Interaction of the Membrane-Bound D-Lactate Dehydrogenase of *Escherichia coli* with Phospholipid Vesicles and Reconstitution of Activity Using a Spin-Labeled Fatty Acid as an Electron Acceptor: A Magnetic Resonance and Biochemical Study†

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ABSTRACT: The interaction with phospholipid vesicles of the membrane-bound respiratory enzyme D-lactate dehydrogenase of *Escherichia coli* has been studied. Proteolytic digestion studies show that D-lactate dehydrogenase is protected from trypsin digestion to a larger extent when it interacts with phosphatidylglycerol than with phosphatidylcholine vesicles. Wild-type D-lactate dehydrogenase and mutants in which an additional tryptophan is substituted in selected areas by site-specific oligonucleotide-directed mutagenesis have been labeled with 5-fluorotryptophan. ^{19}F nuclear magnetic resonance studies of the interaction of these labeled enzymes with small unilamellar phospholipid vesicles show that Trp 243, 340, and 361 are exposed to the lipid phase, while Trp 384, 407, and 567 are accessible to the external aqueous phase. Reconstitution of enzymatic activity in phospholipid vesicles has been studied by adding enzyme and substrate to phospholipid vesicles containing a spin-labeled fatty acid as an electron acceptor. The reduction of the doxyl group of the spin-labeled fatty acid has been monitored indirectly by nuclear magnetic resonance and directly by electron paramagnetic resonance. These results indicate that an artificial electron-transfer system can be created by mixing D-lactate dehydrogenase and D-lactate together with phospholipid vesicles containing spin-labeled fatty acids.

D-Lactate dehydrogenase (D-LDH)[1] is a flavin-containing enzyme with a molecular weight of 65 000, located on the cytoplasmic side of the *Escherichia coli* membrane. It catalyzes the oxidation of D-lactate in an electron-transfer reaction that is coupled to the active transport of various amino acids and sugars into membrane vesicles prepared from *E. coli* (Barnes & Kaback, 1971; Futai, 1973; Kohn & Kaback, 1973). The activity of D-LDH is enhanced by a wide variety of lipids and detergents (Fung et al., 1979; Kovatchev et al., 1981). Detergent is also required for the isolation and purification of the enzyme, indicating that D-LDH is membrane bound. Active transport of amino acids and sugars in membrane vesicles from *E. coli* deficient in D-LDH can be reconstituted by adding the purified enzyme to either right-side-out or inside-out vesicles (Futai, 1974; Short et al., 1974). This suggests that D-LDH is able to interact with elements of the electron-transfer chain from either side of the membrane after binding to the exposed surface of the membrane vesicle.

D-LDH contains 571 amino acids, 5 of which are tryptophans (Campbell et al., 1984; Rule et al., 1985). When a Trp auxotroph of *E. coli* containing a plasmid carrying the gene for D-LDH is grown in the presence of 5-fluorotryptophan (5F-Trp), the ^{19}F-labeled Trp is incorporated into D-LDH (Rule et al., 1987a,b). The ^{19}F-labeled enzyme is active and capable of stimulating D-lactate-driven active transport in *E. coli* membrane vesicles that are deficient in D-LDH (Pratt et al., 1983). The 5F-Trp-labeled wild-type D-LDH shows five peaks by ^{19}F nuclear magnetic resonance (NMR), each of which has been assigned to a specific Trp residue (Rule et al., 1987b). Our previous investigations have focused on the study of D-LDH in the presence of lysophosphatidylcholine (lysoPC), i.e., in micellar-like structures. The smaller size of the protein–micelle complex, as opposed to a protein–liposome complex, gives rise to sharper peaks in the ^{19}F NMR spectra (Rule et al., 1987b; Peersen et al., 1990). These studies using wild-type enzyme show that Trp 387, 407, and 567 are exposed to the aqueous medium. Data from mutant D-LDHs show that the region extending from residues 340 to 361 is close to the lipid phase (Peersen et al., 1990) on the basis of the broadening induced by a spin-labeled fatty acid. This latter region has been postulated to be the hydrophobic binding domain of D-LDH.

Recently, we have expanded our research to investigate the structure and lipid interactions of D-LDH in sonicated unilamellar vesicles (SUVs), which, unlike micelles, form bilayer structures and have distinct inside and outside domains and thus constitute a system closer to the native membrane. Furthermore, the location of spin-labeled fatty acids in micelles

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[1] Abbreviations: D-LDH, D-lactate dehydrogenase; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; 12-doxyl-DMPC, 1-myristoyl-2-[11-(4,4-dimethyl-3-oxy-2-ethyl-2-oxazolidinyl)undecanoyl]-*sn*-glycero-3-phosphocholine; DMPG, 1,2-dimyristoyl-*sn*-glycero-3-phospho-*rac*-1-glycerol; NMR, nuclear magnetic resonance; TFA, trifluoroacetic acid; DSS, 2,2-dimethyl-2-silapentane-5-sulfonate; EPR, electron paramagnetic resonance; lysoPC, egg lysophosphatidylcholine; SUVs, sonicated unilamellar phospholipid vesicles; TEMPOcholine, 4-[*N*,*N*-dimethyl-*N*-(2-hydroxyethyl)ammonium]-2,2,6,6-tetramethylpiperidine-1-oxyl; FAD, flavin adenine dinucleotide; 5F-Trp, 5-fluorotryptophan; F340W, F361W, and Y243W, D-LDH mutants in which Trp is substituted for Phe 340, Phe 361, and Tyr 243, respectively.

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is a subject of controversy, with some studies suggesting that the probe may be located at the water–micelle interface instead of the hydrophobic region (Ramachandran et al., 1982; Shobha et al., 1989). Using SUVs alleviates this problem, since spin-labeled fatty acids (Ellena et al., 1988) and spin-labeled phospholipids (McConnell, 1976) are located in the apolar region of vesicles. We have sought to determine whether the conformation and orientation of D-LDH are the same in phospholipid vesicles as they are in micelles. In addition, we have developed an artificial system in which electron transfer to the membrane can be monitored spectroscopically in phospholipid vesicles.

Materials and Methods

D-Lactate and 5F-Trp were purchased from Sigma (St. Louis, MO) and phospholipids from Avanti (Birmingham, AL); 4-[*N*,*N*-dimethyl-*N*-(2-hydroxyethyl)ammonium]-2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPOcholine) was from Molecular Probes (Junction City, OR) and trypsin (TPCK-treated, Catalog No. TRTPCK OCA) from Worthington (Freehold, NJ). All materials were used without further purification. 1-Myristoyl-2-[11-(4,4-dimethyl-3-oxy-2-ethyl-2-oxazolidinyl)undecanoyl]-*sn*-glycero-3-phosphocholine (12-doxyl-DMPC) was prepared from the corresponding spin-labeled myristic acid (Hubbell & McConnell, 1971) with subsequent acylation of lysophosphatidylcholine (Gupta et al., 1977).

D-LDH, mutant D-LDHs, and 5F-Trp-labeled D-LDHs were obtained, and their activities were measured, as described previously (Rule et al., 1987a,b). In order to establish that the amount of Triton remaining in our preparations was minimal, one purification of D-LDH was carried out in the presence of ^{3}H-labeled Triton. Based on the amount of ^{3}H measured in the final sample, the Triton concentration in our D-LDH preparation was less than 0.003%, or less than one Triton molecule for every five D-LDH molecules. The concentration of D-LDH was determined by measuring the flavin adenine dinucleotide (FAD) concentration after denaturing the enzyme in 6 M guanidine hydrochloride and 0.1 M sodium acetate, pH 4.0, by using an extinction coefficient of 12 000 M^{-1} cm^{-1} at 450 nm. This extinction coefficient was determined by measuring the absorbance of a known concentration of free FAD under the same conditions.

Phospholipid vesicles were prepared by adding buffer to dry phospholipid and sonicating the resulting suspension to clarity in a sonicator bath (Branson 2200) at a temperature above the phase-transition temperature of the lipid. Alternatively, a chloroform/methanol solution of the lipids with any other addition was dried under nitrogen, redissolved in benzene/methanol (9:1 v/v), and then lyophilized to obtain a powder residue before addition of buffer and sonication. Tubes for sonication were acid washed and presonicated to minimize contamination by fluoride from the glass (see Figure 5 and legend).

Trypsin digestions were carried out in 20 mM Tris at pH 8.5 and 22 °C. D-LDH (4 μM final concentration) was incubated with 6 mM lipid at 37 °C for a minimum of 20 min before the addition of trypsin (5 mg/mL final concentration from a stock solution of 100 mg/mL in Tris buffer). Digestions were stopped by adding an equal volume of 0.125 M Tris, 4% SDS, 20% glycerol, 0.03% bromophenol blue, and 10% 2-mercaptoethanol, pH 6.8, and heating at 100 °C for 3 min. Fragments were then separated by SDS–polyacrylamide gel electrophoresis.

NMR samples were prepared by mixing equal volumes of SUVs and D-LDH; they typically contained approximately 1 mM D-LDH and 60 mM phospholipid [80% DMPC and 20% DMPG (mol %)] in 10 mM potassium phosphate and 0.2 mM EDTA, pH 7.2, in 90% D_2O, unless otherwise indicated. TEMPOcholine (0.8 M stock solution in 10 mM potassium phosphate, 0.2 mM EDTA, pH 7.2) and $K_3Fe(CN)_6$ (1 M stock solution in the same buffer) were added to the NMR samples to the indicated final concentrations. NMR measurements were obtained on Bruker WH-300 and AM-300 spectrometers at 282.4 MHz for ^{19}F and 300 MHz for ^{1}H. The ^{19}F NMR spectra of D-LDH labeled with 5F-Trp were obtained with an 8-kHz spectral width and 4K data points in a 5-mm ^{19}F probe by using a 4.4-μs 60° pulse and a relaxation delay of 2 s. Free induction decays (10 000–25 000) were accumulated and Fourier transformed with a 20-Hz line broadening, and the resulting spectra were corrected for phase and base-line distortions. ^{19}F chemical shifts are expressed relative to trifluoroacetic acid (TFA). For ^{1}H NMR measurements, water was suppressed by presaturation with a low-power decoupling pulse; free induction decays were accumulated with a 5-kHz spectral width and 16K data points in a 5-mm probe or in a broad-band probe by using a 90° pulse. ^{1}H chemical shifts are referred to the residual water (HDO) in the sample, which is 4.60 ppm downfield from the methyl-proton resonance of a standard, 2,2-dimethyl-2-silapentane-5-sulfonate (DSS).

Electron paramagnetic resonance (EPR) spectra of samples in 50-μL capillary tubes were taken on a Bruker ER 300 spectrometer operating at 9.3 GHz with a 1-G modulation amplitude and 0.49 mW of power with a 100-G scan range and a scan time of 200 s. The resonance cavity was at room temperature (22 °C).

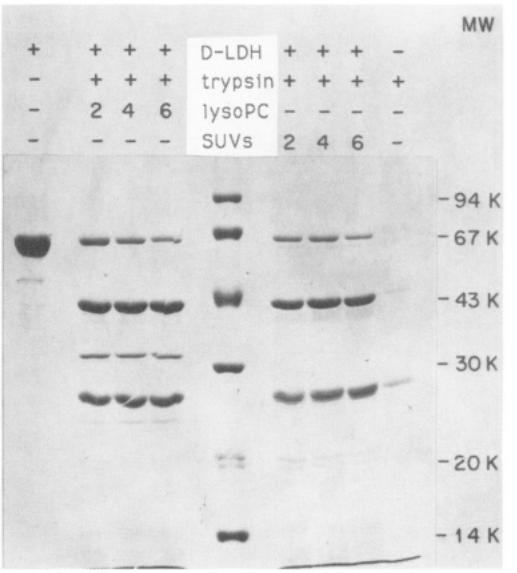

FIGURE 1: Tryptic digestion of D-LDH: D-LDH in lysoPC versus DMPC/DMPG vesicles. Digestion was carried out for 1 h by using 4 μM D-LDH, 5 mg/mL trypsin, and either 0, 2, 4, or 6 mM lysoPC or 0, 2, 4, or 6 mM phospholipid (80% DMPC/20% DMPG) as SUVs.

Results

Proteolytic Digestion. Small unilamellar phospholipid vesicles were mixed with D-LDH. Trypsin was added to the phospholipid vesicle complexes in order to gain information on the location and conformation of the enzyme in the lipid environment. As shown in Figure 1, D-LDH is degraded by the protease, indicating that D-LDH is accessible to trypsin from the outside of the phospholipid vesicles. The tryptic digestion patterns of D-LDH in lysoPC micelles, DMPC/DMPG vesicles, and DMPG vesicles are given in Figures 1

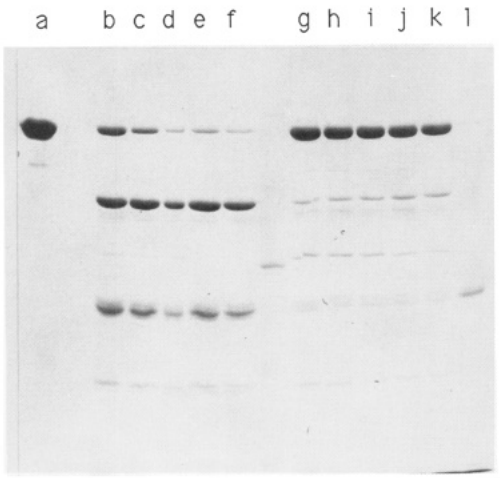

FIGURE 2: Tryptic digestion of D-LDH: D-LDH in DMPC/DMPG vesicles versus DMPG vesicles. D-LDH (4 μM) with SUVs (6 mM phospholipid) containing 80% DMPC and 20% DMPG (b, c, d, e, f) or 100% DMPG (g, h, i, j, k) was incubated with 5 mg/mL trypsin for 30 (b, g), 60 (c, h), 90 (d, i), 120 (e, j), or 300 (f, k) min. The controls were (a) D-LDH alone and (l) trypsin alone.

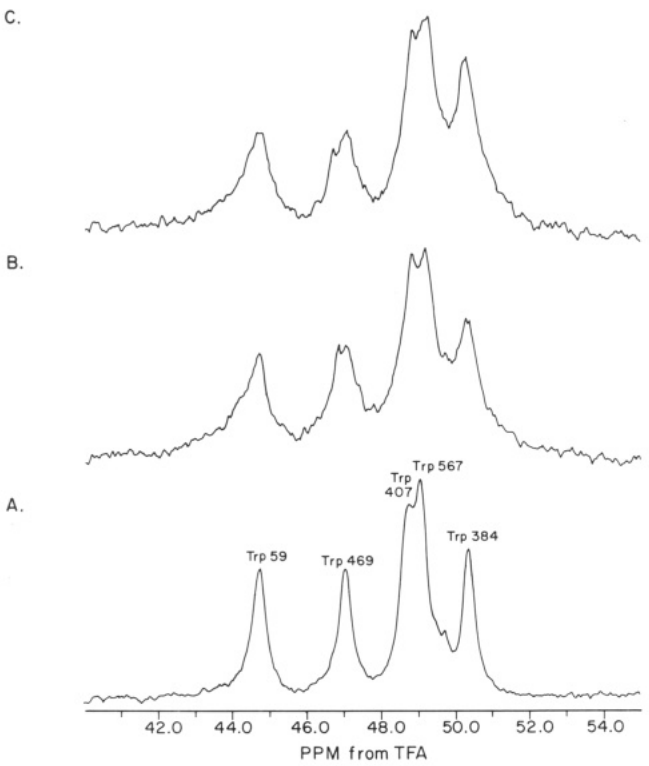

FIGURE 3: 282.4-MHz ^{19}F NMR spectra of 5F-Trp-labeled D-LDH in different lipid environments: (A) 0.3 mM D-LDH with 100 mM lysoPC, (B) 0.9 mM D-LDH with SUVs (78 mM phospholipid, 80% DMPC/20% DMPG), and (C) 1.1 mM D-LDH with SUVs (75 mM DMPG).

and 2. Digestion of D-LDH in lysoPC results in a 32-kDa fragment that is not present in the digestion of D-LDH in DMPC/DMPG vesicles. Furthermore, when D-LDH is mixed with vesicles composed of DMPG, it is less extensively digested by trypsin than when it is mixed with vesicles composed of both DMPC and DMPG. The residual D-LDH activity after a 90-min treatment with trypsin is 27% for D-LDH in DMPC/DMPG and 74% for D-LDH in DMPG. Control studies show that the activity of trypsin on bovine serum albumin is identical in the presence of lysoPC and DMPC and is increased in the presence of DMPG (results not shown). These results show that D-LDH is not identically accessible

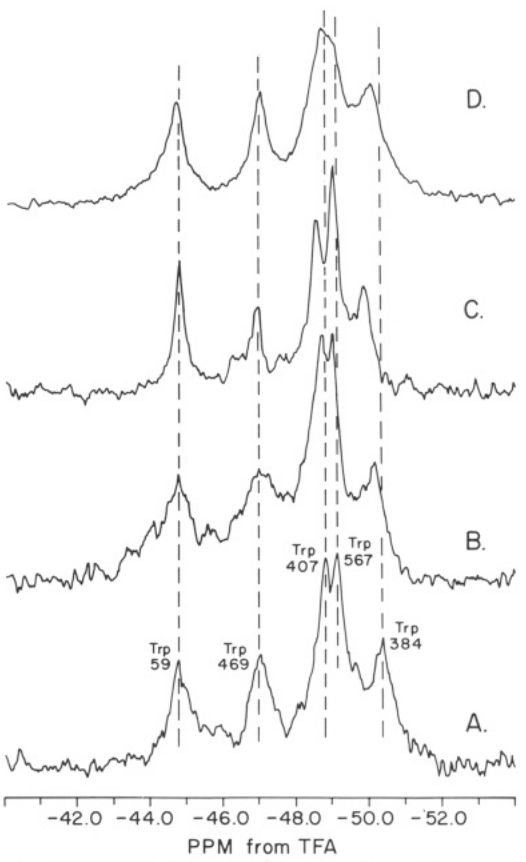

FIGURE 4: 282.4-MHz ^{19}F NMR investigation of the exposure of 5F-Trp-labeled D-LDH in SUVs to the external aqueous environment at 42 °C: (A) 90% D_2O, 0.6 mM D-LDH, and 60 mM phospholipid, (B) 98% H_2O, 0.9 mM D-LDH, and 60 mM phospholipid, (C) 90% D_2O with 0.2 M $K_3Fe(CN)_6$ added to the preformed SUVs, 0.45 mM D-LDH, and 45 mM phospholipid, and (D) 50% D_2O with 130 mM TEMPOcholine added to preformed SUVs, 0.6 mM D-LDH, and 46 mM phospholipid.

to trypsin in the different lipid environments. They also suggest that D-LDH adopts a different conformation in micelles than it does in phospholipid vesicles and that the enzyme interacts differently with DMPG vesicles than it does with DMPC/DMPG vesicles.

^{19}F NMR Studies of D-LDH in Various Lipid Environments. Further studies on the conformation of D-LDH in phospholipid vesicles were carried out by ^{19}F NMR investigations of D-LDH labeled with 5F-Trp. D-LDH has five Trp residues (Campbell et al., 1984; Rule et al., 1985), each of which in labeled D-LDH is resolved by ^{19}F NMR when the enzyme is prepared in lysoPC (Rule et al., 1987b). When the enzyme is prepared with SUVs, the five Trp peaks are still resolved; however, the line widths are broader in SUVs than in lysoPC, consistent with the larger size of D-LDH–phospholipid vesicle complexes relative to D-LDH–micelle complexes. The ^{19}F chemical shift positions of the five Trp residues are identical in lysoPC and in SUVs composed of DMPC/DMPG and of DMPG (Figure 3), indicating that each of the five Trp residues is in an identical microenvironment irrespective of the composition of the lipid phase. This suggests that the conformation of the enzyme, as monitored by these five Trp residues, is the same in the three lipid environments.

Exposure to the Aqueous Medium. The Trp residues that are exposed to the aqueous phase are determined by changing the composition of the aqueous medium. Changing the solvent composition from 90% (Figure 4A) to 2% D_2O (Figure 4B) shifts the positions of Trp 384, 567, and possibly 407. This

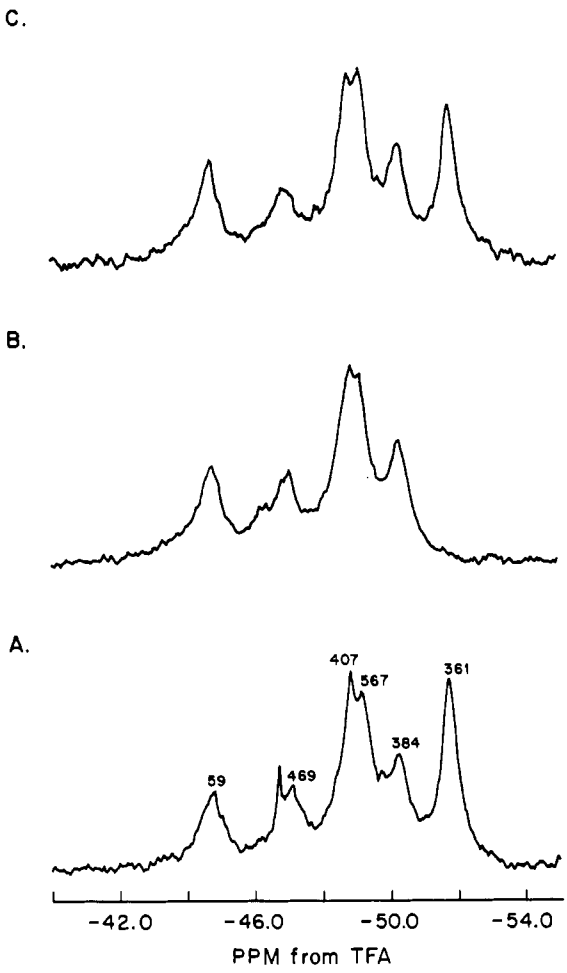

FIGURE 5: Effects of spin-labeled fatty acid and substrate on the 282.4-MHz ^{19}F NMR spectrum of the F361W mutant D-LDH at 42 °C in (A) SUVs, (B) SUVs containing 28 mM 8-doxylpalmitic acid, and (C) SUVs containing 28 mM 8-doxylpalmitic acid with 100 mM D-lactate added. The sharp peak at −46.7 ppm is due to contaminating F^- ion from the glassware used for sonication during the preparation of the samples.

indicates that the same residues are exposed to the aqueous phase in SUVs as in micelles (Rule et al., 1987b; Peersen et al., 1990). By comparing these shifts with the change of the ^{19}F chemical shift of free Trp with respect to solvent composition (Hagen et al., 1979; Rule et al., 1987b; Peersen et al., 1990), we conclude that Trp 384 is fully exposed to the solvent and Trp 567 and 407 are partially exposed in SUVs.

The addition of the shift reagent $K_3Fe(CN)_6$ to the outside of the phospholipid vesicles shifts the resonance position of Trp 384 and to a lesser extent those of Trp 407 and 567, showing that these residues are accessible to ferricyanide and are exposed to the external aqueous phase (Figure 4C). Furthermore, the hydrophilic paramagnetic molecule TEMPOcholine, when added to the external aqueous phase, broadens the resonances of Trp 384, 407, and 567, confirming that these residues are indeed exposed to the external aqueous phase (Figure 4D).

Exposure to the Lipid Phase. D-LDH isolated from a mutant generated by site-specific oligonucleotide-directed mutagensis in which Phe 361 is replaced by a Trp (mutant F361W) shows six peaks by ^{19}F NMR spectroscopy, corresponding to the six Trp residues in the mutant enzyme, as shown in Figure 5A. When this mutant D-LDH is added to SUVs composed of phospholipid that has been cosonicated with a spin-labeled fatty acid, only five peaks corresponding to the spectrum of wild-type D-LDH are seen (Figure 5B). The

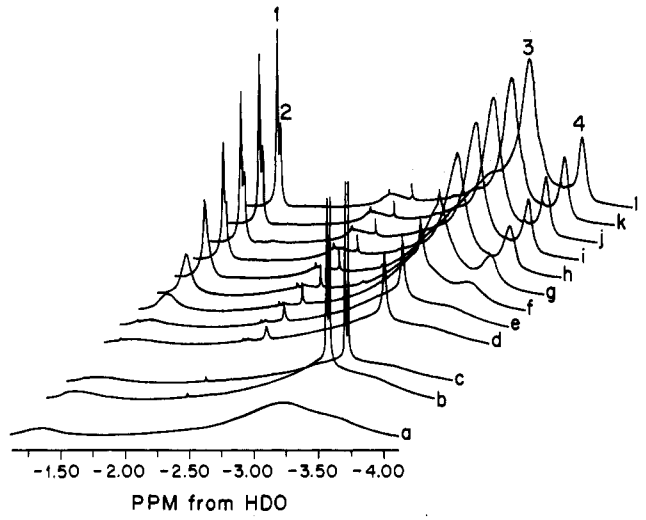

FIGURE 6: Effects of D-LDH and D-lactate on the 300-MHz ^1H NMR spectrum of phospholipid vesicles containing spin-labeled fatty acid, at 42 °C, with (a) no additions, (b) 100 mM D-lactate, (c) 100 mM D-lactate (24 h after addition), and (d–l) 100 mM D-lactate + 0.3 mM D-LDH; spectra were acquired every 15 min. The peaks are (1) outer monolayer choline methyls, (2) inner monolayer choline methyls, (3) acyl methylenes, and (4) acyl methyls. The ^1H chemical shift of H_2O is 4.60 ppm downfield from DSS at 42 °C.

paramagnetic doxyl group broadens the resonance corresponding to Trp 361 beyond detection, showing that, as in the lysoPC environment, Trp 361 is within 10 Å of the doxyl group (Arseniev et al., 1981). A similar result was obtained for a mutant where Phe 340 is replaced by Trp (mutant F340W, results not shown). Since the nitroxide moiety of the spin-labeled fatty acid is located in the hydrocarbon region of the phospholipid vesicles, though not as deeply as the analogous position in the phospholipid chain (Ellena et al., 1988), these results indicate that Trp 340 and 361 are exposed to the lipid phase of the SUVs.

The same results are obtained for mutants F340W and F361W with SUVs containing a spin-labeled DMPC instead of a spin-labeled fatty acid. In addition, the resonance corresponding to Trp 243 from mutant Y243W, which is partially broadened by the spin-labeled fatty acid in lysoPC (Peersen et al., 1990), is also partially broadened by spin-labeled DMPC, albeit to a lesser extent than Trp 340 and 361 (results not shown).

D-LDH-Dependent Reduction of Spin Nitroxide. The resonance corresponding to Trp 361 from mutant F361W, which is broadened when the spin-labeled fatty acid is present in the phospholipid vesicle, reappears upon addition of D-lactate (Figure 5C). The peak recovers its normal height within at most 2 h after the addition of the substrate. Similar results are obtained for mutant F340W (results not shown). These observations suggest that, upon the addition of substrate, either the region of the enzyme exposed to the lipid phase moves away from the lipid or the nitroxide group is affected. It is unlikely that a major change in conformation occurs since the final spectrum is that expected for the mutant D-LDH in the presence of substrate. The ^1H NMR spectrum of SUVs is affected by the presence of spin-labeled fatty acid; i.e., the choline methyl peaks and acyl methylene and methyl peaks are broadened (Figure 6). The addition of D-lactate to the phospholipid vesicles containing spin-labeled fatty acid does not affect the broadened peaks, showing that D-lactate alone does not destroy the broadening effect of the spin label. However, the subsequent addition of D-LDH to the system causes a time-dependent sharpening of the peaks broadened

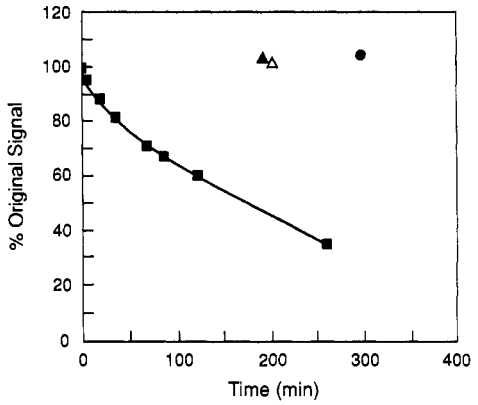

FIGURE 7: Effects of D-lactate and D-LDH on the EPR signal of spin-labeled fatty acid. D-Lactate and D-LDH (1 mM and 0.2 mM final concentrations, respectively) were added to SUVs composed of 60 mM phospholipid (80% DMPC, 20% DMPG) and 0.5 mM 8-doxylpalmitic acid (■). Controls were SUVs with (▲) no additions, (△) D-lactate, and (●) D-LDH.

by the spin-labeled fatty acid. The changes are essentially completed in 2 h, similar to the recovery of the lipid-sensitive ^{19}F resonances. Since a more accurate determination of the rate of recovery in the ^{19}F NMR experiments is not possible, due to the minimum time necessary to acquire a spectrum, a more exact comparison with the ^{1}H NMR results is not possible. Similar results are obtained with spin-labeled lipid. These results suggest that the recovery of the normal peak widths is caused by a specific reduction of the spin nitroxide.

This possibility was verified by electron paramagnetic resonance (EPR) measurements. The addition of D-lactate or D-LDH to phospholipid vesicles containing spin-labeled fatty acid does not cause any decrease in their EPR signal with time (Figure 7). However, when D-LDH and substrate are both added to the spin-labeled phospholipid vesicles, the signal from the doxyl group decreases in a time-dependent fashion, with half of the signal reduced after 3 h. The decrease of the EPR signal appears to be slower than the recovery of the NMR signal. However, a direct comparison is not possible due to the very different conditions of the experiments. Aside from differences in concentrations, the lower temperature at which the EPR experiments were carried out (22 °C as opposed to 42 °C) would result in a lower rate of reduction of the nitroxide groups. Thus, the addition of D-lactate and D-LDH to phospholipid vesicles containing spin-labeled fatty acid reduces the nitroxide groups, causing a narrowing of the broadened peaks in the NMR spectra.

Discussion

By ^{19}F NMR spectroscopy, it appears that the orientation of D-LDH as it interacts with the outer layer of SUVs is similar to its imputed orientation in lysoPC micelles. The experiments using hydrophilic shift and broadening agents added to preformed phospholipid vesicles show that those portions of D-LDH shown to be accessible to the aqueous phase (i.e., those containing Trp 384, 407, and 567) (Rule et al., 1987b; Peersen et al., 1990) are located on the external face of the phospholipid vesicle–protein complexes. When spin-labeled fatty acid or lipid are present in the phospholipid vesicles, where they have been shown to be located in the bilayer and not at the interface (McConnell, 1976; Ellena et al., 1988), the amino acid residues located at positions 340, 361, and, to a lesser extent, 243 are broadened, confirming the conclusion reached with the lysoPC system that these residues are in or near the lipid phase.

The conformation of 5F-Trp-labeled D-LDH, as monitored by the five F-labeled Trp residues, appears to be the same in lysoPC, DMPC/DMPG vesicles, and DMPG vesicles. However, the enzyme is not equally accessible to trypsin in the three different lipid environments, suggesting that there may be differences in the conformation of the enzyme or that the enzyme interacts differently with the various lipids. The difference in tryptic digestion patterns of D-LDH in lysoPC compared to D-LDH in phospholipid vesicles is probably a reflection of the accessibility of peptide loops in the protein to the proteolytic enzyme, loops that do not contain any Trp residues. There are 571 amino acids in D-LDH and only 5 Trp residues in the native enzyme, so it is not surprising that the 5 Trp residues are not a faithful set of reporters of the overall conformation of D-LDH [even though they do report differences in the conformation of D-LDH in Triton versus lysoPC (Rule et al, 1987b)]. The difference in the extent of digestion between D-LDH in DMPG versus in DMPC/DMPG phospholipid vesicles could be ascribed to an electrostatic potential effect, since at physiological pH DMPG head groups carry a negative charge, whereas DMPC head groups have no net electric charge. However, since the p*I* of bovine trypsin is 10.8 (Keil, 1971), it is more likely that the difference in proteolytic digestion is due to a difference in the interaction of D-LDH with the lipid phase. D-LDH is activated more by phosphatidylglycerols than it is by phosphatidylcholines (Kovatchev et al., 1981), which suggests either that phosphatidylglycerol may bind more tightly to the enzyme and thus protect it from proteolysis or that phosphatidylglycerol may induce a more favorable conformation of the enzyme.

D-LDH, when it interacts with SUVs, reduces a spin-labeled fatty acid upon the addition of substrate. Lipophilic nitroxides are reduced in *E. coli* vesicles (Baldassare et al., 1974); in the mitochondria of living cells, reduction occurs in the respiratory chains at the level of ubiquinone (Chen et al., 1988). The spin-labeled fatty acid acts as an electron acceptor, located in the membrane. Thus, the membrane activity of D-LDH has been reconstituted, similar to its role in electron transfer. Our system gives us a spectroscopic probe of electron transfer in an artificial electron-transfer system, where the spin-labeled fatty acid acts as an electron acceptor in the phospholipid vesicles. This is reminiscent of the system developed by Matsushita and Kaback (1986) where electron transfer was reconstituted in artificial proteoliposomes containing a ubiquinone, cytochrome *o*, and D-LDH. In the system described here, D-LDH oxidizes D-lactate, and the resulting electrons become available for the reduction of the nitroxide group located in the lipid phase. Ubiquinone, which in vivo is the intermediate in the electron-transfer chain following D-LDH, is not necessary for electron transfer in our system. Apparently the spin-labeled fatty acid diffuses freely enough so that its nitroxide moiety comes into contact with the enzyme. The rate of nitroxide reduction is low compared to the rate of electron transfer in other reconstituted systems (Reeves et al., 1973; Matsushita et al., 1986; Peersen et al., 1990), perhaps due to inefficient coupling of the enzyme to the phospholipid vesicles or due to rare contact between the nitroxide and the enzyme. Indeed, in mitochondria, the rate-limiting step in the reduction of a nitroxide group buried in the hydrocarbon region of the membrane is diffusion controlled (Chen et al., 1988). However, the fact that some reduction does occur shows that D-LDH in the phospholipid vesicle–protein complex is indeed active and is able to transfer electrons to the lipid phase.

Thus, ^{19}F NMR measurements have given us information about the structure and conformation of a large membrane protein in a phospholipid vesicle environment. D-LDH binds to the outer layer of SUVs with an orientation and confor-

mation similar to that assumed in micelles; it is also active and capable of participating in transferring electrons to the lipid phase.

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Mechanisms for the Facilitated Diffusion of Substrates across Cell Membranes†

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ABSTRACT: Two classes of theoretical mechanisms for protein-mediated, passive, transmembrane substrate transport (facilitated diffusion) are compared. The simple carrier describes a carrier protein that exposes substrate influx and efflux sites alternately but never both sites simultaneously. Two-site models for substrate transport describe carrier proteins containing influx and efflux sites simultaneously. Velocity equations describing transport by these mechanisms are derived. These equations take the same general form, being characterized by five experimental constants. Simple carrier-mediated transport is restricted to hyperbolic kinetics under all conditions. Two-site carrier-mediated transport may deviate from hyperbolic kinetics only under equilibrium exchange conditions. When both simple- and two-site carriers display hyperbolic kinetics under equilibrium exchange conditions, these models are indistinguishable by using steady-state transport data alone. Seven sugar transport systems are analyzed. Five of these systems are consistent with both models for sugar transport. Uridine, leucine, and cAMP transport by human red cells are consistent with both simple- and two-site models for transport. Human erythrocyte sugar transport can be modeled by simple- and two-site carrier mechanisms, allowing for compartmentalization of intracellular sugars. In this instance, resolution of the intrinsic properties of the human red cell sugar carrier at 20 °C requires the use of submillisecond transport measurements.

Several fundamental properties of facilitated diffusion (passive transport) systems remain to be resolved. The most basic of these is an understanding of the catalytic principles involved in substrate transport. A detailed knowledge of intermediate forms of carrier/substrate complexes is unavailable, and the physical mechanism(s) by which the carrier protein translocates bound substrate to the opposite side of the mem-

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