Specific Interaction of (*R*)-3-Hydroxybutyrate Dehydrogenase with Membrane Phosphatidylcholine As Studied by ESR Spectroscopy in Oriented Phospholipid Multibilayers: Coenzyme Binding Enhances the Interaction with Phosphatidylcholine†

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ABSTRACT: The interaction of phospholipid with (*R*)-3-hydroxybutyrate dehydrogenase, a phosphatidylcholine-requiring membrane enzyme, has been studied using ESR spectroscopy of spin-labeled lipids, both as ordered multibilayers and in lipid vesicle suspensions (liposomes). Partially oriented phospholipid multibilayers were prepared from lipid vesicles composed of a 1:1 mixture of phosphatidylcholine (PC) and phosphatidylethanolamine (PE). Vesicles containing (*R*)-3-hydroxybutyrate dehydrogenase yielded active preparations of the enzyme in such multibilayers. With increasing protein/lipid ratio, the order of the multibilayers was disrupted as monitored by ESR spectroscopy with a spin-labeled analogue of PC, 5-doxyl-PC (5 mol %, 10% of total PC) as a probe. The outer peak separation of 5-doxyl-PC varied with the lipid/protein ratio. The lower the ratio, the larger was the separation, with higher activity enzyme being more effective in exerting this effect. When 5-doxylstearic acid was substituted for 5-doxyl-PC or when the enzyme was inactive, the $2A_{zz}$ value stayed practically constant at its lower limit (about 54 G). Multilayers composed of 81% PE, 11% diphosphatidylglycerol (DPG), and 8% 5-doxyl-PC (no unlabeled PC present) gave similar results. With this lipid mixture, the maximal $2A_{zz}$ value (about 61 G) was reached at lower protein/lipid ratios, although the enzymic activity of (*R*)-3-hydroxybutyrate dehydrogenase is reduced to 40% in this system.The outer peak separation also depended on the presence of the coenzyme, NAD^+, and 2-methylmalonate. The latter enhances binding of NAD^+ about 100-fold by forming a ternary complex. With this ternary complex, the $2A_{zz}$ values were increased unless the maximal values had been reached already in the absence of coenzyme. In all these experiments only a single ESR spectral component was observed. Similar results were obtained for the enzyme in liposomes, although the effect was less pronounced apparently due to the higher mobility of the probe. It is concluded that PC is motionally restricted by (*R*)-3-hydroxybutyrate dehydrogenase and yet is in rapid exchange with the bulk lipid on the ESR time scale. PC is required for formation of tight and functional complexes with NAD [Rudy et al. (1989) *Biochemistry 28,* 5354−5366], and such complexes strengthen the interaction of the enzyme with PC.

(*R*)-3-Hydroxybutyrate dehydrogenase catalyzes the NAD-dependent interconversion of acetoacetate and (*R*)-3-hydroxybutyrate. The enzyme from mammalian sources is lipid-requiring, with an absolute specificity of phosphatidylcholine (PC)[1] for function (Jurtshuk et al., 1963; Fleischer et al., 1966). (*R*)-3-Hydroxybutyrate dehydrogenase, purified from beef heart mitochondria, is devoid of phospholipid and is referred to as apodehydrogenase. This apoenzyme is inactive but can be reactivated by forming an enzyme−phospholipid complex. Activation can be achieved with soluble PC below the critical micellar concentration or by reconstitution into phospholipid vesicles containing PC, in which it inserts spontaneously and unidirectionally (Fleischer et al., 1983; Maurer et al., 1985; McIntyre et al., 1979). We have shown previously that PC is required for tight and functional coenzyme binding; *i.e.*, binding of NAD in the absence of PC is weak and PC enhances the binding of NAD by about 50-fold (Rudy et al., 1989). In the studies reported here, ESR spectroscopy of spin-labeled PC has been used to investigate the interaction of PC with (*R*)-3-hydroxybutyrate dehydrogenase. Further, we have studied the effect on the enzyme−PC interaction of binding ligands to the catalytic site by forming a ternary complex with NAD^+ and a substrate analogue.

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[1] Abbreviations: PC, phosphatidylcholine (lecithin); PE, phosphatidylethanolamine; DPG, diphosphatidylglycerol; doxyl, 2,2-dimethyloxazolidinyl-1-oxy, a nitroxide substituent spiro-linked at positions 5, 7, 12, or 16 of stearic acid; doxyl-PC, 1-palmitoyl-2-doxylstearoyl-PC; EDTA, ethylenediaminetetraacetic acid.

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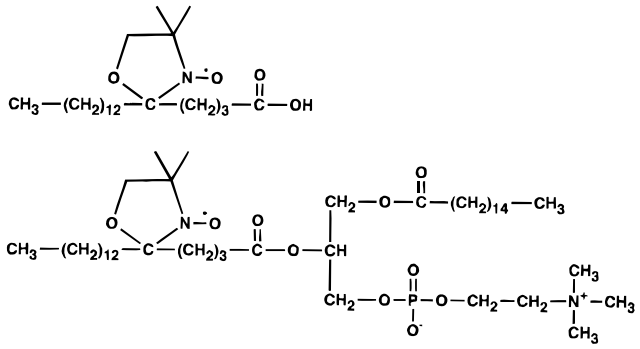

FIGURE 1: Structural formulas of 5-doxylstearic acid and 5-doxyl-PC.

EXPERIMENTAL PROCEDURES

Materials

Doxylstearic acids (Figure 1), 1,2-dioleoylphophatidylcholine, and 1,2-dioleoylphosphatidylethanolamine were purchased from Sigma Chemical Co., St. Louis, MO, and 1-palmitoyllysolecithin was from Serva, Heidelberg, Germany. Diphosphatidylglycerol (DPG) was either purified from bovine heart mitochondria (Fleischer et al., 1967) or purchased from Avanti Polar Lipids Inc., Alabaster, AL, as an isolate from bovine heart.

5-, 7-, 12-, and 16-doxyl-PC were synthesized by condensation of the corresponding doxylstearic acid and 1-palmitoyllysolecithin in the presence of dicyclohexylcarbodiimide and 4(dimethylamino)pyridine following a general and very mild esterification procedure of Neises and Steglich (1978), in order to minimize transesterification at positions 1 and 2 of the glycerol moiety. In earlier syntheses, carbonyldiimidazole had been utilized as condensing reagent (Boss et al., 1975). Purification was achieved by preparative thin layer chromatography on silica plates with chloroform/methanol/ammonia/water (50:50:4:1) as eluent.

Methods

Preparation of (*R*)*-3-Hydroxybutyrate Dehydrogenase and Phospholipid Vesicles.* (*R*)-3-Hydroxybutyrate dehydrogenase was purified to homogeneity from beef heart mitochondria (Bock & Fleischer, 1974; McIntyre et al., 1988) and was stored frozen in a liquid nitrogen refrigerator (1−2 mg/mL in 0.4 M LiBr, 5 mM Hepes (*N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid), and 5 mM dithiothreitol, pH 7.0). Phospholipid vesicles were prepared as described previously (Cortese et al., 1989).

Reconstitution of (R)-3-Hydroxybutyrate Dehydrogenase with Phospholipid Vesicles. The purified enzyme was reconstituted by incubation with phospholipid vesicles at the lipid/protein ratios indicated essentially as described previously (Rudy et al., 1989). Samples for ESR were prepared at 200 μg of P/mL in 10 mM Tris-HCl (tris(hydroxymethyl)-aminomethane hydrochloride), and 0.5 mM EDTA, pH 8.0.

Enzymic Activity. (*R*)-3-Hydroxybutyrate dehydrogenase activity was determined by reduction of NAD^+ with 20 mM (*R*,*S*)-3-hydroxybutyrate as described previously (McIntyre et al., 1983). For the enzyme reconstituted with PC/PE vesicles, the activity was about 90% of that with mitochondrial phospholipid (Gazotti et al., 1975; Sandermann et al., 1986). Vesicles with low PC content (8 mol %) give considerably lower reactivation (compare Tables 1 and 2; see also Gazotti et al., 1975).

Preparation of Ordered Phospholipid Multibilayers. Lipid vesicles or enzyme−lipid complexes (50 μl, 200 μg of P/mL) were applied in 4 μL portions onto the center of an acetone-washed glass coverslip (6 × 20 mm), drying each aliquot under a stream of argon gas. These were kept in a constant humidity chamber over a saturated solution of zinc sulfate (90% relative humidity) at 20 °C for 4−5 days. After this time, a second plate was carefully placed over the sample, and vertical finger pressure was applied in order to evenly distribute the lipid. Ordering of the multibilayers formed by this procedure was monitored under polarized light with a microscope.

ESR experiments were carried out with a Bruker ESP 300 E spectrometer operating in the X-band mode at 100 kHz modulation of 0.8−2.0 G peak-to-peak amplitude and an applied microwave power of 6.3 mW. Vesicle suspensions were measured in micro flat cells in a TE_{102} resonator equipped with a special cell holder to position the cell reproducibly within the cavity as described previously (Wenzel et al., 1976). Ordered multibilayers on glass plates were mounted in a tissue cell holder at a fixed orientation in a cylindrical resonator (Bruker, ER 4106 ZRC). H_0 and the modulation coils were rotated with respect to microwave cavity and spectra were recorded at two angles differing by 90°. One orientation, approximately perpendicular to the membrane surface (parallel to the long molecular axis of doxyl lipid), was selected to yield a maximum hyperfine splitting. Spectra of vesicle suspensions obtained in both cavities were indistinguishable.

RESULTS

(1) Influence of (R)-3-Hydroxybutyrate Dehydrogenase on Doxyl Lipids in Ordered Membranes. Lipid vesicles composed of PC/PE (1:1) and containing 5% 5-doxyl-PC were partially oriented into multibilayers as described in Methods. Figure 2 shows ESR spectra recorded with the magnetic field H_0 perpendicular (⊥, a) or parallel (||, b) with respect to the surface of the membrane. Both spectra contain a major component arising from spin packets with the *z* axis of the nitroxide radical (Griffith & Jost, 1976) either nearly parallel or perpendicular to the external field. The unpaired electron in nitroxide radicals is located mainly in the p_z orbital of the nitrogen atom, and the *z* axis is defined by the spatial orientation of this orbital, as shown in Figure 3. Hence, the hyperfine splitting is largest when the direction of the external field coincides with the *z* axis. Other orientations are represented as well, although to a substantially lower extent. Hence, the degree of order is rather high. Order parameters (*S*) as obtained by ESR spectroscopy are usually expressed as the ratio between the observed and the motionally averaged hyperfine tensors parallel and perpendicular to the external field according to the formula (Smith & Butler, 1976; Schreier-Muccillo et al., 1973):

$$S = (A'_{||} - A'_{\perp})/(A_{||} - A_{\perp}) \quad (1)$$

This value still depends on the overall mobility of the label and on the local polarity, *e.g.*, the hydration of the bilayer (Seelig, 1976). Moreover, even when the multibilayers were perfectly oriented, the spin label would still report a certain disorder as the lipid molecule rotates about its long molecular

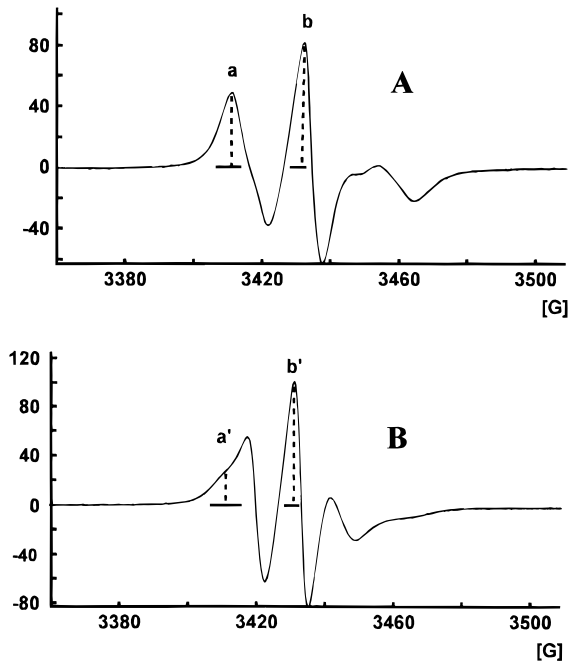

FIGURE 2: ESR spectra of ordered phospholipid multibilayers composed of PC/PE/5-doxyl-PC (1:1:0.1) recorded with H_0 either parallel (A) or perpendicular (B) to the long molecular axis of the lipid. The degree of order S^* as defined by the ratio difference $(a/b - a'/b')$ is 0.33.

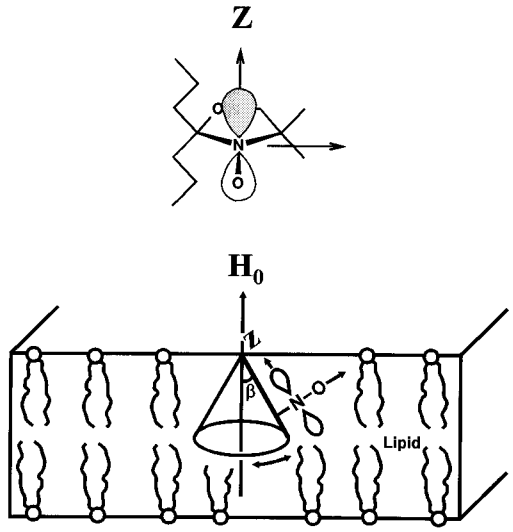

FIGURE 3: Structure of the doxyl group showing the orientation of the nitroxide *z* axis with respect to the hydrocarbon chain of lipid molecules (top). Illustration of the motional distribution of the nitroxide in doxyl lipid with respect to the surface of the membrane (bottom).

axis, resulting in a motion of the label on the surface of a cone with the angle β (Seelig, 1976; Figure 3). We therefore used a simplified expression (formula 2), as defined in Figure 2, which yields a relative value for apparent degree of ordering (S^*) that can be applied for comparison in a given system. At the maxima chosen for this calculation (center-

$$S^* = a/b - a'/b' \qquad (2)$$

field and low-field maximum of the component with H_0 parallel to the *z* axis), there is considerable spectral overlap of the parallel and perpendicular spectral components as well as contributions from randomly oriented labeled lipid present in a varying degree in preparations of different composition.

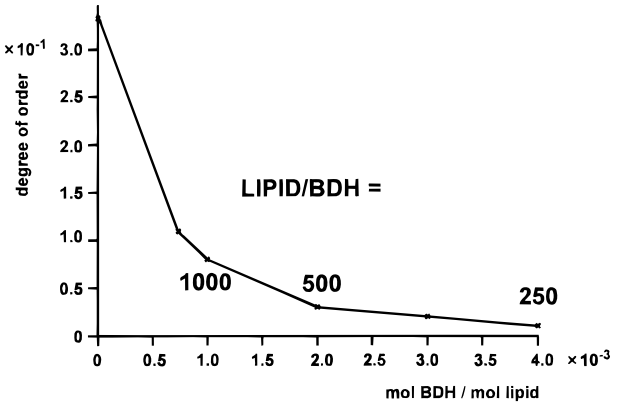

FIGURE 4: Effect of (*R*)-3-hydroxybutyrate dehydrogenase on the degree of order of oriented multibilayers composed of PC/PE/5-doxyl-PC (1:1:0.1) as determined by ESR spectroscopy (Figure 2).

Hence, according to this definition, S^* of 0.33 (see Figure 2) represents a high degree of ordering of the multibilayer.

Ordered multibilayers containing (*R*)-3-hydroxybutyrate dehydrogenase showed progressively less order with decreasing lipid/protein ratios (Figure 4). Spectra of doxyl-PC of samples prepared at less than 250 mol of lipid/mol of enzyme showed virtually no orientation-dependent ESR spectral lineshape. This effect, *i.e.*, total loss of order at lipid/protein ratios of 100, is corroborated by freeze−fracture electron micrographs taken of similar samples prepared without the spin label or when examined in the light microscope under polarized light (Mink, 1985; A. Saito, J. O. McIntyre, and W. E. Trommer, 1987, unpublished). However, for samples containing the enzyme, the ESR spectra of the doxyl-PC differed significantly in their $2A_{zz}$ values from those recorded in its absence. The maximal separation reached 62.0 G with H_0 parallel to z (perpendicular to the membrane surface) and, hence, exceeds the value of this component in the absence of protein (54.0 G) by 8 G (Figure 5).

The outer peak separation in the presence of (*R*)-3-hydroxybutyrate dehydrogenase varied both with the lipid/protein ratio as well as with the specific activity of the enzyme. The lower the lipid/protein ratio, the larger was the separation, with higher activity enzyme being more effective in exerting this effect (Table 1). Whereas enzyme with a specific activity of 75 U/mg reached the maximal separation of 60.5 G at 500 mol of lipid/mol of (*R*)-3-hydroxybutyrate dehydrogenase (Table 1, column 2), lower activity enzyme (40 U/mg) gave a maximal value of only 58.0 G even at 250 mol of lipid/mol of protein (Table 1, column 3). In an early experiment (Rudy, 1990) with an enzyme preparation exhibiting an activity of 95 U/mg, the $2A_{zz}$ value at 500 mol of lipid/mol of protein was the highest achieved, 62 G (Table 1, column 5). When 5-doxyl-stearic acid was substituted for 5-doxyl-PC, the $2A_{zz}$ value stayed practically constant (54.0−55.0 G, Table 1, column 4). In one experiment, an enzyme preparation that had become inactive was employed, and again, $2A_{zz}$ was unchanged.

5-Doxyl-PC activates (*R*)-3-hydroxybutyrate dehydrogenase almost as effectively as PC itself; *i.e.*, the NAD^+-dependent oxidation of (*R*,*S*)-3-hydroxybutyrate in vesicles composed of 5-doxyl-PC/PE is better than 90% that of dioleoyl-PC. However, a preferential interaction of either normal or spin-labeled PC with the enzyme cannot be excluded. Since ESR experiments are complicated at high spin label concentrations due to spin exchange processes

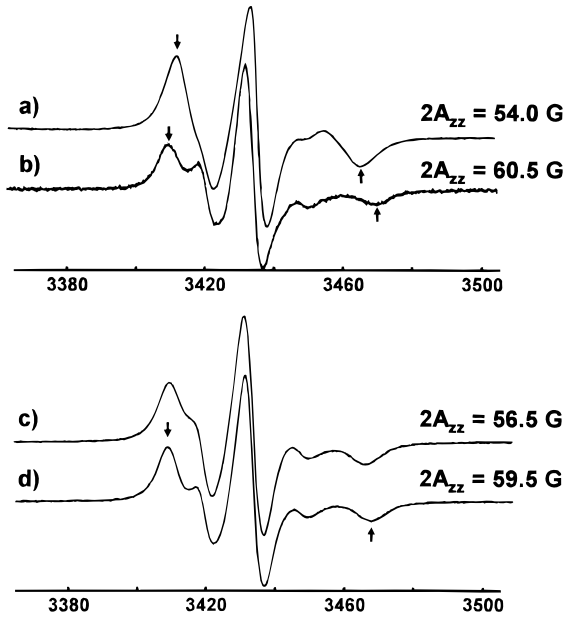

FIGURE 5: ESR spectra of 5-doxyl-PC in ordered multibilayers composed of PC/PE/5-doxyl-PC (1:1:0.1) recorded with H_0 perpendicular to the membrane. Samples were prepared with 0.33 μmol of lipid either alone (a) or in the presence of different amounts of (*R*)-3-hydroxybutyrate dehydrogenase (b: 500 mol of lipid/mol of enzyme; c and d: 1370 mol of lipid/mol of enzyme). For spectrum d, the sample was prepared as the ternary complex in the presence of 4 mM NAD^+ and 60 mM 2-methylmalonate. The $2A_{zz}$ values are summarized in Table 1.

(Pake & Tuttle, 1959), conditions were established under which 5-doxyl-PC would still effectively activate the enzyme, but concentration-dependent line broadening due to spin exchange was minimal. This was achieved by substituting PE and DPG for unlabeled PC and raising the concentration of 5-doxyl-PC to 8% of total phospholipid. The specific activity of the particular (*R*)-3-hydroxybutyrate dehydrogenase preparations under these conditions was reduced to 30% (30 U/mg in a preparation that exhibited 100 U/mg in the standard assay). The degree of order of these multibilayers, *i.e.*, composed of PE/DPG/5-doxyl-PC (81:11:8), was low even in the absence of the enzyme (not shown).

For lipid bilayers devoid of normal PC, the presence of (*R*)-3-hydroxybutyrate dehydrogenase showed the same kind of effect on the outer peak separation ($2A_{zz}$) of the 5-doxyl-PC ESR spectra as described above for conditions where only about 10% of PC was spin-labeled. The same maximal separation of 60.5 G was obtained at the lipid/enzyme ratio of 500. However, at the higher lipid/protein ratio (1370), the effect in the absence of cofactors was more pronounced than in the PC/PE lipid (57.5 G as compared to 56.5 G) in spite of the lower activity of the enzyme.

7-Doxyl-PC when employed under the same conditions (no unlabeled PC present) showed similar behavior. In this case, however, all $2A_{zz}$ values were generally lower, indicative of a somewhat higher mobility of the label and/or a less polar environment (Table 1, column 7).

The outer peak separation also depended on the presence of the coenzyme NAD^+ and a substrate analogue, 2-methylmalonate. The latter profoundly enhances binding of NAD^+ under formation of a ternary complex, with the apparent K_D decreasing from about 200 μM to 1 μM (Rudy et al., 1989). When (*R*)-3-hydroxybutyrate dehydrogenase was present in the form of this ternary complex, the $2A_{zz}$ values were increased unless the maximal values had already been reached by the presence of the enzyme alone (Table 1).

(2) Influence of (R)-3-Hydroxybutyrate Dehydrogenase on Doxyl Lipids in Vesicles. In liposomes, the mobility of the spin label in doxyl-PC derivatives is considerably higher as revealed by much lower $2A_{zz}$ values, ranging from 33.4 G for 16-doxyl-PC to about 50.0 G for 5-doxyl-PC, all in the absence of (*R*)-3-hydroxybutyrate dehydrogenase (Table 2). The presence of (*R*)-3-hydroxybutyrate dehydrogenase at a lipid/protein ratio of 50 had no effect on spectra of 12- and 16-doxyl-PC but did increase the outer peak separation in 5- and 7-doxyl-PC (Figure 6). Ternary complex formation with NAD^+ and 2-methylmalonate did not further increase these values. At a lipid/protein ratio of 200, the enzyme alone had but a small effect, whereas the ternary complex showed almost the same $2A_{zz}$ as for vesicles with higher protein (lipid/protein ratio of 50). As was observed in partially ordered multibilayers, the $2A_{zz}$ value of 5-doxyl-stearic acid was hardly affected by the presence of (*R*)-3-hydroxybutyrate dehydrogenase.

DISCUSSION

Ordered multibilayers were prepared from lipid vesicles in order to study the interaction of (*R*)-3-hydroxybutyrate dehydrogenase with activating PC. By this approach, the enzyme could be reconstituted with the lipid prior to formation of the multibilayers. While this approach does not result in complete ordering of the lipid as can be achieved using, for example, solutions of lipids in organic solvents (Powers & Pershan, 1977; Ge et al., 1994), a reasonably high degree of order can be obtained (Figure 2). The selected conditions assure that the enzyme remains functional within the multibilayer. The presence of protein, however, does disrupt the degree of order of the system.

(*R*)-3-Hydroxybutyrate dehydrogenase strongly enhances the outer peak separation in the ESR spectra of 5-doxyl-PC in ordered lipid multibilayers composed either of PC/PE/5-doxyl-PC (1:1:0.1) or of PE/DPG/5-doxyl-PC (81:11:8), thus revealing a more restricted motion of the lipid in presence of the enzyme. This effect cannot be attributed to changes in the overall structure of the lipid, *e.g.*, an altered state of hydration or lipid molecules being entrapped between protein aggregates, because the mobility of doxyl-labeled stearic acid is not changed. The ESR spectra of doxyl-PC exhibit only a single component, although there is a considerable excess (12- to 110-fold) of doxyl-PC over the enzyme. This is the case independent of whether doxyl-PC has to compete with unlabeled PC for the interaction with (*R*)-3-hydroxybutyrate dehydrogenase, or not. With oriented multibilayers, a relatively small amount of enzyme (molar ratio of 1 enzyme protomer per 1370 lipids, *i.e.*, more than 5000 lipid molecules per enzyme tetramer and equivalent to a lipid/protein weight ratio of >30) has a marked effect on the lipid dynamics detected by perturbation of the ESR spectral lineshape. It is of note that in neither system (membrane dispersion and aligned lipid bilayers) do we detect resolution of the ESR spectral lineshape into two distinct components even with the relatively high protein content preparations used for the vesicle studies (lipid to protein weight ratio of 1.2 at 50 mol of lipid/mol of enzyme protomer). Hence, in accordance with a previously proposed model in which the formation

Table 1: $2A_{zz}$ Values[a] in G of Doxyl-PC and Doxylstearic Acid in Ordered Multibilayers Containing (*R*)-3-Hydroxybutyrate Dehydrogenase (BDH) Alone or BDH in Ternary Complexes with NAD^+ and 2-Methylmalonate

lipid/BDH ratio	PC/PE/5-doxyl-PC (1:1:0.1)	PC/PE/5-doxyl-PC (1:1:0.1)	PC/PE/5-doxyl-SA (1:1:0.1)	PC/PE/5-doxyl-PC (1:1:0.05)	PE/DPG/5-doxyl-PC (81:11:8)	PC/DPG/7-doxyl-PC (81:11:8)
sp act. of BDH (U/mg)	75	40	75	95	30[b]	30[b]
∞; no BDH	54.0	54.0	54.0	54	54.0	53.5
1370; BDH	56.5	54.5	54.5	56	57.5	57.0
1370; ternary complex	59.5	56.5	55.0	61	59.5	58.3
500; BDH	60.5	55.5	55.0	62	60.0	58.5
500; ternary complex	60.5	58.0		62	60.5	59.0
250; BDH		58.0				

[a] $2A_{zz}$ values, measured as illustrated in Figure 5, have an error of ±0.2 G. [b] BDH activity in the standard assay after reconstitution with mitochondrial phospholipid (McIntyre et al., 1983) is 100 U/mg.

Table 2: $2A_{zz}$ Values[a] (in G) of Doxyl-PC Derivatives and 5-Doxylstearic Acid in Lipid Vesicles Containing (*R*)-3-Hydroxybutyrate Dehydrogenase (BDH) Alone, or BDH in Ternary Complexes with NAD^+ and 2-Methylmalonate

lipid/protein ratio	PE/DPG/5-doxyl-SA (81:11:8)	PE/DPG/5-doxyl-PC (81:11:8)	PE/DPG/7-doxyl-PC (81:11:8)	PE/DPG/12-doxyl-PC (81:11:8)	PE/DPG/16-doxyl-PC (81:11:8)
sp. act. of BDH (U/mg)[b]	0	30	30	30	30
∞; no BDH	50.7	50.0	49.8	37.6	33.4
200; BDH		51.2			
200; ternary complex		52.2			
50; BDH	51.5	52.8	52.4	37.8	33.4
50; ternary complex	51.5	52.8	52.4	37.8	33.4

[a] $2A_{zz}$ values, measured as illustrated in Figure, have an error of ±0.2 G. [b] BDH activity in the standard assay after reconstitution with mitochondrial phospholipid (McIntyre et al., 1983) is 100 U/mg.

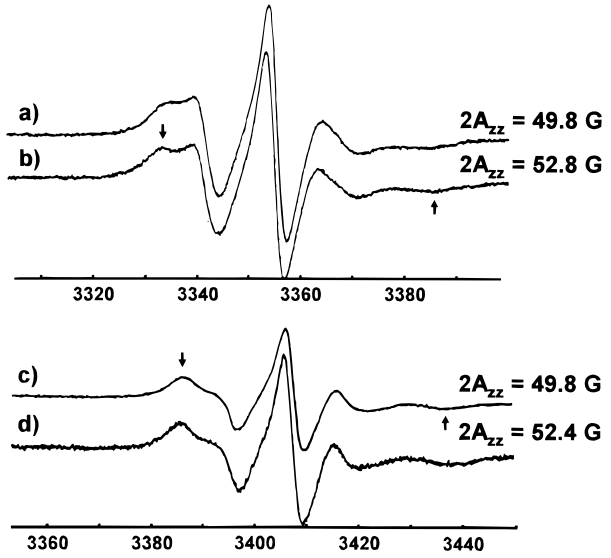

FIGURE 6: ESR spectra of 5-doxyl-PC (a and b) and of 7-doxyl-PC (c and d) in phospholipid vesicles composed of PE/DPG/doxyl-PC (81:11:8) either with (b and d) or without (a and c) (*R*)-3-hydroxybutyrate dehydrogenase (50 mol of lipid/mol of enzyme, 6.4 μM lipid). The $2A_{zz}$ values are summarized in Table 2.

of the active enzyme−PC complex in bilayers is a dynamic process (Sandermann et al., 1986), our data suggest a rapid exchange between PC associated with the enzyme and PC in the bulk lipid. Rapid exchange on the ESR time scale is well below the microsecond range. This rapid exchange process with an averaged environment for the doxyl-PC is in contrast with results obtained studying transmembrane proteins which exhibit two spectral components referable to a boundary layer and bulk lipids (Marsh, 1987). This difference may be attributable to a more peripheral interaction of (*R*)-3-hydroxybutyrate dehydrogenase with the bilayer (Marks et al., 1992) and appears to result in a nonrandom distribution of lipid in the bilayer as suggested previously (Wang et al., 1988). Consistent with this is the lack of effect of the enzyme on the ESR spectra of 12- and 16-doxyl-PC, whereas broadening is detected for both 5- and 7-doxyl-PC (Table 2). In this context, the lipid in the bilayer that is adjacent to the enzyme is proximal rather than a boundary layer of lipid as for transmembrane proteins. Since the ESR spectral broadening afforded by (*R*)-3-hydroxybutyrate dehydrogenase is specific for PC and dependent on the enzymatic activity—the enzyme must be active to affect the ESR spectrum of doxyl-PC—the broadening would appear to be referable to the specific interaction of PC with the enzyme rather than general interaction of proximal lipids with the protein.

The extent of the decrease in mobility of doxyl-PC depends on the lipid/protein ratio as shown by different $2A_{zz}$ values. Also, the more active the enzyme preparation, the stronger are the effects at a given lipid/protein ratio. The presence of a substrate analogue, 2-methylmalonate, strongly enhances the binding of NAD^+ to (*R*)-3-hydroxybutyrate dehydrogenase (Rudy et al., 1989; Fritzsche et al., 1984). Such ternary complexes are more efficient in increasing the PC/enzyme interaction as compared to the enzyme alone. We showed previously that binding of NAD^+ to (*R*)-3-hydroxybutyrate dehydrogenase is very weak in the absence of PC and, especially, that PC is required for the formation of tight and functional complexes (Rudy et al., 1989). The present study now demonstrates the reverse effect, coenzyme binding strengthens the interaction of the enzyme with PC.

Most measurements were carried out in oriented lipid multibilayers because these showed more pronounced effects, *i.e.*, higher orientational sensitivity. However, the decrease in the mobility of doxyl-PC in the presence of (*R*)-3-hydroxybutyrate dehydrogenase was also observed in liposomes, although to a lesser degree. The overall higher mobility of the lipid in vesicles as compared to the multibilayers apparently diminishes the effects (Ge et al., 1994). The lipid/protein ratio in the liposomes was further reduced

to 50 in order to maximize the effect. At this low ratio, the differences between enzyme alone and ternary complexes were no longer observed; *i.e.*, the maximal splitting was reached without adding NAD^+. In these experiments the excess of doxyl-PC over (*R*)-3-hydroxybutyrate dehydrogenase was as low as 4:1. However, for the 5-doxyl-PC at a lipid/protein ratio of 200, the ternary complex increased the hyperfine splitting. Thus, the coenzyme also affects the interaction of (*R*)-3-hydroxybutyrate dehydrogenase with PC in vesicles (Table 2).

The enhanced effect of (*R*)-3-hydroxybutyrate dehydrogenase on the spectral lineshape of doxyl-PC in oriented multibilayers as compared with the lipid vesicle system is consistent with previous studies with other membrane proteins (Jost et al., 1973; Ge et al., 1994). The better spectral resolution obtained with well-aligned samples appears to yield additional information as compared with studies of vesicles. Bacteriorhodopsin was reported to increase lipid ordering in lipid vesicles but to reduce the ordering of the lipid in aligned samples (Ge et al., 1994). Likewise, for the peptide gramicidin A reconstituted with dipalmitoyl-PC, there were notable differences in ESR results between aligned lipid bilayers and membrane dispersions. Studies with oriented samples showed that the boundary layer of lipids associated with gramicidin A exhibited increased ordering but no substantial decrease in mobility except at higher protein concentrations in the bilayer; *i.e.*, the boundary layer is not "immobilized" but rather "motion-restricted" (Ge et al., 1994), as we had suggested previously for lipid interactions with the calcium pump protein of sarcoplasmic reticulum (Fleischer & McIntyre, 1982).

The effect of (*R*)-3-hydroxybutyrate dehydrogenase on lipid dynamics detected by ESR is profound and more pronounced in the ordered multibilayer system. Furthermore, the effect is enhanced by the binding of coenzyme, NAD^+, to the enzyme. As predicted by our previous finding that PC enhances the binding of NAD^+ to (*R*)-3-hydroxybutyrate dehydrogenase by about 50-fold (Rudy et al., 1989), these studies thus show that the interaction of PC with the enzyme is enhanced by NAD^+ bound at the catalytic center.

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