Saturation-Transfer Electron Paramagnetic Resonance Detection of Anisotropic Motion by Sickle Hemoglobin Molecules in the Polymer State†

P. Thiyagarajan‡ and Michael E. Johnson*

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

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ABSTRACT: The motional behavior of spin-labeled deoxygenated sickle hemoglobin has been studied by using both 9- and 35-GHz saturation-transfer electron paramagnetic resonance (EPR). Using spectral subtraction techniques and saturation-transfer EPR parameter correlation plots, we find that the saturation-transfer EPR spectra for the sickle hemoglobin gel state at high temperature and high hemoglobin concentration cannot be described as a simple superposition of spectra from immobilized hemoglobin plus solution-state hemoglobin but instead suggest that the individual sickle hemoglobin molecules exhibit limited, anisotropic, rotational oscillation within the polymer fiber. The spectra also imply that the symmetry axis for sickle hemoglobin rotational oscillation is approximately coincident with the nitroxide *z* axis of the covalently attached spin-label. We suggest that this anisotropic rotational motion may be produced by one or two of the known intermolecular contact sites within the sickle hemoglobin fiber acting as strong intermolecular binding sites, and producing "motional alignment" within the fiber; determining the location of the strong binding site should be important in focusing the future development of antisickling agents.

Electron microscopy and image reconstruction have provided detailed information about the ultrastructure of the deoxy sickle hemoglobin (HbS)[1] polymer fiber. The current view appears to be that, in its most common form, the fiber is about 200 Å in diameter, with an inner helical core of 4 monofilament HbS strands surrounded by an outer helix of 10 monofilament HbS strands to give a total of 14 strands (Dykes et al., 1979; Crepeau & Edelstein, 1984). Recent X-ray work is consistent with a 14-strand structure but suggests that the arrangement of strands may differ somewhat from that predicted by electron microscopy image reconstruction (Rosen & Magdoff-Fairchild, 1985). From symmetry considerations, other workers have also suggested that the fiber should contain 16 strands (Wellems & Josephs, 1979; Potel et al., 1984).

Regardless of the detailed ultrastructure of the fiber, however, there is strong consensus that the fundamental unit of the fiber is a pair of monofilament strands, with the two strands approximately in half-register with each other. The arrangement of the individual HbS molecules within these pairs of monofilament strands has been suggested on the basis of X-ray studies of HbS crystals grown in poly(ethylene glycol) (Wishner et al., 1976; Padlan & Love, 1985). Within the double strand, there are essentially two important types of contacts: the axial or longitudinal contact between the two molecules in the same strand and the side to side or lateral contact between molecules in adjacent strands.

Over the last decade, there has been substantial progress in developing a detailed thermodynamic characterization of the deoxy-HbS aggregation process [see Ferrone et al. (1985a,b) and references cited therein]. However, there is still little information, at a molecular level, on the extent to which the various contacts are energetically important to the *binding* process in polymer formation, as opposed to being merely spatial contacts resulting from molecular packing. Such information will be important in the design of stereochemically specific antisickling agents, in that it will permit design efforts to focus on those regions of the HbS molecule that are energetically important to the HbS polymerization process.

In these experiments, we have used a spin-label that is rigidly attached to the Hb molecule in conjunction with slow-motion spin-label saturation-transfer EPR to monitor HbS molecular motions within the polymer state. We have used both 9- and 35-GHz observation frequencies, in conjunction with ST-EPR spectral parameter correlation plots (Johnson et al., 1982), to evaluate the ST-EPR spectral superposition resulting from the polymeric and monomeric HbS components in the samples and to provide a partial characterization of the apparent HbS anisotropic motion in the polymer state. We have used spectral subtraction methods to obtain approximate ST-EPR spectra for the deoxy-HbS polymer component. The use of both 9- and 35-GHz obsevational frequencies provides enhanced resolution for the characteriztion of anisotropic motion and is useful for discriminating between the existence of anisotropic motion and multiple, overlapping spectral components with differing motional rates (Johnson & Hyde, 1981; Johnson et al., 1982; Fung & Johnson, 1983). We find that deoxy-HbS exhibits very slow but highly anisotropic motion within the polymer state, suggesting that some of the fiber contacts produce very strong "anchoring" of the HbS molecule for some orientations but allow wobbling or flexing of the HbS molecules about the strongly anchored sites.

Materials and Methods

HbS was prepared from sickle blood samples obtained from homozygous donors with less than about 5% HbA and HbF. Membrane-free (carbonmonoxy)-HbA and HbS were prepared and spin-labeled with Mal-6 following procedures in Abraham et al. (1975) and Johnson (1978). IHP was added

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‡ Present address: Chemistry Division, Argonne National Laboratory, Argonne, IL 60439.

[1] Abbreviations: Hb, hemoglobin; COHb, (carbonmonoxy)-liganded ferrous Hb; deoxy-Hb, unliganded ferrous Hb; HbA, normal adult Hb; HbS, sickle Hb; IHP, inositol hexaphosphate; EPR, electron paramagnetic resonance; ST-EPR, saturation-transfer EPR; Mal-6, 4-maleimido-2,2,6,6-tetramethylpiperidinyl-1-oxy.

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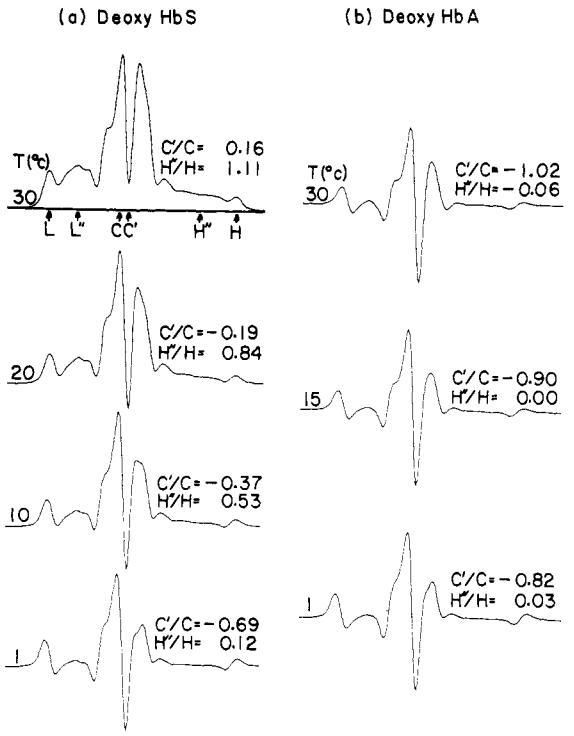

FIGURE 1: (a) X-band V_2' spectra for deoxy-HbS at a concentration of 27 g/dL, as a function of increasing temperature. Samples were prepared, loaded into capillaries, and then allowed to equilibrate for 1–2 h at 1 °C before beginning the temperature increase series. The samples were then equilibrated for about 0.5 h at each temperature before the spectrum was recorded, and increasing the temperature to the next value. Increasing the equilibration time did not produce any significant changes in the spectral shape. Note the relatively rapid increase in spectral intensity in the wings of the spectra, compared with the slow change in the central (C') dip, as the temperature increases. (b) X-band V_2' spectra for deoxy-HbA at a concentration of 30 g/dL, as a function of increasing temperature. The spectra show very little temperature dependence. The C'/C and H''/H ratios are shown for each spectrum to provide further comparison.

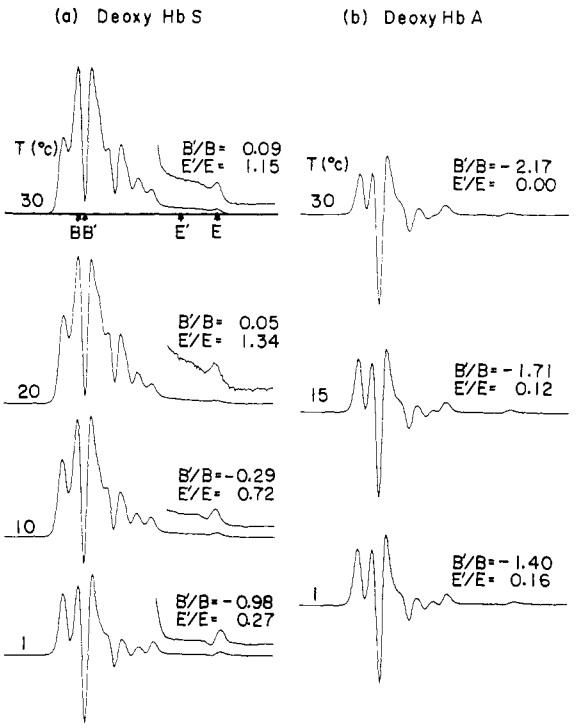

FIGURE 2: (a) Q-band V_2' spectra for deoxy-HbS at a concentration of 30 g/dL, as a function of increasing temperature. Samples were prepared, loaded into capillaries, and then allowed to equilibrate overnight at 1 °C before beginning the temperature increase series. The samples were then equilibrated for about 0.5 h at each temperature before the spectrum was recorded, and increasing the temperature to the next value. Increasing the equilibration time did not produce any significant changes in the spectral shape. Note the relatively rapid increase in spectral intensity in the high-field E' region of the spectra, compared with the slow change in the downfield B' dip, as the temperature increases. (b) Q-band V_2' spectra for deoxy-HbA at a concentration of 27 g/dL, as a function of increasing temperature. The spectra show very little temperature dependence. The B'/B and E'/E ratios are shown for each spectrum to provide further comparison.

to give a constant IHP:Hb molar ratio of 4:1.

For use in deoxygenation studies, the COHb solutions were first fully oxygenated by flushing slowly with oxygen in a round-bottom flask on a rotary evaporator under a flood lamp. After the completeness of Hb oxygenation through the optical spectra was checked, samples were deoxygenated by flushing with nitrogen; completion of deoxygenation was judged first by visual inspection of the change to the characteristic purple color of deoxy-Hb and then checked spectrophotometrically. After deoxygenation, samples were transferred to 50-μL capillaries and sealed for EPR measurement.

Spectra for sedimented polymer samples of deoxy-HbS were obtained by preparing concentrated (ca. 30 g/dL) samples of deoxy-HbS in sealed capillaries and centrifuging the sealed capillary in a Beckman SW50.1 rotor at 45 000 rpm for 1 h at 32 °C (Poillon & Bertles, 1979). After centrifugation, the capillary was transferred to the EPR cavity, and spectra were measured for the sedimented polymer fraction at the bottom of the capillary.

X-band (9-GHz) second harmonic out of phase absorption V_2' ST-EPR spectra were measured by using a Varian E-4 spectrometer and a modulation frequency of 100 kHz; a Princeton Applied Research Model 126 lock-in amplifier was used for phase-sensitive detection at 200 kHz. Phase nulls for the V_2' spectra were obtained at a microwave power attenuation of 26 dB (nominal 0.5 mW); V_2' spectra were obtained at a microwave power attenuation of 6 dB (nominal 50 mW). Temperature control was maintained with a Varian E-257 controller, and nitrogen flow was directed through a quartz Dewar in the EPR cavity. X-band spectra were digitized on a Nicolet Model 1174 time averager and stored on magnetic tape for future analysis. Q-band (35-GHz) V_2' spectra were obtained following previously published procedures (Johnson & Hyde, 1981). Modulation amplitudes of 5 G were used for both X- and Q-band observation. Temperatures were measured by using a digital thermometer and a copper–constantan thermocouple.

Isotropic motion V_2' spectra were obtained at various rotational correlation times from 3% COHb in glycerol–water mixtures with varying proportions of glycerol and water. Calibration of the spectra followed previously published procedures (Johnson & Hyde, 1981). Immobilized Hb samples at high temperature were prepared by mixing labeled and unlabeled Hb in a molar ratio of 1:2 and precipitating the resulting solution with saturated (at 4 °C) ammonium sulfate at pH 7 (Johnson, 1978).

RESULTS

The V_2' ST-EPR spectra for deoxy-HbS undergoing thermally induced aggregation are shown in Figures 1a and 2a for X- and Q-band observational frequencies, respectively. In previous work, we have shown that the Mal-6 label covalently attached to the Hb molecule is essentially immobilized within the protein matrix on the ST-EPR time scale, with the resulting ST-EPR spectra thus reporting the overall rotational tumbling of the Hb molecule (Johnson, 1978). At X band, it can be seen that the spectral intensity in the high-field region

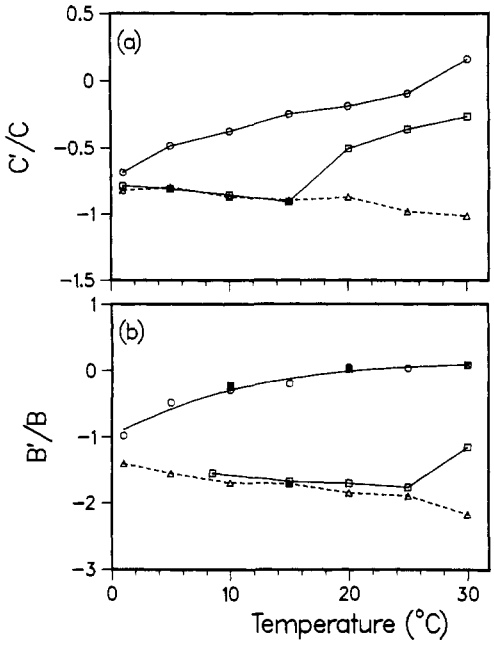

FIGURE 3: (a) Temperature dependence of the X-band C'/C ratio for 30 g/dL deoxy-HbA (△), 24 g/dL deoxy-HbS (□), and 27 g/dL deoxy-HbS (○). (b) Temperature dependence of the Q-band B'/B ratio for 27 g/dL deoxy-HbA (△), for one 22 g/dL deoxy-HbS (□) sample, and two 30 g/dL deoxy-HbS samples (○ and ⊠).

of the spectrum increases relatively rapidly as temperature is increased, with the H''/H ratio going from nearly zero at 1 °C to a value greater than 1 at 30 °C. The central region of the spectrum, however, shows less change, with the C'/C ratio exhibiting a relatively slow increase, going from a value of about −1 at 1 °C to a value only slightly greater than zero at 30 °C. The Q-band spectra exhibit similar behavior, with the high-field E'/E parameter going from a value of approximately zero at 1 °C to a value greater than 1 at 30 °C, while the downfield B'/B parameter increases much more slowly with increasing temperature. In both cases, the samples were fluid at 1 °C, but gelled at 30 °C, indicating that HbS gelation or polymer formation occurred during the temperature increase series. All of these spectral changes indicate that increasing the temperature substantially increases the effective deoxy-HbS rotational correlation time, consistent with the motional restriction that would be expected from deoxy-HbS gelation, but suggest that incorporation of the HbS molecules into the polymer fiber affects the various regions of the deoxy-HbS V_2' spectra differently. For comparison, the effects of temperature on the X- and Q-band spectra of deoxy-HbA are shown in Figures 1b and 2b, respectively, from which it can be seen that deoxy-HbA rotational diffusion exhibits relatively little temperature dependence.

The temperature dependencies of the X-band C'/C and the Q-band B'/B parameters for several experimental systems are shown in more detail in panels a and b of Figure 3, respectively. For deoxy-HbA, the C'/C (Figure 3a) and B'/B (Figure 3b) ratios both decrease slightly with increasing temperature, indicating that the rotational correlation times decrease gradually with increasing temperature (Thomas et al., 1976; Johnson & Hyde, 1981), consistent with the behavior expected from thermally activated rotational diffusion. The X-band C'/C ratio for deoxy-HbS at a concentration of 24 g/dL [(⊠) Figure 3a] exhibits a behavior virtually identical with that of deoxy-HbA up to 15 °C; at 20 °C, however, the ratio jumps substantially and then continues to increase with temperature. Similar behavior is observed for a 22 g/dL sample at Q band [(□) Figure 3b], except that the divergence between HbA and HbS occurs between 25 and 30 °C. In previous work, we have shown that this spectral behavior reflects the HbS transition from the fluid to the gel state (Thiyagarajan & Johnson, 1983). The X-band C'/C ratio for 27 g/dL deoxy-HbS [(○) Figure 3a] exhibits a value close to that of deoxy-HbA at 1 °C but then increases steadily with temperature, indicating that the gelation transition temperature drops by ~10 °C for an increase in deoxy-HbS concentration from 24 to 27 g/dL. The Q-band B'/B ratios for a 22 g/dL deoxy-HbS sample (□) exhibit a behavior similar to that of deoxy-HbA up to 25 °C; at 30 °C, the ratio jumps substantially, apparently indicating the onset of gelation. The qualitative behaviors of the deoxy-HbS systems are similar at both X and Q band, indicating that the two observational frequencies report comparable information. The close correspondence of the data for the two 30 g/dL samples [(○ and ⊠) Figure 3b] also indicates that the measurements are quite reproducible.

X- and Q-band V_2' spectra for Hb undergoing isotropic rotational diffusion in water–glycerol solutions of varying viscosity are shown in Figures 4a and 5a; the individual correlation times are shown for each spectrum. A comparison of these spectra with those for deoxy-HbS shows clear differences in the spectral trends. For isotropic rotational diffusion, greater immobilization (higher viscosity) strongly influences the X-band C'/C ratio before it significantly affects the high-field H''/H ratio; similarly, greater immobilization also strongly influences the Q-band B'/B parameter before it significantly affects the high-field E'/E ratio. In fact, only complete immobilization brings the high-field H''/H and E'/E ratios to values close to 1. In contrast, as noted above, for deoxy-HbS undergoing gelation, the high-field H''/H and E'/E ratios are most strongly affected by the aggregation process. The differences between the deoxy-HbS spectra and those for Hb undergoing isotropic rotational diffusion are particularly pronounced for the 30 °C deoxy-HbS spectra, indicating that the effects of deoxy-HbS aggregation are more complex than simply increasing the overall Hb rotational correlation time.

Thermodynamic analyses (Minton, 1977; Ross et al., 1977; Ferrone et al., 1985a,b, and references cited therein) have suggested that the HbS gel can be regarded as a two-state system, with a polymeric deoxy-HbS phase in equilibrium with a solution phase of monomeric deoxy-HbS. $^{13}C/^{1}H$ NMR double resonance experiments also indicate that the solution phase of monomeric deoxy-HbS molecules behaves as a low-viscosity solution of isotropically mobile molecules, while the polymer phase behaves as a crystalline solid within the time scale of the NMR experiment (Sutherland et al., 1979; Noguchi et al., 1980; Noguchi, 1984). The V_2' ST-EPR spectra for the solution phase of monomeric deoxy-HbS can thus probably be approximated quite accurately by deoxy-HbA spectra at the same temperature and concentration, leading to the following question: What is the spectral behavior of the polymeric phase? A simple first approach to this question is to ask if the polymer structure can produce complete immobilization of the incorporated deoxy-HbS molecules, as suggested by the NMR experiments (Sutherland et al., 1979). If so, the spectra for a gel consisting of immobilized polymeric HbS and solution-phase monomeric HbS would then be simply the superposition of an immobilized Hb spectrum, corresponding to the polymer state, plus an isotropic rotational diffusion spectrum for monomeric Hb, corresponding to the solution phase. To evaluate this possibility, we have added varying ratios of the V_2' spectra for immobilized Hb to those

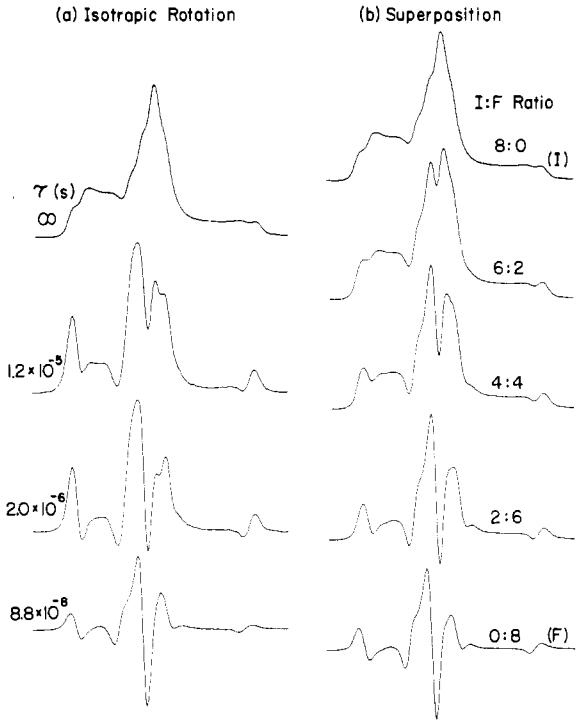

FIGURE 4: (a) X-band V_2' spectra for COHbS undergoing isotropic rotational diffusion in water–glycerol solutions. Note that the increase in the central (C') dip now occurs faster than the increase in the intensity in the spectral wings. The infinite correlation time spectrum is from Mal-6-labeled Hb that has been immobilized by ammonium sulfate precipitation. The overall shape of this spectrum is more of an undifferentiated envelope with less structure than we have observed in previous measurements on immobilized Hb (Johnson, 1978). The difference appears to result from the fact that we are using 100-kHz modulation and detecting at 200 kHz, rather than the 50/100-kHz combination that is more commonly used (Thomas et al., 1976), and that we are also using a somewhat lower effective microwave power than the commonly used 63 mW in order to reduce spectral sensitivity to the power setting. Both of these procedural modifications will tend to produce spectra with higher C'/C and H''/H ratios and more absorption–envelope-type shapes than the standard instrument settings. All of the spectral comparisons in this study used the same instrument settings; thus, all of the comparisons are internally consistent. (b) Superposition of spectra from Hb immobilized by ammonium sulfate precipitation, plus that from deoxy-Hb undergoing free rotational diffusion at a concentration of 30 g/dL. The two spectra were first normalized to provide the same peak to peak maximum amplitude and then added in the ratios shown in the figure; no attempt was made to consider the relative spin concentrations of the two spectral components. For this system, the central (C') dip and the intensity in the spectral wings increase in close correspondence with each other.

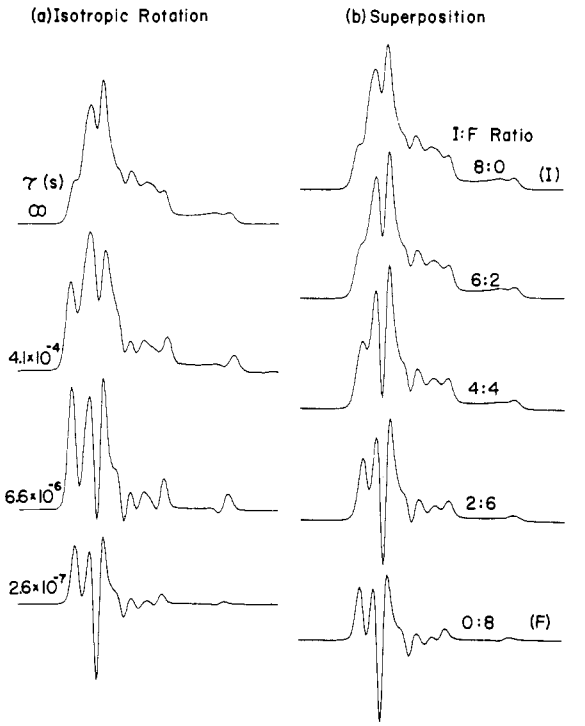

FIGURE 5: (a) Q-band V_2' spectra for COHbS undergoing isotropic rotational diffusion in water–glycerol solutions. The infinite correlation time spectrum is from Mal-6-labeled Hb that has been immobilized by ammonium sulfate precipitation. Note that the increase in the B' dip now occurs faster than the increase in the intensity in the E' region. (b) Superposition of spectra from Hb immobilized by ammonium sulfate precipitation, plus that from deoxy-Hb undergoing free rotational diffusion at a concentration of 27 g/dL. The two spectra were first normalized to provide the same peak to peak maximum amplitude and then added in the ratios shown in the figure; no attempt was made to consider the relative spin concentrations of the two spectral components. For this system, the downfield B' dip and the E' intensity in the high-field region increase in close correspondence with each other.

for deoxy-HbA in solution; the resulting X- and Q-band spectra are shown in Figures 4b and 5b, respectively.

A comparison of the deoxy-HbS spectra of Figures 1a and 2a with the superposition spectra of Figures 4b and 5b indicates that the superposition more closely approximates the observed gel-state deoxy-HbS spectra than do those for simple isotropic rotational diffusion but that there are still substantial discrepancies. For example, the X-band 30 °C deoxy-HbS spectrum (Figure 1a) exhibits a C'/C ratio of about 0.16, and an H''/H ratio of about 1.2, while the superposition spectrum for a 4:4 immobilized:free ratio exhibits a C'/C ratio of almost 0.4 but an H''/H ratio of less than 0.8. Similarly, the Q-band 30 °C deoxy-HbS spectrum exhibits a B'/B ratio of slightly less than 0.1 and an E'/E ratio of nearly 1.2, while the superposition spectrum for a 4:4 immoblized:free ratio exhibits a B'/B ratio of about 0.1 but an E'/E ratio less than 0.6. Thus, polymer-state deoxy-HbS does not appear to be fully immobilized on the ST-EPR time scale.[2]

To obtain further information about the deoxy-HbS motional behavior in the polymer state, we have prepared gelled samples at initial concentrations of about 30 g/dL and then sedimented the polymer deoxy-HbS by ultracentrifugation, as described above. The sedimented material consists primarily of polymer fibers, along with a small amount of monomer deoxy-HbS trapped in the interstices between polymer fibers. This sample was then transferred to the EPR cavity and the V_2' spectrum of the sedimented polymer fraction measured directly. The resultant spectrum is shown in Figure 6a and is very similar to the 30 °C spectrum shown in Figure 1a. The similarity of the sedimented polymer spectrum (Figure 6a) to that of the simple gel superposition spectrum at the same temperature (Figure 1a) is initially surprising. However, Ross et al. (1977) have shown that for 27–30 g/dL deoxy-HbS gels at 30 °C, about 60–65% of the the deoxy-HbS is in the polymer phase, and 35–40% is in the monomer phase. Our samples were also prepared in the presence of IHP, which tends to reduce deoxy-HbS solubility; thus, the polymer fraction for our samples may be even higher. Furthermore, ST-EPR is

[2] It might also be asked whether residual spin-label motion at the Hb surface might be contributing to the spectral appearance of anisotropic motion. However, several methods of Hb immobilization, including lyophilization, siloxane polymer entrapment, and the ammonium sulfate precipitation described here, all produce ST-EPR spectra that indicate complete spin-label immobilization. Thus, residual label motion does not appear to contribute significantly to the Hb ST-EPR spectra in this system.

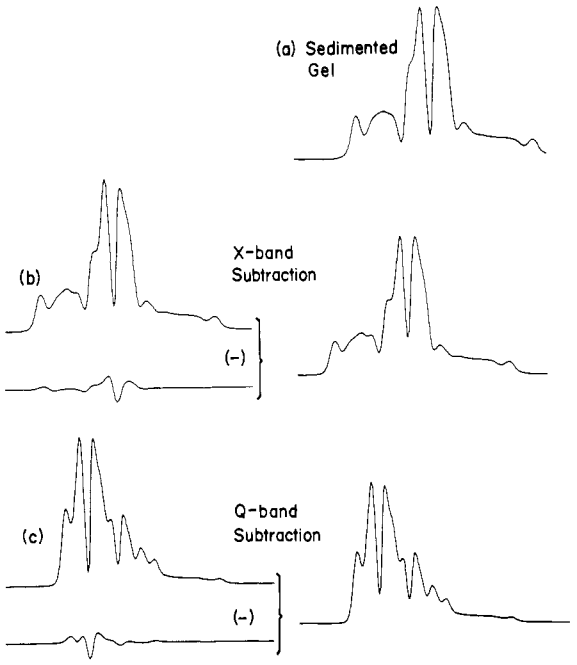

FIGURE 6: (a) X-band V_2' spectrum for deoxy-HbS that has been sedimented by ultracentrifugation. The sample was prepared in a capillary and transferred, intact, directly from the ultracentrifuge rotor to the EPR cavity for ST-EPR measurement. (b) X-band subtraction of the V_2' spectrum for deoxy-HbA (Figure 1b, top) from that of a deoxy-HbS gel sample (Figure 1a, top) at 30 °C. (c) Q-band subtraction of the V_2' spectrum for deoxy-HbA (Figure 2b, top) from that of a deoxy-HbS gel sample (Figure 2a, top) at 30 °C. The HbS:HbA spectral amplitude ratios were set at 1.0:0.175 before subtraction for both the X and the Q band.

a nonlinear technique in which the V_2' signal intensity for very slow motion components ($\sim 10^{-3}$ s) is about 3 times higher than that for components with correlation times of $\sim 10^{-7}$ s (Johnson & Hyde, 1982). Thus, for the 30 °C spectra in Figures 1a and 2a, only about 15–20% of the total spectral intensity should arise from the monomer deoxy-HbS component.

In Figure 6b,c, we have subtracted X- and Q-band deoxy-HbA spectra from the corresponding composite gel-phase deoxy-HbS spectra (Figures 1a and 2a, respectively). The peak to peak amplitudes of the deoxy-HbA spectra were reduced to 0.175 times the amplitude of the gel-phase deoxy-HbS spectra (midway, in the 15–20% estimated range) before the subtraction. The resulting difference spectra are shown in the right column of Figure 6, from which it can be seen that the subtractions produce only slight increases in the C'/C and B'/B ratios for the X- and Q-band spectra, respectively. The close correspondence between the X- and Q-band subtraction spectra provides further confirmation that the ST-EPR spectra are reporting the motional behavior of the deoxy-HbS polymer fraction. Thus, to a good approximation, the spectra in the right column of Figure 6 can be regarded as reporting the motional behavior of polymeric deoxy-HbS.

In previous work, we have shown that parameter correlation plots provide a systematic method for empirically evaluating complex motion (Johnson et al., 1982; Fung & Johnson, 1983). In such plots, ST-EPR parameters that are sensitive to the loss of correlation between the magnetic field and one of the nitroxide magnetic axes are plotted against parameters that are sensitive to the loss of correlation between the magnetic field and another of the nitroxide magnetic axes, rather than against extrinsic variables such as temperature. For example, the X-band H''/H ratio primarily senses loss of correlation between the nitroxide z axis and the external magnetic field,

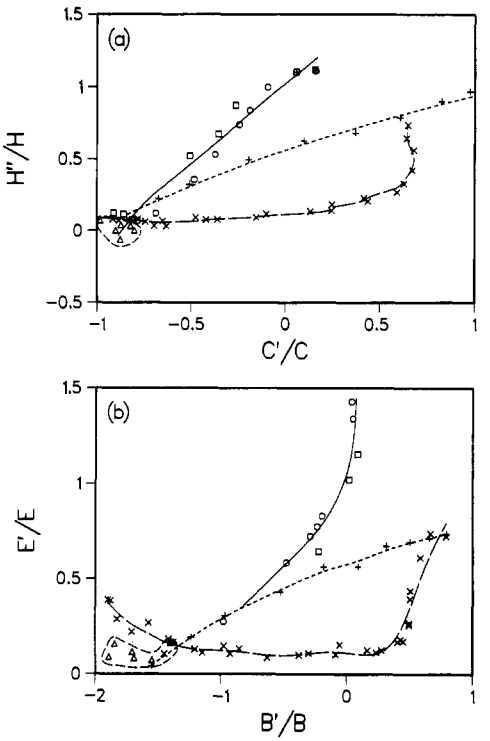

FIGURE 7: Parameter correlation plots for the system shown in Figures 1, 2, 4, and 5. (a) X-band H''/H vs. C'/C plot for 30 g/dL deoxy-HbA (△), deoxy-HbS [(□) 24 g/dL; (○) 27 g/dL; (⊞) sedimented polymer fraction], isotropic rotational diffusion (×), and the superposition of immobilized/free rotation (+). (b) Q-band E'/E vs. B'/B plot for 27 g/dL deoxy-HbA (△), 30 g/dL deoxy-HbS (□ and ○), isotropic rotational diffusion (×), and the superposition series (+). At both frequencies, deoxy-HbA exhibits relatively little temperature dependence and occupies only a small region of the plot; a contour is draw around the HbA data. The smooth curves through the data in both plots are spline fits for visual organization and have no theoretical significance.

while the C'/C ratio is sensitive to loss of correlation between the magnetic field and both the x and y axes of the nitroxide; the H''/H ratio is sensitive to both x–z and y–z rotational averaging, while the C'/C ratio is particularly sensitive to x–y rotational averaging. Variation of temperature, viscosity, etc. for an H''/H vs. C'/C plot will thus generate curves characteristic for the rotational diffusion of the system of interest.

An X-band H''/H vs. C'/C plot for deoxy-HbS, deoxy-HbA, isotropic rotational diffusion, and spectral superposition is shown in Figure 7a. The corresponding Q-band E'/E vs. B'/B plot is given in Figure 7b. The Q-band E'/E parameter is primarily sensitive to loss of correlation of the nitroxide magnetic z axis with the magnetic field, while the B'/B parameter should be particularly sensitive to x–y averaging (Johnson & Hyde, 1982). The X- and Q-band plots should thus provide qualitatively similar information about these systems.

An examination of the two correlation plots indicates that the spectral behavior of the deoxy-HbS systems at low temperature corresponds quite closely to that of the immobilized/free rotation superposition series but that the deoxy-HbS curve diverges quite rapidly from the superposition curve at higher temperatures where the deoxy-HbS polymer fraction becomes significant. For both plots, the high-field spectral ratios increase quite rapidly, while the C'/C ratio increases more gradually (X band), and the B'/B ratio appears to reach a nearly constant value that is slightly larger than zero (Q band). These results, plus the spectra in Figure 6, suggest that deoxy-HbS polymer formation produces almost complete im-

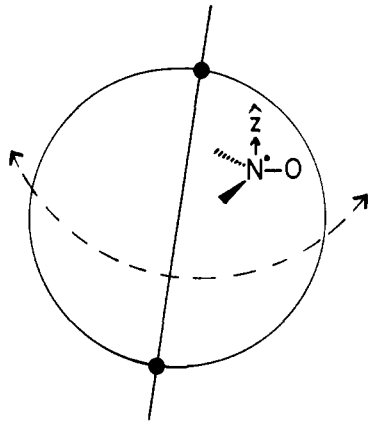

FIGURE 8: Schematic representation of the deoxy-HbS anisotropic oscillation within the HbS polymer fiber. The strong binding sites are assumed to form a preferred axis for rotation, with other contact sites allowing more flexibility. The nitroxide magnetic z axis of the tightly bound Mal-6 label also appears to be oriented in approximate coincidence with the rotational symmetry axis, as shown.

mobilization of the nitroxide z axis of Mal-6-labeled deoxy-HbS but that at least limited x–y rotational averaging continues even in the polymer state.

Discussion

$^{13}C/^{1}H$ NMR double resonance spectroscopy has shown that the deoxy-HbS gel is composed of two distinct subfractions: one phase that behaves as a low-viscosity solution of isotropically mobile molecules and a second polymeric phase that behaves essentially as a crystalline solid within the sensitivity limits and time domain of the NMR experiment (Sutherland et al., 1979; Noguchi et al., 1980; Noguchi, 1984). ST-EPR, however, appears to provide higher sensitivity to motion in the very slow motion time domain and indicates that deoxy-HbS continues to exhibit a limited motional flexing or wobbling after incorporation into polymer fibers.

The results described above also strongly indicate that deoxy-HbS molecular motion within the polymer state is highly anisotropic. Comparisons of the deoxy-HbS spectra at high temperature where the polymer fraction is relatively high (Figures 1a and 2a) with those for a superposition of isotropic rotation plus immobilized Hb (Figures 4b and 5b) suggest that the binding of the deoxy-HbS molecules within the polymer fiber occurs in a fashion such that the nitroxide z axis of the tightly bound Mal-6 label is nearly immobilized but that at least limited rotational oscillation or wobbling occurs in the nitroxide x–y plane. This is shown schematically in Figure 8.

In previous work, we have shown that the use of both X- and Q-band observation, along with parameter correlation plots, provides sensitive methods for discriminating between the existence of anisotropic motion and the presence of multiple motional rates within a system of interest (Johnson et al., 1982; Fung & Johnson, 1983). Thus, the consistency of the results betwen the X- and Q-band observational frequencies provides further evidence for the existence of limited anisotropic motion by the polymeric deoxy-HbS molecules.

These results thus suggest that one or more of the contact sites within the polymer fiber form strong binding sites that induce partial motional "alignment" or motional ordering of the deoxy-HbS molecules within the fibers. Within the double-strand unit of the polymer fiber, the deoxy-HbS molecules are in both axial and lateral contact with neighboring HbS molecules (Wishner et al., 1976; Padlan & Love, 1985). If all of these contacts were equally strong, the HbS motional restrictions would be essentially isotropic, restricting motion in all orientations. These results thus suggest that either the axial or the lateral contact must exhibit relatively strong binding, while the other contact is more flexible, allowing at least limited HbS motion.

It should also be recognized that HbS molecules within the fiber may exhibit differing degrees of motional restriction, depending on their location within the fiber. The 14-strand model consists of an inner helical core of 2 pairs of HbS double strands surrounded by an outer helix of 5 pairs of HbS double strands (Dykes et al., 1979; Crepeau & Edelstein, 1984). Thus, it might be expected that HbS molecules in the outer layer might exhibit more motional freedom than those in the inner core. However, regardless of differences in flexibility that might occur between the inner and outer regions of the fiber, the fibers and the individual double-strand subunits are too long to exhibit independent motions on the submillisecond time scale, and the observed motions must be from the individual HbS molecules within the polymer fibers.

In early work, Ohnishi et al. (1966) have shown that the nitroxide x and z axes of Mal-6-labeled horse HbO_2 are nearly parallel to the Hb molecular a and b axes, respectively. However, there appears to be no published information on the orientation of the Mal-6 nitroxide within the human deoxy-Hb molecule. In preliminary fiber alignment studies with Mal-6-labeled deoxy-HbS, we have found that the hyperfine separations in conventional EPR spectra for Mal-6-labeled deoxy-HbS are slightly larger when the partially oriented fibers are parallel to the magnetic field than when they are perpendicular to the magnetic field, suggesting that the nitroxide z axis may be oriented close to the polymer fiber axis (M. Johnson, unpublished work). Thus, we speculate that the axial contact within the double strand may be the stronger binding site. However, further work will be required to confirm this point. Determining which of these contacts is providing the stronger binding site will permit drug design efforts to focus on the design of compounds that will competitively bind at that specific site, hopefully allowing greater specificity in inhibiting deoxy-HbS aggregation.

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Thermodynamics of Glycophorin in Phospholipid Bilayer Membranes†

A. Leo MacDonald and David A. Pink*

Theoretical Physics Institute, Center for Mathematical Simulation, St. Francis Xavier University, Antigonish, Nova Scotia, Canada B2G 1C0

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ABSTRACT: We have developed a model of glycophorin in a phospholipid bilayer membrane in order to study the thermodynamics of this system and to understand the detailed behavior of recent calorimetric data. We assume that the larger glycophorin polar group can be considered as either adopting a pancakelike conformation at the bilayer interface (D state) or be directed generally away from the interface (U state) [Ruppel, D., Kapitza, H. G., Galla, H. J., Sixl, F., & Sackmann, E. (1982) *Biochim. Biophys. Acta 692*, 1–17]. Lipid hydrocarbon chains are described either as excited (e state) with high energy and relatively many gauche conformers or as generally extended (g state) with low energy. We performed a Monte-Carlo simulation using the Glauber and Kawasaki procedures on a triangular lattice which represents the plane of half of the bilayer. Lattice sites can be occupied either by lipid hydrocarbon chains or by model glycophorin α-helical hydrophobic cores. The states D and U are represented by hexagons of different sizes in the plane of the lattice, and the hard core repulsion between two such polar groups is accounted for by forbidding hexagon–hexagon overlap. We have studied the effect of having the glycophorin polar group interact in various ways with the lipid bilayer. We find that the protein polar group in its D state interacts, either directly or indirectly, with the lipid bilayer so as to reduce the effective lateral pressure acting on the lipid hydrocarbon chains by about 3 dyn/cm. Polar groups in their U states do not reduce this lateral pressure. We find that the region of reduced lateral pressure has a smaller area, in the plane of the bilayer, than the area of the region excluded by the protein polar group to penetration by other such polar groups, due to hard-core interactions. We find that as the protein concentration, c, increases from $c = 0$, the specific heat curves first broaden to a maximum at $c \approx c_1$ while the transition enthalpy decreases approximately linearly. At this concentration, the plane of the bilayer is essentially covered with protein polar groups in their D states. As c increases further, the specific heat curves narrow while the transition enthalpy stays approximately constant. As c increases still further, the specific heat peak broadens, and the transition enthalpy decreases. Our simulations show that this behavior is understood as a consequence of the bilayer behaving like a mixture of two kinds of lipids, one with the unperturbed lateral pressure acting on the chains and the other perturbed with a reduced lateral pressure acting on the chains. We calculate a phase diagram and show that it is remarkably similar to one deduced from measurements. Finally, we make predictions about the dependence of ^{2}H NMR line splitting upon protein concentration and propose experiments that may be performed to test predictions of the model.

A recent study of the effect of glycophorin upon the transition enthalpy of dimyristoylphosphatidylcholine (DMPC) bilayer membranes showed quite unexpected results (Ruppel et al., 1982), and there are indications that the concanavalin receptor in DMPC bilayers has a similar effect (Chicken & Sharom, 1984). It is possible that a similar effect may be associated with many glycoproteins, and it is our intention to present a theoretical model which, while it describes the behavior of glycophorin in DMPC, may have applications to glycoproteins in general.

There has been considerable interest in the interaction between membrane-spanning integral proteins and the phospholipids making up the quasi-two-dimensional lipid bilayer

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