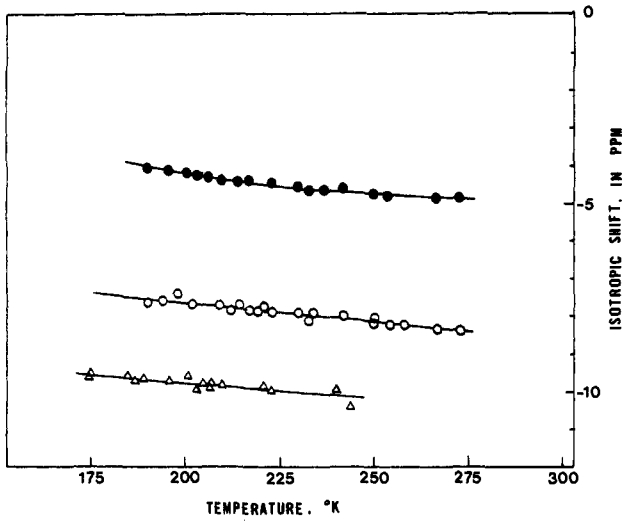

Figure 2. Graph of pyrrole-H isotropic shift vs. temperature for PFe-O-FeP, ●; PFe-OO-FeP, ○; and PFe-OO-FeP', △. The decrease in isotropic shift on lowering the temperature 273 to 200 K is 14% for PFe-O-FeP and only 9% for PFe-OO-FeP.

stability of a dioxygen vs. a peroxo bridge and thereby determine whether the step A → C is reversible.

Acknowledgments. This research was supported by grants from the National Institute of Health, HL-16087 (G.N.L.) and GM-18357 (A.L.B.). We thank C. A. Reed for a gift of PFe and J. P. Collman for a preprint of reference 5.

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^{15}N Nuclear Magnetic Resonance as a Probe of Residual Structure in the Backbone of Unfolded Hemoglobin

Sir:

An important aspect of dynamic studies of protein folding is the determination of the degree of order that exists in unfolded polypeptides.[1-3] Optical rotation, UV absorption, viscosity, enzyme activity, NMR, and Raman spectroscopic measurements[4-8] on unfolded proteins have provided information on the existence of highly ordered residual structures that contain stable side-chain-side-chain interactions. However, simpler residual structures consisting of residues whose available conformations have been restricted by short range interactions with neighboring residues have proved to be more difficult to detect.[9] NMR is a tool remarkably well suited for assaying the degree of time averaged randomness of each residue in the backbone of unfolded proteins since ^{1}H, ^{13}C, and ^{15}N chemical shifts of peptide nuclei are sensitive to conformation and solvation effects.[10] Unfortunately, the ^{1}H, ^{13}C, and ^{15}N NMR spectra protein backbones display notoriously little resolution. We here report that exchange of labile N-H hydrogens for deuterium is a convenient method for considerably improving resolution in the ^{15}N NMR spectra of protein amide groups. Using this procedure, we have been able to observe a number of glycyl ^{15}N resonances, spread over 20 ppm, in the ^{15}N NMR spectrum of [Gly-^{15}N] hemoglobin in D_2O. Upon acid and alkaline denaturation of hemoglobin and globin, not all resonances shift to the random coil position, which provides evidence that in denaturated globins there are glycyl residues, whose conformations have not been completely randomized.

A Me_2SO-treated Friend virus induced murine leukemic cell culture grown in medium containing [^{15}N]glycine (95% ^{15}N) was used to prepare hemoglobin, Hb-[Gly-^{15}N], whose glycyl residues and heme groups were labeled to 50% with ^{15}N.[11] The Friend luekemic cell hemoglobin mixture studied[11] consists of hemoglobins composed of DBA/2 mouse α^{major} globin chains, containing 11 glycyl residues at A2, A13, A16, AB1, B3, B6, D7, E7, E6, E20, and EF7, β^{major} globin chains with 14 glycyl residues at A10, A16, B4, B6, B7, CD5, D7, E8, E18, EF7, G9, G17, GH2, and H14, and β^{minor} globin chains, which lack the A16 glycyl residue.[12] This heterogeneous group of labeled glycyl resides consists of residues with a variety of ϕ-ψ values and hydrogen-bonding modes and reside in the middle and terminal regions of regular and irregular helices, in interhelical bends, as well as at the $\alpha_1\beta_1$ contact.

The proton-coupled 9.12-MHz ^{15}N NMR spectrum of carbomonoxy [^{15}N-Gly]hemoglobin (CO-Hb-[Gly-^{15}N]) displays a set of three broad, poorly resolved resonances centered at 80.9 ppm when measured at a concentration of 3.7 mM in aqueous 0.05 M, pH 7.5 phosphate buffer, which were similar to those reported previously.[11] Proton broad-band noise decoupling produces a ^{15}N spectrum of lower intensity (NOE ~ 0.25) consisting of a major resonance at 80.9 and a minor resonance at 88.2 ppm. An improvement in resolution could be obtained by dilution to 0.8 mM, which allowed the 80.9-ppm resonance to be observed as doublet with $^{1}J_{NH}$ = 95.2 ppm in the proton-coupled ^{15}N spectrum.

Exchange of the hemoglobin amide protons with deuterium at 10 °C over the course of several days had a pronounced effect on the proton coupled ^{15}N NMR spectrum (Figure 1a) of CO-Hb-[Gly-^{15}N] (0.8 mM in D_2O phosphate buffer, pD 7.5, 0.05 M). The spectrum displays no less than seven clearly resolved, narrow, and reproducible resonances spanning a chemical shift range of 20 ppm. *This spectrum demonstrates the marked improvement in the resolution of ^{15}N spectra of proteins which can be obtained simply by exchanging the labile amide hydrogen with deuterium.*

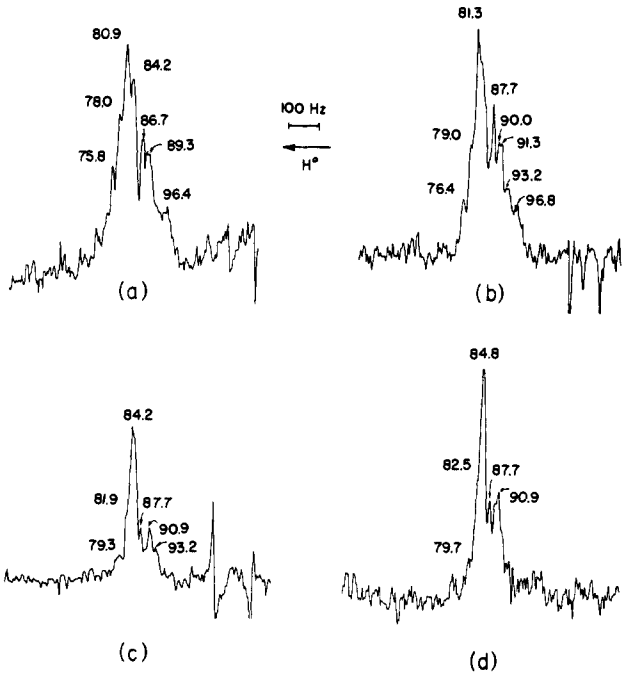

Figure 1. ^{15}N NMR (9.12 MHz) of Friend leukemic cell carbomonoxy[Gly-^{15}N]hemoglobin (2.5 cm^3, 0.8 mM in D_2O phosphate buffer, 0.05 M): (a) pD 7.0, (b) pD 10.5, (c) pD 12.5, and (d) pD 1.2. Spectral conditions: pulse angle, 90°; accumulations, 50 000; spectral width, 3000 Hz; resolution, ±0.08 ppm; 27 °C; field stabilization on the ^2H signal of D_2O. Chemical shifts in parts per million downfield from 4 M NH_4Cl in 2 M HCl. Measurements made on Bruker HFX-90 Fourier transform spectrometer.

The improvement in resolution is similar to that reported and discussed for ^{13}C resonances of amino acids[13] and results from the fact that the ^{15}N spin-spin relaxation time (T_2) and the line width of the ^{15}N resonance ($\Delta\nu_{1/2} = 1/\pi T_2$) are dependent on the nuclear properties (γ_S and S) of the nuclei with which the ^{15}N nucleus undergoes dipolar interaction as given in eq 1,[13] where

$$T_{2,\text{dipolar}}^{-1} = \frac{4}{15}\frac{\gamma_N^2\gamma_S^2h^2S(S+1)\tau_c}{r^6} \times \left[1 + \frac{0.25}{1+(\omega_N-\omega_S)^2\tau_c^2} + \frac{0.75}{1+\omega_N^2\tau_c^2} + \frac{1.5}{1+\omega_S^2\tau_c^2} + \frac{1.5}{1+(\omega_N+\omega_S)^2\tau_c^2}\right] \quad (1)$$

where r is the N-H bond length (0.80 Å), ω_N and ω_S are Larmor frequencies of ^{15}N and the interacting nucleus, respectively, and τ_c is the isotopic rotational correlation time of the protein. As seen in Figure 2, substitution of deuterium (γ_D = 6720 rad s^{-1} G^{-1}, S = 1), for hydrogen (γ_H = 26753 rad s^{-1} G^{-1}, S = ½, leads to an ~12-fold decrease in the dipolar relaxation rate for τ_c = 30 ns. However, the rapid quadrapol spin lattice relaxation rate of the amide deuterium given in eq 2,[13] where (e^2Qq/h) for acetamide is 196 KHz,[14] leads to a scalar interaction of the second kind between ^{15}N and ^2H, given in eq 3[13]

$$T_{1,Q}^{-1} = \frac{3}{40}\left(\frac{2S+3}{S^2(2S-1)}\right)4\pi^2\left(\frac{e^2Qq}{h}\right)^2 \times \left[\frac{\tau_c}{1+\omega_D^2\tau_c^2} + \frac{4\tau_c}{1+2\omega_D^2\tau_c^2}\right] \quad (2)$$

$$T_{2,\text{scalar}}^{-1} = \frac{8}{3}\frac{(2\pi J)^2S(S+1)T_{1,Q}}{3} \quad (3)$$

where $^1J_{^{15}N^2H}$ is 15 Hz for [^2H,^{15}N]glycylglycine.[11] The observed ^{15}N spin-spin relaxation time, $T_{2,\text{total}}^{-1}$ is the sum of

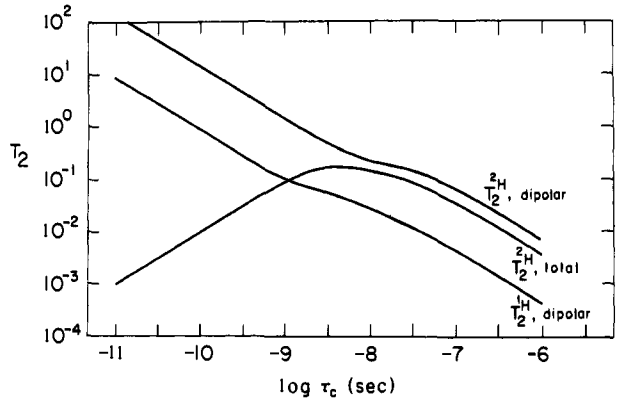

Figure 2. The calculated effect of the substitution of ^2H for ^1H on ^{15}N spin-spin relaxation rates of an N-H group with r = 0.8 Å. The $T_2{}^1H$ dipolar and $T_2{}^2H$ dipolar values were calculated from eq 1 and $T_2{}^2H$ total was calculated as the sum of $T_2{}^2H$ dipolar and $T_2{}^2H$ scalar, which was estimated from eq 2 and 3.

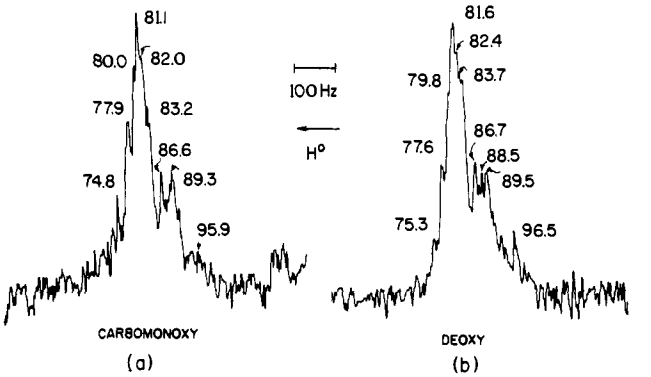

Figure 3. ^{15}N NMR (9.12 MHz) of Friend leukemic cell: (a) carbomonoxy- and (b) deoxy[Gly-^{15}N]hemoglobin (2.5 cm^3, 0.8 mM in D_2O phosphate buffer, 0.05 M, pH 7.0). Spectral conditions as in Figure 1.

$T_{2,\text{dipolar}}^{-1}$ and $T_{2,\text{scalar}}^{-1}$. The gain in the resolution achieved by reducing the ^{15}N dipolar interactions is then offset by scalar interactions. As seen in Figure 2, for proteins with $\tau_c \sim$ 10-30 ns, the increase in $T_{2,\text{total}}$, that is achieved by substituting ^2H for ^1H, is still significant. The rapid spin-lattice relaxation of amide deuterium eliminates the ^{15}N-^2H scalar splitting and the ^{15}N resonances remain singlets.

The improved resolution of hemoglobin ^{15}N NMR spectra allowed us to study the effect of dissociation, ligand binding, and denaturation on the ^{15}N chemical shifts of glycyl residues. Dissociation of CO-Hb-[Gly-^{15}N] into α,β dimers by increasing the pD to 10.5[15] resulted in only subtle changes in the ^{15}N NMR spectrum of the hemoglobin sample (Figure 1B). Ligand binding to hemoglobin has no effect on the ^{15}N spectrum (Figure 3). However, both acid and alkaline denaturation of hemoglobin is reflected in significant changes in the ^{15}N NMR spectra (Figures 1c, d). The major resonance, which is at 80.9 ppm in native hemoglobin has shifted downfield to ~84.5 ppm in unfolded hemoglobin. More remarkable is the fact that not all of the resonances have coalesced into a single resonance. ^{15}N resonances at 79, 82, 88, and 91 ppm are observed in denatured hemoglobins in addition to the 84.5-ppm resonance. Further changes in the ^{15}N spectra of denatured hemoglobins were not observed upon removal of hemin or upon addition of 5 M urea.

The Gly^2 residue of *N*-acetyltriglycine (AcGly^{1}Gly^{2}Gly^3) in aqueous solution was taken as a model of a water solvated glycyl residue with a random conformation. The proton-decoupled natural abundance ^{15}N NMR spectrum of *N*-acetyltriglycine (1 M, pH 3.0) in H_2O displays resonances at 85.1, 89.5, and 90.3 ppm. The 85.1-ppm resonance is assigned to Gly^2 residue, since this resonance is absent in *N*-acetyldi-

glycine, which displays resonances at 88.0 and 90.15 ppm. On the basis of these observations, the major resonance at 84.5 ppm in unfolded hemoglobins is assigned to glycyl residues, whose conformations and modes of solvation have been randomized.

The effect of primary structure on the ^{15}N chemical shifts of polypeptide residues is a subject of current controversy. Nearest neighbor residue effects on ^{15}N chemical shifts have been reported for some glycyl peptides[10e] and not for others.[16] the side-chain groups of nearest neighbor residues are probably too distant to alter significantly, via through-bond electronic effects, the degree of polarization of the peptide bond or charge density on nitrogen. The reported nearest neighbor effects on the ^{15}N chemical shifts are most likely due to conformation and solvation effects which would be averaged out in a true random coil.

We conclude that the ^{15}N resonances in denatured hemoglobin that do not have glycyl random coil chemical shifts represent glycyl residues, whose conformations or hydrogen bonding modes have not been completely averaged in the unfolded polypeptide. These residues could be part of residual structures that may constitute nucleation sites for protein folding.

Acknowledgment. We wish to thank Professor Charlotte Friend for kindly sending us Friend cell (clone no. 707).

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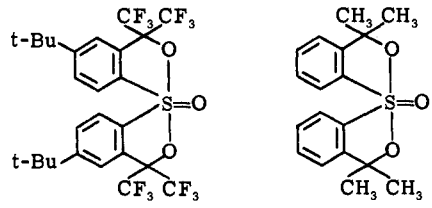
An Alkylaryldialkoxysulfurane Oxide with Labile α Protons. A New Kind of Pentacoordinated Sulfur Ylide[1]

Sir:

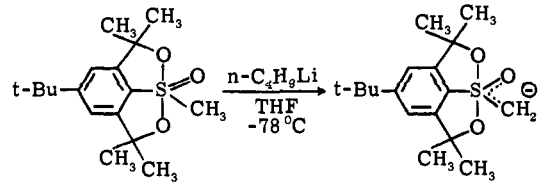
Although a number of sulfurane oxides, including **1** and **2**, have been prepared,[2] none of them contains hydrogens α to sulfur. Compound **1** was found to be inert toward aqueous base



1 **2**

or acid.[2i,j] In contrast, **2** rapidly fragments to give a sulfone enol upon addition of acid.[2l]

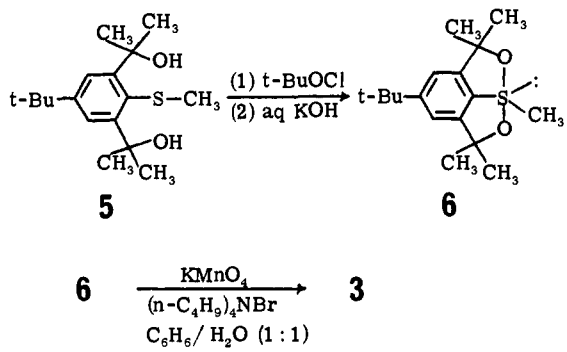
We now report the synthesis of the first alkylaryldialkoxysulfurane oxide (**3**) and evidence for a remarkable lability of its α protons via a pentacoordinated sulfur ylide (**4**) formed by loss of a methyl proton to base.



3 **4**

The target molecule **3**,[3] mp 195–197 °C, was prepared in 57% yield from diol **5**[4] using phase-transfer oxidation,[5] as shown in Scheme I, in the final step. Sulfurane **6** is a stable white crystalline solid, mp 155–156 °C.[6] Slow deuterium exchange of the *S*-methyl protons was observed by NMR when a sample of **6** was dissolved in 0.3 mL of pyridine-d_5 containing 4 drops of 20% NaOD-D_2O.[7]

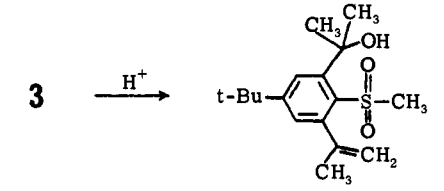
Scheme I



5 **6**

6 **3**

As expected,[2l] sulfurane oxide **3** is acid sensitive, quickly giving sulfone enol **7**[8] (mp 172–173 °C) in $CHCl_3$ containing a trace of HCl. Compound **3**, unlike **2**, is stable in pyridine solution for at least 3 months without any change. Deuterium exchange of the methyl protons is complete within minutes at room temperature, even in the absence of base, when excess D_2O is added to an acetone-d_6 solution of sulfurane oxide **3**. In pyridine-d_5 solution with D_2O, methyl proton exchange is complete within seconds for **3** under conditions which show negligible exchange of the methanesulfonyl protons of **7**.



7

When 1 equiv of *n*-butyllithium is added to a THF solution of **3** at −78 °C, a pale yellowish solution of **4** is obtained. Sulfur