Interaction of a Spin-Labeled Phenylalanine Analogue with Normal and Sickle Hemoglobins: Detection of Site-Specific Interactions through Spin-Label-Induced ^{1}H NMR Relaxation†

Yu-Hwei Lee, Bruce L. Currie, and Michael E. Johnson*

Department of Medicinal Chemistry and Pharmacognosy, University of Illinois at Chicago, Chicago, Illinois 60680

Received January 16, 1986; Revised Manuscript Received May 6, 1986

ABSTRACT: In a preliminary report, we have previously shown that *N*-[(2,2,5,5-tetramethyl-1-oxypyrrolidin-3-yl)carbonyl]-L-phenylalanine *tert*-butyl ester (SL-Phe) exhibits specific binding to hemoglobin and an antiaggregation activity more than 2 orders of magnitude greater than that of phenylalanine [Lu, H.-Z., Currie, B. L., & Johnson, M. E. (1984) *FEBS Lett. 173*, 259–263]. Transverse ^{1}H NMR relaxation measurements have been used to investigate the interaction of SL-Phe with hemoglobin molecules by use of the resonances assigned to the C2 protons of the β2 His, the β143 His, and the β146 or β97 His residues as intrinsic probes. Distance calculations using the paramagnetically induced relaxation data suggest that the SL-Phe binding site is ~12–16 Å away from the C2 protons of the β2 His and the β146 or β97 His residues in the (carbonmonoxy)hemoglobin tetramer; for deoxyhemoglobin, the distances are ~14–17 Å between the SL-Phe binding site and the C2 protons of the β2 His, the β143 His, and the β146 His residues. Calculations using the (carbonmonoxy)hemoglobin crystal atomic coordinates only restrict the probable SL-Phe binding region to the full F and H helices of the β-chain and a small section of the α-chain. For deoxyhemoglobin, the distance calculations provide greater restrictions on the probable binding region, limiting it to small sections of the β-chain F, G, and H helices near the EF bend and to a few residues on the α-chain. The coincidence between the probable binding regions for both (carbonmonoxy)hemoglobin and deoxyhemoglobin suggests that the binding site is probably the same for both hemoglobin forms. Most of the residues whose coordinates are consistent with the distance calculations for deoxygenated hemoglobin are at or near the lateral contact site that is complementary to the β6 mutation site within the sickle hemoglobin double-strand structure that is considered to be the fundamental unit of the sickle hemoglobin polymer fiber. Binding of SL-Phe at this region could thus explain its strong inhibitory activity. Further work in defining the binding stereochemistry should be helpful in developing antisickling agents with higher activity and specificity.

Sickle cell anemia results from the substitution of a valine residue for glutamic acid at the β6 position in the Hb[1] molecule. Upon deoxygenation, the HbS molecules aggregate together to form long helical fibers that deform and rigidify the erythrocytes. [For a review, see Dean and Schechter (1978a–c).]

The basic unit of the sickle hemoglobin fiber appears to be a pair of monofilament strands, with the two strands approximately in half-register with each other (Edelstein, 1980, 1981; Potel et al., 1984). The arrangement of the individual HbS molecules within these strands has been suggested by Love and co-workers from X-ray studies of HbS crystals (Wishner et al., 1976; Padlan & Love, 1985). The intermolecular contact regions within the deoxy-HbS double strand include two axial and two lateral sites. The β6 mutation is located within one of the lateral contact sites. In the deoxy-HbS double-strand structure, the β6 Val residue from a tetramer in one strand fits into a hydrophobic pocket formed by EF helices of a β-chain in a tetramer from the neighboring strand. That molecule, in turn, has the β6 Val from its other β-chain inserted into the hydrophobic EF pocket in the next molecule up or down the chain in the original strand. Thus, each tetramer contributes one β6 mutation site and one hydrophobic pocket to the double-strand structure.

A variety of compounds has been shown to inhibit deoxy-HbS aggregation through noncovalent interactions, presumably by competitive binding at one of the important HbS–HbS intermolecular contact sites. In vitro studies utilizing amino acids (Noguchi & Schechter, 1978), various phenyl derivatives (Behe & Englander, 1979), alkylureas (Poillon, 1980), small peptides (Gorecki et al., 1980), aryl-substituted alanines (Poillon, 1982), halogenated aryl derivatives of oxy acids (Abraham et al., 1983), disubstituted benzoic acids (Abraham et al., 1984), and biaromatic phenylalanyl derivatives (Votano et al., 1984) suggest several structural correlations with antigelation activity. Bicyclic and biaromatic compounds appear to be more effective than monocyclic compounds, and aromatic moieties are more effective than aliphatic (Votano et al., 1984). For aromatic compounds, the higher the hydrophobicity and ring polarizability, the higher will be the gelation inhibitory activity (Gorecki et al., 1980). However, aromaticity alone is not a sufficient condition for inhibition of deoxy-HbS ag-

† Supported in part by grants from the National Institutes of Health (HL-23697 and HL-15168). This paper represents a portion of the dissertation of Y.-H.L. submitted in partial fulfillment of requirements for the Ph.D. degree at the University of Illinois at Chicago and was done in part during the tenure of an Established Investigatorship of the American Heart Association to M.E.J.

[1] Abbreviations: Hb, hemoglobin; COHb, carbonmonoxy-liganded ferrous Hb; deoxy-Hb, unliganded ferrous Hb; HbA, normal adult Hb; HbS, sickle Hb; NMR, nuclear magnetic resonance; EPR, electron paramagnetic resonance; SL-Phe, *N*-[(2,2,5,5-tetramethyl-1-oxypyrrolidin-3-yl)carbonyl]-L-phenylalanine *tert*-butyl ester; Tempone, 2,2,6,6-tetramethyl-4-oxopiperidine-1-oxyl; Bis-Tris, [bis(2-hydroxyethyl)amino]tris(hydroxymethyl)methane; EDTA, ethylenediaminetetraacetic acid; TLC, thin-layer chromatography.

0006-2960/86/0425-5647$01.50/0

gregation as shown by the fact that several aromatic compounds also enhance gelation (Noguchi & Schechter, 1978).

To fully correlate inhibitor structure with inhibitory activity, a detailed knowledge of the binding site stereochemistry on the Hb molecule is also needed. ^{1}H NMR relaxation studies (Novak et al., 1977, 1979) have suggested that there is a binding site for aromatic compounds near the heme iron of Hb, but no detailed binding site has been proposed. This site would probably be consistent with inhibitor binding in the hydrophobic pocket at the lateral contact region that is complementary to the β6 mutation site within the deoxy-HbS polymer structure (Ross & Subramanian, 1977). X-ray studies have shown that three pairs of clofibric acid molecules cocrystalize with deoxy-HbA at sites inside the internal cavity of the deoxy-Hb tetramer, and one pair cocrystallizes with COHb at a site near α14 Trp (Abraham et al., 1983). Recent ^{1}H NMR studies on the interaction of phenylalanine (and tryptophan) with deoxy-Hb also suggest that phenylalanine binds to deoxy-HbS at locations close to the β-heme cleft and to the β2 His residues (Russu et al., 1986). Binding studies of biaromatic peptides with deoxy-Hb have suggested the existence of one symmetric binding site with moderate affinity (i.e., two identical sites per Hb tetramer) and one or more sites with weaker affinity (Votano & Rich, 1985). Recent ^{1}H NMR studies with SL-Phe, a spin-labeled analogue of phenylalanine with high antigelation activity, also suggest that there may be binding site(s) near the β-chain N- and C-termini of COHbS (Lu et al., 1984). Thus, current information suggests that different inhibitors may bind at different locations and/or that there may be more than one binding site per Hb tetramer for some inhibitors. However, there is still little detailed information on inhibitor binding site location(s) or binding stereochemistry under solution conditions.

In this paper, we report the use of ^{1}H NMR relaxation measurements to study the interaction of SL-Phe with Hb. Using the published ^{1}H NMR assignments for several of the His residues of COHb and deoxy-Hb (Kilmartin et al., 1973; Fung et al., 1975; Russu et al., 1980, 1982; Russu & Ho, 1982; Perutz et al., 1985), we have used the proton resonances assigned to the β2 His, the β143 His, and the β146 or β97 His residues as intrinsic probes to monitor their relaxation behaviors in the presence of the paramagnetic SL-Phe. From the paramagnetically induced contributions to the relaxations of these resonances, we have calculated the probable distances between these histidine resonances and the SL-Phe binding site. Those regions of the Hb molecule that are consistent with these distances were then determined from the Hb crystal coordinates.

Materials and Methods

General. Optical spectra were obtained on a Cary 118C spectrophotometer. ^{1}H NMR spectra were obtained at 200 MHz, on a Nicolet NMC200WB spectrometer locked on the internal solvent ^{2}H signal. EPR spectra were taken on an IBM ER-200 spectrometer interfaced to a Nicolet 1174 time averager. The spin probe Tempone and all other chemicals used for the synthesis of SL-Phe were purchased from Aldrich Chemical Co. All glassware used in preparing samples for NMR relaxation measurements was soaked in alkaline EDTA solution overnight and washed with deionized water to remove paramagnetic impurities before use in Chelex 100 treatment of solutions. Solution pH values were measured on a Corning pH meter (Model 130) equipped with an Ingold microcombination electrode (3 mm × 180 mm). The pH values (pH_M) were obtained directly from the pH meter reading without correction for the deuterium isotope effect on the glass electrode (Glasoe & Long, 1960).

Synthesis of N-[(2,2,5,5-Tetramethyl-1-oxypyrrolidin-3-yl)carbonyl]-L-phenylalanine tert-Butyl Ester (SL-Phe). A mixed carboxylic acid anhydride method similar to that reported for other *N*-acylphenylalanine derivatives was used to prepare SL-Phe from 3-carboxy-2,2,5,5-tetramethylpyrrolidine-1-oxyl (2.69 mmol) and L-phenylalanine *tert*-butyl ester (2.69 mmol) (Goebel et al., 1982). Slightly yellow needles of SL-Phe were obtained that showed one spot on TLC with an R_f of 0.67 in chloroform–methanol (9:1) on silica gel. EPR analysis showed the typical three-line spectrum of a nitroxyl free radical. The ^{1}H NMR spectrum was consistent with the expected product. Anal. Calcd for $C_{22}H_{33}N_2O_4$: C, 67.84; H, 8.54; N, 7.19. Found: C, 67.94; H, 8.56; N, 7.15.

Sample Preparation. HbS was prepared from whole blood samples obtained from donors homozygous for HbS; Hb composition was checked by electrophoresis (Helena Laboratories). Blood samples with more than trace amounts (~5%) of HbA or HbF were discarded. Normal blood samples for HbA were obtained from the University of Illinois Hospital blood bank. Membrane-free solutions of HbA and HbS were prepared by the methods of Rossi-Fanelli et al. (1961) and maintained under CO atmosphere. Hb solutions were then desalted on a Sephadex G-25 column with CO-saturated 5 mM sodium phosphate buffer at pH 7.2. All Hb and D_2O buffer solutions were treated with Chelex 100 (Bio-Rad) to remove paramagnetic ions (Willard et al., 1969).

Hb samples for NMR studies were prepared by exchanging the Hb solution 4 times with Bis-Tris buffer in D_2O, pH_M 7.4, so that the final water content in the Hb solution was less than 5%. Then, the D_2O-exchanged COHb solutions were concentrated to 20 g/dL by ultrafiltration and fully flushed with CO before being loaded into 5-mm NMR tubes. D_2O-exchanged Hb solutions used for the preparation of deoxy-Hb samples were first fully oxygenated by flushing slowly with oxygen in a pear-shaped flask on a rotary evaporator under a flood lamp. After the completeness of Hb oxygenation from the optical spectra was checked, solutions were deoxygenated by flushing with nitrogen gas until a characteristic purple solution formed at a concentration of about 20 g/dL. Stock solutions (1.4 mM) of Tempone and SL-Phe were prepared by dissolving the respective compounds in nitrogen-saturated 100 atom % D deuterium oxide with low paramagnetic impurities (Aldrich) under a nitrogen atmosphere.

D_2O-exchanged deoxy-Hb and COHb solutions (20 g/dL) were mixed with 1.4 mM stock solutions of Tempone and SL-Phe, respectively, in 1:1 volume ratios in the 5-mm NMR tubes under nitrogen atmosphere. Blank control samples were prepared by diluting the 20 g/dL Hb solutions with equal volumes of D_2O. The final samples with 10 g/dL hemoglobin and no spin probe, with 0.7 mM Tempone, or with 0.7 mM SL-Phe, respectively, were in 0.1 M Bis-Tris, pH_M 7.3 ± 0.1 (COHb) or pH_M 7.4 ± 0.1 (deoxy-Hb). These NMR samples were sealed with serum caps (Wilmad) and coated with epoxy (Hardman Inc.) to prevent oxygen permeation. Hb samples containing SL-Phe or Tempone were checked by EPR following the NMR measurements to ensure that no significant reduction of the nitroxide occurred during the NMR measurement. For measurements with the reduced form of SL-Phe, sodium dithionite was added to the COHb + SL-Phe or deoxy-Hb + SL-Phe samples under nitrogen atmosphere.

T_2 Relaxation Measurements. T_2 relaxation times for the protons of the histidine residues on the surface of the Hb molecule were measured by a Hahn spin-echo pulse sequence, (equilibration time–90_x–τ–90_x–180_y–90_x–τ–accumulation)$_n$

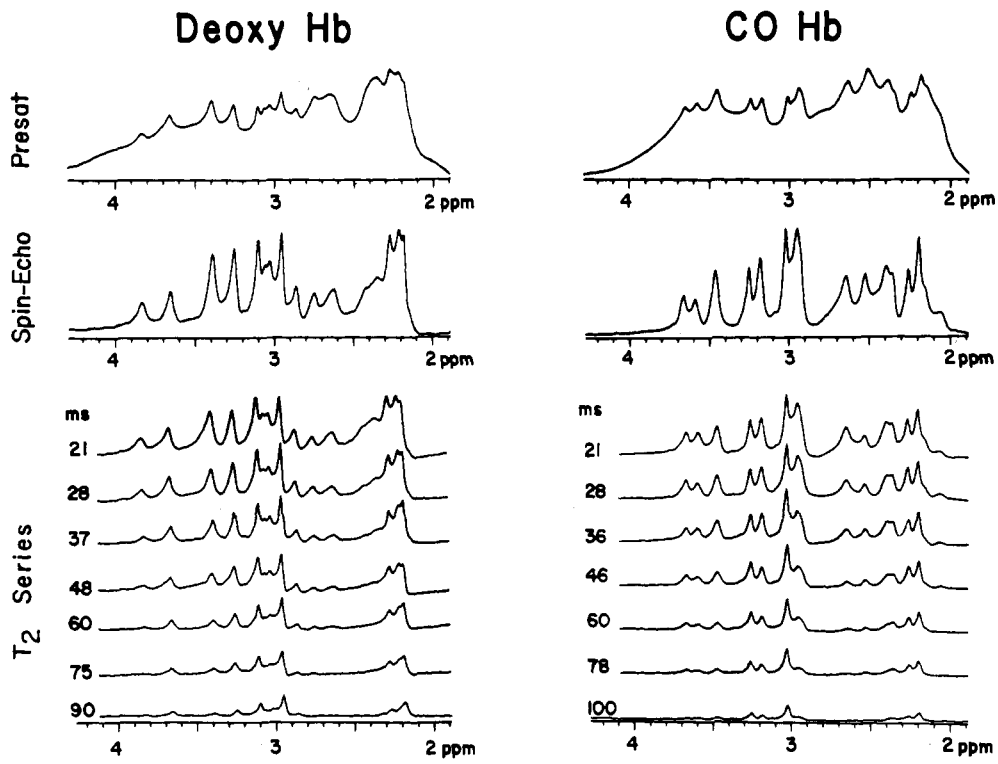

FIGURE 1: Comparison of ^{1}H NMR spectra of aromatic residue regions for Hb using conventional one pulse with solvent presaturation (presat) methods and spin-echo methods (top). Stacked plots with increasing delay times, τ, using the spin-echo pulse sequence are shown at the bottom of the figure.

(Campbell et al., 1975), with quadrature detection. The 90_x–180_y–90_x composite pulse was used in place of a simple 180° pulse to compensate for off-resonance effects (Levitt & Freeman, 1981). To decrease the dynamic range problem arising from the residual HDO resonance, the decoupler was set to the frequency of the HDO signal and turned on during the initial equilibration time to presaturate the HDO signal. The pulse delay was set longer than 5 times the longest T_1 in the sample. The decoupler power was optimized to be large enough to reduce the HDO resonance to an intensity comparable to that of the Bis-Tris buffer resonances and to avoid interfering with the aromatic region of interest in the ^{1}H NMR spectrum. No detailed T_1 measurements were done since cross-relaxation effects would make their interpretation difficult or impossible (Kalk & Berendson, 1976).

The spectral width was set for 3000 Hz, and several hundred scans were accumulated for each spectrum. Data were collected in 8K data blocks and processed with floating point routines to retain dynamic range. The probe temperature of the instrument was kept at 27 ± 1 °C. The chemical shifts of the aromatic proton resonances were referenced with respect to the residual HDO signal. The intensities of the individual resonances at various delay times were measured, and T_2 relaxation times were calculated, with the two-parameter nonlinear regression method in the Nicolet 1280 NMR software analysis routines.

RESULTS

Comparison between Presat and Spin-Echo Methods. Figure 1 shows the ^{1}H NMR spectra of the aromatic region for COHb and deoxy-Hb over the range from 4.3 ppm to 1.8 ppm downfield from the residual HDO resonance at 200 MHz and 27 °C. The "conventional" spectra of COHb and deoxy-Hb, obtained with a 90° pulse and presaturation of the solvent resonance (presat), are shown at the top of Figure 1, with the broad envelope in the aromatic region resulting from a number of overlapping signals that are produced by buried aromatic residues within the Hb structure. Although the C2 and C4 proton resonances of the His residues arise from relatively isolated environments and have longer T_2 relaxation times than other aromatic protons, the spectrum is not sufficiently resolved for detailed quantitative analysis. The spin-echo method has been used in ^{1}H NMR studies of biological systems by Campbell et al. (1975) and has been shown to selectively suppress the broad background from the buried aromatic residues due to their short T_2 relaxation times. More recent work has shown that substantial information about molecular interactions may be obtained through spin-echo methods (Lee et al., 1982; Sharp & Frasch, 1985; Frasch & Sharp, 1985).

We use a combination of spin-echo methods and presaturation of the solvent resonance, as described under Materials and Methods, to produce a considerable suppression of both the solvent peak and the broad underlying envelope of aromatic resonances. Spectra obtained with a pulse spacing, τ, of 16 ms for COHb and deoxy-Hb are shown below the presat spectra in Figure 1 (noted as spin echo). Compared with the presat method alone, the spectra obtained with the combined presat/spin–echo method are well-resolved and contain histidine proton resonance and amplitude information that would be difficult to obtain from the former method. Stacked plots of 200-MHz ^{1}H NMR spectra for COHb and deoxy-Hb obtained with this method are shown at the bottom of Figure 1 for a series of delay times ranging from 21 to 100 ms.

Effect of Tempone and SL-Phe on His C2 Proton Resonances of Hb. The 200-MHz ^{1}H NMR aromatic region spectra for COHbS and COHbA and for deoxy-HbS and deoxy-HbA are shown at the tops of Figures 2 and 3, respectively. The spectra were obtained by using the spin-echo pulse sequence with a delay time, τ, of 16 ms. In the spectra of COHb (Figure 2), the C2 and C4 proton resonances from the histidyl residues are labeled from A to S, following the

Table I: Effect of 0.7 mM Tempone and SL-Phe on T_2 Relaxation Times of Histidine Proton Resonances for COHb

COHb + spin probe	Hb type	T_2 (ms)							
		A	C	B + D	E	F	G	I + J + K + L	S
none	A	75	73	59	119	79	134	59	92
none	S	71	67	55	119	92	120	58	82
Tempone	A	70	74	53	108	89	146	53	67
Tempone	S	64	72	51	105	86	120	56	70
SL-Phe	A	44	46[a]	41	83	30	66	40	37
SL-Phe	S	48	*b*	42	91	37	52	40	35

[a] This T_2 was determined in the presence of 0.1 mM SL-Phe. [b] T_2 not obtainable.

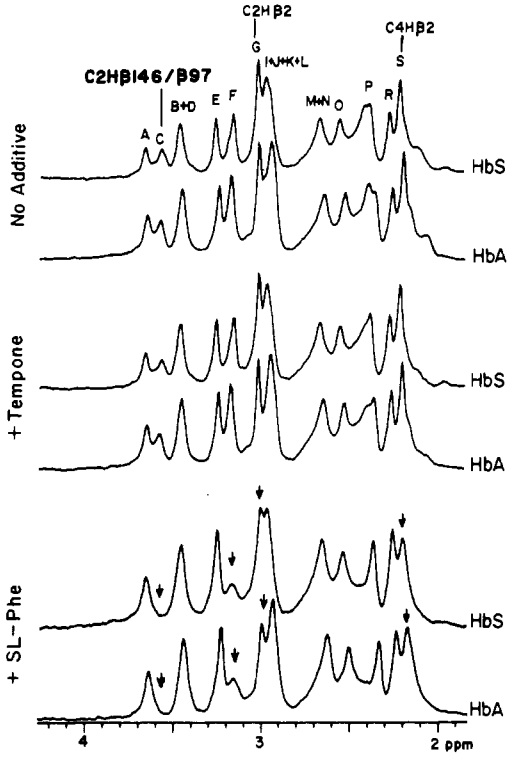

FIGURE 2: Comparison of histidine proton resonances for COHbS and COHbA in the 200-MHz ^{1}H NMR spectra of COHb at a concentration of 10 g/dL in 0.1 M Bis-Tris buffer in D_2O, pH_M 7.3, at 27 °C, with no added spin probe, with 0.7 mM Tempone, and with 0.7 mM SL-Phe. Resonance assignments in the top spectrum are from Russu et al. (1980, 1982), Russu and Ho (1982), and Perutz et al. (1985).

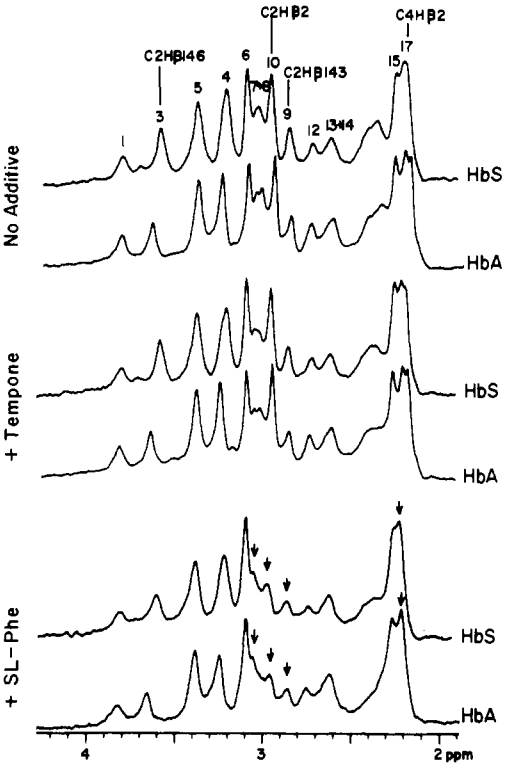

FIGURE 3: Comparison of histidine proton resonances for deoxy-HbS and deoxy-HbA in the 200-MHz ^{1}H NMR spectra of deoxy-Hb at a concentration of 10 g/dL in 0.1 M Bis-Tris buffer in D_2O, pH_M 7.4, at 27 °C, with no added spin probe, with 0.7 mM Tempone, and with 0.7 mM SL-Phe. Resonance assignments are from Russu et al. (1982) and Russu and Ho (1982).

published convention (Russu et al., 1980, 1982; Russu & Ho, 1982). Peak G has been assigned to the C2 proton of the β2 His (Russu & Ho, 1980). Resonance C was initially assigned to the C2 proton of the β146 His residue (Russu et al., 1980), but more recent work suggests that it should be assigned to the C2 proton of the β97 His residue (Perutz et al., 1985). Figure 3 shows the spectra of deoxy-Hb, with resonances labeled from 1 to 17 following the published convention (Russu & Ho, 1982). Among these resonances, peaks 3, 10, and 9 have been assigned to the C2 protons of the β146, β2, and β143 His residues, respectively (Russu et al., 1982; Russu & Ho, 1982). On the whole, the ^{1}H NMR spectra of HbA and HbS appear to be nearly identical under these conditions for both the CO and deoxy forms of Hb.

Also shown in Figures 2 and 3 are Hb spectra with 0.7 mM Tempone and with 0.7 mM SL-Phe. A qualitative comparison of the spectra for COHb (Figure 2) indicates that the addition of 0.7 mM Tempone produces no significant change in the spectrum. However, in the presence of 0.7 mM SL-Phe, resonance C completely disappears, and resonances F, G, and S are partially suppressed. The spectra of COHbA and COHbS under the same conditions are virtually identical. For deoxy-Hb (Figure 3), the addition of SL-Phe causes resonances 9 and 10 to be largely suppressed, whereas the addition of Tempone produces virtually no change, as compared with the spectra of deoxy-Hb in the absence of any nitroxide. Similarly, there is no significant difference between the spectra of deoxy-HbA and deoxy-HbS under equal conditions. The spectra for COHb and deoxy-Hb in the presence of the diamagnetic reduced form of SL-Phe are equivalent to those of COHb and deoxy-Hb in the absence of any additive (data not shown), indicating that the observed broadening is produced by the nitroxide moiety of SL-Phe.

T_2 relaxation times for the aromatic proton resonances of COHb and deoxy-Hb are listed in Tables I and II, respectively. A conservative estimate of the uncertainty in the measurements is 20%. This uncertainty is partly due to the existence of overlapping resonances whose chemical shifts are pH-dependent and partly due to limits in the resolution of the spectra where some residual intensity from the broad envelope still exists so that T_2 values shorter than 20 ms cannot be reliably measured.

Table I shows that the T_2 values of the proton resonances for the histidine residues are essentially equivalent for COHb without added spin probe and for COHb in the presence of

Table II: Effect of 0.7 mM Tempone and SL-Phe on T_2 Relaxation Times of Histidine Proton Resonances for Deoxy-Hb

		T_2 (ms)								
deoxy-Hb + spin probe	Hb type	1	3	5	4	6	7 + 8	10	9	17
none	A	42	75	58	86	113	90	137	66	88
none	S	43	70	61	83	105	76	123	54	82
Tempone	A	49	75	55	85	112	*a*	110	62	77
Tempone	S	43	67	52	74	86	58	87	39	70
SL-Phe	A	35	59	45	58	87	*a*	49	27	*a*
SL-Phe	S	27	42	39	48	75	36	36	22	42

[a] T_2 is not obtainable.

Table III: Effect of 0.7 mM Tempone and SL-Phe on T_2 Ratios of Histidine Proton Resonances for COHb[a]

	T_2 ratio							
COHb + spin probe	A	C	B + D	E	F	G	I + J + K + L	S
Tempone	0.92	1.04	0.91	0.90	1.02	1.04	0.92	0.79
SL-Phe	0.63	0.66[b]	0.74	0.73	0.40	0.46	0.68	0.41

[a] Ratios listed are the average values for COHbA and COHbS combined together. [b] The value is in the presence of 0.1 mM SL-Phe; in the presence of 0.7 mM SL-Phe, the resonance is completely suppressed.

Table IV: Effect of 0.7 mM Tempone and SL-Phe on T_2 Ratios of Histidine Proton Resonances for Deoxy-Hb[a]

	T_2 ratio								
deoxy-Hb + spin probe	1	3	5	4	6	7 + 8	10	9	17
Tempone	1.09	0.97	0.90	0.94	0.91	0.70	0.75	0.85	0.86
SL-Phe	0.74	0.68	0.71	0.62	0.74	0.43	0.32	0.41	0.49

[a] Ratios listed are the average values for COHbA and COHbS combined together.

Tempone. However, the addition of 0.7 mM SL-Phe produces profound effects on the T_2 value for resonance C, assigned to the C2 proton of β146 or β97 His, which is completely suppressed. However, when the SL-Phe concentration is reduced to 0.1 mM, resonance C is only partially suppressed, permitting measurements of T_2, which decreases from 70 to 46 ms. For resonance G, assigned to the C2 proton of β2 His, T_2 decreases from 130 to 60 ms in the presence of 0.7 mM SL-Phe; the unassigned resonance F also shows a large T_2 decrease, i.e., from 85 to 35 ms. The rest of the resonances appear to be slightly influenced by the presence of SL-Phe. The change of T_2 values in the presence of Tempone and SL-Phe can be compared through T_2 ratios, i.e., T_2(spin probe)/T_2(control); these ratios are listed in Table III. The ratio of most resonances in the presence of Tempone are close to 1. However, the effect of SL-Phe on the resonances can be categorized into three levels: the completely suppressed resonance C is heavily affected such that the T_2 ratios are not obtainable at 0.7 mM SL-Phe (but the ratio is 0.66 with 0.1 mM SL-Phe); the partially suppressed resonances F, G, and S with T_2 ratios of $\sim$0.4 are moderately affected, while resonances A and E and the combined resonances B + D and I + J + K + L are only slightly affected with T_2 ratios of $\sim$0.7.

For the T_2 relaxation times of the proton resonances of the histidine residues of deoxy-Hb (Table II), no significant difference is observed in T_2 values between samples in the presence or absence of Tempone. Again, in the presence of SL-Phe, the paramagnetic effect on the T_2 relaxation times can be classified into two levels with respect to the T_2 ratio listed in Table IV. Resonances with a T_2 ratio of $\sim$0.4, such as 7 + 8 (not assigned), 9 (assigned to C2-H β143 His), 10 (assigned to C2-H β2 His) and 17 (assigned to C4-H β2 His), are moderately affected; other resonances with T_2 ratios of $\sim$0.7 are slightly affected.

Estimates of Distances from SL-Phe Binding Site to His Residues of Hb. To estimate the distances from the SL-Phe binding site to the assigned histidine residues of Hb, we assume that the relaxation enhancement of the C2 or C4 protons of histidine residues is due to the binding of SL-Phe to Hb and that the observed relaxation rate is the sum of components resulting from both paramagnetic and diamagnetic contributions (Krugh, 1976):

$$1/T_{2\text{obsd}} = B/T_{2\text{b}}^{\text{p}} + (1 - B)/T_{2\text{f}}^{\text{p}} + 1/T_2^{\text{d}} \qquad (1)$$

The K_d of the SL-Phe–Hb complex is estimated to be $\sim$10 mM, as obtained from ultrafiltration studies, assuming two equivalent SL-Phe binding sites per Hb molecule (Lu et al., 1984). B is the fraction of binding sites occupied by SL-Phe, and $T_{2\text{obsd}}$, $T_{2\text{b}}$, and $T_{2\text{f}}$ are respectively the observed, the bound, and the free spin–spin relaxation times. The superscripts p and d represent the paramagnetic and diamagnetic contributions, respectively. From the mass action expression, the value of B is estimated to be 0.05 for samples in the presence of 0.7 mM SL-Phe and 0.0076 for samples in the presence of 0.1 mM SL-Phe, under the conditions of this study. Furthermore, it is assumed that relaxation in the presence of Tempone, which interacts nonspecifically with Hb, will include both the "intrinsic" diamagnetic relaxation (third term in eq 1) and the effect of random (nonbinding) collisions of the nitroxide with the Hb molecule (second term in eq 1). With these assumptions, the paramagnetically induced contributions of the bound SL-Phe to the relaxation of protons assigned to histidine residues are estimated to be as follows: $T_{2\text{b}}^{\text{p}}$ (C2-H β146 or β97, COHb), $\sim$0.94 ms; $T_{2\text{b}}^{\text{p}}$ (C2-H β2, COHb), $\sim$5.4 ms; $T_{2\text{b}}^{\text{p}}$ (C2-H β146, deoxy-Hb) $\sim$9.2 ms; $T_{2\text{b}}^{\text{p}}$ (C2-H β2, deoxy-Hb), $\sim$3.9 ms; $T_{2\text{b}}^{\text{p}}$ (C2-H β143, deoxy-Hb), $\sim$2.5 ms.

The distance from the aromatic protons of histidine residues to the nitroxide of the bound SL-Phe can be calculated from (Krugh, 1976)

$$r\ (\text{Å}) = 540[f(\tau_c)T_{2\text{b}}^{\text{p}}]^{1/6} \qquad (2)$$

where

$$f(\tau_c) = 4\tau_c + \frac{3\tau_c}{1 + \omega_I^2\tau_c^2} + \frac{13\tau_c}{1 + \omega_s^2\tau_c^2} \qquad (3)$$

Table V: Estimates of Distances between C2 Protons of Histidine Residues and Nitroxide of Bound SL-Phe

resonance	Hb	assignment	distance (Å)
C	COHb	C2-H, β97/β146 His	12 ± 1
G	COHb	C2-H, β2 His	16 ± 2
3	deoxy-Hb	C2-H, β146 His	17 ± 2
10	deoxy-Hb	C2-H, β2 His	15 ± 2
9	deoxy-Hb	C2-H, β143 His	14 ± 2

and τ_c, ω_I, and ω_s are the correlation time and the proton and electron Larmor frequency, respectively. The correlation time is given by

$$1/\tau_c = 1/\tau_r + 1/\tau_s + 1/\tau_b \quad (4)$$

where τ_c is the rotational correlation time of the vector between the unpaired electron of the nitroxide and the protons of Hb, τ_s is the electronic spin relaxation time, and τ_b is the SL-Phe-bound state lifetime. τ_r is the correlation time for the nitroxide of SL-Phe bound to the Hb molecule. Since SL-Phe bound to Hb has been shown to exhibit an "immobilized" EPR spectrum with a correlation time comparable to that of the Hb molecule itself (Lu et al., 1984), τ_r can be estimated as being equivalent to the rotational correlation time of the Hb molecule, i.e., $\tau_r \sim 3 \times 10^{-8}$ s (McCalley et al., 1972). The electronic relaxation time is $\tau_s \sim 6.6 \times 10^{-6}$ s (Thomas et al., 1976). τ_b can be estimated indirectly by assuming diffusion controlled binding and that the "radius" of the SL-Phe binding site is approximately 7 Å, yielding $\tau_b \sim 3.3 \times 10^{-7}$ s. Thus, τ_c is primarily determined by τ_r, which is the shortest of the three times τ_r, τ_s, and τ_b and which is also known fairly accurately. These values give $\tau_c \sim 2.7 \times 10^{-8}$ s, and $f(\tau_c) \sim 1.1 \times 10^{-7}$ s.

Including uncertainties in the binding model, the estimate of K_d, and the T_2 measurements, a conservative estimate of the uncertainty in T_{2b}^{β} is probably a factor of 2. With these estimates, the calculated distances and uncertainties from the protons of the assigned histidine residues to the nitroxide of the bound SL-Phe are listed in Table V.

Probable Binding Regions. Those regions of the Hb molecule that are consistent with these distances were then determined by first calculating the distances from the ε-carbon of the β2 His side chain to the side chains of all the other Hb amino acid residues and selecting those that were within the sum of the estimated distance plus uncertainty for that residue. The "uncertainty" range used in the calculation was also increased by an additional 2 Å beyond that listed in Table V to account for conformational fluctuations of the histidine side chains that will occur in the solution state. Then, the distances from a second His residue affected by SL-Phe to the side chains of all the residues selected in the first step were calculated, and the group of selected residues was reduced to those that satisfied both distance constraints, etc. Distances were calculated with the atomic coordinates from X-ray structure determinations of COHb and deoxy-Hb (Baldwin, 1980; Fermi, 1975).

For the COHb form, the conflicting assignments for resonance C (Russu et al., 1980; Perutz et al., 1985) add some uncertainty to this calculation. However, the β97 His residue is located at the end of the F helix and is within about 13 Å of the C-terminal β146 His residue. Performing these calculations using both the β97 and β146 assignments for resonance C (alternately) and including all residues indicated by either assignment produce three regions that satisfy the COHb distance constraints: (1) from the β77 His in the EF helix bend through the full F helix to the β104 Arg residue, (2) the H helix from β130 Tyr to the β145 Tyr residue, and (3) the α34 Leu to α39 Thr residues near the N- and C-termini of the β-chain.

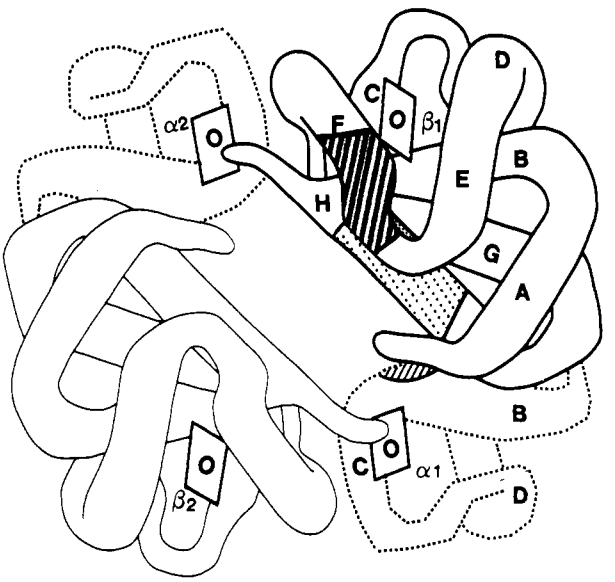

FIGURE 4: Schematic arrangement of β-subunits and part of the α-subunits of deoxy-Hb, with residues that are within the calculated distances from the SL-Phe binding site shown as cross-hatched regions for the F and H helices of the β-chain and for the α-chain. A small section of the G helix is also within the calculated distances but is located "behind" the F and H helices and is thus not shown. Adapted from Dickerson and Geis (1983).

For the deoxy form, the addition of the β143 His residue to the distance calculations adds further constraint, narrowing the three regions to (1) the F helix region from the β80 Asn to the β87 Thr residues, plus the β103 Phe and β104 Arg residues in the G helix, (2) the H helix ranging from β128 Ala to the β141 Leu residues, and (3) the α35 Ser and α36 Phe residues. These three regions are shown schematically for deoxy-Hb in Figure 4.

Discussion

In a previous preliminary report using rapid-scan cross-correlation NMR techniques, we have shown qualitatively that SL-Phe interacts specifically with COHbS at a site or sites that must be relatively close to the β2 and β146 or β97 His residues (Lu et al., 1984). In that work, the broad envelope of underlying aromatic resonances limited the quantitative information that could be obtained from the spectra. In this work, we demonstrate that the use of spin-echo techniques provides substantially enhanced resolution for the surface histidine resonances, as shown in Figure 1. These techniques also allow us to obtain quantitative information about the magnitude of the interaction through direct measurements of the T_2 relaxation times; in contrast to T_1 measurements, T_2 measurements are not significantly affected by cross-relaxation and can be reliably used in macromolecular systems.

In analyzing the relaxation data to obtain the location of any probable binding regions, we have assumed the existence of one unique primary binding site on each αβ dimer or two equivalent binding sites per Hb tetramer. This assumption is consistent with the results of binding studies by Votano and Rich (1985), who have shown that a series of biaromatic peptides associate with Hb at one preferred site and one or more sites with much lower affinity. If SL-Phe were to bind to two or more nonequivalent sites with approximately equal affinity, then only the qualitative conclusion that it binds near the affected resonances would be appropriate. However, the work of Votano and Rich (1985) indicates that compounds with significantly differing structures bind to one preferred

site, with other sites having significantly lower affinity. Thus, the assumption of one primary binding site per $\alpha\beta$ dimer appears reasonable. At the relatively low SL-Phe occupancy ratio used in this study, only the primary binding site should be significantly affected.

It should also be recognized that the resolution of these measurements is limited by conformational fluctuations of the histidine side chains at the Hb surface, plus the variety of possible SL-Phe conformations. The calculated distance is an estimate of the "average" separation between the histidine C2 proton and the nitroxide moiety of SL-Phe while SL-Phe is in the bound state and yields no information about the overall SL-Phe conformation. Even with the most precise relaxation measurements, there is probably still an intrinsic net uncertainty of 4–5 Å for each individual measurement. However, the use of "triangulation" with measurements for several assigned resonances can be used to substantially localize the binding interaction, and molecular modeling techniques can then be used to further refine the binding stereochemistry.

For COHb, the distance calculations provide only moderate restrictions on the probable binding region, including essentially the full F and H helices of the β-chain and a small section of the α-chain. Additional assignments for resonances affected by SL-Phe will be required to further restrict the probable binding region.

For deoxy-Hb, the distance calculations restrict the probable binding region to relatively small sections of the β-chain F and H helices, plus a few residues on the α-chain (Figure 4). The similarity between the probable binding regions for COHb and deoxy-Hb suggests that the binding region is probably the same for both Hb forms. The lack of any differences between the effects of SL-Phe on HbS and on HbA also indicates that the β6 mutation site itself plays no apparent role in SL-Phe binding. The majority of the residues that are calculated to fall within the probable binding region for deoxy-Hb are at or near the β-chain EF bend region that is complementary to the β6 mutation site within the deoxy-HbS double strand. Thus, it appears likely that the primary SL-Phe binding site is one in which binding would inhibit deoxy-HbS aggregation, consistent with its strong antigelation activity (Lu et al., 1984). Work is in progress to further define the binding site and stereochemistry; such information should be of substantial benefit in the design of selective antisickling agents.

ACKNOWLEDGMENTS

The Nicolet NMC200 used in these studies was purchased in part with funds from a research grant to M.E.J. (USPHS HL-15168) and is operated and maintained by the Research Resources Center of the University of Illinois at Chicago. Drs. R. A. Kleps and J. A. Anderson are thanked for instrumental assistance in various phases of this work. Crystal coordinates were obtained from the Brookhaven data bank.

Registry No. HbA, 9034-51-9; HbS, 55963-97-8; SL-Phe, 92455-23-7.

REFERENCES

Abraham, D. J., Perutz, M. F., & Phillips, S. E. V. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 324–328.

Abraham, D. J., Gazze, D. M., Kennedy, P. E., & Mokotoff, M. (1984) *J. Med. Chem. 27*, 1549–1559.

Baldwin, J. M. (1980) *J. Mol. Biol. 136*, 103–128.

Behe, M. J., & Englander, S. W. (1979) *Biochemistry 18*, 4196–4201.

Campbell, I. D., Dobson, C. M., Williams, R. J. P., & Wright, P. E. (1975) *FEBS Lett. 57*, 96–99.

Dean, J., & Schechter, A. N. (1978a) *N. Engl. J. Med. 299*, 752–763.

Dean, J., & Schechter, A. N. (1978b) *N. Engl. J. Med. 299*, 804–811.

Dean, J., & Schechter, A. N. (1978c) *N. Engl. J. Med. 299*, 863–870.

Dickerson, R. E., & Geis, I. (1983) *Hemoglobin: Structure, Function, Evolution, and Pathology*, Benjamin Cummings, Menlo Park, CA.

Edelstein, S. J. (1980) *Biophys. J. 32*, 347–360.

Edelstein, S. J. (1981) *J. Mol. Biol. 150*, 557–575.

Fermi, G. (1975) *J. Mol. Biol. 97*, 237–256.

Frasch, W. D., & Sharp, R. R. (1985) *Biochemistry 24*, 5454–5458.

Fung, L. W. M., Ho, C., Roth, E. F., & Nagel, R. L. (1975) *J. Biol. Chem. 250*, 4786–4789.

Glasoe, P. K., & Long, F. A. (1960) *J. Phys. Chem. 64*, 188–190.

Goebel, R. J., Currie, B. L., & Bowers, C. Y. (1982) *J. Pharm. Sci. 71*, 1062–1064.

Gorecki, M., Votano, J. R., & Rich, A. (1980) *Biochemistry 19*, 1564–1568.

Kalk, A., & Berendson, H. J. C. (1976) *J. Magn. Reson. 24*, 343–366.

Kilmartin, J. V., Breen, J. J., Roberts, G. C. K., & Ho, C. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 1246–1249.

Krugh, T. R. (1976) *Spin Labeling: Theory and Applications* (Berliner, L. J., Ed.) pp 339–372, Academic, New York.

Lee, L., Kossak, M. N., & Fung, L. W. M. (1982) *Biophys. J. 37*, 372a.

Levitt, M. H., & Freeman, R. (1981) *J. Magn. Reson. 43*, 65–80.

Lu, H.-Z., Currie, B. L., & Johnson, M. E. (1984) *FEBS Lett. 173*, 259–263.

McCalley, R. C., Shimshick, E. J., & McConnell, H. M. (1972) *Chem. Phys. Lett. 13*, 115–119.

Noguchi, C. T., & Schechter, A. N. (1978) *Biochemistry 17*, 5455–5459.

Novak, R. F., Kapetanovic, I. M., & Mieyal, J. J. (1977) *Mol. Pharmacol. 13*, 15–30.

Novak, R. F., Dershwitz, M., & Novak, F. C. (1979) *Mol. Pharmacol. 16*, 1046–1058.

Padlan, E. A., & Love, W. E. (1985) *J. Biol. Chem. 260*, 8280–8291.

Perutz, M. F., Gronenborn, A. M., Clore, G. M., Fogg, J. H., & Shih, D. T. (1985) *J. Mol. Biol. 183*, 491–498.

Poillon, W. N. (1980) *Biochemistry 19*, 3194–3199.

Poillon, W. N. (1982) *Biochemistry 21*, 1400–1406.

Potel, M. J., Wellems, T. E., Vassar, R. J., Deer, B., & Josephs, R. (1984) *J. Mol. Biol. 177*, 819–839.

Ross, P. D., & Subramanian, S. (1977) *Biochem. Biophys. Res. Commun. 77*, 1217–1223.

Rossi-Fanelli, A., Antonini, E., & Caputo, A. (1961) *J. Biol. Chem. 236*, 391–396.

Russu, I. M., & Ho, C. (1982) *Biochemistry 21*, 5044–5051.

Russu, I. M., Ho, N. T., & Ho, C. (1980) *Biochemistry 19*, 1043–1052.

Russu, I. M., Ho, N. T., & Ho, C. (1982) *Biochemistry 21*, 5031–5043.

Russu, I. M., Lin, A. K.-L. C., Yang, C.-P., & Ho, C. (1986) *Biochemistry 25*, 808–815.

Sharp, R. R., & Frasch, W. D. (1985) *Biochemistry 24*, 5449–5454.

Thomas, D. D., Dalton, L. R., & Hyde, J. S. (1976) *J. Chem. Phys. 65*, 3006–3024.

Votano, J. R., & Rich, A. (1985) *Biochemistry 24*, 1966–1970.

Votano, J. R., Altman, J., Wilchek, M., Gorecki, M., & Rich, A. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 3190–3194.
Willard, J. M., Davis, J. J., & Wood, H. G. (1969) *Biochemistry 8*, 3137–3144.
Wishner, B. C., Hanson, J. C., Ringle, W. M., & Love, W. E. (1976) *Proceedings of the Symposium on Molecular and Cellular Aspects of Sickle Cell Disease* (Hercules, J. I., Cottam, G. L., Waterman, M. R., & Schechter, A. N., Eds.) pp 1–29, DHEW Publication No. 76-1007, NIH, Bethesda, MD.

Observation of Manganese(II)–Ligand Superhyperfine Couplings in Complexes with Proteins by Electron Spin-Echo Spectroscopy†

Russell LoBrutto,‡ Geoffrey W. Smithers,§ George H. Reed,*,§ William H. Orme-Johnson,∥ Swee L. Tan,∥ and John S. Leigh, Jr.*,‡

Department of Biochemistry and Biophysics, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104, Institute for Enzyme Research, Graduate School, and Department of Biochemistry, College of Agricultural and Life Sciences, University of Wisconsin, Madison, Wisconsin 53705, and Department of Chemistry, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139

Received November 20, 1985; Revised Manuscript Received March 26, 1986

ABSTRACT: Pulsed electron paramagnetic resonance spectroscopy has been used to detect Mn(II)–ligand superhyperfine couplings in complexes with creatine kinase and in the Mn(II) metalloprotein concanavalin A. Electron spin-echo envelopes from Mn(II), bound in these complexes, are modulated by superhyperfine interactions between Mn(II) and nearby, weakly coupled nuclear spins. The characteristic frequencies of the modulations were obtained by Fourier transformation of the three-pulse, spin-echo envelopes. In transition-state analogue complexes of creatine kinase (enzyme–Mn^{II}ADP–anion–creatine), superhyperfine interactions from the directly coordinated nitrogen of the thiocyanate ligand give envelope modulations. The source of the modulations was confirmed by measurements with the ^{14}N and ^{15}N forms of thiocyanate. On the other hand, the nitrogen of coordinated nitrate, which is two bonds removed from the paramagnetic center, does not produce detectable modulations. In spectra for Mn(II) concanavalin A, envelope modulations are detected due to the nitrogen of the coordinated histidine residue. Complexes prepared in ^2H_2O give strong signals due to weakly coupled ^2H. For Mn(II)-doped single crystals of sodium pyrophosphate, signals are observed in the frequency domain spectra that are due to coupling from ^{31}P. Phosphorus signals from the ADP ligand in complexes with creatine kinase show approximately the same coupling constant but have a much broader line width.

Divalent manganese is a popular paramagnetic probe for both NMR[1] and EPR investigations of metal ion binding sites and liganding interactions in enzymes and other proteins (Mildvan & Gupta, 1978; Reed & Markham, 1984). While Mn(II) is a natural constituent or specific activator of relatively few proteins (McEuen, 1982), it does function virtually isomorphically with respect to Mg(II) as an activator of many other enzymes (Reed & Markham, 1984). The long-lived spin states of this S-state ion are conducive to EPR measurements at room temperature and provide for enhanced rates of relaxation for nearby nuclear spins in macromolecular complexes. Superhyperfine interactions between the unpaired electron spin of Mn(II) and nuclear spins in the immediate vicinity of the ion provide a means for the unequivocal identification of ligands. For example, the high nuclear spin multiplicity ($I = {}^5/_2$) and the relatively strong, scalar superhyperfine coupling from ^{17}O of directly coordinated oxygen ligands result in significant changes in the line widths of Mn(II) EPR signals relative to those observed for ligands with ^{16}O (Reed & Leyh, 1980). Despite the generally favorable electron spin relaxation properties of the ion, however, EPR signals for complexes of Mn(II) with proteins typically have line widths that are too broad to permit detection of superhyperfine splittings from more weakly coupled nuclei. Thus, superhyperfine coupling between Mn(II) and nuclei such as ^{14}N or ^{15}N is normally concealed in the line widths of the EPR signals (Reed & Markham, 1984).

ENDOR (Scholes, 1979) and ESEEM (Norris et al., 1980; Mims & Peisach, 1981; Thomann et al., 1984) spectroscopies have the potential for detection of nuclear spin couplings that remain unresolved in a conventional EPR spectrum. In particular, the success of ESEEM spectroscopy, exemplified by recent studies of iron and copper metalloproteins (Mims & Peisach, 1976, 1979), prompted an investigation of the applicability of this methodology to studies of Mn(II) in complexes with proteins. The present paper reports the results of ESE measurements for Mn(II) in transition-state analogue complexes with creatine kinase and for the Mn(II) metalloprotein, concanavalin A.

† This work was supported by Grants AM 17517, AM 36842, and GM 35752 (G.H.R.), GM 25052 (J.S.L.), and GM 28358 (W.H.O.-J.) from the National Institutes of Health.

* Address correspondence to these authors.
‡ University of Pennsylvania.
§ University of Wisconsin.
∥ Massachusetts Institute of Technology.

[1] Abbreviations: NMR, nuclear magnetic resonance; EPR, electron paramagnetic resonance; ENDOR, electron nuclear double resonance; ESEEM, electron spin-echo envelope modulation; ESE, electron spin-echo; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; S/N, signal-to-noise ratio; FFT, fast Fourier transform; H_1, magnetic field component of microwave radiation.

0006-2960/86/0425-5654$01.50/0