A New Bifunctional Spin-Label Suitable for Saturation-Transfer EPR Studies of Protein Rotational Motion†

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ABSTRACT: A new bifunctional spin-label (BSL) has been synthesized that can be immobilized on the surface of proteins, allowing measurement of rotational motion of proteins by saturation-transfer electron paramagnetic resonance (STEPR). The spin-label contains a photoactivatable azido moiety, a cleavable disulfide, and a nitroxide spin with restricted mobility relative to the rest of the label. The label reacts with surface lysine residues modified with β-mercaptopropionate. Bifunctional attachment is achieved by photoactivation of the azido group. Any spin-label that remains monofunctionally attached after photolysis is removed by reduction of the disulfide. Only bifunctionally attached BSL remains on the protein. Hemoglobin was used to test the utility of the BSL in STEPR by comparison with hemoglobin modified with maleimide spin-label (MSL), a commonly used standard for the STEPR technique. MSL is a monofunctional spin-label which is fortuitously immobilized by local protein structure within hemoglobin. The BSL labeling of hemoglobin did not significantly affect the quaternary structure of hemoglobin as determined by gel filtration chromatography. The conventional EPR spectra of the mono- and bifunctionally attached BSL-hemoglobin were similar to the MSL-hemoglobin spectrum, indicating that both forms of BSL were rigidly bound to hemoglobin. In contrast, the spectrum obtained by reaction of modified hemoglobin lysine residues with MSL indicated that these labels were highly mobile. The monofunctionally attached BSL was mobilized upon octyl glucoside addition whereas bifunctionally attached BSL was only slightly mobilized, suggesting that hydrophobic interactions immobilize the monofunctionally attached label on hemoglobin. The response of STEPR spectra of mono- and bifunctionally attached BSL-hemoglobin to changes in hemoglobin rotational correlation time was similar to the MSL-hemoglobin over the range of 10^{-5}–10^{-3} s. The spectra of bifunctionally attached BSL indicated slightly less motion than corresponding spectra for MSL or monofunctionally attached BSL. The new BSL is a good reporter of protein rotation and does not require unique protein structures for its immobilization on the protein. Thus, the BSL should be more generally applicable for STEPR studies of membrane protein rotation than existing monofunctional spin-labels.

Interactions among membrane proteins can be investigated by measuring changes in molecular rotational mobilities. Protein mobility is important in the mechanisms of electron transport, active transport, and transmembrane signaling (Cherry, 1979; Thomas, 1985, 1986; Robinson et al., 1985). Rotational mobility is dependent on the size and shape of the protein as well as on interactions with lipids and other proteins. The rotational motion of membrane proteins is relatively slow with rotational correlation times, τ_r, between 10^{-6} and 10^{-3} s and can be measured primarily through only two techniques, transient optical anisotropy [TOA;[1] reviewed by Cherry (1979) and Thomas (1986)] or saturation-transfer electron paramagnetic resonance [STEPR; reviewed by Hemminga and deJager (1989) and Beth and Robinson (1989)]. Both techniques usually require the use of tightly bound molecular probes. However, STEPR is more sensitive to errors from weakly immobilized probes than TOA since the motion of the probe cannot be easily resolved from the motion of the protein. Existing monofunctional spin-labels require fortuitous local protein structure for their immobilization and are not widely applicable for STEPR measurements of membrane protein rotation. Bifunctional spin-labels can potentially overcome this limitation, since attachment of the label at two points would prevent rotation of the label with respect to the protein. Bifunctional labels have been synthesized that are capable of reaction with proteins by a variety of mechanisms [reviewed by Hideg and Hankovsky (1989)]. Despite the presence of two reactive groups, effective immobilization of bifunctional labels is complicated by several factors. Homobifunctional spin-labels have been synthesized that require the proximity of two reactive groups on the protein so that both ends of the label can attach to the protein (Wenzel et al., 1978; Gaffney et al., 1983; Willingham & Gaffney, 1983; Beth et al., 1986; Anjaneyulu et al., 1988, 1989). This problem can be circumvented by heterobifunctional labels. For example, Keana et al. (1982) synthesized spin-labels that formed the first attachment to a protein through an α-β-unsaturated ketone and the second through the widely reactive nitrene generated from a photoactivatable phenyl azide. A second problem is that incomplete reaction between the label and the protein can

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[1] Abbreviations: BSL, bifunctional spin-label; BSL-Hb, hemoglobin modified with bifunctional spin-label; DTPA, diethylenetriaminepentaacetic acid; EDTA, ethylenediaminetetraacetic acid; Hb, hemoglobin; MSL, maleimide spin-label (4-maleimido-2,2,6,6-tetramethyl-1-piperidine-*N*-oxyl); MSL-Hb, maleimide spin-labeled hemoglobin; NEM, *N*-ethylmaleimide; NEM-Hb, hemoglobin modified with *N*-ethylmaleimide; OG, octyl glucoside; PBE, sodium phosphate buffer (100 mM) with 1 mM EDTA; SDS, sodium dodecyl sulfate; SPDP, succinimidyl (pyridyldithio)propionate; STEPR, saturation-transfer electron paramagnetic resonance; TEMPO, 2,2,6,6-tetramethyl-1-piperidine-*N*-oxyl; τ_r, rotational correlation time; TOA, transient optical anisotropy.

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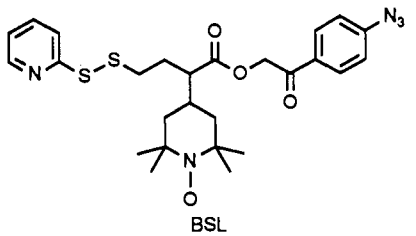
lead to monofunctional attachment in which the label still has significant freedom of rotation. Finally, rotation of the nitroxide-containing ring relative to the backbone of the label should not occur.

We have synthesized a new bifunctional spin-label with the following structure:



BSL

One linkage to the protein is formed by the reaction of the BSL with protein thiols that either are native or result from chemical modification. The other end of the label contains a phenacyl azide which upon photoactivation reacts with the protein to form the second linkage. Any label that does not form the second linkage to the protein can be removed by reduction of the label's disulfide. Free rotation of the nitroxide-containing ring is prevented by attachment of the ring by a double bond to the label.

The utility of the bifunctional spin-label in STEPR was examined by using human hemoglobin so that the results could be compared with oxygenated maleimide spin-labeled (MSL)-hemoglobin, which is a commonly used standard for the STEPR technique (Thomas et al., 1976). MSL is a monofunctional label that binds to an accessible cysteine thiol within the hemoglobin structure and is fortuitously immobilized through hydrogen bonding of the nitroxide with hemoglobin and steric constraints imposed by local protein structure (McCalley et al., 1972; Johnson, 1981). BSL was examined using hemoglobin whose accessible cysteine thiols were blocked by treatment with *N*-ethylmaleimide. Conventional EPR revealed that the mono- and bifunctionally attached BSLs were as immobile when attached to hemoglobin as the MSL. The monofunctionally attached label was probably immobilized due to hydrophobic interactions of the label with the protein surface. Although there were slight differences among the labeled hemoglobin species, the STEPR spectra for mono- and bifunctionally attached BSL showed responses to changes in hemoglobin τ_r similar to those of maleimide spin-labeled hemoglobin. Unique protein structures are not necessary for immobilization of the bifunctionally attached BSL. Thus, the new BSL is a good reporter of protein rotation and should be more generally applicable for STEPR studies of protein rotation than existing monofunctional or bifunctional spin-labels.

Materials and Methods

General Techniques and Reagents. The synthesis of the BSL will be described elsewhere. The structure of the BSL was verified by nuclear magnetic resonance (after reduction of the nitroxide), mass spectroscopy, and infrared absorbance analysis. Maleimide spin-label (4-maleimido-2,2,6,6-tetramethyl-1-piperidine-*N*-oxyl) and TEMPOL (4-hydroxy-2,2,6,6-tetramethyl-1-piperidine-*N*-oxyl) were obtained from Sigma Chemical Co. (St. Louis, MO), and SPDP [succinimidyl (pyridyldithio)propionate] was obtained from Pierce (Rockford, IL). Spectrophotometric grade glycerol was purchased from Aldrich Chemical Co. (Milwaukee, WI) and was stored with 3-Å molecular sieves (Alfa Products, Danvers, MA) under an argon atmosphere to maintain anhydrous conditions. All other solvents were obtained from J. T. Baker Inc. (Phillipsburg, NJ). Ultraviolet and visible spectra were obtained with a Bausch and Lomb Spectronic 2000 (East Rochester, NY). SDS–polyacrylamide gel electrophoresis was performed following the procedure of Laemmli (1970), and protein bands were visualized by silver staining (Wray et al., 1981).

EPR Spectroscopy. EPR experiments were performed on a Bruker ER-200D X-band spectrometer equipped with a 4103 TM cavity. Samples were contained in a large quartz flat cell (0.3 mm × 13.4 mm × 115 mm) with a maximum sample volume of 0.4 mL. Sample temperature was controlled by using a Bruker ER 4111 VT variable-temperature controller which regulated the temperature of nitrogen gas flowing over the sample. All experiments were obtained in the absorption mode (V). The spectrometer was equipped with a phase-sensitive detector, and signals were detected either in-phase (V) or 90° out-of-phase (V') with respect to the modulation of the Zeeman field. Conventional EPR spectra (V_1) were obtained by using nonsaturating power levels and using a field modulation frequency of 100 kHz with a peak to peak modulation amplitude of 1 G. STEPR spectra were obtained from the second harmonic of the out-of-phase signal (V_2') using a field modulation frequency of 50 kHz with a peak to peak modulation amplitude of 5 G (Thomas et al., 1976). Phase adjustments were performed by using the procedure of Squier and Thomas (1986). In order to minimize phase drifts during the acquisition of spectra, the spectrometer was warmed up 3 h before STEPR experiments were started, and the cavity was allowed to equilibrate for 30 min whenever adjustments to sample temperature were made. For all STEPR experiments, the reference arm of the spectrophotometer was on and the cavity was critically coupled. All spectra were digitized with 8000 points per 100-G scan width.

Spin concentrations were determined by comparison of the conventional EPR spectral area of the unknown spin concentration with that of 20 μM MSL. The amount of error in the spectral area determination was examined by measuring the area of the MSL standard, removing the sample, and then refilling with the same concentration of MSL standard. The error from each area determination was less than 10%.

In addition to the dependence on rotational motion, the intensity and shape of the STEPR spectra are dependent on the intensity of the microwave power level, H_1 (Squier & Thomas, 1986). To obtain the standard setting of H_1 equal to 0.25 G in the cavity, the instrument was calibrated to determine the relationship between the power setting on the instrument and the true H_1 in the sample cavity using a peroxylamine disulfonate standard according to the procedure of Fajer and Marsh (1982). Further adjustments to the power level were made for each sample to account for sample-dependent differences in the Q of the cavity (Squier & Thomas, 1986).

Calculation of Hb Rotational Times. Hb is roughly spherical and undergoes isotropic Brownian rotational diffusion in solution (McCalley et al., 1972). Since chemical modification of Hb increased its Stokes radius, the rotational correlation times (τ_r) of the spin-labeled Hb species were calculated from the Debye equation using Stokes radii determined by gel filtration chromatography through Sephadex G-200 (Pharmacia, Inc., Piscataway, NJ). The viscosities of the 95% glycerol solutions at the various temperatures were determined from published tables (Weast, 1961; Ibert, 1970).

Reaction of Hemoglobin with N-Ethylmaleimide (NEM). Hb was isolated by using the procedure of Benesch and Benesch (1962). The concentration of Hb was obtained from its visible absorption spectrum (Benesch et al., 1965). The

native cysteine thiols of Hb were blocked with NEM. Twenty-five-microliter aliquots of an NEM solution (62.8 mg, 502 μmol, in 200 μL of ethanol) were added to stirred Hb (1.5 g, 23.1 μmol) in PBE, pH 6.8, every 10 min for 80 min. The Hb solution was reacted overnight at 4 °C. Excess NEM was removed by dialyzing against four changes of PBE, pH 7.6. The blocking efficiency of this reaction was measured by treatment of a 3 μM Hb-NEM solution with 300 μM 2,2′-dithiodipyridine which reacts with free thiols to release pyridine-2-thione. The concentration of accessible thiols on Hb was measured from the increase in absorbance at 343 nm due to the released pyridine-2-thione [ϵ_{343nm} = 8080 L/(m·cm); Stuchbury et al., 1975] to give 0.009 thiol per Hb subunit. The extinction coefficient at 343 nm for the dithiothreitol-treated Hb was measured on a heme basis as 28 000 L/(m·cm).

Reaction of Hb with Maleimide Spin-Label (MSL). Labeling essentially followed the procedure of McCalley et al. (1972). MSL (3.1 mg, 12.3 μmol) in 40 μL of tetrahydrofuran was added with stirring at 23 °C to Hb (50 mg, 0.8 μmol) in 1 mL of PBE, pH 6.1. The solution was reacted 30 min, and excess MSL was removed by gel filtration chromatography on Sephadex G-25 (Pharmacia, Inc., Piscataway, NJ) in PBE, pH 7.6. MSL-Hb was analyzed by EPR to give the expected strongly immobilized spin-label spectrum (rotational correlation time, $\tau_r > 10^{-8}$ s). The number of spins per protein was found to be 2.3 spins/Hb (expected 2). The labeled Hb solution was concentrated under nitrogen using a Model 3 ultrafiltration cell with a YM10 membrane (Amicon Corp., Danvers, MA). The STEPR samples were prepared by diluting the concentrated Hb solution under an argon atmosphere with glycerol to make the final solution 95% glycerol (w/w) and 18 μM Hb. The MSL-Hb solutions were stored at 4 °C.

Reaction of Hb with Succinimidyl (Pyridyldithio)-propionate (SPDP). SPDP (0.72 mg, 2.3 μmol) in 18.5 μL of 50% tetrahydrofuran/ethanol was added with stirring to NEM-Hb (19 mg, 0.29 μmol) in 1 mL of PBE at 23 °C (Carlsson et al., 1978). After 45 min, the reaction was terminated by removal of the excess SPDP by G25 gel filtration chromatography. The average number of SPDP groups was 0.93 per subunit as determined by treatment of the modified Hb with 25 mM dithiothreitol for 20 min and measurement of the absorbance increase at 343 nm due to the release of pyridine-2-thione. The stock solution was stored at 4 °C.

Reaction of MSL with Hb Surface Thiols. NEM-Hb was modified with SPDP to yield an average of 1.2 modifications per subunit. The modified Hb (19 mg, 0.29 μmol) in 1 mL of PBE was treated with 25 mM dithiothreitol for 20 min and passed through a G-25 gel filtration column from which the oxygen had been purged with buffer equilibrated with argon. The NEM-Hb was collected under an argon atmosphere in 4.8 mL of PBE. MSL (0.6 mg, 2.4 μmol) in 39 μL of 50% tetrahydrofuran/ethanol was added to the stirred Hb solution and reacted for 12 h at 4 °C. The labeled Hb solution was concentrated to 1 mL, and excess MSL was removed by using deoxygenated G-25 column chromatography with collection under an argon atmosphere. The solution was concentrated to 1 mL, and the number of MSL per Hb was determined by EPR spin counting to yield 0.7 MSL per Hb subunit. The EPR spectrum showed a weakly immobilized label ($\tau_r < 10^{-8}$ s). The labeled Hb solution was concentrated by using a Centricon 10 concentrator (Amicon Corp., Danvers, MA). The STEPR sample was prepared by diluting the concentrated sample with glycerol to make the final solution 95% glycerol (w/w) and 82 μM Hb. The modified Hb was stored under an argon atmosphere at 4 °C.

BSL Labeling of Hb Surface Thiols. NEM-Hb was modified with SPDP to yield 0.93 label per Hb subunit. The modified Hb (7 mg, 110 nmol) in 1 mL of PBE was treated with 25 mM dithiothreitol for 20 min and then passed through a deoxygenated G25 column and collected in 4.4 mL of PBE, pH 7.6, under an argon atmosphere. The concentration of Hb was adjusted to less than 26 μM. BSL (0.36 mg, 670 nmol) in 75 μL of ethanol was added to the stirred Hb solution with 100 mM octyl glucoside to form a red-brown cloudy solution. The solution was reacted 12 h at 4 °C. Octyl glucoside and excess BSL were removed by using deoxygenated G-25 column chromatography. Noncovalently bound BSL was removed by treatment with 100 mM octyl glucoside for 12 h at 4 °C. The octyl glucoside was removed by using deoxygenated G-25 column chromatography. Analysis of the EPR spectrum showed a strongly immobilized spin-label ($\tau_r > 10^{-8}$ s) with very little free or weakly immobilized BSL. Spin counting gave an average of 0.8 BSL per Hb subunit. For STEPR analysis, the labeled Hb was concentrated by using the Amicon Model 3 miniultrafiltration cell with a YM10 membrane and diluted with glycerol to make the final solution 95% glycerol (w/w) and 29 μM Hb.

Photolysis of Monofunctionally Attached BSL-Hb. The monofunctionally attached BSL-Hb solution (2.8 μM, 2-mL total volume) in a quartz cuvette was photolyzed for 2 min with stirring 5.1 cm from a medium-pressure mercury lamp (Hanovia 679A36, Ace Glass, Vineland, NJ) using a 300-nm interference filter with a 10-nm bandwidth at half-height of the transmission peak (Oriel Corp., Stratford, CT). A 4-cm path-length quartz cuvette containing deionized water was placed between the lamp and the optical filter to prevent excessive heating of the filter. After photolysis, the Hb solutions were concentrated to 72 μM. Photolysis kinetics were followed by obtaining the labeled Hb absorbance spectrum (390–240 nm) after irradiation for a specific time interval.

Removal of Monofunctionally Attached BSL-Hb. To remove any BSL that did not form a second attachment to the protein after photolysis, the Hb solution (72 μM) was treated with 25 mM dithiothreitol for 35 min. The solution was passed through a deoxygenated G25 column equilibrated with 100 mM phosphate buffer (no EDTA) and collected under an argon atmosphere. Regeneration of the disulfide and the nitroxide was achieved by dilution of the labeled Hb to 2.6 μM and treatment with 21 μM potassium ferricyanide for 4 min. Oxidation was halted with addition of 10 mM diethylenetriaminepentaacetic acid and 1 μM dithiothreitol. The Hb solution was passed through a deoxygenated Sephadex G-200 gel filtration column to remove any heavily cross-linked protein. The peak Hb fractions were collected under an argon atmosphere, pooled, and concentrated to 1 mL. Noncovalently bound label was removed by treatment of the Hb solution with 100 mM octyl glucoside for 12 h at 4 °C and passage of the solution through a deoxygenated G-25 column with collection under an argon atmosphere. The concentration of labeled Hb was determined by using an average of the extinction coefficients for deoxy- and metHb at 532 nm (Benesch et al., 1965) with an estimated error in calculated Hb concentration of less than 10%.

The EPR spectrum showed a strongly immobilized spin-label ($\tau_r > 10^{-8}$ s) with a small amount of free or weakly immobilized spins. Spin counting gave an average of 0.25 label per Hb subunit. For STEPR analysis, the labeled Hb was concentrated under nitrogen using the Centricon 10 unit and diluted with glycerol to make the final solution 95% glycerol (w/w) and 75 μM Hb. The bifunctionally attached BSL-Hb

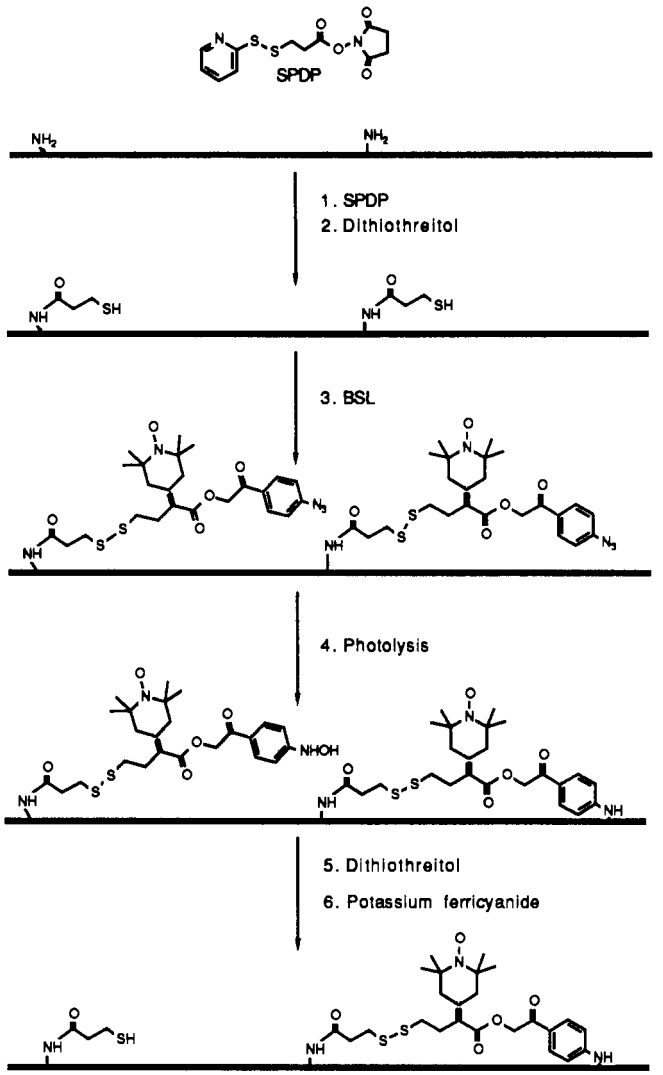


FIGURE 1: BSL labeling protocol. Steps 1 and 2, thiolation of hemoglobin. Thiols were added to hemoglobin by reaction with succinimidyl dithiopropionate (SPDP) and treatment with dithiothreitol. Step 3, reaction of BSL with hemoglobin. The BSL was added in octyl glucoside to yield the monofunctionally attached label. Step 4, photolysis of the BSL. After removal of noncovalently bound BSL, the hemoglobin solution was photolyzed at 300 nm for 90 s. Steps 5 and 6, the hemoglobin modified with BSL was treated with dithiothreitol to remove any monofunctionally attached label. The intact bifunctionally attached BSL was recovered by treatment with potassium ferricyanide to regenerate the label's disulfide and nitroxide to form the bifunctionally attached BSL.

was stored under argon at 4 °C.

Results

The general procedure for labeling a protein with the bifunctional spin-label is given in Figure 1. Hb was used as the model for the labeling, and as the standard to determine the accuracy of the BSL as a reporter of slow microsecond rotational motion. This was accomplished by comparing BSL-labeled Hb to maleimide spin-labeled hemoglobin (MSL-Hb) using the STEPR technique. The spin-label must be immobilized with respect to the protein for accurate measurements of rotational motion. The native Hb tetramer ($\alpha_2\beta_2$) contains two accessible cysteine thiols (β93) which react with the monofunctional reagent, MSL, resulting in fortuitously immobilized label due to local protein structure. For this reason, MSL-Hb is used as the standard for STEPR measurements of protein rotational motion. Since the goal of this project was to develop a spin-label that would be immobilized on the surface of a protein without the need for a special protein structure, the accessible Hb thiols were blocked by using *N*-ethylmaleimide (NEM) prior to labeling with BSL. This prevents immobilization of BSL by reaction with the same thiols as MSL. NEM treatment will not be necessary for BSL labeling of other proteins. The completion of NEM reaction with Hb thiols was confirmed by using the thiol-indicating reagent 2,2′-dithiodipyridine and was found to be greater than 98% complete.

Labeling of Hemoglobin with BSL. The NEM-Hb was treated with SPDP (Figure 1, step 1) to nonspecifically modify amino groups of lysine side chains or the protein's amino terminus with pyridyl disulfides. Surface thiols were generated by reduction of these disulfides with dithiothreitol (step 2). The number of thiols generated per protein was calculated from the absorbance from released pyridine-2-thione and found to be 0.9 per subunit. When NEM-Hb is modified with SPDP to give four thiols per subunit, intermolecular disulfide cross-linking occurs in the presence of oxygen or after treatment with potassium ferricyanide in step 6. Thus, modification of Hb was limited to one to two thiols per subunit, and oxygen was excluded from all Hb solutions containing thiol modifications.

NEM-Hb modified with SPDP, to give an average of 0.9 thiol per subunit, was incubated with BSL in 100 mM octyl glucoside for 12 h (Figure 1, step 3). In preliminary experiments, it was found that the insolubility of BSL in aqueous solution interfered with labeling of Hb. This problem was overcome by solubilizing the label with the detergent octyl glucoside. Furthermore, the concentration of Hb was maintained at 26 μM or less since higher concentrations of Hb resulted in precipitation of the protein during labeling. Excess BSL and octyl glucoside were removed by gel chromatography. Some noncovalently bound label remained associated with the hemoglobin. This was shown by the presence of free spins in the EPR spectrum. The unbound BSL was removed by a second treatment of the Hb with 100 mM octyl glucoside and gel chromatography. The EPR spectrum showed only a small percentage of free or weakly immobilized spins (see below, Figure 5). Spin counting of the labeled Hb indicated an average of 0.8 BSL per Hb subunit.

The BSL-Hb solution was irradiated at 300 nm to photoactivate the azido group to the reactive nitrene (Figure 1, step 4). The short-lived nitrene either reacts with Hb to form the label's second attachment or else reacts with the water shell surrounding the protein to leave monofunctionally attached label. The changes in the BSL-Hb absorbance spectrum during irradiation times from 0 to 480 s are shown in Figure 2. The BSL-Hb solution was routinely diluted to less than 3 μM to reduce potential intermolecular cross-linking during the lifetime of the nitrene. The sample in Figure 2 was more concentrated (3.6 μM) so that the photolysis kinetics could be observed. The absorbance decrease at 284 nm mirrors the absorbance increase at 324 nm with apparent isosbestic points at 261 and 312 nm. The absorbance decrease at 284 nm stops at 240 s whereas the increase at 328 nm continues. This is probably due to changes in the heme absorption spectrum during prolonged irradiation. As expected, the reaction kinetics appear to be first order as shown by the linearity of the semi-log plot of the absorbance change at 284 nm (Figure 2, inset). However, after 120 s, the absorbance decrease deviates slightly from linearity, probably due to changes in heme absorbance. The first-order process for the first 120 s of irradiation has a rate constant $k_1 = 0.023\ s^{-1}$. A plot of the increase in the absorbance at 328 nm yielded a rate constant of 0.019 s^{-1}. After photolysis, EPR analysis showed the

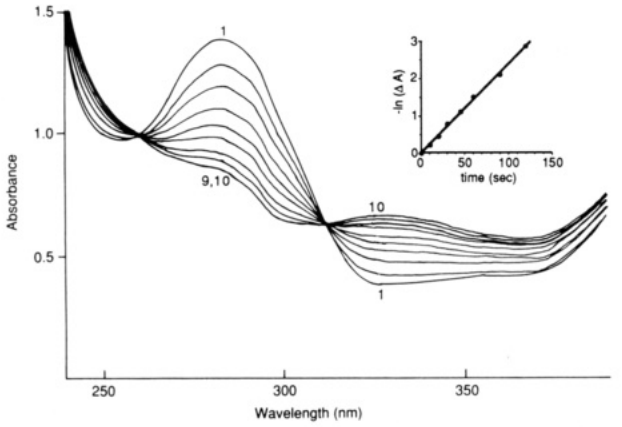


FIGURE 2: Effect of photolysis time on the BSL-hemoglobin absorbance spectrum. Monofunctionally attached BSL-hemoglobin solution (3.6 μM) was irradiated at 300 nm with a medium-pressure mercury lamp as described under Materials and Methods for 0 (1), 10 (2), 20 (3), 30 (4), 45 (5), 60 (6), 90 (7), 120 (8), 240 (9), and 480 (10). Inset: First-order plot of absorbance change at 284 nm. $\Delta A = [A(t) - A(240\ s)]/[A(0\ s) - A(240\ s)]$.

presence of a small amount of free spins. The mechanism of the release of these spins was not investigated but probably occurred by photoreduction of the label's disulfide. There was some photoreduction of the nitroxide upon prolonged irradiation (>10 min) as judged by the decrease in signal intensity of the EPR spectrum. Labeled Hb was routinely photolyzed for 90 s.

BSL that reacts with water upon photolysis and is thus monofunctionally attached was removed by treatment with dithiothreitol to reduce the label's internal disulfide (Figure 1, step 5). After removal of dithiothreitol by gel filtration chromatography, the BSL-Hb solution was diluted to less than 3 μM (to prevent cross-linking) and treated with potassium ferricyanide to reoxidize the nitroxide and regenerate the label's disulfide (Figure 1, step 6). The ability to remove label that did not form a second linkage to the protein upon photolysis was investigated by omitting the photolysis step. The sample was treated with dithiothreitol followed by potassium ferricyanide. The released spin-label was removed by treatment with octyl glucoside and ultrafiltration. Spin counting showed that greater than 98% of the spin-label had been removed. In contrast, 19% of the spin-label remained attached to Hb following photolysis, reduction, reoxidation, and removal of the free label. Less than 2% of these remaining spins were free or weakly immobilized as judged from the conventional EPR spectrum (see below, Figure 5a).

In preliminary experiments, it was found that the oxidation reaction can result in cross-linking of the Hb if the labeled Hb concentration is too high (>10 μM) or if the potassium ferricyanide concentration is high relative to the amount required to oxidize the nitroxide and label's thiols (2-fold excess). Cross-linking was minimal under the reaction conditions routinely used as shown in Figure 3. The degree of cross-linking was analyzed by SDS gel electrophoresis under nonreducing conditions. The unphotolyzed BSL-Hb (lane 3) shows a small amount of dimer, but the majority of the protein migrates as a monomer. Following photolysis (lane 4), monomer, dimer, and trimer species are apparent with a small amount of high molecular weight material that did not enter the gel. The distribution of cross-linked species did not change following reduction and oxidation of the label's disulfide (lane 5). Most of the cross-linking resulting from labeling with BSL was intramolecular rather than intermolecular as shown by gel filtration chromatography on Sephadex G-200 (see below,

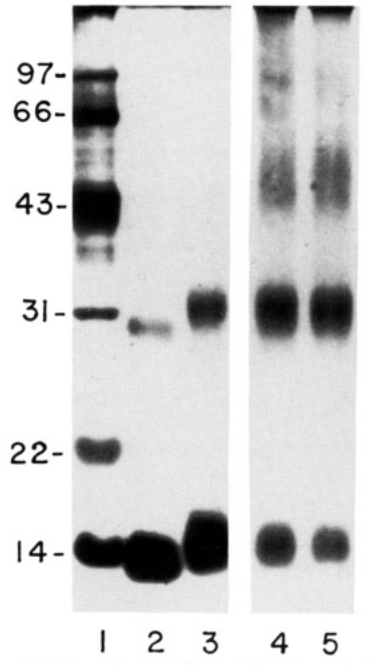


FIGURE 3: Cross-linking of hemoglobin following labeling with BSL. Hemoglobin was labeled with BSL, photolyzed to form a bifunctional attachment, and treated with dithiothreitol to remove labels with a monofunctional attachment. The label's disulfide and nitroxide were generated by treatment with an equimolar amount of potassium ferricyanide. The samples were examined by SDS–polyacrylamide gel electrophoresis under nonreducing conditions. Protein bands were visualized with silver staining. Molecular weight standards (lane 1), hemoglobin (lane 2), unphotolyzed, monofunctionally attached BSL-hemoglobin (lane 3), and BSL-hemoglobin treated with potassium ferricyanide for 0 min (lane 4) or 4 min (lane 5).

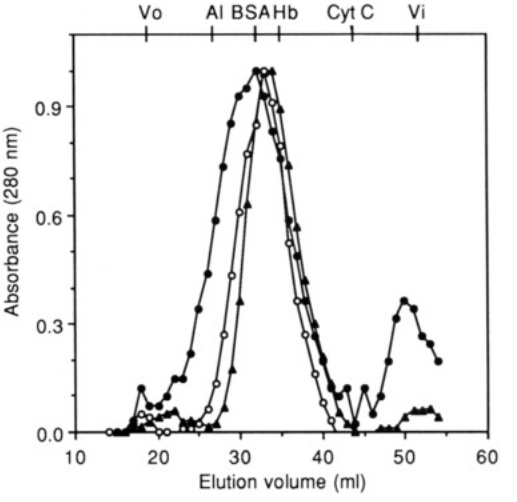


FIGURE 4: Effects of chemical modification on hemoglobin quaternary structure. Labeled hemoglobin species were examined by gel filtration chromatography on Sephadex G-200 to determine their apparent Stokes radii: *N*-ethylmaleimide-treated hemoglobin (▲), 34 Å; monofunctionally attached BSL-hemoglobin (○), 36 Å; bifunctionally attached BSL-hemoglobin (●), 37 Å. Each elution profile was normalized to the fraction with maximum absorption. The peak absorbances are 0.527 (▲), 0.099 (○), and 0.041 (●), respectively. The column was calibrated by using proteins of known Stokes radius: aldolase (Al, 50 Å), bovine serum albumin (BSA, 35 Å), unmodified hemoglobin (Hb, 29 Å), and cytochrome c (Cyt *c*, 17 Å). The void volume (V_o) and inclusion volume (V_i) are denoted.

Figure 4). This chromatography step was used to remove the higher molecular weight material resulting from intermolecular cross-linking.

The effects of the various chemical modifications on the Stokes radius of hemoglobin were determined by gel filtration

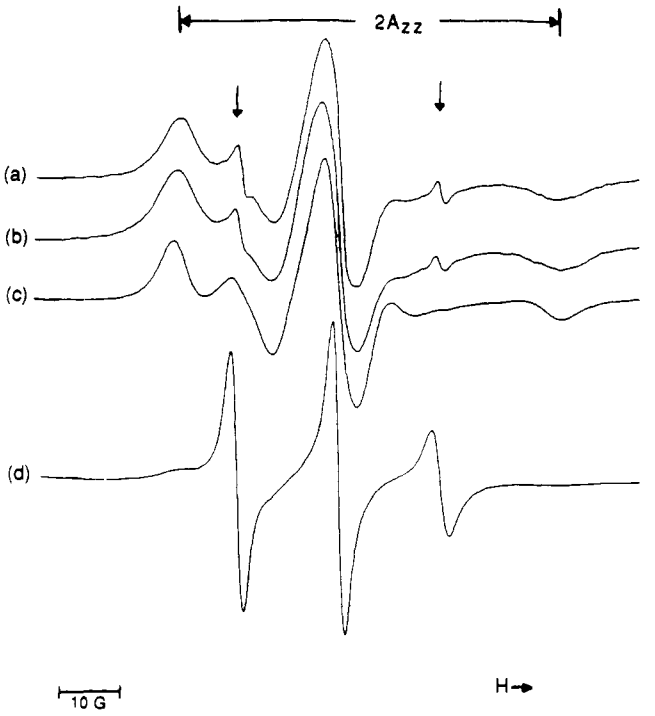


FIGURE 5: Conventional EPR spectra of spin-labeled hemoglobin species. BSL-hemoglobin with bifunctional attachment (a), BSL-hemoglobin with monofunctional attachment (b), hemoglobin modified with maleimide spin-label at amino acid β93 (c), and surface maleimide spin-labeled hemoglobin (d) were examined by conventional EPR (V_1 display) at 25 °C. The hyperfine splitting ($2A_{zz}$) for the BSL-hemoglobin with bifunctional attachment is shown. The arrows indicate free or weakly immobilized spin-labels present in the EPR spectra.

chromatography on Sephadex G-200 (Figure 4). The NEM-Hb eluted with an apparent Stokes radius of 34 Å, compared to 29 Å for unmodified Hb, indicating a small change in the hydrodynamic properties of Hb. The monofunctionally attached BSL-Hb eluted with an apparent Stokes radius of 36 Å, similar to NEM-Hb, indicating BSL labeling has only small effects on Hb structure. The bifunctionally attached BSL-Hb had an apparent Stokes radius of 37 Å after chromatography to remove higher molecular weight cross-linked species. This is higher than the NEM-Hb (34 Å) and unphotolyzed BSL-Hb (36 Å) and reflects either changes in the protein structure or the continued presence of a small amount of cross-linked Hb. The biological activity of hemoglobin after BSL labeling was not examined.

Conventional EPR Analysis of BSL-Hb. The EPR spectra of bifunctionally attached BSL-Hb, monofunctionally attached BSL-Hb (unphotolyzed), and MSL-Hb are shown in Figure 5a–c. The BSL-Hb species displayed a highly immobilized label with a hyperfine splitting ($2A_{zz}$) of 64 G whether the label was attached bifunctionally (Figure 5a) or monofunctionally (Figure 5b). The spectra were similar to the spectrum of immobilized MSL-Hb (Figure 5c) with a splitting of 64 G. However, there were slight spectral differences among the labeled Hb species. The monofunctionally attached BSL probably undergoes a broader range of mobilities as shown by the broadened high-field dip indicating heterogeneity in the label environment. Free or weakly immobilized labels (arrows in Figure 5) contributed approximately 2% of the total label in BSL-Hb [derived by comparison with EPR spectra from Squier and Thomas (1986)]. This may be label that is noncovalently bound to the Hb but not removed from the Hb during the purification due to the hydrophobicity of the label. Alternatively, a small percentage of the labels may be attached

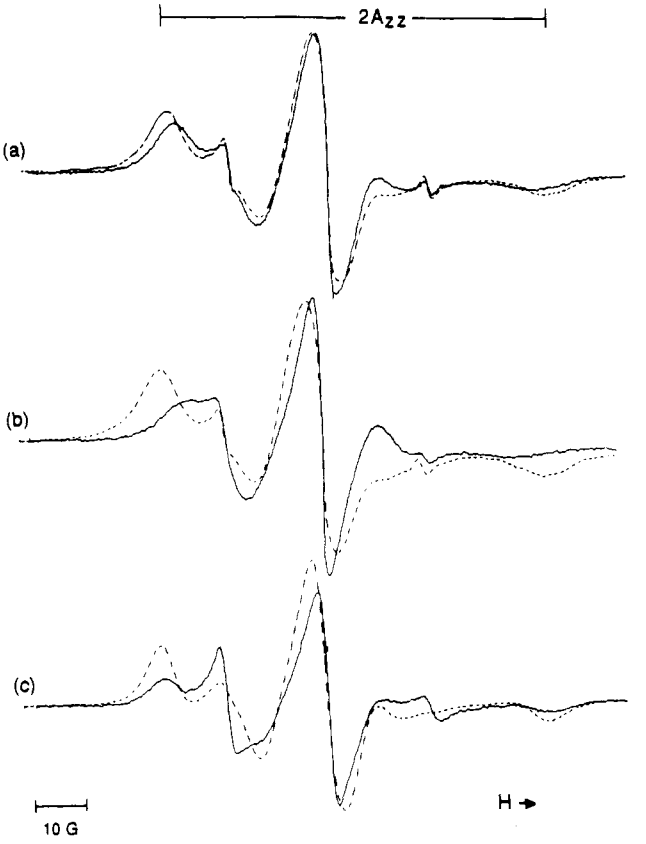


FIGURE 6: Effect of octyl glucoside on spin-labeled hemoglobin. BSL-hemoglobin with bifunctional attachment (a), BSL-hemoglobin with monofunctional attachment (b), and hemoglobin modified with maleimide spin-label at amino acid β93 (c) were examined by conventional EPR (V_1 display) at 25 °C in the presence (solid line) or absence (dashed line) of the detergent octyl glucoside. The hyperfine splitting ($2A_{zz}$) for the BSL-hemoglobin with bifunctional attachment is indicated.

to a mobile domain of the protein.

The relative immobility of the monofunctionally attached label is probably due to the hydrophobicity of the label which may cause it to lie flat on the protein surface or to be buried in the protein interior. This hypothesis was supported by modification of surface thiols on Hb with MSL instead of BSL. NEM-Hb was reacted with SPDP and then with MSL to label surface thiols. In contrast to the monofunctionally attached BSL, the EPR spectrum of the surface MSL shows a weakly immobilized spin-label (Figure 5d). The mobility of the surface MSL also suggests that the BSL does not bind to a special conformation of thiolated Hb that results in an immobile spin-label.

Effect of Octyl Glucoside on Spin-Label Mobility. If BSL is immobilized on the Hb surface through hydrophobic interactions, then addition of detergent might disrupt those interactions and increase the label's mobility. EPR spectra of the different labeled Hb species in the presence or absence of octyl glucoside are shown in Figure 6. The bifunctionally attached label showed the least overall increase in motion upon octyl glucoside addition (Figure 6a). The hyperfine splitting was reduced from 64 to 60 G in the presence of octyl glucoside. The low-field peak and high-field dip were broadened in the presence of octyl glucoside, indicating an increase in the range of mobilities of the label. The increase could result from an increase in the mobility of the label on Hb or a loosening of the protein structure in the vicinity of the label. This effect is reversible since removal of octyl glucoside yields the more immobile spectrum.

The addition of octyl glucoside to monofunctionally attached

BSL-Hb greatly increases the mobility of the label (Figure 6b). However, the spectrum does not resemble the spectrum of weakly immobilized surface MSL on Hb (Figure 5d). The hyperfine splitting decreases to ~54 G. Thus, the addition of octyl glucoside mobilizes the BSL on the surface of the Hb, but the motion of the label is still partially restricted.

The addition of octyl glucoside to MSL-Hb appeared to give rise to two different populations of spin-label environments (Figure 6c). The outer hyperfine splitting was 63 G which is slightly less than that in the absence of octyl glucoside. However, the fraction of weakly immobilized spins increased dramatically (Figure 5d). The detergent may loosen the protein structure such that the special local structures no longer immobilize the MSL. Additionally, the dissociation constant for oxy-Hb is relatively high (1 μM; Antonini & Brunori, 1971), and the octyl glucoside may greatly shift the equilibrium toward the formation of dimer. Thus, the two populations of spins in the presence of the octyl glucoside spectrum may represent dimer and tetramer forms of Hb. Gel filtration data (not shown) supported the conclusion that octyl glucoside promotes dissociation to dimer since the apparent Stokes radius of MSL-Hb shifted from 34 to 28 Å in the presence of octyl glucoside. The effect of octyl glucoside on the Hb quaternary structure was reversible upon removal of the detergent. The effects on the spectral properties of hemoglobin were not examined. The effect of octyl glucoside on the dissociation of the BSL-Hb species may be much less for two reasons. The BSL-Hb species are deoxygenated to prevent the formation of intermolecular disulfide linkages, deoxyhemoglobin has a much lower dissociation constant (1 pM; Ip et al., 1976), and the dissociation of BSL-Hb or loosening of protein structure could be prevented by intramolecular cross-linking of subunits upon photolysis of the label.

STEPR Spectra of the Spin-Labeled Hb Species. The STEPR spectra of the spin-labeled Hb species were obtained over the range of rotational correlation times (τ_r) from 10^{-5} to 10^{-3} s. The τ_r of the Hb was controlled by varying the temperature of the labeled Hb in 95% glycerol. Representative spectra for the fast (3×10^{-5} s) and slow (1×10^{-3} s) rotational correlation times for mono- and bifunctionally attached BSL-Hb are shown in Figure 7A,B. STEPR spectra for immobilized MSL-Hb are shown in Figure 7C for comparison with published data (Thomas et al., 1976) and as a standard for comparison with BSL-Hb species. The dependence of the STEPR spectra on τ_r was similar to published results (Thomas et al., 1976). When the τ_r is increased, the area of the spectrum above the base line increases. The peak at the low-field turning point (L'') increased relative to L as did the high-field peak H'' relative to H and the center-field dip (C') relative to C. Spectra of BSL-Hb with bifunctional versus monofunctional attachment (Figure 7A,B) showed a similar dependence on τ_r. However, there were some differences in the spectra. The center-field dip, C', was not as deep relative to C for the bifunctionally attached BSL-Hb spectra at the longest τ_r. The center-field feature labeled "a" had a different dependence on τ_r for the monofunctionally attached BSL-Hb than for bifunctionally attached BSL-Hb. Spectra of MSL-Hb most clearly resembled those of the monofunctionally attached BSL-Hb.

STEPR spectra of the surface MSL on Hb (Figure 7D) were not as responsive to changes in τ_r as the more immobilized labels. At the longest τ_r, the STEPR spectrum resembles the shorter τ_r spectra for the BSL species or immobile MSL on Hb. This clearly shows that the motion of the surface MSL obscures the slow motion of Hb. The spectra show decreased

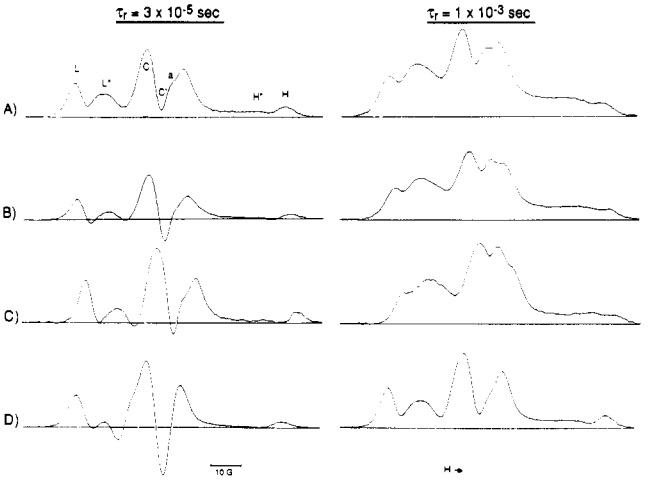


FIGURE 7: Saturation-transfer EPR spectra of various spin-labeled hemoglobin species at two different hemoglobin rotational correlation times (τ_r). Hemoglobin rotational correlation time (τ_r) was manipulated by decreasing the temperature of the spin-labeled hemoglobin species in solutions of 95% glycerol. (A) Bifunctionally attached BSL-hemoglobin; (B) monofunctionally attached BSL-hemoglobin; (C) hemoglobin modified with maleimide spin-label at amino acid β93; (D) surface maleimide spin-labels on hemoglobin. The spectral parameter L is the height above the base line of the low-field peak, and L'' is the height above the base line of the spectrum 10 G to the right of the L peak. The spectral parameters C and C' are the heights of the center-field peak and center-field dip, respectively. The "a" denotes a peak that increases with decreasing τ_r. The spectral parameter H is the height above the base line of the high-field peak, and H'' is the height of the spectrum above the base line 10 G to the left of peak H.

responsiveness to τ_r at all spectral positions.

The ratios of specific peak heights in the STEPR spectra are a convenient way to determine τ_r by comparison with a known standard such as MSL-Hb. The dependence on τ_r of low-, center-, and high-field peaks for the different labeled species is shown in Figure 8A–C. The τ_r's of the different Hb species were calculated by using Stokes radii determined by gel filtration to account for the changes in hydrodynamic radius that accompany chemical modification. The ratio L''/L (Figure 8A) is the most widely measured parameter in STEPR for τ_r determination. The L''/L values for bifunctionally attached BSL-Hb were generally higher than those of MSL-Hb over most of the range of τ_r examined. However, L''/L for bifunctionally attached BSL-Hb was slightly lower at the longest τ_r. The dependence of L''/L on τ_r for monofunctionally attached BSL-Hb resembled that of MSL-Hb. The L''/L values for the surface MSL on Hb were small relative to those for MSL-Hb, reflecting the greater mobility of the surface MSL.

A plot of H''/H versus τ_r for the spin-labeled species is shown in Figure 8B. The H''/H ratio reflects most accurately the τ_r of the spin-label under conditions of high signal to noise ratios, and it has the least sensitivity to experimental variables such as weakly immobilized labels and experimental error (Squier & Thomas, 1986). However, this parameter is difficult to measure at low signal to noise ratios. The H''/H values for the bifunctionally attached BSL-Hb over the τ_r range examined were slightly higher than for the MSL-Hb similar to the L''/L parameter. The value of H''/H for the monofunctionally attached BSL-Hb was generally similar to that of MSL-Hb, as was the case for the L''/L parameter. The plot of the center-field parameter C'/C versus τ_r for the spin-labeled species is shown in Figure 8C. This parameter is more sensitive to experimental error than the others and is not as widely used for spectral comparisons. However, changes

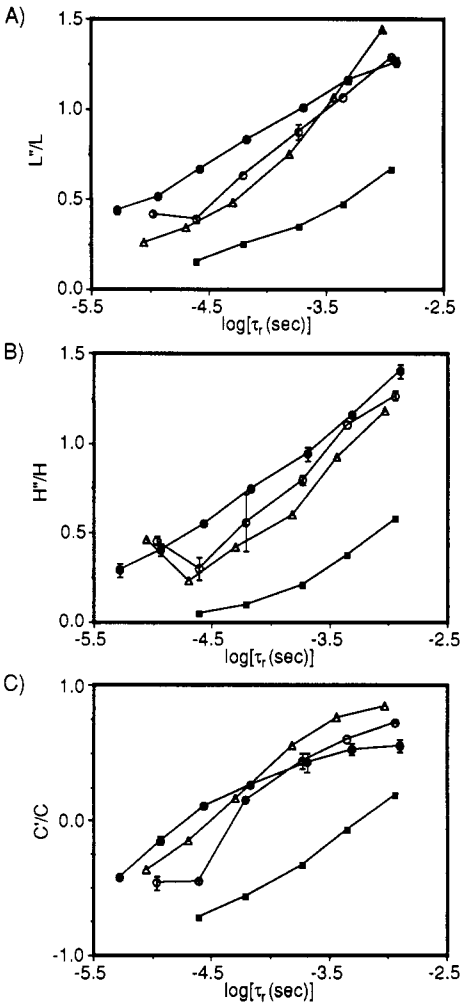


FIGURE 8: Dependence of saturation-transfer EPR spectral parameters on the rotational correlation time of the spin-labeled hemoglobin species. Ratios of spectral parameters were determined from saturation-transfer EPR spectra as indicated in Figure 7. L''/L (A), H''/H (B), and C'/C (C) are shown as a function of the rotational correlation time in semi-log plots for bifunctionally attached BSL-hemoglobin (●), monofunctionally attached BSL-hemoglobin (○), maleimide spin-labeled hemoglobin (△), and surface maleimide spin-labeled hemoglobin (■). Each data point for the mono- and bifunctionally attached BSL-hemoglobin represents the average of the parameter from two spectra. Range bars are shown.

in the C'/C ratio were similar to those of L''/L and H''/H. This similarity in all three spectral parameters shows that both bifunctional and monofunctional attachment of BSL-Hb resulted in good reporters of Hb rotational diffusion. This label should therefore be generally applicable to the study of protein rotational motion.

Discussion

For accurate measurements of protein rotational motion using the STEPR technique, the spin-label must be immobilized with respect to the protein; otherwise, the motion of the spin-label will obscure the measurement of rotational motion for the protein. Several bifunctional spin-labels have been synthesized to try to overcome this problem (Gaffney, 1985; Hideg & Hankovsky, 1989). Most of these are homobifunctional labels that require the proximity of appropriately reactive groups on the protein. An advantage, however, is the simplicity of the labeling protocol, since only one reaction is required for attachment to the protein. Most homobifunctional labels are not site-specific, and in general, not all sites of attachment would be bifunctional. An exception is the series of labels synthesized by Beth and co-workers (Beth et al., 1986; Anjaneyulu et al., 1988, 1989), which modify the erythrocyte anion channel specifically by virtue of their anionic character. Heterobifunctional labels have been synthesized that form the first attachment to the protein through an α-β-unsaturated ketone and the second through a photoactivatable phenyl azide (Keana et al., 1982). However, there was no mechanism to release any spin-label that remained monofunctionally attached to the protein. The BSL used in this study was designed to have more general applicability than existing homobifunctional labels, combined with the ability to remove monofunctional label, which would provide an advantage over most existing hetero- or homobifunctional spin-labels.

BSL was used to spin-label Hb so that its utility could be tested by direct comparisons with MSL-Hb. Although widely used as a model, the interpretation of STEPR spectra of spin-labeled Hb is complicated by several properties of the Hb structure. The protein is a tetramer consisting of two α and two β chains. The tetramer can dissociate into $\alpha\beta$ dimers, but not monomers under normal conditions. Oxyhemoglobin can readily dissociate into dimers ($K_d \sim 1$ μM; Antonini & Brunori, 1971), whereas deoxyhemoglobin is locked into the tetramer form ($K_d \sim 1$ pM; Ip et al., 1976). Modification of the oxyhemoglobin cysteine (β93) with NEM slightly decreases the dissociation constant (Parkhurst & Flamig, 1978) whereas octyl glucoside can increase it (data not shown). The BSL labeling protocol did not significantly disrupt the quaternary structure of Hb since there was no detectable dissociation to dimers or monomers. There were small increases in the apparent Stokes radius of Hb as measured by gel filtration upon modification of Hb with NEM, or mono- or bifunctionally attached BSL. Thomas et al. (1976) found that maleimide modification of Hb did not change the Hb Stokes radius, whereas in our hands a slight increase in Stokes radius was observed. Although we do not know the reason for these differences, they probably result from minor changes in the hydrodynamic properties of Hb or subunit packing in the Hb tetramer. Further contributing to the increased Stokes radius of bifunctionally attached BSL-Hb may be the presence of small amounts of cross-linked Hb in the sample which were not removed during the preparative gel filtration. Cross-linking of the Hb tetramers can occur through the formation of intermolecular disulfides from the oxidation of the free thiols placed on Hb. The cross-linking was controlled by exclusion of oxygen from the thiolated Hb solutions and by limiting thiol modifications of Hb to one per subunit. However, even under these conditions, some Hb cross-linking occurred so that the final bifunctionally attached BSL-Hb sample required purification by gel filtration.

The conventional EPR spectrum for the monofunctionally attached BSL-Hb resembled the spectrum of the immobilized MSL-Hb rather than the highly mobile surface MSL on Hb. The monofunctionally bound BSL is probably rigidly held on Hb by hydrophobic interactions of the label with the protein which agrees with the findings of Naber and Cooke (1989) using hydrophobic monofunctional spin-labels. The behavior of the monofunctionally attached BSL contrasts with the more hydrophilic surface MSL on Hb which displays a highly mobile EPR spectrum. Thus, monofunctional attachment of BSL may provide a suitably immobilized label for STEPR in many applications. However, the immobilization of the monofunctionally attached BSL may also be dependent on the properties of a particular protein. Thus, for other proteins, the monofunctionally bound BSL may not be immobilized,

especially if the label is bound to a membrane protein near or within a hydrophobic environment such as a lipid bilayer. The hydrophobic environment of a membrane interior may mobilize the monofunctionally attached BSL and thus require bifunctionally attached BSL.

Bifunctional attachment of the label is achieved by photolysis of the BSL phenyl azide to the highly reactive nitrene [photoaffinity labeling reviewed by Bayley and Staros (1984)]. Nitrenes react preferentially with amino, thiol, or hydroxy groups primarily through the nitrene's singlet intermediate, either indirectly after rearrangement of the singlet or directly if the nitrene is immobilized on the protein with a favorable orientation. The nitrene will insert less readily into carbon–hydrogen bonds through singlet or triplet intermediates by hydrogen abstraction and rearrangement for singlet nitrenes or radical recombination for triplet nitrenes. This insertion reaction is more efficient when the nitrene is immobilized on the protein and favorably oriented. The lack of specificity of the BSL's second attachment is advantageous since the proximity of two specific functional groups on the protein is not required for bifunctional attachment of the label. However, this labeling strategy is unlikely to result in unique labeling of the protein by BSL. Another feature of the photolysis is that the BSL will remain monofunctionally attached to the protein if the nitrene's singlet or triplet intermediate reacts with water or the triplet intermediate forms an aniline by abstraction of two protons from the protein. These monofunctionally attached labels were released after reduction of the BSL's disulfide attachment to the protein whereas bifunctionally attached labels remain bound. The bifunctionally attached label is then regenerated by mild oxidation.

The STEPR spectra for mono- and bifunctionally attached BSL-Hb show a response to changes in rotational diffusion of hemoglobin similar to the MSL-Hb, although there are small differences in the amplitude changes of specific peak heights. The L''/L and H''/H spectral parameter plots for the monofunctionally attached BSL-Hb are very similar to those for the MSL-Hb, whereas the L''/L and H''/H parameter values for the bifunctionally attached BSL-Hb are shifted to higher values relative to the parameter values of MSL-Hb over most of the range of rotation times examined. This suggests that the bifunctionally attached label undergoes slower motion than MSL-Hb. Only at the slowest rotation times (10^{-3} s) does the MSL-Hb have a higher value of L''/L (though not of H''/H) than BSL-Hb. The difference in behavior between the L''/L and H''/H parameters near the rigid limit may be due to differences in anisotropic motion between the labels in MSL-Hb versus BSL-Hb. Thus, the effective range of τ_r's measurable with BSL labeling is from a few microseconds to several hundred microseconds. This is similar to the range detectable with MSL-Hb, which is limited by the internal dynamics of the MSL (Johnson, 1978, 1981).

On the basis of the presented data, the bifunctionally attached BSL-Hb is a good reporter of protein rotation and is as responsive to changes in Hb rotation as the MSL-Hb, a commonly used standard for the STEPR technique. Unexpectedly, the monofunctionally attached BSL was also a good reporter of Hb rotational motion. The monofunctionally bound label is immobilized on Hb through hydrophobic interactions which may not occur with all proteins. The monofunctionally attached BSL might be useful in cases where other monofunctional spin-labels are mobile and the protein is sensitive to the bifunctional attachment of the label. Monofunctional spin-labels, such as MSL, and existing bifunctional reagents have simpler protein labeling protocols and require less manipulation of the protein than the BSL labeling protocol. However, the BSL does not require specialized protein structures for its immobilization on the protein, and any BSL that is not bifunctionally attached to the protein can be removed; thus, the BSL should be more generally applicable for STEPR studies of protein rotation than existing spin-labels.

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Sequence-Specific ^{1}H NMR Assignments and Secondary Structure of Porcine Motilin†

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ABSTRACT: The solution structure of the 22-residue peptide hormone motilin has been studied by circular dichroism and two-dimensional ^{1}H nuclear magnetic resonance spectroscopy. Circular dichroism spectra indicate the presence of α-helical secondary structure in aqueous solution, and the secondary structure can be stabilized with hexafluoro-2-propanol. Sequence-specific assignments of the proton NMR spectrum of porcine motilin in 30% hexafluoro-2-propanol have been made by using two-dimensional NMR techniques. All backbone proton resonances (NH and αCH) and most of the side-chain resonances have been assigned by using double-quantum-filtered COSY, RELAYED-COSY, and NOESY experiments. Simulations of NOESY cross-peak intensities as a function of mixing time indicate that spin diffusion has a relatively small effect in peptides the size of motilin, thereby allowing the use of long mixing times to confidently make assignments and delineate secondary structure. Sequential αCH–NH and NH–NH NOESY connectivities were observed over a significant portion of the length of the peptide. A number of medium-range NOESY cross-peaks indicate that the peptide is folded into α-helix from Glu9 to Lys20, which agrees favorably with the 50% helical content determined from CD measurements. The intensities of selected NOESY cross-peaks relative to corresponding diagonal peaks were used to estimate a rotational correlation time of approximately 2.5 ns for the peptide, indicating that the peptide exists as a monomer in solution under the conditions used here.

Motilin is a small gastrointestinal peptide hormone (M_r = 2699) found in blood and in some endocrine cells situated in the gut (Usellini et al., 1984). The hormone stimulates gastrointestinal peristalsis, notably during the interdigestive period (Mutt, 1982; McIntosh & Brown, 1988; Schubert & Brown, 1974), and it has been associated with phase III of migrating myoelectric complexes (Jacobowitz et al., 1981). The receptors for the hormone are located primarily in the smooth muscle of the duodenum (Kondo et al., 1988). Elevated levels of the hormone are observed in many disorders, particularly diarrhea (Ohe et al., 1980; Imura et al., 1980; Christofides & Bloom, 1981).

Although numerous physiological and clinical studies have been performed on motilin, no crystallographic or solution structural data are available for this peptide hormone. Motilin is known to consist of a single peptide chain of 22 amino acids with the sequence FVPIFTYGELQRMQEKERNKGQ (Brown et al., 1973; Schubert & Brown, 1974). Recently it has been shown from corresponding cDNA sequences that human and porcine motilin have identical sequences (Dea et al., 1989). These studies also suggest that motilin is synthesized as a higher molecular weight precursor from which the 22 amino acid peptide is subsequently cleaved. The eight amino terminal residues are largely hydrophobic, while the remainder of the molecule can be classified as hydrophilic. Physiological studies on canine motilin reveal that the active portion of the molecule lies between residues 6 and 16 (Poitras et al., 1987). However, studies conducted with synthetic porcine motilin indicate that the whole molecule is responsible for biological activity (Ueda et al., 1977).

Two-dimensional nuclear magnetic resonance techniques have proven to be useful in the study of structural properties of small proteins and peptides (Wagner, 1983; Wuthrich, 1986; Wright et al., 1988). Through-bond or through-space connectivities are established either via spin–spin scalar coupling or through dipole–dipole interactions. Spin–spin scalar connectivities are visualized as correlation peaks in two-dimen-

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