Distance Estimate of the Active Center of D-β-Hydroxybutyrate Dehydrogenase from the Membrane Surface†

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ABSTRACT: D-β-Hydroxybutyrate dehydrogenase (EC 1.1.1.30) is a membrane-bound, lipid-requiring enzyme which has a reactive sulfhydryl in the vicinity of the active center. The spin-probe–spin-label technique has been used to estimate the distance of separation of the reactive sulfhydryl of D-β-hydroxybutyrate dehydrogenase from the bilayer surface. The reactive sulfhydryl of the enzyme was derivatized with the maleimide spin-label reagent 4-maleimido-2,2,6,6-tetramethylpiperidinyl-1-oxy in the presence of the cofactor NAD^+. The derivatized enzyme, inserted (inlaid orientation) into phospholipid vesicles, was titrated with spin probes, either Mn^{2+} or Gd^{3+}, until the spin-label EPR spectrum was reduced in amplitude to its residual (limiting) value. From this limiting amplitude, the dipolar interaction coefficient was obtained, which is related to the reciprocal of the distance to the sixth power. The radial distances of closest approach of the paramagnetic Mn^{2+} and Gd^{3+} ions to the spin-label nitroxide on the enzyme were found to be 18 and 16 Å, respectively. These calculated distances were in accord with those determined by comparison with a phosphatidylcholine calibration system having 2,2-dimethyloxazolidinyl-1-oxy spin-labels located at selected positions along the *sn*-2 fatty acyl chain. Since the distal nitroxide moiety of the maleimide spin-label (17 Å from the bilayer surface) is 8 Å from the sulfhydryl addition site, the two limiting distances of immersion of the reactive sulfhydryl within the bilayer are 9 and 25 Å. The shorter distance is considered more compatible with facile access of the coenzyme to the active site of the enzyme.

D-β-Hydroxybutyrate dehydrogenase is a lipid-requiring enzyme with an absolute and specific requirement of lecithin for function [for reviews, see Fleischer et al. (1974, 1983) and Fleischer and McIntyre (1985)]. The apodehydrogenase, i.e., the enzyme purified from mitochondria and devoid of lipid, is inactive but spontaneously inserts into phospholipid vesicles (McIntyre et al., 1979). The enzyme inserted into mitochondrial phospholipid vesicles has characteristics similar to those of the native enzyme in the mitochondrial inner membrane in that it is enzymically active with the same ordered sequential reaction mechanism and comparable kinetic constants (Nielsen et al., 1973) and exists as a tetramer in the membrane (McIntyre et al., 1983). The spontaneous insertion characteristic suggests that the enzyme is amphipathic and inlaid in orientation, i.e., inserted into the bilayer but not transmembrane (McIntyre et al., 1978). This is supported by proton NMR studies which show perturbation of only the phosphatidylcholine in the outer leaflet (Deese et al., 1986) as well as by the decreased susceptibility of the enzyme to proteases when the enzyme is inserted into the bilayer (Berrez et al., 1984; Maurer et al., 1985). Nonetheless, the extent of immersion of the enzyme within the bilayer has not yet been determined. D-β-Hydroxybutyrate dehydrogenase has a reactive sulfhydryl which is located in the vicinity of the reactive center of the enzyme (Latruffe et al., 1980; McIntyre et al., 1984a; Dubois et al., 1986). The aim of the present study is to estimate the distance of the reactive sulfhydryl in the enzyme with respect to the membrane surface. The spin-label–spin-probe method has been employed for these measurements [see Eaton and Eaton (1978) and Hyde et al. (1979) for reviews].

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Experimental Procedures

Chemicals. Doxylstearic acids and 2,2,6,6-tetramethyl-4-aminopiperidine-1-oxyl, which were used in the synthesis of spin-labeled phospholipids, were obtained from Syva (Palo Alto, CA) or Molecular Probes (Eugene, OR). The doxylstearic acids used for the synthesis of phospholipids, spin-labeled in the *sn*-2 acyl chain (see below), contained the doxyl spin-label moiety at either carbon 5, 7, 10, 12, or 16 (see Figure 1). The maleimide spin-label protein derivatizing reagent 4-maleimido-2,2,6,6-tetramethylpiperidinyl-1-oxy was obtained from Syva. NAD^+ and dithiothreitol were obtained from Chemical Dynamics Corp. (South Plainfield, NJ). The buffers, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (Hepes)[1] and Pipes, were Ultrol grade, from Calbiochem (La Jolla, CA), and Tris-HCl was from Sigma (St. Louis, MO). Gadolinium chloride was from Alfa Inorganics (Beverly, MA). EDTA and inorganic salts, including manganous chloride, magnesium chloride, and lanthanum nitrate, were from Fisher Scientific (Fair Lawn, NJ). Chloroform and methanol (Fisher

[1] Abbreviations: AMPPCP, adenosine 5′-(β,γ-methylenetriphosphate); BDH, D-β-hydroxybutyrate dehydrogenase; DPG, diphosphatidylglycerol (cardiolipin); doxyl, 2,2-dimethyloxazolidinyl-1-oxy, a nitroxide substituent at positions "*n*" of *sn*-2 stearoyl chains of phosphatidylcholine; EDTA, ethylenediaminetetraacetic acid; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; MSL, 4-maleimido-2,2,6,6-tetramethylpiperidinyl-1-oxy; MPL, phospholipids extracted from bovine heart mitochondria; PC, phosphatidylcholine (lecithin); PE, phosphatidylethanolamine; Pipes, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); ST-EPR, saturation-transfer electron paramagnetic resonance; Tempo, 2,2,6,6-tetramethylpiperidinyl-1-oxy, a nitroxide substituent; Tempo-PC, phosphatidylcholine "polar" spin-label with the Tempo group attached to the quaternary ammonium moiety; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; ΔH_{pp}, line width of EPR spectrum, measured peak to peak; I_0, initial amplitude of the EPR spectrum; I_{lim}, limiting residual EPR amplitude in the presence of paramagnetic ion.

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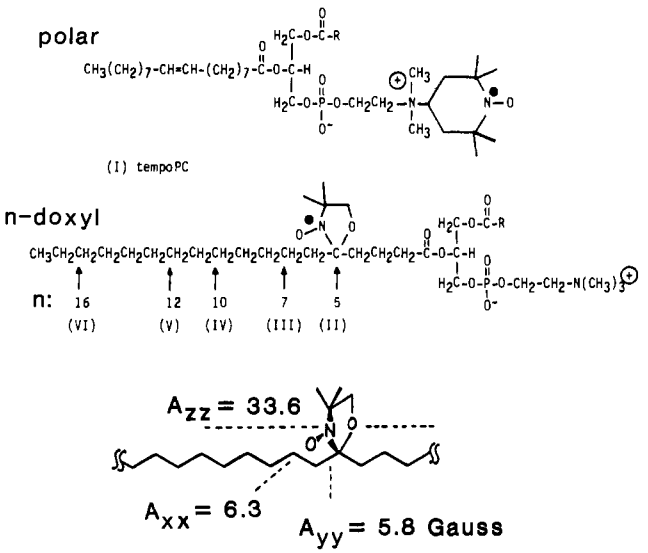

FIGURE 1: Structures of spin-labeled analogues of phosphatidylcholine. The polar phospholipid (I) contains the 2,2,6,6-tetramethylpiperidinyl-1-oxy (Tempo) substitutent in place of one *N*-methyl group of the choline in lecithin. The second group of spin-labeled (*n*-doxyl) analogues of lecithin contains doxylstearates in the *sn*-2 position. The substituent in the *sn*-1 chain position (R) is palmitate. The spin-label moiety (doxyl ring shown at position 5) is located at the 5-, 7-, 10-, 12-, or 16-positions of the stearate as indicated in compounds II–VI, respectively. The orientation and values of the magnetic tensor elements (A_{zz}, A_{xx}, and A_{yy}) relative to the nitroxide of the spin-label are also shown.

Scientific) were freshly distilled before use. Column chromatography materials, Sephadex G-25 (coarse) and Bio-Sil A (100–200 mesh), for purification of mitochondrial phospholipids (see below), were obtained from Pharmacia (Piscataway, NJ) and Bio-Rad (Richmond, CA), respectively. Other chemicals were reagent grade. Solutions were prepared in deionized water.

Assays. Protein was determined by the method of Lowry et al. (1951). Phospholipid phosphorus was determined by a modification (Fleischer et al., 1967) of the method of Chen et al. (1956). D-β-Hydroxybutyrate dehydrogenase activity was measured spectrophotometrically according to Bock and Fleischer (1975).

Spin-Labeled Phospholipids. The lecithin analogue having the piperidino nitroxide substituent on the ammonium moiety (I; Figure 1) was synthesized by amination of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoric acid bromoethyl ester with 2,2,6,6-tetramethyl-4-aminopiperidinyl-1-oxy according to the procedure of Eibl et al. (1983). Lecithins having the oxazolidinyl (doxyl) substituent on the *sn*-2 stearoyl chain (II–VI; Figure 1) were synthesized from 1-palmitoyl-*sn*-glycero-3-phosphoric acid bromoethyl ester and doxylstearic acid according to the method of Eibl et al. (1983).

Preparation of Phospholipid Vesicles. Lipids were extracted from bovine heart mitochondria as described previously (Fleischer et al., 1967), and the phospholipid fraction (MPL)[2] was purified by Sephadex and silicic acid chromatography (Rouser et al., 1967). DPG was also obtained by this method. Aliquots of mitochondrial phospholipids (MPL) (containing 250 μg of P) and the appropriate spin-labeled phospholipid (2.5 μg of P) in chloroform/methanol (2:1) solution were mixed, evaporated under argon, and dried under vacuum for 0.5 h to remove residual solvent. The dried phospholipids were resuspended in 0.20 mL of buffer (10 mM Pipes, 10 mM Hepes, and 1 mM EDTA, pH 7.0, previously degassed and saturated with argon) by using a vortex mixer and then dispersed under argon by sonication in a bath sonicator (Laboratory Supplies Co., Hicksville, NY) for 15 min. The mixing and sonication were repeated 2 or 3 times until the dispersion appeared uniform and opalescent. Five microliters of 2 M NaCl was added to bring the final concentration of NaCl to 50 mM so as to minimize the effect of the subsequent addition of paramagnetic ions on the total ion concentration. The sample was then sonicated briefly (~5 min) since prolonged sonication (or storage) in the presence of salt increased the turbidity of the phospholipid vesicle (liposome) preparations. Dioleoyl-PC and -PE, obtained from Avanti Polar Lipids, Inc. (Birmingham, AL), were also used.

Spin-Labeling of D-β-*Hydroxybutyrate Dehydrogenase.* The enzyme–MPL complex was formed by addition of the apoenzyme (2.5 mg) to MPL vesicles (100 μg of phosphorus/mg of protein) in 6.25 mL of 20 mM Tris-HCl (pH 8.1), 1 mM EDTA, 67 mM LiBr, and 1 mM dithiothreitol followed by incubation for 2.5 h at room temperature (Churchill et al., 1983). The enzyme–MPL complexes used in these studies consisted of 100 mol of phospholipid/mol of enzyme monomer, and the initial specific activity ranged from 130 to 140 μmol of NAD^+ reduced min^{-1} (mg of enzyme)$^{-1}$. After dialysis (total of 18 h) vs. 3 × 100 volumes of 50 mM NaCl, 5 mM Hepes, and 1 mM EDTA (pH 7.0) to remove dithiothreitol, the enzyme–MPL complex was reacted, in the presence of 5 mM NAD^+, with the sulfhydryl spin-labeling reagent 4-maleimido-2,2,6,6-tetramethylpiperidinyl-1-oxy (MSL) at 6 mol of MSL/mol of enzyme monomer. Under these labeling conditions, the derivatization of 0.5 equiv of sulfhydryl per enzyme monomer was obtained (McIntyre et al., 1984a). The enzyme became inactivated to ~2% residual activity, and the spin-label was incorporated selectively at the rapidly reacting ("essential") sulfhydryl, yielding MSL–enzyme–MPL (McIntyre et al., 1984a). Excess label was removed by dialysis (24 h) vs. 2 × 100 volumes of 50 mM NaCl, 5 mM Hepes, and 1 mM EDTA (pH 7.0) and concentrated in a Micro-ProDiCon (Bio-Molecular Dynamics, Beaverton, OR) under argon to a final volume of ~0.20 mL. The concentrated MPL vesicles, with inserted spin-labeled enzyme (~12 mg of protein/mL, 340 μM monomer), were suspended in 10 mM Pipes, 10 mM Hepes, 1 mM EDTA, and 50 mM NaCl (pH 7.0) previously degassed and saturated with argon and titrated with paramagnetic ions as described below.

EPR Spectroscopy. EPR spectra were recorded in the absorption mode by using a Varian E-112 spectrometer with an X-band (9.5-GHz) E-101 microwave bridge and a TM_{110} cavity. For signal averaging, the spectrometer was interfaced to a Digital Equipment Corp. LSI-11 computer so that the magnetic field was swept under computer control. Each accumulated spectrum was referenced to the field marker signal on the Varian E-272B field-frequency lock accessory. The data points (2040) were acquired over a 128-G sweep range (~0.06-G step size) centered near 3350 G. At each magnetic field point, signal amplitudes were digitized by an ADAC 1000 analog-to-digital converter to 12-bits resolution. For comparison of signal intensities, the digitized and averaged EPR spectra (first-derivative format) were integrated, making use of a Simpson's rule algorithm. A Varian E-257 temperature-controller accessory was used to regulate temperatures to ±1 °C of the desired temperature. The temperature varied less than 1 °C between the bottom and top of the flat cell.

[2] MPL consists mainly of three classes of phospholipids, i.e., phosphatidylcholine, phosphatidylethanolamine, and diphosphatidylglycerol in a ratio of 43:37:20 on a phosphorus basis (Fleischer et al., 1967).

Samples of liposome dispersions (containing either spin-labeled phospholipids or MSL–enzyme–MPL) were contained under an argon atmosphere in a standard EPR quartz flat cell (Wilmad Glass Co., Buena, NJ) having 0.25-mm internal thickness and 0.18-mL volume. After each addition of aliquots of buffered paramagnetic ion stock solution to the 0.20-mL initial volume of the liposome dispersion (see below), care was taken to reposition the flat cell in the cavity in the same orientation. The reproducibility of alignment was checked by observing the leakage current (adjusted initially at 250 μA for 5-mW incident microwave power) on the diode detector and by checking that the paramagnetic ion EPR amplitudes corresponded to the increased ion concentrations. A modulation amplitude of 1.0 G was selected, since this is approximately half the width of the narrowest EPR line (1.9 G for 16-doxylphosphatidylcholine) so as to minimize modulation broadening (<0.05 G for 1.9-G line width) (Poole, 1983).

Titration of Liposomes and Spin-Labeled D-β-*Hydroxybutyrate Dehydrogenase with Paramagnetic Ions.* A series of phosphatidylcholines with nitroxide spin-labels either at the polar head group or at positions 5, 7, 10, 12, or 16 along the *sn*-2 stearoyl moiety, each codispersed (1 mol % of spin-label) with MPL to form phospholipid vesicles, was used to calibrate the reduction in amplitude vs. the distance from the aqueous interface of the liposome. The structures of the phospholipids containing spin-labels in different positions, designated I–VI, respectively, are shown in Figure 1. The polar phosphatidylcholine analogue Tempo-PC (I) was used as a control for measuring interactions at the surface of the bilayer.

The first-derivative EPR signal was recorded digitally, and the absorption integral (proportional to signal intensity) was computed in the absence of metal ions in order to compare the signal intensities of the various labels I–VI and to compare recovery of signal after the addition of EDTA at the conclusion of each titration series. Aliquots of paramagnetic ion stock solutions (200 mM Mn^{2+} or Gd^{3+}), in 100 mM Hepes and 100 mM Pipes, pH 7.0, were added and carefully mixed with minimum exposure to air. After each addition, both the full EPR spectrum of the spin-label (128-G scan) and a 10-G-wide scan of the central $m_I = 0$ line of the nitroxide spectrum were recorded for each concentration of metal ion. The complete spectrum (128-G scan) of the nitroxide provided the spectral shape characteristics including the ratio of amplitudes of the ($m_I = -1$; high field)/($m_I = 0$; central) lines. Major changes in these spectral parameters would have been indicative of the formation of multilayers or domain segregation of spin-labeled phospholipid distribution in the lateral plane. No such spectral line-shape changes were observed, indicating that the integrity of the bilayer of the phospholipid vesicles was maintained throughout the titration. In the presence of Gd^{3+} (up to 30 mM), we did not observe, by freeze–fracture electron microscopy, any change in the morphology of the phospholipid vesicles. The line widths of the low- and center-field resonances were measured from spectra recorded with a 10-G field sweep. The line widths of the nitroxide EPR spectra for structures II–VI increased slightly in the presence of paramagnetic ions (see Results), thereby indicating that dipolar interaction rather than Heisenberg spin exchange is the dominant mechanism contributing to the reduction in amplitude of the EPR spectra (Eaton & Eaton, 1978; Poole, 1983). For Tempo-PC (structure I), the line width of the residual EPR spectra in the presence of paramagnetic ions was significantly broadened (20–30% increase) compared to the initial line width. After each addition of Mn^{2+} in the titration, a corresponding higher field portion of the Mn^{2+} spectrum (see Figure 8A) was recorded in order to (a) correlate the paramagnetic ion signal amplitude with concentration (this was used as an internal check for the alignment of the flat cell) and (b) verify the base-line corrections applied to the 128-G nitroxide spectrum (see Figures 3 and 8). For Gd^{3+}, the base-line correction simply involved subtraction of a sloping line since the Gd^{3+} spectrum consisted of a single line of 470-G width (see Figure 3). At the highest concentration of added Mn^{2+} (7 mM), 1 μL of 500 μM ionophore A23187 in ethanol was added to the sample to give a final concentration of 0.4–0.5 μM ionophore (equivalent to four to five molecules per phospholipid vesicle). The A23187 was used to facilitate the penetration of the divalent metal ions to the internal compartment of the vesicles. After the EPR spectra were recorded in the presence of ionophore, the vesicle suspension was sonicated for 3 min to ensure penetration of Mn^{2+} to the inside of the vesicles, and the EPR measurements were repeated. The ionophore and sonication did not markedly alter the EPR spectra, indicating that, during the experiment, the metal ions had permeated to the inside compartment of the vesicles. Since D-β-hydroxybutyrate dehydrogenase inserts unidirectionally into vesicles (McIntyre et al., 1979), it was not necessary to ensure penetration of metal ions to the inside of the vesicles. After completion of each titration series, an amount of EDTA sufficient to chelate the di- or trivalent paramagnetic ions was introduced into the vesicle suspension in order to test whether the reduction in EPR spectral amplitude was reversible.

Calculation of Distances from Limiting Reduction in EPR Amplitude. For two different paramagnetic species, spin "S" and relaxer "R", separated by a distance (r) large enough to preclude orbital overlap, the reduction in amplitude of the EPR spectrum of the more slowly relaxing species (S, with the longer T_{1e}) is governed by dipolar interaction (Leigh, 1970). The magnitude of the dipolar interaction between these species is inversely proportional to the sixth power of the distance of separation. In the presence of the relaxer, R, the observed composite nitroxide resonance line consists of the superposition of individual component resonances ("spin packets"), each corresponding to a given angle (θ_R) of the S → R vector with respect to the external magnetic field. Dipolar interaction between the slowly relaxing spin S and the rapidly relaxing spin R results in a selective broadening of a subset of spin packets according to the relationship $\Delta H_{dd} \propto C(1 - 3\cos^2\theta_R)^2$, where ΔH_{dd} is the dipole–dipole broadened component line width and C is the dipolar interaction coefficient. As an example, for close approach of S and R (large C), the spin packets which contribute to the observed composite resonance line correspond to those packets with S → R oriented near the "magic angle" with respect to the magnetic field. Spin packet components at other orientations are broadened significantly so that they become essentially indistinguishable from the base line. Thus, for an isotropic absorption line, dipole–dipole interaction causes a decrease in the observed amplitude of the EPR spectrum with little observable increase in line width; only spins within a range of orientations close to the magic angle with respect to the magnetic field are detected (Leigh, 1970). The residual observed spectrum therefore corresponds to a reduction in amplitude of the composite nitroxide resonance line. In general, both reduction in amplitude and changes in residual line shape were predicted by Leigh (1970) for EPR spectra of spins with lower symmetries (e.g., nitroxides). The apparent reduction in amplitude attributable to dipolar interaction also depends upon the initial line width of the composite (inhomogeneously broadened) nitroxide resonance, ΔH_{pp}. Leigh (1970) has calculated the relationship

between I_{lim}/I_0 (where I_{lim} is the residual EPR amplitude in the presence of paramagnetic ions and I_0 is the initial amplitude) and C for a range of initial peak to peak widths of the nitroxide resonance line. For a random distribution of orientations of the interacting dipoles with respect to the external magnetic field, there is a specific relationship between I_{lim}/I_0 and $C/\Delta H_{pp}$ (Leigh, 1970). C is inversely related to r^6. In these experiments, C was determined from measured I_{lim}/I_0 and ΔH_{pp} values by using the published data of Leigh (1970), and $1/C$ was used to calculate the radial distance r according to the relationship:

$$r = \left[\left(\frac{g_S \mu_B \mu_R^2 T_{1eR}}{\hbar}\right)\left(\frac{1}{C}\right)\right]^{1/6} \quad (1)$$

where g_S is the Lande g factor for the nitroxide, μ_B is the Bohr magneton, μ_R is the magnetic moment of the transition ion spin probe, T_{1eR} is the spin–lattice relaxation time of the ion, $\hbar$ is $1/2\pi \times$ Planck's constant, and r is the radial distance (in centimeters) of separation between the nitroxide and the transition ion.

The theory by Leigh (1970) was developed to describe the influence on the EPR line shape by dipolar coupling between a single paramagnetic ion and a spin-label with rigid-lattice EPR line shape; it was shown that, although changes in EPR line shape can occur, the amplitude reduction of the EPR signal afforded by the paramagnetic ion allows calculation of the distance between the two spins. For our studies of the reduction in amplitude of the EPR spectrum of a nitroxide spin-label at a unique site in D-β-hydroxybutyrate dehydrogenase, the experimental system differs from that considered by Leigh in that, although the EPR spectrum is close to the rigid-lattice line shape, interaction with multiple paramagnetic ions may pertain. The theoretical considerations of Hyde and Rao (1978) indicate that dipolar effects are strongly dependent on the distance of the paramagnetic ion which most closely approaches the nitroxide, suggesting that the treatment described by Leigh appears to be applicable in our studies of D-β-hydroxybutyrate dehydrogenase. We tested the applicability of the theory of Leigh (1970) with a series of spin-labeled phospholipids where the distance from the bilayer surface can be estimated by independent methods.

Results

Calibration of Distance in the Transverse Plane of the Bilayer. A series of spin-labeled phospholipids with the nitroxide located at defined positions in the transverse plane of the bilayer (see Figure 1) was used for calibration. The EPR spectra of these spin-labeled phospholipids, codispersed with MPL into small unilamellar vesicles, are shown in Figure 2. As the distance from the polar moiety increases, there is increased motional freedom of the labeled segment of the hydrocarbon chain, as reflected by a decrease in the order parameter, S^{app}, and an increase in γ, the corresponding half-angle of a cone describing possible wobble orientations of the doxyl ring. The values calculated for this n-doxyl series in MPL (see Figure 2) are comparable to those reported previously (Griffith & Jost, 1976).

Spectra of the spin-labeled phospholipids dispersed in MPL liposomes were recorded in the presence of increasing amounts of paramagnetic metal ions. The nitroxide spectrum was superimposed on the broader spectra of Gd^{3+} or Mn^{2+} (see Figures 3 and 8, respectively). The amplitudes of the nitroxide resonance lines in the presence of increasing amounts of paramagnetic ions (I) were measured after subtraction of the paramagnetic ion spectrum and were then normalized relative to the amplitude in the absence of paramagnetic ions (I_0).

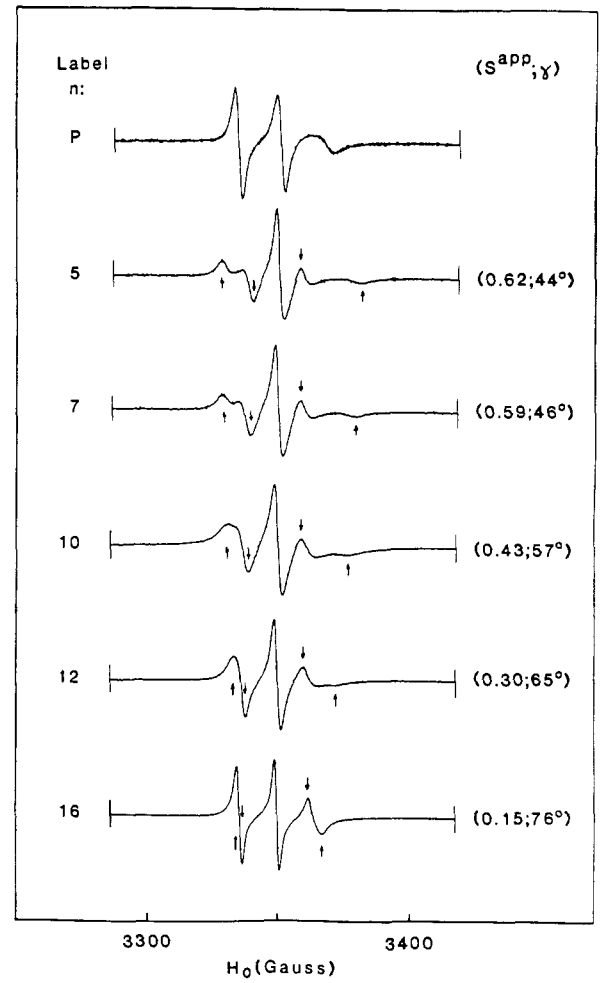

FIGURE 2: EPR spectra of the spin-labeled phospholipids dispersed as 1% of the total lipid phosphorus in unilamellar MPL vesicles. Structures of the spin-labeled phosphatidylcholine analogues, with label positions P (polar) and 5–16, are shown in Figure 1 (compounds I–VI, respectively). Codispersions of the spin-labeled phospholipids in MPL were prepared by sonication in 10 mM Pipes, 10 mM Hepes, and 1 mM EDTA, pH 7.0, at 1.25 mg of P/mL, and NaCl was then added to a final concentration of 50 mM (see Experimental Procedures). Each sample was sealed in a standard EPR quartz flat cell, and the spectrum was recorded at 25 °C using 5-mW incident microwave power, 1-G modulation amplitude, and a field sweep of 128 G. The spectra are scaled to the same amplitude for the central ($m_I = 0$) line. The upward arrows indicate the hyperfine extrema; their separation in gauss is referred to as $2A_{max}$ (twice the hyperfine splitting). Downward arrows indicate $2A_{min}$. The approximate order parameters, S^{app}, and the corresponding half-angle of wobble orientations (γ) calculated for the n-doxyl series are given at the right. $S^{app} = (A_{max} - A_{min})/[A_{zz} - {}^1/_2(A_{xx} + A_{yy})]$; γ was obtained according to Griffith and Jost (1976).

A number of factors in the design of experiments were found to be important for reliable measurement of distances by the spin-probe–spin-label method, especially with respect to measurements in membrane systems. First, the formalism for obtaining distance information from dipolar interactions between paramagnetic species is strictly applicable to spin-label–spin-probe separations greater than the radii of these species; i.e., direct orbital overlap must be precluded. If the species come into direct contact with one another, then the reduction in signal amplitude results from both Heisenberg spin exchange and dipolar relaxation; both mechanisms lead to reduction in the EPR spectral amplitude. Dipolar relaxation gives a reduction in amplitude of the resonance line with little observable broadening (Leigh, 1970), while Heisenberg spin

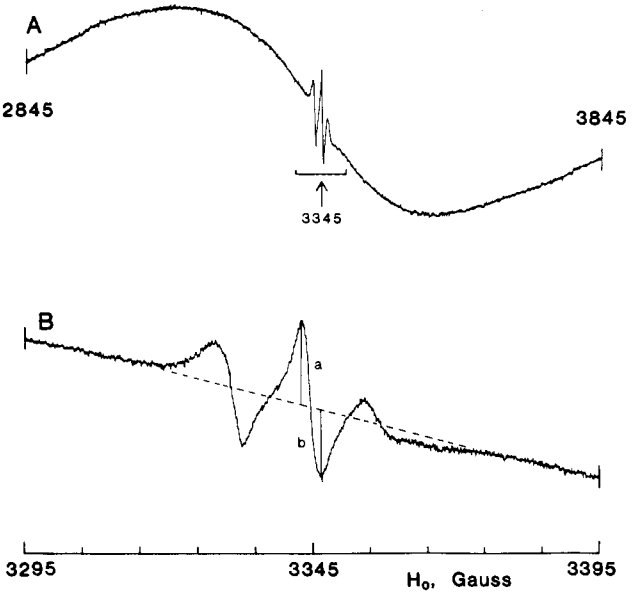

FIGURE 3: EPR spectrum of spin-labeled phospholipid in the presence of gadolinium ion. The overlap of the EPR spectra and measurement of the residual amplitude of the nitroxide spectrum are illustrated. Spin-labeled phospholipid vesicles containing 1 mol % 12-doxyl-PC in MPL (see Figure 2) were titrated with $GdCl_3$ to 20 mM, and the EPR spectrum was recorded by using the same conditions given in Figure 2. (A) The upper spectrum (1000-G width) shows a portion of the broad Gd^{3+} spectrum with the narrow three-line nitroxide spectrum centered at ~3345 G. (B) The lower spectrum (100-G width) depicts the expanded central region of the upper spectrum. The dashed line is used as the reference to subtract the gadolinium spectrum from the nitroxide spectrum. The amplitude of the m_I = 0 (central) line of the nitroxide spectrum is taken as the sum of a + b and is expressed relative to the initial amplitude in the absence of paramagnetic ion (see Figure 4). Spectra in the presence of gadolinium were recorded with several receiver gains so that the residual amplitude of the nitroxide spectrum could be more accurately quantitated.

exchange results in a general broadening of the spectrum (with conservation of integrated signal intensity in both cases). When a solution of nitroxide (0.25 or 0.5 mM 3-carboxy-1-oxy-2,2,5,5-tetramethylpyrroline in buffer) was titrated with paramagnetic ions varied over a range of concentrations similar to those used in the liposome titrations, we observed marked increases in line width reflecting Heisenberg spin exchange; e.g., in the presence of 5 mM Mn^{2+} or 30 mM Gd^{3+}, the nitroxide line width was increased to more than twice its original value (not shown). By contrast, the spectra of the *n*-doxyl phospholipids did not exhibit marked line broadening (≤15% increase) in the presence of paramagnetic ions, indicating that dipole–dipole interaction dominates the amplitude reduction in the EPR spectra. However, some line broadening (see Table I, footnote *h*), indicative of Heisenberg spin exchange, was observed for Tempo-PC, in which the nitroxide moiety is accessible to the bulk solvent. Such Heisenberg spin exchange is likely a factor which contributes to the deviation of the calculated distance from the predicted distance for this spin-label (see Table I).

A second important aspect of the spin-label–spin-probe methodology, as applied in these studies, is that the paramagnetic ions be bound to the membrane surface with a residence time longer than the spin–lattice relaxation time (T_{1eR}). The bound paramagnetic ions have a greater dipolar interaction with spin-labels buried in the membrane than do ions in the bulk solution, where rotation of the hydrated ions results in negligible net dipolar interaction (Cohn et al., 1971). In MPL, the predominant negatively charged lipid is DPG (Fleischer et al., 1967), which binds cations tightly (Hegner et al., 1973). The influence of residence time of the paramagnetic ion bound at sites on the surface of the liposome was tested by conducting titrations with Mn^{2+} of 7- and 16-doxyl-PC in liposomes prepared from mixtures of the purified lipids phosphatidylcholine (PC), phosphatidylethanolamine (PE), and diphosphatidylglycerol (DPG). This mixture was prepared in a 1.0:0.8:0.45 PC:PE:DPG ratio (phosphorus ratio basis) to match that of MPL, i.e., the composition of the mitochondrial inner membrane (Fleischer et al., 1967). A smaller I_{lim}/I_0 was obtained for *n*-doxyl-PC labels dispersed in DPG/PC/PE as compared with the same labels in PC/PE (Figure 4). This indicates that negatively charged DPG provides a binding site for paramagnetic metal ions which enhances the dipolar interaction. These results are consistent with the binding of divalent cations to negatively charged lipids such as DPG (Hegner et al., 1973) and phosphatidylglycerol (Wagner et al., 1980).

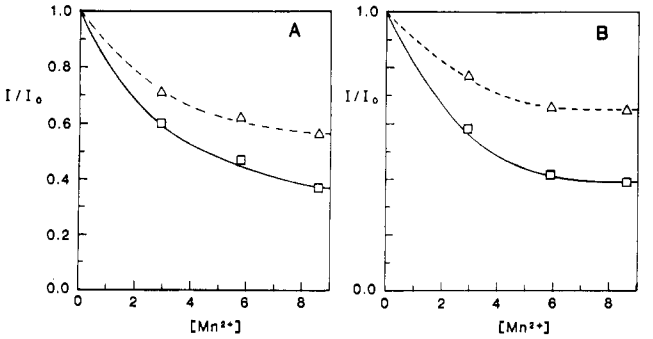

FIGURE 4: Effect of negatively charged phospholipids (DPG) on the amplitude reduction of the EPR spectra of spin-labeled phospholipids by titration with Mn^{2+}. The phospholipid vesicles contained either PC and PE (1.0:0.8 lipid phosphorus ratio) (△) or PC/PE/DPG (1.0:0.8:0.45) (□). The latter mixture is similar in composition to MPL. Data are shown for phospholipid vesicles containing either (A) 7-doxyl-PC or (B) 16-doxyl-PC which exhibited initial line widths, ΔH_{pp}, of 3.1 and 1.9 G, respectively. The EPR spectral amplitude (I) (see Figures 3 and 8) was measured at each concentration of Mn^{2+} and is expressed relative to the initial amplitude (I_0). Radial distances, calculated from the limiting amplitude (I_{lim}/I_0), and line-width data (see eq 1 and Table I) for the central line (m_I = 0) of the 7- and 16-doxyl-PC nitroxides in PC/PE/DPG are 15 and 19 Å, respectively. The I_{lim}/I_0 values for these labels in PC/PE vesicles result in calculated radial distances about 3–4 Å larger than their respective values in PC/PE/DPG.

The choice of paramagnetic ion is a key consideration in design of spin-label–spin-probe experiments. For optimal resolution of distance in the transverse plane of the bilayer, the paramagnetic ion selected as spin probe should possess a high magnetic moment and a long spin–lattice relaxation time (see eq 1). Compatibility with the biological system is yet another factor in the choice of paramagnetic ions. Mn^{2+} and Gd^{3+} were selected due to their high magnetic moments and adequately long spin–lattice relaxation times (long in comparison to the microwave radiation period; Eaton & Eaton, 1978). Moreover, aquo complexes of these ions are stable at neutral pH, a characteristic desirable for enzyme stability and for binding of cations to the negatively charged surface of the MPL vesicles.

Plots of I/I_0 vs. paramagnetic ion concentration (Mn^{2+} and Gd^{3+}) are presented in Figure 5 for the series of phosphatidylcholines having nitroxide spin-labels located at defined positions in the transverse plane of the bilayer. Control titrations with diamagnetic Mg^{2+} and La^{3+} were conducted to ascertain whether small changes in amplitude were ascribable to changes in ionic strength or binding of di- and trivalent ions to charged groups on the surface of the liposome. Little or no reduction in amplitude occurred upon addition of dia-

Table I: Calibration and Estimate of Distances from the Membrane Surface to Spin-Labels on Phospholipid and the Essential Sulfhydryl of D-β-Hydroxybutyrate Dehydrogenase (BDH)[a]

nitroxide spin-label	line width, ΔH_{pp} (G)[b]	EPR amplitude ratio (I_{lim}/I_0)[c]		dipolar coefficient[d] (G)	distance from membrane surface (Å)			
		+Mn/−Mn	+Gd/−Gd		calculated[d] Mn[g]	calculated[d] Gd[g]	X-ray[e]	X-ray/NMR[f]
(A) lecithin								
polar PC[h]	3.1	0.13	0.28	31	11.7	13.9	6–8	6–8
5-doxyl-PC	3.1	0.21		13	13.6		8.1	8.1
7-doxyl-PC	3.1	0.28	0.36	7.8	15.2[i]	15.1	10.5	10.5
10-doxyl-PC	3.0	0.47		2.7	17.6		14.5	14.0
12-doxyl-PC	2.8	0.54	0.42	1.8	18.8	16.0	17	16.0
16-doxyl-PC	1.9	0.56		1.0	20.3[i]		22	18.6
(B) reactive sulfhydryl on BDH[j]								
MSL–enzyme–MPL	5.70 (s)	0.66		2.2	18.3			
MSL–enzyme–MPL	2.05 (w)	0.47		1.8	18.7			
MSL–enzyme–MPL	3.6		0.48	2.9		16.2		

[a] Data for ΔH_{pp} and I_{lim}/I_0 are taken either from Figures 2 and 5 (for spin-labeled phospholipids) or from Figures 8C, 7, and 5 (for MSL–BDH–MPL), respectively. BDH was reconstituted with MPL at 100 mol of phospholipid per BDH monomer, and the BDH–MPL complex was derivatized with 2 equiv of MSL per BDH tetramer to obtain MSL–BDH–MPL (see Experimental Procedures). [b] Peak to peak line widths of first-derivative EPR spectra in the absence of paramagnetic metal ions. The widths quoted are for the central line ($m_I = 0$) of the spin-labeled phospholipids. In the BDH titration with Mn^{2+}, the amplitudes of both strongly immobilized (s) and weakly immobilized (w) (Figures 7 and 8) components of the low-field ($m_I = +1$) resonance were monitored as a function of [Mn^{2+}]; the central $m_I = 0$ line was monitored in the titration with Gd^{3+}. In the presence of paramagnetic ions, the EPR spectra of the *n*-doxyl-PCs were slightly broadened (≤15%) whereas no significant change in line width was detected for MSL–BDH–MPL. Quantitation of the spectral broadening was of limited precision due to a poor signal to noise ratio of the residual spectrum after subtraction of the paramagnetic ion spectrum. [c] The limiting residual amplitude, I_{lim}, in the presence of Mn^{2+} or Gd^{3+} divided by the original amplitude in the absence of paramagnetic ions, I_0, was obtained from linear regression analysis of double-reciprocal plots of the data shown in Figure 5. [d] Distance was calculated from titration with paramagnetic ions to give I_{lim}/I_0 residual amplitude (Figure 5), from which the dipolar interaction coefficient, *C*, was determined. Input parameters into the Leigh equation (eq 1, Experimental Procedures) are as follows: μ_{Mn} = 5.9 Bohr magnetons (μ_B) or 5.47×10^{-20} erg/G (Pake, 1962); $T_{1eMn} = 1.5 \times 10^{-9}$ s (Cohn et al., 1971); $\mu_{Gd} = 8.0\ \mu_B$ or 7.42×10^{-20} erg/G (Pake, 1962); $T_{1eGd} = 5.3 \times 10^{-10}$ s (Dwek et al., 1975); $g_{doxyl} = 2.0059$ (Gaffney & McConnell, 1974); and $g_{MSL} = 2.00574$ (Thomas et al., 1976). The calculated distances are given to three significant figures in order to show trends. However, estimated error is ±1.5 Å, considering the maximum uncertainty in T_{1eR} to be a factor of 3 (Bloembergen, 1956). [e] Distance from the polar surface of the bilayer (phosphorus) to the nitrogen of the Tempo-PC nitroxide or specific carbon atom (*n*) in spiro linkage to the *n*-doxyl group of the *sn*-2 fatty acyl chain. Distances were calculated for the *sn*-2 acyl chain in the all-trans conformation based on X-ray diffraction data of dimyristoyl-PC crystals obtained by Pearson and Pascher (1979). [f] Distances from the polar surface of the bilayer (phosphorus ≡ 0) to the nitrogen of the Tempo-PC nitroxide, or to the 5-doxyl- and 7-doxyl-PCs, were calculated from the X-ray diffraction data of Pearson and Pascher (1979) as indicated in footnote *e*. Distances of the 10-, 12-, and 16-doxyl-PCs were calculated from the X-ray diffraction data but scaled according to the order parameter vs. *sn*-2 carbon atom data derived from ^2H NMR data of Seelig and Seelig (1980) as described in the text. [g] Radial distances were measured from the amplitude reduction arising from dipolar interaction which is dominated by paramagnetic ions bound to the surface of the phospholipid vesicle. In MPL liposomes, the phosphate groups of DPG appear to be the tightest binding site for cations (see Figure 4 and Discussion). Thus, for the 5- and 7-doxyl-PC labels, the distance of closest approach of the spin-label to the paramagnetic ion (bound to adjacent DPG lipid molecules rather than to PC itself) would be expected to be longer than the distances listed under "X-ray" and "X-ray/NMR" which are based on PC phosphorus as the origin. For the more distal labels (12- and 16-doxyl), this factor will contribute less to the distance of closest approach. [h] For the polar PC, the amplitude reduction of the EPR signal by the paramagnetic ion may be influenced by several factors. Since the nitroxide is exposed to the bulk solvent, it is reasonable to expect that Heisenberg spin exchange will occur in addition to dipolar interaction. For Tempo-PC (polar PC), the residual EPR spectra in the presence of paramagnetic ions exhibited significant broadening of the line width (~30% and ~20% increase in line width with 7 mM Mn^{2+} and 30 mM Gd^{3+}, respectively) indicative of Heisenberg spin exchange. By contrast, for the *n*-doxyl-PCs, the EPR spectra were broadened ≤15%. For Tempo-PC, EDTA restored ~65% of the original EPR amplitude (see legend of Figure 5) which is a much larger restoration than that obtained for soluble nitroxide (not shown), indicating that even for Tempo-PC there is significant dipolar interaction between metal ions bound to DPG and the nitroxide moiety. However, the effects of both Heisenberg spin exchange and the rapid tethered motion of the nitroxide (which would shorten the dipolar interaction period) give limited confidence in the values for distance calculated from the EPR amplitude reduction. The distance given in the X-ray/NMR column (estimated from molecular models) is likely an underestimate of the distance from the Tempo-PC nitroxide to the nearest bound paramagnetic ion; i.e., the PC to DPG distance is not included in the estimate (see footnote *g*). [i] Distances calculated from I_{lim}/I_0 data for 7-doxyl- and 16-doxyl-PC in DPG/PC/PE (see Figure 4) are 15 and 19 Å, respectively, comparable with distances obtained with MPL. Corresponding I_{lim}/I_0 data for 7- and 16-doxyl-PC in PC/PE liposomes yielded distances of 18 and 22 Å, respectively. [j] For the titration of MSL–enzyme–MPL with Mn^{2+}, the relative amplitudes of both the strongly immobilized (s) and weakly immobilized (w) components of the low-field resonance ($m_I = +1$) were monitored (Figures 6 and 7). For titration with Gd^{3+}, the amplitude of the central resonance ($m_I = 0$) was monitored.

magnetic ions (Figure 5). From the limiting residual amplitudes obtained at 6 mM Mn^{2+} (Figure 5A), it can be seen that the Tempo-PC polar spin-label is reduced to the lowest I_{lim}/I_0. For this label position, the residual EPR spectrum was broadened in the presence of paramagnetic ion (see Table I, footnote *h*), indicating that for this label, Heisenberg spin exchange is a contributing mechanism to the total spin–spin interaction and resulting amplitude reduction of the EPR spectrum. For the doxyl labels on the *sn*-2 acyl chain at increasing distance from the polar moiety, the value of I_{lim}/I_0 is progressively larger as the distance from the bilayer surface increases (Table I). The increase between the I_{lim}/I_0 values for 12-doxyl- and 16-doxyl-PC is less than the increases for 5-, 7-, and 10-doxyl labels in this series. The similar values of I_{lim}/I_0 for 12- and 16-doxyl PC can be attributed to increased segmental flexibility in the distal region of the *sn*-2 chain (Seelig & Seelig, 1980) (reflected in the values of S^{app} and γ shown in Figure 2) as well as the differences in line width (see below).

A similar calibration of I_{lim}/I_0 vs. position of the nitroxide group on spin-labeled phospholipids was carried out with gadolinium (spectra shown in Figure 3). In Figure 5B, the plots of relative amplitude of the nitroxide resonance vs. gadolinium and lanthanum (diamagnetic control) concentration are shown. At 30 mM Gd^{3+}, the I_{lim}/I_0 amplitude ratios correspond to distance of separation from the bilayer surface (glycerol or phosphate moieties): 12-doxyl > 7-doxyl > Tempo-PC. The resolution of I_{lim}/I_0 with nitroxide location is not as marked with Gd^{3+} as with Mn^{2+}. Cu^{2+} was also used as the paramagnetic ion in the titration of liposomes (data not shown) with good resolution of I_{lim}/I_0 vs. label position. However, Cu^{2+} was less useful than Mn^{2+} and Gd^{3+} for studies involving

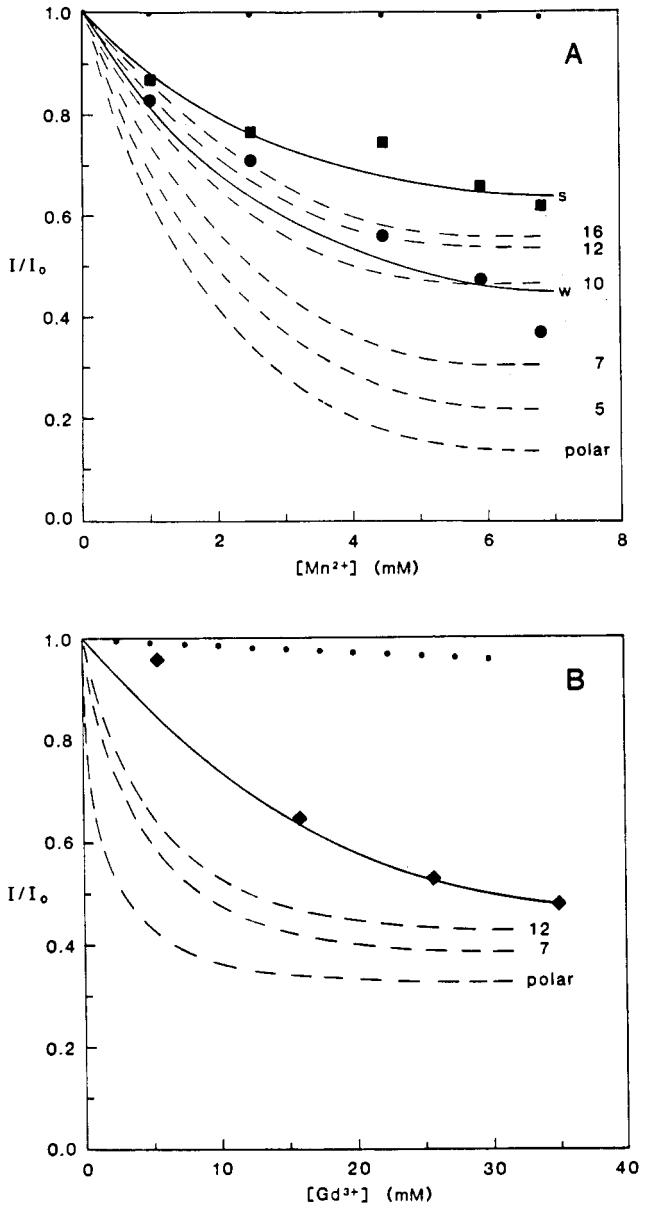

FIGURE 5: Titration of phospholipid vesicles containing either spin-labeled phospholipid or MSL–enzyme–MPL with paramagnetic ions. The EPR spectral amplitude, expressed as (I/I_0) (see Figure 4), was measured at each concentration of ion. (A) Titration of MPL liposomes containing either 1% Tempo-PC ("polar") or 5-, 7-, 10-, 12-, or 16-doxyl-PC (see Figure 2) with Mn^{2+} is shown (---). The solid lines with closed symbols represent the data for the amplitude reduction of the strongly (■) and weakly (●) immobilized components of the spin-labeled D-β-hydroxybutyrate dehydrogenase (MSL–enzyme–MPL) EPR spectrum (see Figure 7). The limiting amplitudes were obtained from $m_I = 0$ and $m_I = +1$ resonance lines (see Figures 3 and 7) for the spin-labeled phospholipid and MSL–enzyme, respectively. (B) Titration of MPL liposomes containing either 1% Tempo-PC or 7- or 12-doxyl-PC with Gd^{3+} is shown (---). The solid line (♦) indicates the relative EPR amplitude of the MSL–enzyme–MPL EPR spectrum. For both spin-labeled phospholipid and MSL–enzyme, amplitudes of the central ($m_I = 0$) line were measured. The solid lines were calculated on the basis of the limiting residual amplitudes (I_{lim}/I_0 values, given in Table I) determined from double-reciprocal plots of the data. With the *n*-doxyl-PCs, the addition of excess EDTA (1.8 mol/mol of Mn^{2+} or Gd^{3+}) restored ≥80% of the original EPR amplitude. After titration of Tempo-PC with 30 mM Gd^{3+}, the addition of excess EDTA restored ~65% of the original EPR amplitude. At the end of the titration of MSL–enzyme–MPL, addition of EDTA to a molar ratio of 1.8 EDTA/Gd^{3+} restored the amplitude of the nitroxide EPR spectrum to 95% of its original value (not shown). Titration with the diamagnetic Mg^{2+} (A) or La^{3+} (B) did not significantly decrease the signal amplitude (dotted line at $I/I_0 \sim 1.0$).

the enzyme since a lower pH was required to maintain $Cu(H_2O)_6^{2+}$ in solution. For this reason, studies with Cu^{2+} were not pursued.

For each of the *n*-doxyl labels, the EPR spectral amplitude was restored to ≥80% of the original amplitude by the subsequent addition of EDTA (1.8 mol/mol of Mn^{2+} or Gd^{3+}) (not shown), confirming that (1) the amplitude reduction was a result of spin–spin interaction rather than chemical processes and (2) the binding of paramagnetic ion to the membrane surface was necessary for the observed effects. By contrast, for Tempo-PC, we found that addition of excess EDTA gave less restoration of the EPR amplitude (~65% after titration with 30 mM Gd^{3+}), indicating that freely diffusing ions also contribute to the amplitude reduction of the spectra for this label location (this is consistent with the spectral broadening of the residual EPR signal for this spin-label in the presence of paramagnetic ions, noted in Table I, footnote *h*).

For the phosphatidylcholines with the doxyl spin-labels at different positions in the *sn*-2 stearoyl moiety, the limiting I/I_0 ratios, obtained by titration with Mn^{2+}, are plotted as a function of distance of the spin-label from the surface of the bilayer (Figure 6). The distances of the spin-labels from the phosphate on the lecithin, shown on the abscissa, were determined from a combination of X-ray diffraction (Pearson & Pascher, 1979; Hauser et al., 1981) and NMR (Seelig & Seelig, 1980) data (see Table I, footnote *f*). The limiting residual amplitude for the *n*-doxyl-PC labels increases monotonically with *n*, which is proportional to distance from the phosphate moiety, except that the I_{lim}/I_0 value for 16-doxyl-PC lies below the extrapolated linear fit of the 5-, 7-, 10-, and 12-doxyl-PC calibration points (Figure 6, dashed line in upper curve). Also plotted in Figure 6 is the reciprocal of the dipolar interaction coefficient to the one-sixth power, a parameter which is proportional to distance (eq 1); $(1/C)^{1/6}$ was calculated from I_{lim}/I_0 and line width according to Leigh (1970). When differences in line width (which affects the *apparent* reduction in amplitude of the EPR spectrum) are taken into account by calculation of the dipolar interaction coefficient *C*, a linear plot of $(1/C)^{1/6}$ (Figure 6, lower curve) with distance is obtained.

Distance Estimate for the Spin-Label Bound to the Reactive Sulfhydryl of D-*β-Hydroxybutyrate Dehydrogenase in MPL.* The EPR spectrum of the MSL–enzyme–MPL complex is shown in Figure 7. It consists predominantly of strongly immobilized spin-label (the broad line designated s) and a small fraction of weakly immobilized spin-label (w). The w signal was not removed by dialysis, which suggests that labels in the w environment are covalently bound, but not firmly constrained, by linkage to the protein. The relative amounts of label in s and w environments were estimated from the relative areas in the computed integral display. The amount of the weakly immobilized signal was less than 5%.

The spin-labeled enzyme was titrated with buffered $MnCl_2$ stock solution to a final $[Mn^{2+}]$ of 6.8 mM. The Mn^{2+} and nitroxide spectra overlap (Figure 8A) so that the residual amplitude of the nitroxide spectrum was obtained after subtraction of the intense broad Mn^{2+} spectrum (see Figure 8B,C). The relative amplitudes of s and w lines are plotted as a function of $[Mn^{2+}]$ in Figure 5A. Although the residual amplitude is different for the slow and fast components (I_{lim}/I_0 = 0.64 and 0.45 for s and w, respectively), the dipolar interaction coefficient, *C*, was calculated to be the same, 1.9 ± 0.1 G, for both components. Using this value for the dipolar interaction coefficient, and the published value of $T_{1eMn} = 1.5 \times 10^{-9}$ s (Cohn et al., 1971), we calculated the radial distance

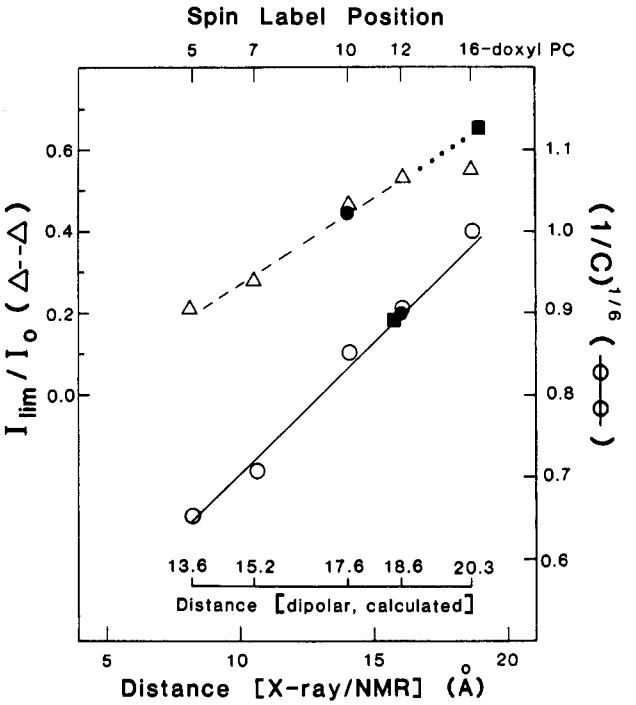

FIGURE 6: Calibration of transbilayer distance for spin-labeled doxyl-PCs and its use to localize the active center of D-β-hydroxybutyrate dehydrogenase. The limiting residual EPR amplitude, I_{lim}/I_0, of the spin-labeled phospholipids, obtained by titration with Mn^{2+} (see Figure 5), is plotted with respect to distance of the spin-label from the phosphate on lecithin (Δ). From the I_{lim}/I_0 value together with the measured initial line widths (Table I), the dipolar interaction coefficient, C, was obtained as described under Experimental Procedures. The inverse sixth root of C, proportional to the radial distance of closest approach of the nitroxide and paramagnetic ion, is shown for each of the spin-labeled phospholipids (O). The distances indicated on the abscissa were obtained from X-ray diffraction and NMR data for phosphatidylcholine (see Table I, "X-ray/NMR" column). On the inset abscissa are indicated the radial distances (r) calculated, by using eq 1, from the dipolar interaction coefficient (C) determined for the n-doxyl-PC labels. The difference in the two distance scales is mainly attributable to the parallax of the DPG with respect to the phosphate of the doxyl-PC; thus, they become more similar with increasing distance (see Table I, footnote g). Data for MSL–enzyme–MPL are indicated by closed symbols on each curve; squares (■) denote the strongly immobilized component (s), and circles (●) denote the weakly immobilized component (w) (see Figures 5 and 7). The I_{lim}/I_0 values for MSL–enzyme–MPL are superimposed on the least-squares fit of the data for 5-, 7-, 10-, and 12-doxyl-PC (dashed line). The $(1/C)^{1/6}$ values for MSL–enzyme are superimposed upon the least-squares fit of data from all five n-doxyl-PC spin-labels (solid line).

of closest approach of the Mn^{2+} and nitroxide to be 18.6 ± 0.4 Å (see Table I).

Spin-Labeled Enzyme Amplitude Reduction by Gd^{3+}. A calibration system using Gd^{3+} as relaxer ion was also constructed and gave results complementary to those obtained with Mn^{2+}. The I/I_0 vs. [Gd^{3+}] titration plots for the polar, 7-doxyl-PC, and 12-doxyl-PC spin-labeled phospholipids are shown in Figure 5B. The corresponding titration plot for Gd^{3+} added to MSL–enzyme–MPL is also shown (solid symbols) superimposed onto the titration curves for lipid spin-labels. The residual I_{lim}/I_0 values for MSL–enzyme is higher (0.48) than for the nearest 12-doxyl-PC phospholipid label (0.42) used for calibration. At the end of the titration with Gd^{3+}, the addition of EDTA restored the amplitude of the EPR spectrum to ~95% of its original value, indicating that metal ions, bound to the surface of membrane, were responsible for the observed amplitude reduction. The dipolar interaction coefficient for MSL–enzyme is 3.1 G, and taking T_{1eGd} to be 5.3×10^{-10} s (Dwek et al., 1975), a calculated radial distance

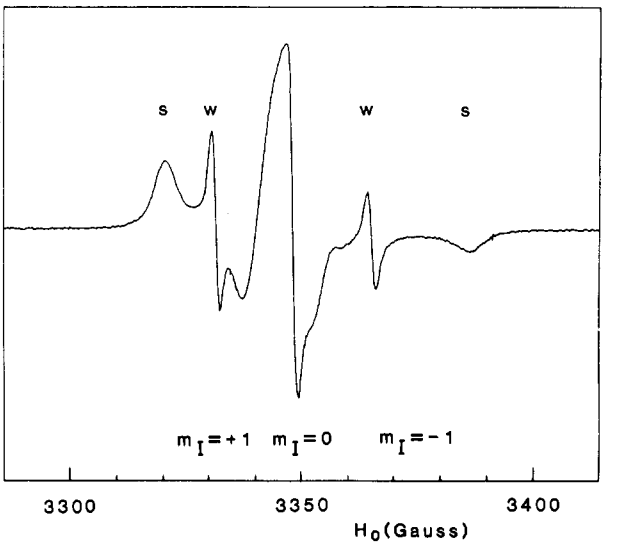

FIGURE 7: EPR spectrum of maleimide-spin-labeled D-β-hydroxybutyrate dehydrogenase inserted into MPL vesicles (MSL–enzyme–MPL). The MSL–enzyme–MPL was prepared at ~12 mg/mL (340 μM enzyme monomer with 100 mol of phospholipid/mol of enzyme monomer) in 10 mM Pipes, 10 mM Hepes, 1 mM EDTA, and 50 mM NaCl, pH 7.0, and the spectrum was recorded under the same conditions as in Figure 2, except at 20 °C. Low- and high-field hyperfine components of the EPR spectrum of strongly and weakly immobilized spin-label are designated s and w. Integration of this spectrum (not shown) indicated that the weakly immobilized spin-label corresponded to 5% or less of the total integrated signal amplitude. The strongly (s) and weakly (w) immobilized labels are clearly resolved for the $m_I = +1$ and -1 (low- and high-field) nuclear spin components, whereas these signals overlap in the center of the spectrum.

of 16 Å is obtained (see Table I).

Discussion

This study is concerned with the orientation of D-β-hydroxybutyrate dehydrogenase in the membrane. The distance of the reactive sulfhydryl, near the active center of the enzyme, to the membrane surface was estimated by the spin-label–spin-probe method. Several technical achievements were required for this study: (1) unidirectional insertion of the purified enzyme into the phospholipid bilayer (McIntyre et al., 1979); (2) unique-site derivatization of the reactive sulfhydryl of D-β-hydroxybutyrate dehydrogenase with a spin-label, which was achieved by using conditions which give derivatization of 0.5 equiv (i.e., two per tetramer) (McIntyre et al., 1984a); (3) synthesis of a series of spin-labeled phosphatidylcholines with the doxyl label placed at specific positions in the *sn*-2-stearoyl moiety (Eibl et al., 1983). This series provided a molecular ruler for calibration of distance in the transverse plane of the bilayer. Acidic phospholipids were required to increase the residence time of the paramagnetic ions on the bilayer surface to optimize the dipolar interaction. The spin-labeled phosphatidylcholines provided a useful empirical calibration to demonstrate the validity of the approach which yielded distance values comparable with those obtained by independent methods. With these conditions established, the dipolar interaction between the paramagnetic ion, Mn^{2+} or Gd^{3+}, and the spin-label on the enzyme, inserted into bilayer vesicles of mitochondrial phospholipids (MSL–enzyme–MPL), was measured. The dipolar interaction coefficients (C) were calculated from the reduction in amplitude and the line width of the EPR spectrum so that the radial distance of the nitroxide group of the spin-label on the enzyme from the membrane surface could be obtained. The Mn^{2+} and Gd^{3+} spin probes, which have different charge and magnetic properties, gave

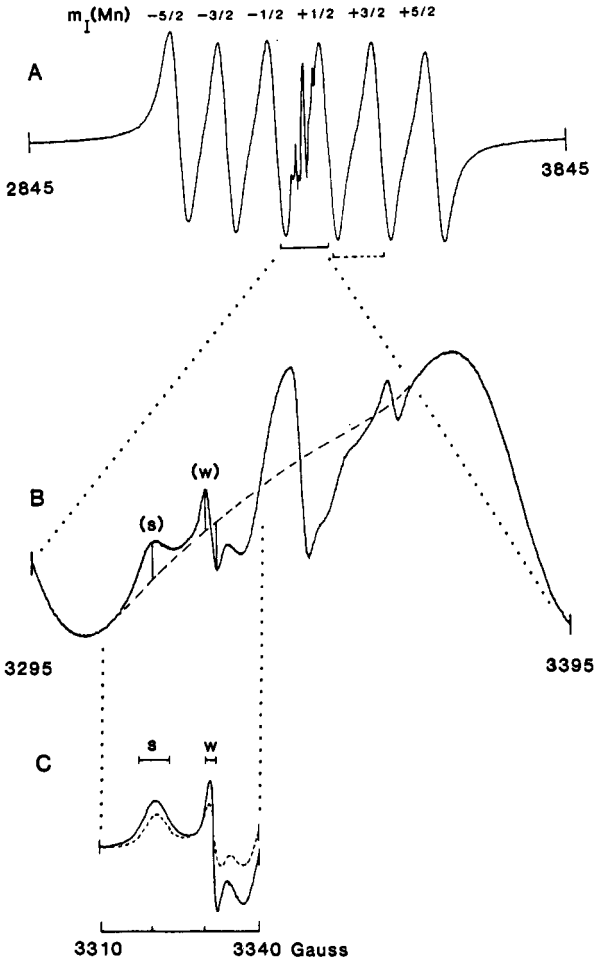

FIGURE 8: EPR spectrum of MSL–enzyme–MPL in the presence of $MnCl_2$, EPR spectra, recorded (see Figure 2) with a field sweep of 1000 G (A) or 100 G (B), are shown for MSL–enzyme–MPL in the presence of 3 mM $MnCl_2$. The spectrum (A) is dominated by the six-line Mn^{2+} component with the nitroxide spectrum overlapping in the $-^1/_2$ to $+^1/_2$ region (solid bar). The expanded region (B) of this spectrum depicts the base line [dashed line obtained from a scan of the Mn^{2+} spectrum in the $+^1/_2$ to $+^3/_2$ region, indicated by the dashed bar in (A)] which was used to determine the residual amplitude of the nitroxide $m_I = +1$ resonance for both the strongly immobilized (s) and weakly immobilized (w) components. The measured residual amplitudes as a function of $MnCl_2$ concentration and expressed relative to the initial amplitude in the absence of paramagnetic ions are shown in Figure 5A. The low-field ($m_I = +1$) portion of the EPR spectrum of MSL–enzyme–MPL in the absence (solid line) and presence of 6 mM Mn^{2+} (dashed line) (after subtraction of the Mn^{2+} spectrum) is shown in (C). The line widths of the s and w spectral components are 5.7 and 2.05 G, respectively. Spectra were recorded at a number of receiver gains so that the residual amplitude of the nitroxide spectrum could be more accurately quantitated. Limiting residual amplitudes for the s and w components are 0.66 and 0.47, respectively (see Figure 5 and Table I).

comparable values for this distance, i.e., 18 and 16 Å for manganese(II) and gadolinium(III), respectively. These values are the same within experimental uncertainty (see below) and were consistent with distances estimated by comparison with results obtained for the depth of immersion of selectively located doxyl spin-labels in the phospholipid bilayer.

A key aspect of our study is that the *n*-doxyl phospholipids provide an empirical calibration that gives more confidence in the calculated distance obtained for the MSL–enzyme. First, we found that titration with paramagnetic ions resulted in an amplitude reduction of the EPR spectra of *n*-doxyl phospholipids that was directly related to the predicted average distance of the nitroxide from the surface of the bilayer (with the exception of the 16-doxyl-PC). When the difference in line width for 16-doxyl-PC was taken into consideration by utilizing the formalism developed by Leigh (1970) to calculate the dipolar interaction coefficient, *C*, we found a direct relationship with distance for all the *n*-doxyl phospholipids (Figure 6). These results therefore provide an empirical calibration for distance using this methodology. Second, when this same theory was used to treat the data for the *n*-doxyl phospholipids, the calculated distances were comparable to those predicted from independent X-ray and NMR data, taking into consideration that the predominant effect of the paramagnetic ions is from ions bound to DPG rather than to PC. Third, the amplitude reduction obtained by titrating the spin-labeled enzyme with Mn^{2+} or Gd^{3+} gave comparable values for distance utilizing the theory of Leigh (1970). Lastly, we obtained the same values for the dipolar interaction coefficient and thereby distance for the immobilized and more mobile spectral components for spin-label on the enzyme only when the initial line width of the two components was taken into account utilizing Leigh theory. The results of our studies are therefore consistent with the predictions based on the formalism developed by Leigh (1970).

The theory that we utilized was developed for a static model in which the geometry of the spin probe (relaxer) and that of the spin-label were taken to be constant in time (Leigh, 1970). In the studies described here, the spin-label (on either the phospholipid or the enzyme) and spin probe were not fixed with respect to each other, yet we find that, within the limits of error of the experiments, the formalism developed by Leigh provides a good approximation of distances for the *n*-doxyl-PCs. The absence of effects of motion of either the paramagnetic ion or the spin-label on the dipolar interaction (and thereby measured distances) can be understood by considering the motional processes in the experimental system. We find that binding of the paramagnetic ion to the membrane surface is necessary since distances larger than expected were obtained in the absence of negatively charged lipids (see Figure 4 and Table I, footnote *i*). Further, the amplitude of the EPR spectra is restored by the addition of excess EDTA which chelated the paramagnetic ion so that the ion–EDTA complex diffused freely in the bulk solution. This confirms that binding of the metal ion to the membrane surface is necessary for effective dipole–dipole interaction and resultant reduction in amplitude of the EPR spectra. Such binding to the membrane surface enhances the dipolar interaction probably by reducing the rate of rotation of the paramagnetic ion and increasing its spin–lattice relaxation time (Bloembergen, 1956; Cohn et al., 1971). It appears that the paramagnetic ions, bound to the surface, are effectively "pseudostatic" and dominate the dipolar interaction with the nitroxide spin-label so that contributions of freely diffusing ions (Weinstein et al., 1980) need not be considered. This interpretation is consistent with the theoretical treatment developed by Hyde and Rao (1978) which stated that, for a system where all radial separations are allowed, the interaction is strongly dependent on the distance of closest approach between free radical and metal ion. In our studies, if more than one paramagnetic ion contributes to the amplitude reduction of the EPR spectrum, the calculated distances would be an underestimate of the true distance of closest approach. However, for the spin-labeled phospholipids in MPL, paramagnetic ion bound to DPG dominates the amplitude reduction of the EPR spectra. Since DPG constitutes ~20% of the phospholipid surface area (20% of the lipid phosphorus), for hexagonal packing, statistically each spin-labeled phospholipid would be adjacent to 1.2 DPG molecules so that dipolar interaction from multiple ions is not considered

to be a major factor in the distance determination. Paramagnetic ions bound to lipids further removed can be neglected since the dipolar interaction coefficient C is proportional to $1/r^6$ (eq 1).

Limited motion of the spin-label did not appear to significantly influence the dipolar interaction, since the rotational correlation times for the labels used in these studies were equal to or greater than the spin–lattice relaxation times of the paramagnetic ions. In the spin-labeled phospholipid calibration series used here, the influence of spin-label motion upon dipolar interaction can be evaluated. As distance from the glycerol moiety increases in the *n*-doxyl-PC series, there is increasing disorder accompanied by an increase in chain flexibility characterized both by EPR (Taylor & Smith, 1980; Kar et al., 1985) and by NMR methods (Seelig & Seelig, 1980; Brown & Williams, 1985). Fast and slow motional processes of the acyl chains of the phospholipid have been reported to be resolved by multifrequency NMR studies of spin–lattice relaxation times (Brown, 1984a,b; Brown & Williams, 1985). "Fast" motions [such as local twisting of a methylene segment (libration) and trans–gauche interconversions] appear to be approximately equivalent at progressive segments along the chain. These types of motion do not change appreciably the orientation of the backbone with respect to the bilayer (i.e., it remains parallel to the transverse plane of the bilayer). By contrast, "slow" motions (such as collective tilting of domains of alkyl chains in a nematic phase) increase with progression toward the center of the bilayer (Brown, 1984a,b). For the *n*-doxyl phospholipids used in our studies, the unique axes of the magnetic tensor system (g_{zz}, A_{zz}) are aligned with an average position parallel to the transverse plane of the bilayer so that the slow rather than fast motional processes [as defined by Brown and Williams (1985)] would vary the orientation of the nitroxide with respect to the plane of the membrane. These slow motional processes are slow on the NMR time scale and are therefore effectively static with respect to even the longest relaxation time in the EPR studies reported here. Thus, motional processes and resulting changes in orientation of the nitroxide would not be expected to be significant in our studies. We find that, despite the increased disorder and chain flexibility for the 12- and 16-doxyl-PC labels, the dipolar interaction coefficient decreased (see Table I) in accordance with depth of immersion of the label in the phospholipid bilayer; that is, $(1/C)^{1/6}$ was directly proportional to distance (see Figure 6), and calculated distances for the *n*-doxyl phospholipids were consistent with distances determined independently from structural studies of PC (Table I, "X-ray/NMR" and "X-ray" columns). Further, comparable values for distance were obtained for the strongly and weakly immobilized spin-label attached to the enzyme (see Table I). Thus, the motion of the nitroxide label per se appears not to adversely affect distance estimates.

The largest uncertainty in the calculation of distance (see eq 1) is the value taken for T_{1eR}, the spin–lattice relaxation time of the paramagnetic ion (Cohn et al., 1971; Azzi et al., 1973; Dwek et al., 1975), which might vary by a factor of 3 between solution and solid-state values (Bloembergen, 1956). However, since the calculated distance is proportional to the sixth root of T_{1eR}, this large uncertainty in T_{1eR} contributes an error of only ±1.5 Å in our studies. The distances obtained by using Mn^{2+} and Gd^{3+} (18 and 16 Å, respectively) are the same within this experimental uncertainty.

Model for the Orientation of D-β-*Hydroxybutyrate Dehydrogenase in the Membrane.* A model is presented for the orientation of D-β-hydroxybutyrate dehydrogenase in the transverse plane of the membrane based on the radial distance of the nitroxide moiety on the enzyme from the membrane surface. The distance of closest approach of the paramagnetic ions to the nitroxide is 17 Å (average of Mn^{2+} and Gd^{3+} data). The data indicate that the nitroxide is located 17 Å from the surface of the membrane. This is based on the assumption that the enzyme does not have a high-affinity site that can bind either Mn^{2+} or Gd^{3+}. The data are consistent with the absence of such a high-affinity binding site on the enzyme. If ion binding site(s) on the protein were a major contributor to the observed amplitude reduction of the EPR spectrum, such sites would require a higher affinity than that of the lipid binding site(s) since, in the reconstituted enzyme–MPL complex, the DPG is present at 20 mol/mol of enzyme monomer. The results show that the amplitude reduction of the EPR spectrum of MSL–enzyme–MPL occurs in a concentration range comparable to that for the spin-labeled lipids (Figure 5); this is consistent with the absence of a high-affinity binding site for paramagnetic ion which would be predicted to saturate at lower concentration of probe. By contrast with the spin-labeled phospholipids (see above), it is possible that more than one paramagnetic ion bound to the surface of the bilayer could contribute to the dipolar relaxation of the spin-label on D-β-hydroxybutyrate dehydrogenase especially if the label is located in the center of the tetramer so that the polar moieties of lipids in the boundary layer would be equidistant. If, for each nitroxide, more than one ion contributes to the dipolar relaxation, the amplitude reduction would be larger than that obtained with a single paramagnetic probe, and the calculated distance of closest approach (~17 Å) would be an underestimate (by ~2 Å if two paramagnetic ions were equidistant).

The known distance of the nitrogen of the maleimide spin-label to the sulfur at the site of addition is ~8 Å (Figure 9B) (Lajzerowicz-Bonneateau, 1968, 1976). With the nitroxide 17 Å from the membrane surface, the reactive sulfhydryl moiety could be located between 9 and 25 Å from the surface, depending upon the angle at which the MSL molecule is positioned with respect to the transverse plane of the bilayer (Figure 10). Location of the nitroxide above the membrane surface is unlikely since the reconstituted enzyme is relatively inaccessible to proteolysis (Berrez et al., 1984; Maurer et al., 1985), indicating that the enzyme is largely immersed within the phospholipid bilayer. The orientation of the nitroxide with respect to the transverse plane has previously been evaluated by simulation of the saturation-transfer EPR spectra of [^{15}N]MSL–enzyme in MPL vesicles (McIntyre et al., 1984b) using an anisotropic diffusion model (Robinson & Dalton, 1980). Best fit of calculated and experimental spectra was achieved when the angle, θ, between the major elements of the magnetic and diffusion tensor axes (A_{zz} and D_{zz}) (Figure 9A) was taken to be 90°; i.e., the magnetic tensor (A_{zz}) of MSL on the reactive sulfhydryl of D-β-hydroxybutyrate dehydrogenase is orthogonal to the major diffusion axis (D_{zz}) (McIntyre et al., 1984b). Three possible orientations of MSL with respect to the enzyme in the bilayer can satisfy orthogonality of the magnetic and diffusion tensors (Figure 10). The line shapes of the saturation-transfer EPR spectra (McIntyre et al., 1984b), analyzed according to the parameters given by Beth et al. (1983), are more consistent with orientations A and B (Figure 10). Although other orientations of the spin-label cannot as yet be excluded, our best interpretation is that the long axis of the MSL is oriented parallel to the major diffusion axis, with the sulfur link to MSL located either above or below the nitroxide, i.e., either 9 Å (Figure 10A) or 25 Å (Figure 10B) from the bilayer surface, respectively.

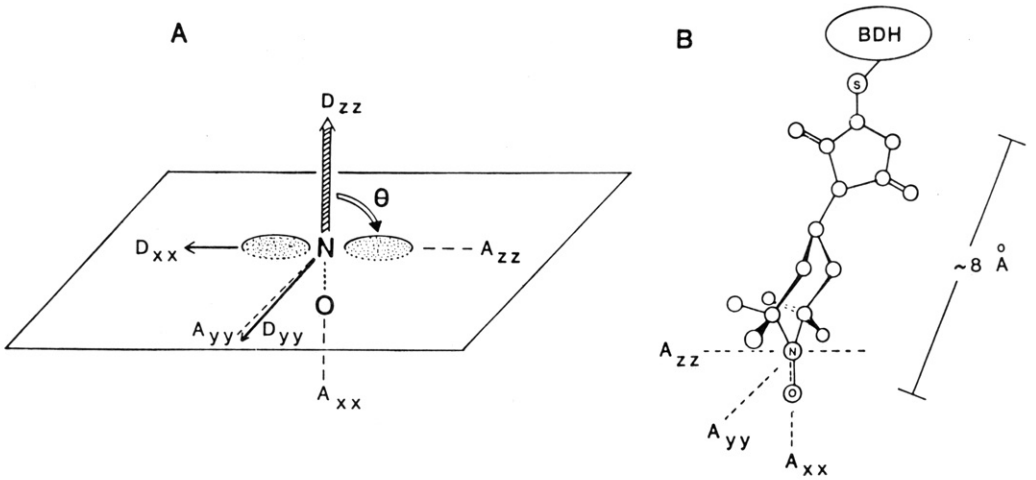

FIGURE 9: (A) Relationship between coordinate systems for the MSL magnetic tensors (A, g) and the D-β-hydroxybutyrate dehydrogenase tetramer diffusion tensor (D) is shown. The unpaired electron of the nitroxide radical is localized in a p–π molecular orbital shared by N and O; for purposes of indicating direction of the largest hyperfine tensor element, A_{zz}, the electron is shown as residing in a nitrogen p_z orbital. The g and A spin-label molecular tensor systems are assumed coincident, as are the diffusion and inertial tensor systems for enzyme tetramer in MPL. The two coordinate systems are related by θ; in this diagram, θ is shown as generated by a rotation about the coincident axes D_{yy} and A_{yy} (Robinson & Dalton, 1980). (B) Structure of the piperidine ring conformation of MSL attached to BDH. The molecular magnetic tensor coordinate system is represented in the same orientation as that shown in Figure 11. A_{zz} is parallel to the nitrogen p_z orbital, A_{xx} is parallel to the N–O bond direction, and A_{yy} is orthogonal to A_{zz} and A_{xx}. In this orientation, A_{zz} is orthogonal to D_{zz}, the major axis of the diffusion tensor (see Figure 11). The distance from the nitroxide N to the S of the sulfhydryl is 8 Å, an estimate based on data reported by Lazjerowicz-Bonneteau (1976). Magnitudes of the hyperfine tensor elements, A_{xx}, A_{yy}, and A_{zz}, are 7.9, 7.7, and 34.8 G, respectively (Beth et al., 1983).

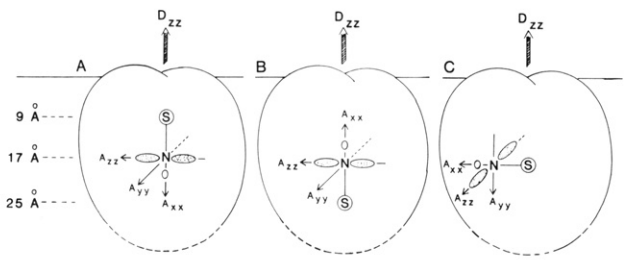

FIGURE 10: Three possible orientations of the maleimide nitroxide with respect to the diffusion tensor of the D-β-hydroxybutyrate dehydrogenase (BDH) tetramer, shown in cross-section in the phospholipid bilayer. The orientation of BDH in MPL is shown with respect to the transverse plane of the phospholipid bilayer. The thickness of the bilayer (the separation of phosphorus to phosphorus) is taken to be ~40 Å. BDH is shown inserted unidirectionally into the outer face of the phospholipid vesicle. The nitroxide moiety of the maleimide spin-label was found to be 17 Å from the aqueous domain (see Figure 6 and Table I). The radial length of MSL which connects from the proximal site of sulfhydryl (Ⓢ) across the maleimide double bond to the distal nitroxide nitrogen is approximately 8 Å (Figure 9). The nitroxide nitrogen p_z orbital (A_{zz}) is shown orthogonal to the axis of rapid diffusion, D_{zz}, so that the sulfhydryl site of derivatization can be located at either 17 – 8 Å or 17 + 8 Å, i.e., 9 or 25 Å, from the bilayer surface as shown in (A) and (B), respectively. In a third orientation of maleimide spin-label (Figure 10C), the orthogonality of A_{zz} and D_{zz} is maintained only when the maleimide spin-label is rigidly fixed so that it cannot librate about its long axis.

A diagram showing key features of the orientation of D-β-hydroxybutyrate dehydrogenase in the membrane is presented in Figure 11. This model is based on the radial distance determined in this study as well as several additional lines of evidence. From this EPR study, the distance of the nitroxide moiety of MSL–enzyme from the membrane surface was found to be about 17 Å. The enzyme is an amphipathic protein which is inserted unidirectionally into the bilayer (McIntyre et al., 1979) but does not traverse the bilayer to the polar region at the inner face as shown by proton NMR studies (Deese et al., 1986). Yet, the enzyme in the membrane is protected from proteolysis as compared with the apoenzyme in aqueous buffer (Berrez et al., 1984; Maurer et al., 1985), suggesting that the protein is immersed to an appreciable extent within the bilayer. The organization of the enzyme in the bilayer (MPL) is tetrameric (McIntyre et al., 1983). The spin-label appears to be within the bilayer since the enzyme is largely immersed within the phospholipid bilayer and the mechanism of reduction of nitroxide signal amplitude is dominated by dipolar interaction; i.e., the nitroxide label is inaccessible to the paramagnetic ion probe.

The reactive sulfhydryl of D-β-hydroxybutyrate dehydrogenase is in the vicinity of the nucleotide binding site since derivatization of the sulfhydryl modifies the NADH binding characteristics (Latruffe et al., 1980; McIntyre et al., 1984a; Fleer et al., 1984) and the rate of derivatization of the reactive sulfhydryl by *N*-ethylmaleimide is reduced in the presence of nucleotide (McIntyre et al., 1984a). The binding site for cofactor NAD(H) should be readily accessible from the aqueous phase to facilitate efficient catalysis and rapid turnover rate. Therefore, the reactive sulfhydryl is near the nucleotide binding site, and the distance closer to the bilayer surface is the more likely; i.e., the sulfhydryl is immersed about 9–10 Å below the membrane surface, as shown in Figures 10A and 11, rather than at 25 Å as in Figure 10B. The reactive sulfhydryl appears to be immersed within the bilayer to a depth equivalent to carbons 5 or 6 of the *sn*-2 fatty acyl chain of phospholipid. This is equivalent to the level of carbons 3 or 4 of the *sn*-1 fatty acyl moiety.

There are two high-affinity NAD(H) binding sites per enzyme tetramer. The phosphatidylcholine is required for proper binding of the coenzyme (Gazzotti et al., 1974). The association of the phosphatidylcholine with the enzyme would have to be at the enzyme–phospholipid interface. The width of NAD(H) in the anti conformation is about 15 Å (Rossman et al., 1975) (see Figure 11). The pyrophosphate is depicted in a position oriented close to the protein–water interface with the nicotinamide and adenine rings of the dinucleotide extending 7–8 Å deeper into the nucleotide binding domain of the protein. The reactive sulfhydryl is shown in close proximity

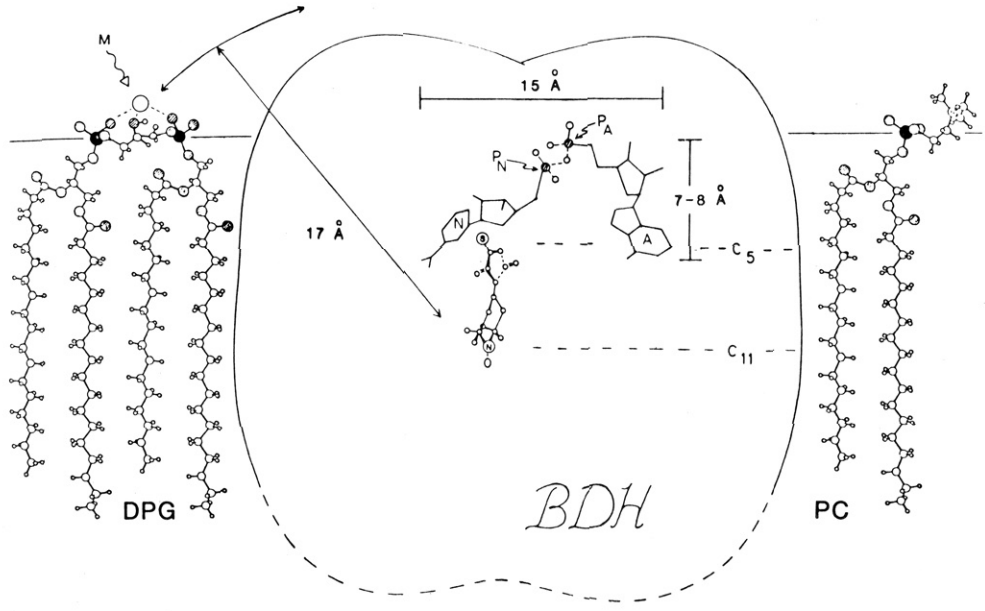

FIGURE 11: Diagrammatic representation of D-β-hydroxybutyrate dehydrogenase (BDH) in the MPL bilayer. The enzyme is depicted as inserted into the outer face. The extent of immersion of the enzyme into the bilayer is uncertain, as depicted by the dashed line at the bottom of the enzyme structure. Two of the four subunits of the tetramer are shown in cross section. The level of the phosphate (●) of PC (or DPG) is defined as the reference point for the membrane surface (solid line). The nitroxide moiety of the spin-label is immersed 17 Å (radial or vectorial distance from paramagnetic ion binding site on DPG) within the membrane, comparable to carbon 11 of the *sn*-2 fatty acyl chain of PC (Figure 6 and Table I). The maleimide spin-label is attached to the enzyme at the sulfur bridge, with the distal piperidine ring containing the nitroxide moiety oriented parallel to the transverse plane of the bilayer. The sulfhydryl is depicted 9 Å (see Figure 10A) from the membrane surface, a distance equivalent to carbon 5 of the *sn*-2 fatty acyl chain of PC (equivalent to carbon 3 of the *sn*-1 fatty acyl chain). The direction of the major axis of the diffusion tensor (D_{zz}) is assumed to be perpendicular to the membrane surface. An interpretation of the spatial arrangement of the NAD(H) and the location of the sulfhydryl with respect to depth in the bilayer is provided. The nicotinamide (N) and adenine (A) moieties of NAD(H), in the anti conformation, are separated by ~15 Å (Rossman et al., 1975) and immersed ~7–8 Å below the pyrophosphate.

to the nicotinamide and 9 Å from the surface. Thus, a crevice at the surface of the enzyme (protein–water interface) would provide facile access for NAD(H) to its binding site as already observed for other dehydrogenases (Rossman et al., 1975).

Comparison of This Study with Previous Distance Measurements in Enzymes. The methodology to measure distances by determining dipolar interaction coefficients was first developed by Leigh, Cohn, and their co-workers (Leigh, 1970; Cohn et al., 1971; Taylor et al., 1969) for the study of immobilized nitroxides and Mn–nucleotide complexes bound to the soluble enzyme creatine kinase. The methodology has since been applied to the determination of separation of sites on several soluble metalloenzymes and enzymes with nucleotide cofactors (Eaton & Eaton, 1978) and to selectively labeled ATP synthetase in the mitochondrial membrane (Azzi et al., 1973). The feasibility of applying the spin-probe–spin-label method to phospholipid bilayer systems using *n*-doxylstearate methyl ester spin-labels was demonstrated by Hyde et al. (1978), who found the correct ordering of I_{lim}/I_0 values for probes at different distances from the carboxyl of the stearates. Herz et al. (1983) also used an *n*-doxylstearic acid system for qualitative comparison of residual amplitude vs. position of the spin-label from the carboxylic acid moiety. For a spin-label on bacteriorhodopsin, Herz et al. (1983) found a greater residual amplitude in the presence of Cu^{2+} as compared with that for the 16-doxylstearic acid. On the basis of this observation, it was concluded that the buried carboxyl groups of the protein, to which the label was attached, were located at a distance from the membrane surface greater than that of the 16-doxyl moiety. This distance estimate is of limited accuracy, since the line width, an important factor which influences the amplitude reduction by dipolar relaxation (Leigh, 1970), was not taken into account by Herz et al. (1983). In our studies with D-β-hydroxybutyrate dehydrogenase, the I_{lim}/I_0 value, taken alone, would suggest that the MSL label on the reactive sulfhydryl was located at a distance from the membrane surface greater than that of the label on 16-doxyl-PC. However, when the line width is taken into account using the formalism of Leigh (1970), the distance to the nitroxide was found to be equivalent to carbon 11 of the *sn*-2 chain.

Spin-labels or paramagnetic ions have been used to measure distance in combination with proton NMR (Krugh, 1976). In such studies, the spin probe, in close proximity (up to 30 Å) to the proton, gives rise to line broadening. This method requires resolution and identification of the specific proton resonance together with detailed line-shape analysis. In one such study, an Fab fragment of a monoclonal antibody specifically directed against a nitroxide spin-label hapten yielded measured distances up to 15 Å as quantitated by paramagnetic broadening of specific tyrosine protons of the Fab fragment by the spin-labeled hapten (Anglister et al., 1984). In a second study, the active site of protein kinase was probed with inert cobalt(III) and chromium(III) complexes of AMPPCP. Intersubunit distances in the range of 5–10 Å were determined from the paramagnetic effects on the relaxation rates of specific protons of a peptide substrate (Granot et al., 1980). These types of proton NMR studies provide estimates of internuclear distance in a range comparable to that using the spin-label–spin-probe method.

The studies reported here address the localization of a specific site on a membrane protein using the spin-probe–spin-label method and differ from the previous studies of Hyde et al. (1978) and Herz et al. (1983) in several important respects. We have used a series of *phospholipid* spin-labels to demonstrate the relationship between residual amplitude of the EPR spectrum with distance from the surface of the bilayer. These phosphatidylcholine-type labels offer the advantage of being held firmly in vertical register with respect to the bilayer in contrast with fatty acids which appear to

exhibit marked vertical fluctuations as detected by electron–electron double-resonance (ELDOR) studies (Feix et al., 1984). Markedly less spin–spin interaction is detected when the spin-label is incorporated into a phospholipid (Seigneuret et al., 1981). In our studies using the *n*-doxyl-labeled phospholipids, measured distances correspond to the distances predicted from the structure of the phospholipid (Pearson & Pascher, 1979; Seelig & Seelig, 1980; Hauser et al., 1981). These results show that reliable distances can be determined by this approach, and we have applied the same methodology to study D-β-hydroxybutyrate dehydrogenase, where the MSL spin-label is located in a *unique site* on the protein. We find that the distance measured for the separation of the nitroxide-derivatized sulfhydryl on the enzyme from the aqueous milieu lies within the measured range covered by the phospholipid calibration system. In the studies of bacteriorhodopsin (Herz et al., 1983), although sensitivity of the measurements to distance was observed, unique site labeling of the protein was not demonstrated, and the amplitude reduction of the EPR spectrum was evaluated qualitatively so that only limited interpretation was possible. Preliminary data regarding the orientation of the spin-label in D-β-hydroxybutyrate dehydrogenase (McIntyre et al., 1984b), together with additional information about the active center of the enzyme (Dubois et al., 1986), indicate that the reactive sulfhydryl is near the active center of the enzyme. In previous studies of spin-labeled membrane proteins (Azzi et al., 1973; Herz et al., 1983), the size and orientation of the spin-label were not addressed.

The present studies provide further insight into the orientation of the membrane-inlaid enzyme, D-β-hydroxybutyrate dehydrogenase, with respect to the bilayer. We find that the reactive sulfhydryl, near the active center of the enzyme, is immersed within the bilayer. Our best estimate is 9 Å from the bilayer surface.

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Unfolding Pathway of Myoglobin: Effect of Denaturants on Solvent Accessibility to Tyrosyl Residues Detected by Second-Derivative Spectroscopy

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ABSTRACT: The effects of denaturants on the solvent accessibility to tyrosyl residues of apomyoglobin have been examined by means of second-derivative spectroscopy in the near-ultraviolet. Three apomyoglobins, i.e., sperm whale, horse, and tuna, were selected because of the different distribution of tyrosyl residues in their primary structure. The results are consistent with the occurrence of two independent consecutive events in the guanidine-induced denaturation pattern of apomyoglobin. The first event, which is responsible for the lack of the ability to bind the heme, has been proved to involve conformational changes in both the domains, i.e., segments 1–79 and 80–153, identified in the myoglobin molecule. However, the conformational changes are not of the same type. In fact, the solvent accessibility to tyrosine HC2 is increased probably because of a partial unfolding of the 80–153 domain. Conversely, the solvent accessibility to tyrosine B2 is decreased, thus indicating that a refolding occurs in some region of the N-terminal moiety (1–79 domain) of the molecule.

The absorption of tyrosyl residues in proteins is known to be largely masked by the stronger absorption of tryptophanyl residues (Wetlaufer, 1962). Althouth it has been proved that second-derivative spectra may contribute to the resolution of overlapping bands, the mutual interference between the two chromophores could not be eliminated at all by this technique (Balestrieri et al., 1978a, 1980). More recently, the overlap between the absorption bands of tyrosine and tryptophan has been utilized for detecting the degree of exposure of tyrosyl residues in proteins as well as for the simultaneous determination of the two residues (Ragone et al., 1984; Servillo et al., 1982).

The data reported in this paper show the effects of two denaturants, i.e., guanidine hydrochloride and acid, on the second-derivative spectra of three apomyoglobins, i.e., tuna, horse, and sperm whale, selected because of the different distribution of tyrosyl residues in their primary structure. This finding made it possible to analyze structural changes oc-

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