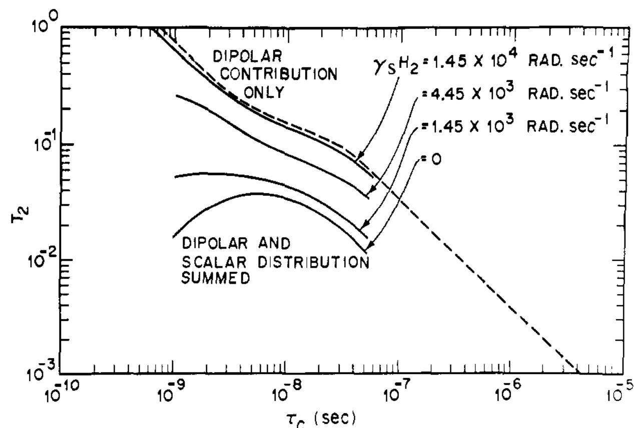
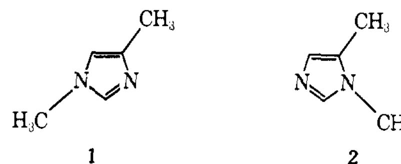


Figure 9. The sum of dipolar and scalar contributions to T_2 for a ^{13}C 1.09 Å from deuterium as a function of τ_c and deuterium decoupling power ($\gamma_S H_2$).



which corresponds to a 15-Hz contribution to the widths of signals from individual C_2 carbons. In the presence of an external, coherent decoupling field of peak amplitude $2H_2$ (in gauss), one replaces[54] T_1^S by $(1/\tau + 1/T_1^S)^{-1}$ where $1/\tau = \gamma_S^2 H_2^2 T_2^S$. In Figure 9, we give the calculated T_2 for a ^{13}C nucleus directly bonded to deuterium as a function of τ_c and decoupling power assuming that T_2 is determined exclusively by dipolar and scalar relaxation with the deuterium. For the present Varian XL-100 spectrometer, $\gamma_S H_2 \simeq 1.45 \times 10^3$ radians sec^{-1}. An H_2 approximately a factor of 10 larger than that presently commercially available is therefore needed to decouple the carbon to the theoretical dipolar line width.

The C_2 carbons are also coupled to ^{14}N atoms, which also have a spin of 1 and a quadrupole moment. However, the contribution to C_2 carbon line widths due to scalar coupling with ^{14}N can be estimated to be at least two orders of magnitude smaller than the contribution from coupling to deuterium.[48]

The histidine residues in ribonuclease have pK_a values ranging from 5.8 to 6.7.[34] It is reasonable to assume that the histidine residues in α subunit will have a similar range of pK_a values. At pH or pD 7, the histidine residues in α subunit are therefore probably largely in the neutral form. The neutral form of the histidine side chain exists as a mixture of two tautomeric forms because the single proton may be attached to either of the nitrogen atoms of the imidazole ring. However, exchange between the two tautomeric forms[55,56] does not appear to be a significant source of line broadening because the C_2 carbons of 1,4- and 1,5-dimethylimidazole (**1** and **2**), which serve as spectroscopic models for the two tautomeric forms of the neutral form of the histidine side chain, have essentially identical chemical shifts.

The nmr signal from the histidine C_2 carbon shifts approximately 2.5 ppm upfield upon protonation.[57] While the histidine residues in α subunit should be largely in the neutral form at pH or pD 7, significant amounts of the protonated (cationic) form will be present, and the possible effect of exchange between the neutral and cationic forms of histidine on C_2 carbon line width must be considered. This potential contribution is difficult to estimate, but we feel that the relatively small observed dependence of line width on field strength makes it unlikely that this exchange is a major source of line broadening for signals from the C_2 carbons.[58]

The observed 50 ± 5 Hz line width[61] of the signal from the C_2 histidine carbons in tryptophan synthetase α subunit can be explained if one assumes that it is a composite of four carbon resonances, each approximately 20 Hz wide (15 Hz for scalar relaxation and 5 Hz from dipole–dipole relaxation) and having a chemical shift range of *ca.* 1 ppm. This range of chemical shifts could result from conformational factors such as ring currents or from differing degrees of protonation of individual histidine side chains. Relatively small contributions from other relaxation mechanisms cannot be excluded but do not have to be invoked in order to explain our observations.

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(56) G. C. Y. Lee and S. I. Chan, *ibid.*, **94**, 3218 (1972).

(57) F. R. N. Gurd, private communication.

(58) The observations of Patel, *et al.*,[48] on the exchange of histidine ring nitrogen protons in ribonuclease suggest that the rate of exchange between the neutral and protonated forms of histidine side chains in α subunit is unlikely to be moderately slow.[59,60] Line broadening due to exchange in the intermediate to fast exchange region shows a dependence on field strength ranging approximately from linear to quadratic.[55,56,60]

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(61) The apparent width of the signal from ^{13}C–^{2}H moieties in proton-decoupled spectra is increased slightly due to overlap with the corresponding broader signal from ^{13}C–^{1}H groups. In proton-coupled spectra, the signal is broadened by *ca.* 6 Hz by *J* coupling to the proton at C_4.[17] The apparent line width in all spectra is also increased slightly by exponential filtering as indicated in the figure captions.

In conclusion, we feel that our results convincingly demonstrate the potential of the selective ^{13}C enrichment technique for study of the dynamic properties of macromolecules in solution, an area of investigation to which X-ray diffraction techniques, for example, are not applicable. The selective enrichment approach in principle permits a detailed study of the dynamic state of any region of a native enzyme or other biological macromolecule by ^{13}C nmr. The conclusion that ^{13}C nmr line widths in deuterated macromolecules appear to be determined largely by scalar relaxation is important for realizing the potential of ^{13}C nmr spectroscopy in providing well-resolved nmr spectra of macromolecules. Deuterium decoupling using high power can eliminate the scalar relaxation of the second kind and thus give narrow signals whose widths are determined primarily by ^{13}C–^{2}H dipolar relaxation in favorable cases. This approach should permit detection of small chemical shift differences which reflect nonequivalence of side chains and changes in conformation.

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Specific and Fluorescent Modifications of Cytidine

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Abstract: Specific fluorescent modifications of cytidine have been obtained by reaction with several pyridinium and quinolinium hydrazides at pH 4.2 and 37°. In alkaline solution, and in general in neutral solution, the products showed a characteristic large absorption maximum at long wavelength where cytidine exhibited no absorption. A bathochromic effect was observed in the lowest energy transition of IIIa, as an example, and the wavelength was found to increase from neutral aqueous solution to 10% ethanol–chloroform. Methylation of IIIa by methyl iodide in sulfolane gave a 3-methylcytidine derivative, V, the structure of which was established by independent synthesis of V from nicotinic acid hydrazide methiodide (IIa) and 3-methylcytidine. The fluorescence of the modified cytidines showed structure and environment dependence. Compounds IIIc and IIId, by their ultraviolet absorption and fluorescent emission characteristics, present favorable possibilities for energy transfer studies with other fluorescing molecules, particularly in single-stranded oligo- and polynucleotides and nucleic acids.

Much of the current research on nucleic acid structure has centered on transfer ribonucleic acids. This attention is understandable since tRNAs constitute perhaps the most versatile class of nucleic acids in terms of the variety and complexity of the reactions in which they participate. The chemical reactions, moreover, can be valuable in sequence analysis, investigation of structure–function relationships, and primary, secondary, and tertiary structure analysis.[2]

Semicarbazide[3,4] and acyl hydrazides[5–7] react specifically with cytidine at pH 4.2 and 37°. In the interest of extending this useful reaction to realize specific fluorescent modification of cytidine and in seeking to achieve the goal of producing specific fluorescent modification of each tRNA base,[8–14] we have synthesized a series of pyridinium and quinolinium hydrazides, IIa–h, and examined the spectroscopic properties of their products with cytidine, IIIa–h.[15]

Experimental Section

Materials and Methods. Ethyl nicotinate (Aldrich), methyl isonicotinate (Chemicals Procurement Laboratories), 2,6-pyridine-

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