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A ^{19}F-NMR study of the membrane-binding region of D-lactate dehydrogenase of *Escherichia coli*

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Abstract

D-Lactate dehydrogenase (D-LDH) is a membrane-associated respiratory enzyme of *Escherichia coli*. The protein is composed of 571 amino acid residues with a flavin adenine dinucleotide (FAD) cofactor, has a molecular weight of approximately 65,000, and requires lipids or detergents for full activity. We used NMR spectroscopy to investigate the structure of D-LDH and its interaction with phospholipids. We incorporated 5-fluorotryptophan (5F-Trp) into the native enzyme, which contains five tryptophan residues, and into mutant enzymes, where a sixth tryptophan is substituted into a specific site by oligonucleotide-directed mutagenesis, and studied the 5F-Trp-labeled enzymes using ^{19}F-NMR spectroscopy. In this way, information was obtained about the local environment at each native and substituted tryptophan site. Using a nitroxide spin-labeled fatty acid, which broadens the resonance from any residue within 15 Å, we have established that the membrane-binding area of the protein includes the region between Tyr 228 and Phe 369, but is not continuous within this region. This conclusion is strengthened by the results of ^{19}F-NMR spectroscopy of wild-type enzyme labeled with fluorotyrosine or fluorophenylalanine in the presence and absence of a nitroxide spin-labeled fatty acid. These experiments indicate that 9–10 Phe and 3–4 Tyr residues are located near the lipid phase.

Keywords: D-lactate dehydrogenase; fluorophenylalanines; 5-fluorotryptophan; fluorotyrosine; ^{19}F-NMR; membrane-associated proteins; NMR; site-specific mutagenesis

D-Lactate dehydrogenase (D-LDH) of *Escherichia coli* is a membrane-associated respiratory enzyme involved in electron transfer, located on the cytoplasmic side of the inner membrane of *E. coli*. The enzyme (65,000 MW) contains 571 amino acid residues with flavin adenine dinucleotide (FAD) as a cofactor (Barnes & Kaback, 1971; Futai, 1973; Kohn & Kaback, 1973; Rule et al., 1985). Detergent is required for isolation and purification of the enzyme, and its activity is enhanced by a wide variety of lipids and detergents (Tanaka et al., 1976; Fung et al., 1979; Kovatchev et al., 1981). These properties indicate that D-LDH is a membrane-associated protein. However, D-LDH can be eluted from *E. coli* membranes by washing with 0.6 M guanidine hydrochloride (Reeves et al., 1973). This suggests that D-LDH, like other primary dehydrogenases involved in electron transfer in *E. coli* (Cronan et al., 1987), is not an integral membrane protein (Steck & Yu, 1973). Furthermore, the amino acid composition of D-LDH is not particularly hydrophobic, and the molecule does not appear to contain any of the transmembrane hydrophobic structures that are typical of integral membrane proteins (Ho et al., 1988; Fasman, 1989). Active transport of amino acids and sugars into 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 membrane vesicles (Futai, 1974; Short et al., 1974).

As with many membrane-associated proteins, it has proven difficult to produce satisfactory crystals of D-LDH for X-ray crystallography, and the size of D-LDH pre-

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cludes the determination of its structure by two- or three-dimensional NMR spectroscopy. We have been using a combination of site-specific mutagenesis and ^{19}F-NMR spectroscopy to investigate the structure, dynamics, and interactions of D-LDH labeled with fluorinated amino acids (Rule et al., 1985, 1987a,b; Ho et al., 1989; Peersen et al., 1990; Truong et al., 1991a,b). The wild-type native enzyme contains five tryptophan residues (Rule et al., 1985; Ho et al., 1988), and the ^{19}F-NMR spectrum of 5-fluorotryptophan (5F-Trp)-labeled wild-type D-LDH shows five peaks (Rule et al., 1987a,b). Tryptophan has been substituted for phenylalanine, tyrosine, isoleucine, and leucine at various positions throughout the enzyme, and the fluorinated native and substituted tryptophan residues of the active mutants have been used as probes of the local environment. These studies have led to the proposal of a three-domain structure for D-LDH, in which this membrane-associated enzyme may have the two-domain structure of many cytoplasmic dehydrogenases, but with the addition of a membrane-binding domain between the catalytic and cofactor-binding domains (Peersen et al., 1990). The low number of lysolecithin molecules (40–60) required to solubilize one D-LDH molecule suggests that the membrane-binding region of the enzyme is small (Rule et al., 1987b).

Our investigation of the membrane-binding region of D-LDH makes use of the observation that a nitroxide spin label can cause broadening of the resonance from any residue within a 15-Å radius, with the amount of broadening inversely proportional to the distance raised to the sixth power (Arseniev et al., 1981). Wild-type D-LDH and mutant D-LDHs, in which a sixth Trp was added by site-specific mutagenesis at locations throughout the molecule, were labeled with 5F-Trp, and a nitroxide spin-labeled fatty acid was incorporated into the lipid phase used to solubilize D-LDHs. None of the five native Trp residues of D-LDH are found to be affected by the spin-labeled fatty acid (Rule et al., 1987b); of the additional sites studied this way, only Phe 340 and Phe 361 are in close proximity to the lipid phase as indicated by the complete broadening of the Trp-substituted resonances in the ^{19}F-NMR spectrum in the presence of a nitroxide spin-labeled fatty acid or lipid (Peersen et al., 1990; Truong et al., 1991b). The sites closest to Phe 340 and Phe 361 that had been studied previously are Tyr 243, the Trp-substituted resonance of which is only slightly broadened by the lipophilic spin label and which is partially exposed to the aqueous solvent (Peersen et al., 1990), and the native Trp 384, which is completely exposed to the solvent and the resonance of which is not affected by the lipophilic spin label (Rule et al., 1987b). The lipid-binding domain appears to include the region between Tyr 243 and Phe 361, and no site outside of this region that had been studied previously is affected by the lipophilic spin label. We herein present additional attempts to define the membrane-binding region of D-LDH.

Results

5F-Trp-labeled tryptophan substitution mutants

To further define the membrane-binding region of D-LDH, we have studied nine additional Trp-substitution mutants: Y226W, Y228W, F251W, F270W, Y280W, F300W, Y309W, Y315W, and F369W. (Trp-substitution mutants are expressed as the original amino acid, the location of the residue in the protein, and the substituted amino acid, e.g., Y226W = the mutant in which Trp is substituted for Tyr at position 226.) The mutants Y226W, Y228W, F300W, Y315W, and F369W give high yields of enzyme, whereas the mutants F251W, F270W, Y280W, and Y309W are low-yield, temperature-sensitive folding mutants (Truong et al., 1991b). All nine mutants are active, and their K_m and V_{max} values are within three- and fourfold of those of the wild-type D-LDH, respectively (Table 1; Rule et al., 1987b; Peersen et al., 1990; Truong et al., 1991b).

We had previously believed that Tyr 243 is at the N-terminal boundary of the membrane-binding region, due to its partial exposure both to the lipid and the aqueous phase (Peersen et al., 1990). The mutants Y226W and Y228W were made to further define this boundary. The ^{19}F-NMR spectrum of 5F-Trp-labeled mutant Y226W shows a resolved resonance peak at −48.4 ppm (Fig. 1). This resonance is not broadened in the presence of a nitroxide spin-labeled fatty acid, suggesting that the Trp-substituted site at position 226 is not in the lipid-binding region. However, when the solvent is changed from D_2O buffer to H_2O buffer, the Trp 226 peak exhibits a −0.4 ppm downfield shift (results not shown), similar to the −0.2 ppm solvent-induced isotopic shift (SIIS) effect of the free 5F-Trp in solution (Rule et al., 1987b), but

Table 1. *Kinetics parameters of wild-type D-LDH and D-LDH mutants used in this study*

D-LDH	Label	K_m [a]	V_{max} [b]
Wild type[c]	None	1.5	13.0
Mutant Y226W	5F-Trp	1.5	11.7
Mutant Y228W	5F-Trp	5.1	10.5
Mutant F251W	5F-Trp	1.9	5.3
Mutant F270W	5F-Trp	3.6	12.5
Mutant Y280W	5F-Trp	1.8	18.3
Mutant F300W	5F-Trp	2.5	9.2
Mutant Y309W	5F-Trp	4.7	10.0
Mutant Y315W	5F-Trp	1.4	7.9
Mutant F369W	5F-Trp	3.0	17.2
Wild type	*o*-F-Phe	1.1	3.6
Wild type	*m*-F-Phe	3.0	13.0
Wild type	*p*-F-Phe	2.0	5.9
Wild type	*m*-F-Tyr	6.6	22.4

[a] Units: [D-lactate] × 10^{-4} M.
[b] Units: mol MTT/min/mg protein × 10^{-5}.
[c] From Peersen et al. (1990).

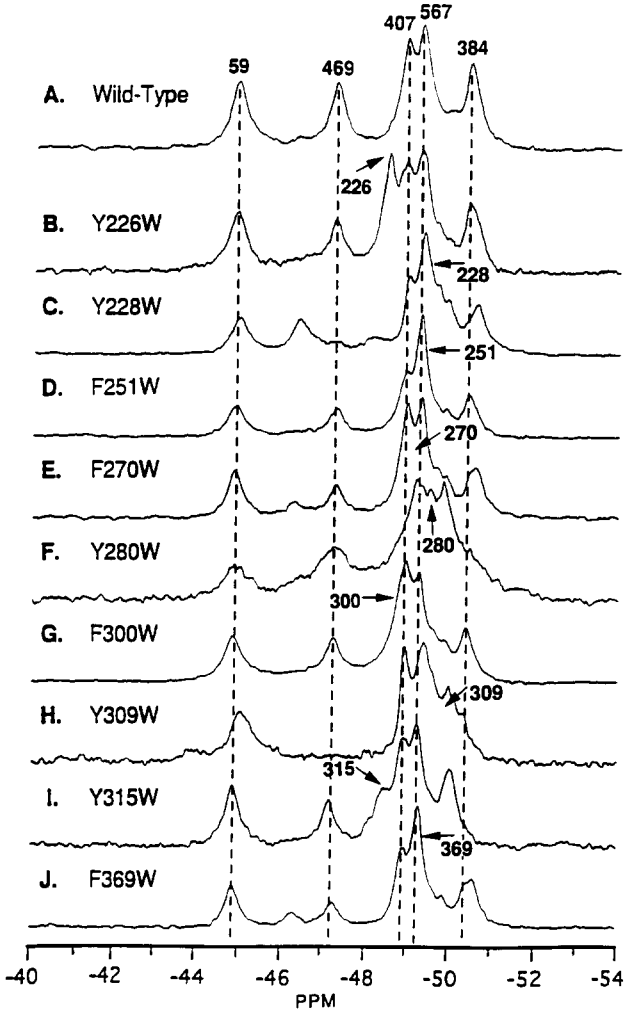

Fig. 1. The 282.4-MHz ^{19}F-NMR spectra (un-normalized) of ~1 mM 5-fluorotryptophan (5F-Trp)-labeled wild-type and mutant D-lactate dehydrogenases (D-LDHs) in 10 mM phosphate buffer, 0.2 mM EDTA, pH 7.2, with 100 mM lysophosphatidylcholine (lysoPC): (**A**) wild-type D-LDH; (**B**) mutant Y226W; (**C**) mutant Y228W; (**D**) mutant F251W; (**E**) mutant F270W; (**F**) mutant Y280W; (**G**) mutant F300W; (**H**) mutant Y309W; (**I**) mutant Y315W; (**J**) mutant F369W. Arrow indicates the approximate position of the resonance from the Trp-substituted residue in each mutant (see text for details).

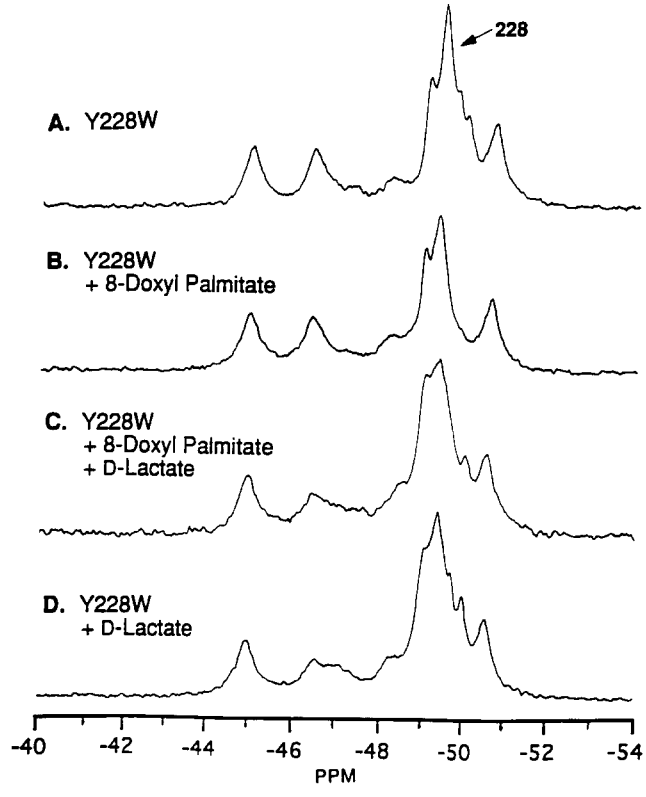

Fig. 2. The 282.4-MHz ^{19}F-NMR spectra of ~1 mM 5F-Trp-labeled Y228W mutant D-LDH in 10 mM phosphate buffer, 0.2 mM EDTA, pH 7.2, with 100 mM lysoPC: (**A**) mutant Y228W; (**B**) mutant Y228W + 28 mM 8-doxyl palmitic acid; (**C**) mutant Y228W + 28 mM 8-doxyl palmitic acid + 100 mM D-lactate; (**D**) mutant Y228W + 100 mM D-lactate only.

−0.2 ppm further downfield. The Trp 226 peak is also found to be shifted downfield in various degrees (−0.1 to −0.2 ppm) in the presence of oxalate, D-lactate, and 8-doxyl palmitic acid. It appears that the Trp 226 residue is exposed to the aqueous phase at the surface of the protein and thus is sensitive to various changes in the solution.

In the ^{19}F-NMR spectrum of the mutant Y228W (Fig. 1), the native Trp 469 peak is shifted from the wild-type position at −47.1 to −46.3 ppm, indicating a change in the local environment. The addition of the substrate D-lactate causes the Trp 469 peak to be broadened and shifted to −46.7 ppm (Fig. 2D), the same as for the wild-type D-LDH (Rule et al., 1987b). The new resonance peak from Trp 228 is unresolved from the Trp 407 and 567 peaks, and although there is change in this region on addition of D-lactate, the results, along with those of SIIS experiments, are not readily interpreted. The spectrum of Y228W in the presence of a nitroxide spin-labeled fatty acid shows that a peak centered around −49.5 ppm is broadened completely (Fig. 2B). Neither Trp 407 nor Trp 567 is in the lipid-binding region in the wild-type protein, and both are partially exposed to solvent (Rule et al., 1987b; Peersen et al., 1990). It is unlikely that either of these two residues in the mutant Y228W would be near the lipid phase because that would necessitate a major change in the conformation that is not detected by ^{19}F-NMR spectroscopy and does not affect the kinetics parameters of the mutant enzyme. Thus, we conclude that the broadened peak is from Trp 228. After the reduction of the nitroxide spin label by adding D-lactate, the lost area is recovered and the spectrum resembles that in the presence of D-lactate only (Fig. 2C,D). Thus, the amino acid residue at position 228 appears to be in the lipid-binding region.

In the ^{19}F-NMR spectrum of the mutant F251W, the new resonance peak from Trp 251 is apparently on top

of the native Trp 567 peak at −49.2 ppm (Fig. 1). There is no change of this peak on addition of D-lactate or a nitroxide spin-labeled fatty acid. An SIIS effect of the Trp 251 resonance is observed when the solvent is changed from D_2O to H_2O buffer. Thus, the amino acid residue at position 251 is not in the lipid-binding region, is not sensitive to substrate binding or oxidation, and is exposed to the aqueous solvent phase.

In the ^{19}F-NMR spectrum of the mutant F270W (Fig. 1), the native Trp 469 resonance splits into two peaks at −46.2 ppm and −47.1 ppm, which then merge into one broadened peak on addition of D-lactate. The new resonance peak arising from Trp 270 is unresolved from the native Trp 407 and 567 peaks, at about −49 ppm. It does not appear to be broadened in the presence of a nitroxide spin-labeled fatty acid or sensitive to substrate addition. Thus, the amino acid residue at position 270 is not in the lipid-binding region.

The ^{19}F-NMR spectrum of the mutant Y280W is perturbed when compared to that of the wild-type D-LDH (Fig. 1), as is typical of many of the temperature-sensitive folding mutants of D-LDH (Truong et al., 1991b). The resonances from the native Trps 59, 469, and 384 are broad, and the region containing the Trp 407 and 567 resonances is unresolved. Therefore, the results of SIIS experiments and substrate addition cannot be interpreted readily. However, on addition of a nitroxide spin-labeled fatty acid, there is a decrease in area centered around −49.6 ppm (Fig. 3A,B), which is reversed when the nitroxide spin label is reduced by the addition of D-lactate (results not shown). Thus, it appears that the new peak from Trp 280 is at −49.6 ppm, and the amino acid residue at position 280 is in the lipid-binding region.

The ^{19}F-NMR spectrum of the mutant F300W has an unresolved peak located beneath the native Trp 407 and 567 peaks (Fig. 1). By spectral subtraction, the position of this additional peak arising from Trp 300 is at −48.8 ppm. This peak is not affected by the presence of a nitroxide spin-labeled fatty acid, by changing the solvent composition, or by the addition of D-lactate. These results indicate that the amino acid residue at position 300 is not close to the lipid region, is not exposed to the solvent, and is not sensitive to substrate binding or oxidation. Thus, the amino acid residue at position 300 appears to be located in the protein core.

The ^{19}F-NMR spectrum of the temperature-sensitive folding mutant Y309W is also perturbed (Fig. 1). The non-native Trp 309 gives rise to a peak between −48.5 and −50.5 ppm, where the resonances from Trp 407 and 567 are also located, and the resonance from Trp 384 is not well resolved from this group. The resonance arising from Trp 469 is missing from its wild-type position. It is apparently divided, part forming the small peak at −43.9 ppm and part under the Trp 59 resonance. On addition of D-lactate, the part at −43.9 ppm disappears, and the Trp 59 peak appears to have a broadened base (results not shown). In the spectra of a number of mutants where the Trp 469 resonance peak is also perturbed, e.g., mutant Y228W, as well as the wild-type D-LDH, the addition of D-lactate results in one broad peak from Trp 469, whereas the resonance from Trp 59 does not appear to be affected (Truong et al., 1991b). Thus, the two parts of the Trp 469 resonance have apparently merged into one single broad peak under the Trp 59 peak. The addition of D-lactate has no effect on other regions of the spectrum. In contrast, the addition of a nitroxide spin-labeled fatty acid results in a partial loss of intensity at approximately −50 ppm (Fig. 3C,D), indicating that one of the residues Trp 309, 407, or 567 is within 15 Å of the nitroxide spin-label. Because neither Trp 407 nor Trp 567 is near the lipid bilayer in the wild-type protein, the amino acid residue that is affected by the lipophilic spin label is most likely the mutated Trp 309. Thus, the amino acid residue at position 309 is not sensitive to addition of substrate and is at least partially exposed to the lipid phase.

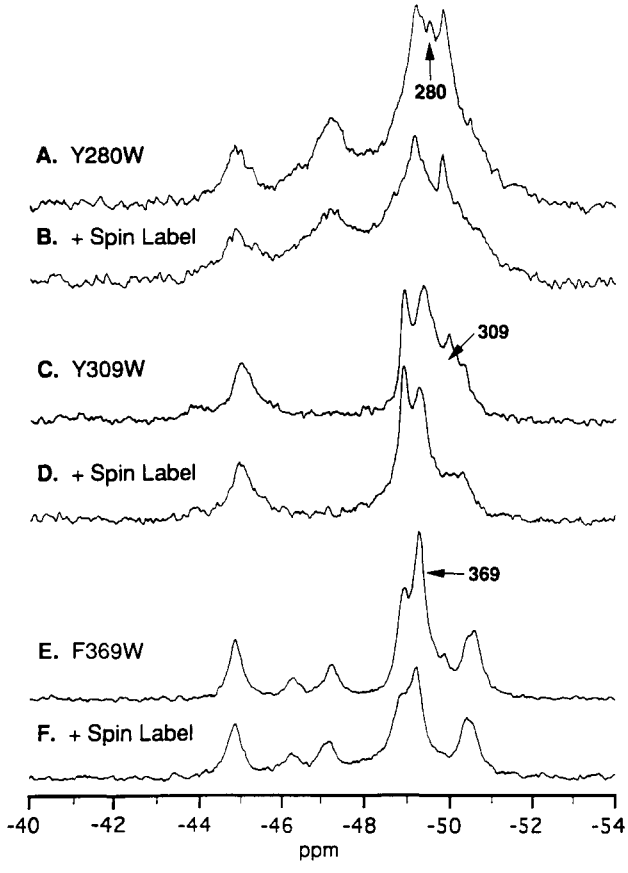

Fig. 3. The 282.4-MHz ^{19}F-NMR spectra of ~1 mM 5F-Trp-labeled mutant D-LDHs in 10 mM phosphate buffer, 0.2 mM EDTA, pH 7.2, with 100 mM lysoPC, in the absence (A, C, E), or presence (B, D, F) of 28 mM 8-doxyl palmitic acid: (**A, B**) mutant Y280W; (**C, D**) mutant Y309W; (**E, F**) mutant F369W.

The ^{19}F-NMR spectrum of the mutant Y315W shows an additional peak at −48.5 ppm that is resolved from

the native Trp peaks (Fig. 1). This peak is not affected significantly by a nitroxide spin-labeled fatty acid, nor does it display any SIIS effect. However, the addition of D-lactate shifts the position of the Trp 315 resonance to −48.2 ppm (Fig. 4). The competitive inhibitor, oxalate ($K_i = 2 \times 10^{-5}$ M), does not have such an effect. Because the K_i of D-LDH for oxalate is less than the K_m for D-lactate, the enzyme binds oxalate more tightly than it does D-lactate. If the shift of the resonance from Trp 315 upon the addition of the substrate were due to a binding effect alone, one would expect oxalate to have the same effect. Thus, Trp 315 must be sensitive to the oxidation state of FAD and not the binding of substrate. In summary, the amino acid residue at position 315 is not exposed to either the lipid phase or the solvent, but is sensitive to the reduction of the FAD cofactor.

In the ^{19}F-NMR spectrum of the mutant F369W (Fig. 1), the native Trp 469 resonance splits into two peaks at −46.25 and −47.2 ppm, which then merge into one broad peak on addition of D-lactate. The new resonance from Trp 369 is located on top of the native Trp 567 peak at −49.3 ppm and does not appear to be sensitive to substrate binding. In the presence of a nitroxide spin-labeled fatty acid, the Trp 369 peak disappears (Fig. 3E,F); it is recovered after the reduction of the nitroxide spin label by adding D-lactate (results not shown). Thus, the amino acid residue at position 369 appears to be in the membrane.

These results from Trp-substitution mutants indicate that the membrane-binding domain includes amino acid residues located between Tyr 228 and Phe 369, but not all residues within this region are located in the membrane. Thus, the course of the polypeptide chain in the membrane-binding region is not in the membrane continuously.

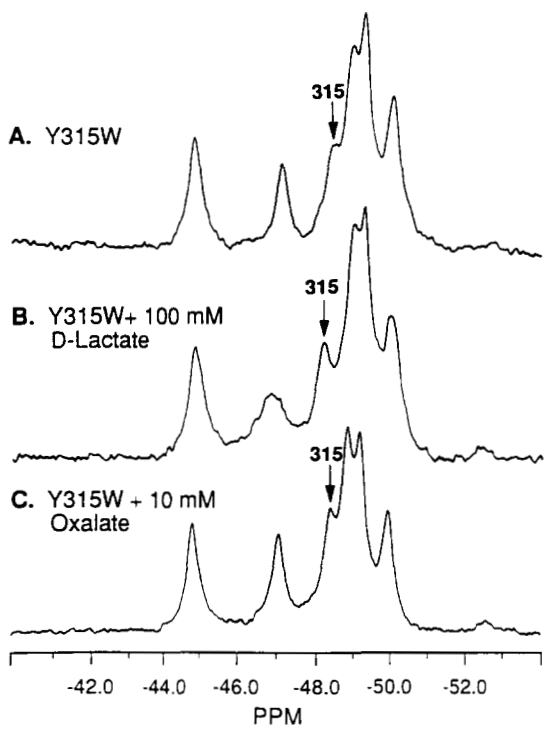

Fig. 4. The 282.4-MHz ^{19}F-NMR spectra of 5F-Trp-labeled mutant Y315W D-LDH in 10 mM phosphate buffer, 0.2 mM EDTA, pH 7.2, with 100 mM lysoPC: **(A)** 0.4 mM mutant Y315W; **(B)** 0.8 mM mutant Y315W + 100 mM D-lactate; **(C)** 0.7 mM mutant Y315W + 50 mM oxalate.

Wild-type D-LDH labeled with F-Phe and F-Tyr

Finding the extent of the lipid-binding region of D-LDH by substituting a Trp for a native residue is a fairly lengthy process, which, if the substitution results in a purifiable enzyme, yields information on only one additional residue. An alternate approach is to label a greater number of hydrophobic residues simultaneously. The size of the D-LDH membrane-binding region can then be gauged by the number of amino acid residues affected by the lipid probe.

D-Lactate dehydrogenase can be labeled with *o*-, *m*-, or *p*-fluorophenylalanine (F-Phe), or *m*-fluorotyrosine (F-Tyr); the resulting enzyme is active (Table 1; Rule et al., 1987a). There are 26 native Phe and 23 native Tyr residues in the wild-type enzyme (Rule et al., 1985). The ^{19}F-NMR spectra of the *o*-, *m*-, and *p*-F-Phe-labeled D-LDH are complex (Fig. 5) and very few of the resonances are resolved. In the presence of a nitroxide spin-labeled fatty acid, the spectra are changed dramatically, due to the broadening of the peaks arising from Phe residues that are located within 15 Å of the nitroxide spin label. Most of the peaks that are broadened resonate in the same region of the spectra, indicating that the Phe residues that are affected by the lipophilic spin label are in similar environments. The ^{19}F-NMR spectra were also obtained of *m*-F-Tyr-labeled D-LDH. The resonances arising from the 23 F-Tyr residues are even less resolved than the peaks from the 26 F-Phe residues (Fig. 6). Estimates of the number of Phe or Tyr residues broadened by the lipophilic spin label were reached by comparing the areas of the spectra in the presence and absence of a nitroxide spin-labeled fatty acid (Table 2). From these estimates, it appears that 9–10 Phe and 3–4 Tyr residues are located near the lipid phase.

Discussion

The ^{19}F-NMR spectroscopy studies of D-LDH proteins labeled with fluorinated native and substituted tryptophan residues provide information about the local environment at these sites that is otherwise unavailable. To minimize the perturbation to the protein structure caused by the tryptophan substitution, our studies have been focused on replacing the amino acid residues of phenylalanine and tyrosine, of which the hydrophobicity and total

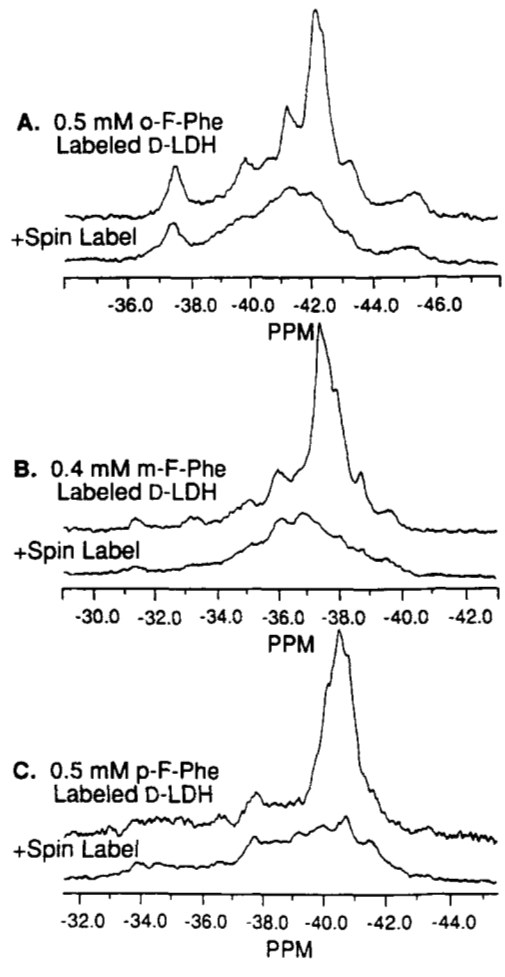

Fig. 5. The 282.4-MHz ^{19}F-NMR spectra of F-Phe-labeled wild-type D-LDHs in 10 mM phosphate buffer, 0.2 mM EDTA, pH 7.2, with 100 mM lysoPC, in the absence (top) or presence (bottom) of 28 mM 8-doxyl palmitic acid: **(A)** 0.5 mM *o*-F-Phe-labeled D-LDH; **(B)** 0.4 mM *m*-F-Phe-labeled D-LDH; **(C)** 0.5 mM *p*-F-Phe-labeled D-LDH.

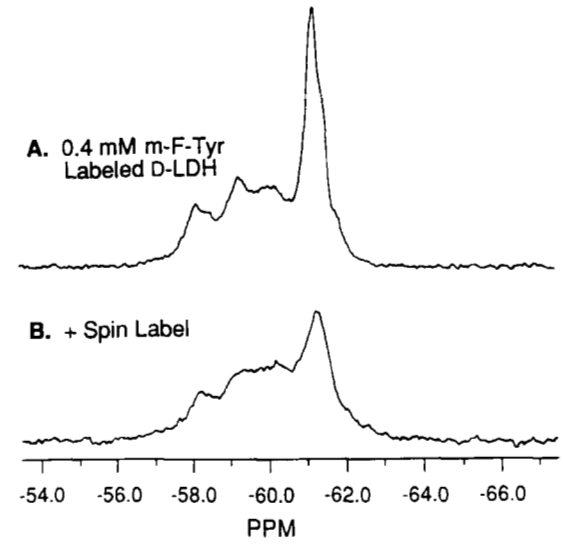

Fig. 6. The 282.4-MHz ^{19}F-NMR spectra of 0.4 mM *m*-F-Tyr-labeled wild-type D-LDH in 10 mM phosphate buffer, 0.2 mM EDTA, pH 7.2, with 100 mM lysoPC, in the absence **(A)** or presence **(B)** of 28 mM 8-doxyl palmitic acid.

buried volume are comparable to those of tryptophan (Chothia, 1975; Matsumura et al., 1988). Although in some cases, tryptophan substitutions in the protein core or at some key positions may significantly destabilize the protein or perturb the folding pathway, yielding inactive mutants (Peersen et al., 1990; Truong et al., 1991b), the mutants studied in this paper are all active, and have kinetics properties close to those of the wild-type D-LDH. The native tryptophans in general are not disturbed by the mutations, as shown by the chemical-shift values of the native 5F-Trp resonances in the ^{19}F-NMR spectra (Fig. 1), with the exception of Trp 469. The resonance from Trp 469 has been found at the wild-type position at −47.1 ppm, and a second position at −46.3 ppm, in the mutants Y228W, F270W, and F369W. When the substrate D-lactate is added, the Trp 469 resonance becomes a single broadened peak at −46.7 ppm, which may be a result of exchanging of the two conformations (Rule et al., 1987b; Peersen et al., 1990); when an inhibitor, oxalate, is added, the Trp 469 resonance becomes a single peak at the wild-type position at −47.1 ppm (results not shown). Thus, it appears that (in these three mutants) the changes of the Trp 469 resonance are not caused directly by the Trp-substitution but rather the perturbation to the protein-folding pathway. These results suggest a preservation of the global functional structure in these Trp-substituted D-LDH mutants.

We have chosen 8-doxyl palmitic acid as the lipid probe in this work. Because the doxyl group is in the middle, about 7 Å to either end of the fatty acid molecule, the fluorinated residue has to be in or near the lipid phase to be affected by the nitroxide spin label. These residues are thus likely to be located near or at the surface of the protein and would have more tolerance toward the tryptophan substitution than the residues located in the core

Table 2. *Estimate of the number of amino acid residues in F-Phe- and F-Tyr-labeled wild-type D-LDH that are broadened by spin-labeled fatty acid*[a]

Label	A_1, area without spin label, preset	A_2, area with spin label	$A_1 - A_2$
o-F-Phe	26.0	16.7	9.3
m-F-Phe	26.0	17.0	9.0
p-F-Phe	26.0	16.5	9.5
F-Tyr	23.0	19.5	3.5

[a] Areas were measured by integration. The areas of the spectra without addition of spin-labeled fatty acid were set to be 26 for F-Phe and 23 for F-Tyr, the number of Phe or Tyr residues in wild-type D-LDH.

(Alber, 1989). In ^{19}F-NMR experiments with 5F-Trp-labeled mutant D-LDHs containing these residues, the peak broadened in the presence of a nitroxide spin-labeled fatty acid is recovered when the lipophilic spin label is reduced by adding D-lactate (Truong et al., 1991a), or a reducing agent, ascorbate (results not shown). This indicates a specific interaction between the nitroxide spin label and the affected fluorinated residue.

In the ^{19}F-NMR spectra of 5F-Trp-labeled wild-type and mutant D-LDHs, small peaks at −49.6 and −49.9 ppm have been observed (Fig. 1), the intensities of which increase with the aging of the D-LDH samples. The ^{19}F-NMR spectrum of wild-type D-LDH denatured by 0.66 M guanidine hydrochloride shows four peaks at −48.9, −49.2, −49.6, and −49.9 ppm (results not shown). Thus, we believe that the small peaks at −49.6 and −49.9 ppm observed in the spectra shown in Figure 1 are from a small amount of denatured D-LDH protein.

The 9–10 Phe residues, the resonances of which are broadened in the presence of a nitroxide spin-labeled fatty acid, represent more than a third of the number of Phe in D-LDH. This is surprising in view of our previous results showing that the membrane-binding region is small, based on the number of lysolecithin molecules (40–60) that bind to D-LDH (Rule et al., 1987b), and that only a small portion of D-LDH appears to reside in the membrane-binding domain, based on the studies of the Trp-substitution mutants (Peersen et al., 1990). Figure 7 shows the distribution of the 26 Phe and 23 Tyr residues in the sequence of D-LDH, as well as the distribution and properties of the native Trp and substituted Trp residues that we have studied (Rule et al., 1987b; Peersen et al., 1990). It is apparent that the Phe residues are not distributed evenly. There are 14 Phe residues in the region from 226 to 384, which our present and previous results suggest to be the limits of the membrane-binding region. Phe 340, 361, 369, and Tyr 280 have been shown to be in the membrane because the Trp-substituted residues at these sites are affected by the lipophilic spin label. There are three Phe residues that are within two residues of these four locations, at positions 339, 341, and 279; along with Phe 340, 361, and 369, these account for a total of six Phe residues inside the putative membrane-binding region that would likely be affected by the lipophilic spin label. The residues of

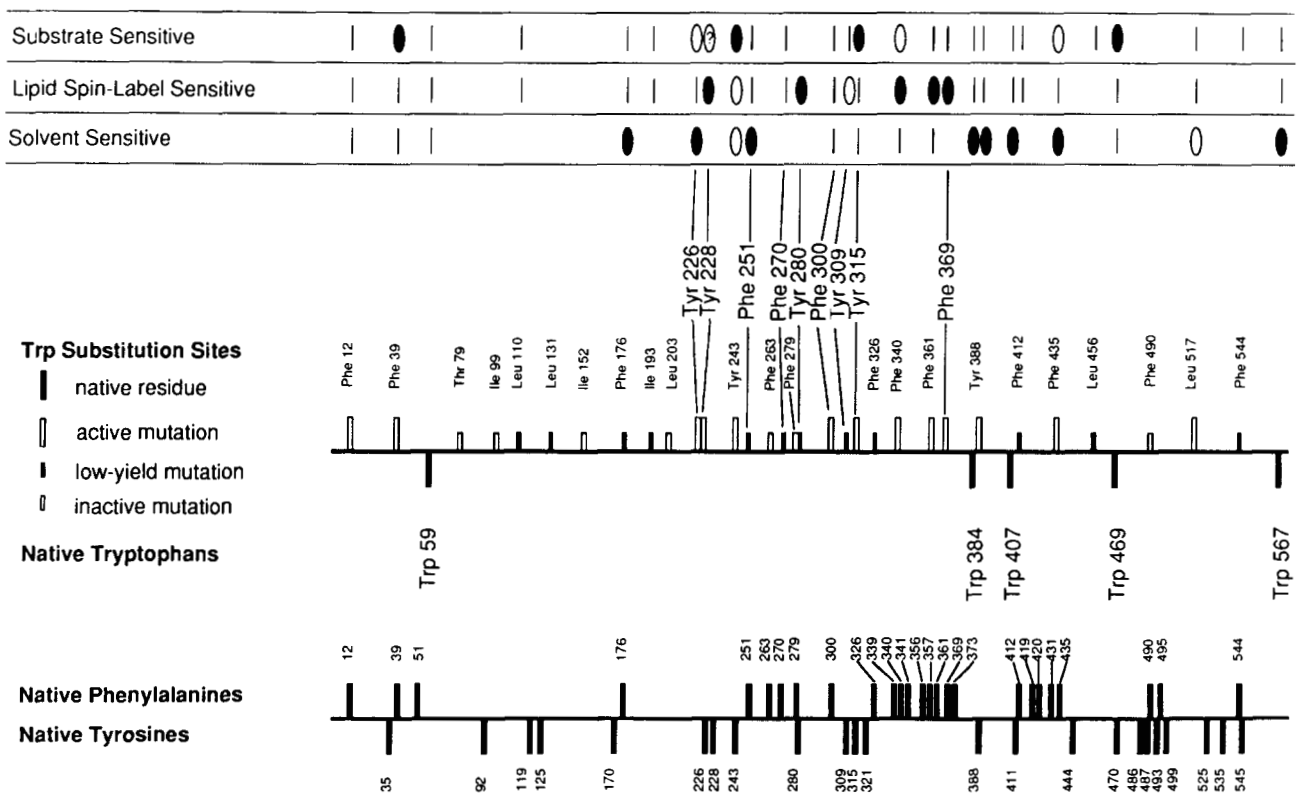

Fig. 7. Distribution of Phe, Tyr, and Trp in wild-type D-LDH and a summary of results from ^{19}F-NMR observations of 5F-Trp-labeled wild-type D-LDH and D-LDHs containing a Trp-substitution mutation. In general, open ovals indicate observed, but small, effects, and filled ovals indicate major effects. Substrate sensitivity: (0) 20–50% broadening, or ≤0.2 ppm shift; (●) >50% broadening, or >0.2 ppm shift. Spin label sensitivity: (0) slight broadening; (●) loss of resonance from spectrum. Solvent sensitivity: (0) exposure index ≤0.25; (●) index >0.25 (index of 1.0 reflects shift of free 5F-Trp in solution). A vertical line indicates that no effect was seen.

Phe 251, 270, and 300 are ruled out by the Trp-substitution mutant studies. The remaining 3–4 Phe residues that are affected by the lipophilic spin label could conceivably be Phe 356 and 357 (5 and 4 residues from Phe 361), Phe 326 (14 residues from Phe 340, 4 residues from Tyr 321 [see below]), Phe 373 (4 residues from Phe 369), or less likely, Phe 263 (between Phe 251 and 270).

The results from the *m*-F-Tyr-labeled D-LDH give further clarification on the extent of the membrane-binding region. Resonances from only 3–4 Tyr (out of 23 Tyr) residues are broadened in the presence of a nitroxide spin-labeled fatty acid, confirming that a small region of the D-LDH molecule is involved in binding to the membrane. There are six Tyr residues located within the limits of the putative membrane-binding region from 226 to 384, namely Tyr 228, 243, 280, 309, 315, and 321. Of these, three to four are presumably in or near the lipid phase. From the results with the Trp-substitution mutants discussed above, the resonances from Tyr 228 and 280 are apparently affected by the lipophilic spin label, whereas that from Tyr 315 is not. In the 5F-Trp-labeled mutants Y243W and Y309W, the resonances arising from Trp 243 and Trp 309 are only partially broadened by the lipophilic spin label, i.e., the line width of the resonance is larger in the presence of a nitroxide spin-labeled fatty acid, but the peak is not broadened beyond detection (Peersen et al., 1990; Fig. 3). Thus, the resonances from Tyr 243 and 309 are unlikely to make significant contributions to the area change in the spectra of the *m*-F-Tyr-labeled D-LDH. It appears that three Tyr residues, the resonances of which are completely broadened by the lipophilic spin label, could be accounted for by Tyr 228, 280, and 321.

Based on the experimental results and inferences derived from them, we can construct a schematic model for the path of the polypeptide chain of D-LDH between amino acid residues at positions 226 and 384 (Fig. 8). Tyr 226 is at the surface of the enzyme molecule and is completely exposed to the solvent; it is followed by Tyr 228, which is in the membrane. The polypeptide chain remains at the surface of the protein so that Tyr 243 is near the water–membrane interface, and Phe 251 is exposed to the solvent. Tyr 243 may also lie close to the flavin cofactor because upon reduction of the FAD, the resonance arising from the 5F-Trp-substituted residue is broadened and exhibits a large shift (Peersen et al., 1990). The polypeptide chain stays away from the membrane for a certain length (Phe 251, 270), then winds back into the membrane, so that Phe 279 and Tyr 280 are near or in the lipid phase, then goes into the core of the protein (Phe 300). Next, a short dip into the membrane (Tyr 309) is followed by another loop away from the bilayer (Tyr 315), then by a long stretch in the membrane (Tyr 321 and Phe 339, 340, 341, 356, 357, 361, and 369). Finally, the polypeptide chain emerges from the membrane, remaining on the surface of the enzyme molecule in the aqueous phase (amino acid residues 384, 388, 407, and 435).

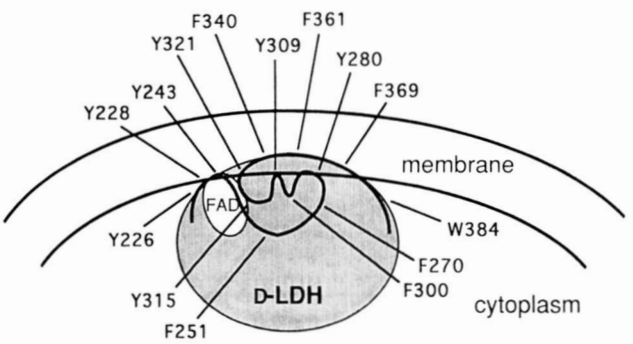

Fig. 8. A schematic model for the path of the polypeptide chain in the membrane-binding region of D-LDH.

It is important to note that our model for the membrane-binding region is consistent with the experimental results, but still needs fine adjustment. Our goal is to obtain a "low-resolution" picture of the protein–lipid interface, in order to help understand the protein–membrane interaction and its role in the enzymatic reaction. Thus, our present results can help us design further experiments to understand the molecular basis of the action of D-LDH.

In summary, the use of site-directed mutagenesis, fluorine-labeled protein, and ^{19}F-NMR has allowed us to propose and delimit a putative membrane-binding region in D-LDH. The membrane-binding region appears to be in the area including Tyr 228 and Phe 369, but not extending much beyond either 228 or 369. The path of the polypeptide chain between these amino acid residues is not continuously in the membrane, suggesting that D-LDH is anchored in the membrane by several separated structural elements.

Materials and methods

Site-directed mutagenesis and expression of 5F-Trp-(Sigma) labeled mutant D-LDHs have been described previously (Rule et al., 1987a,b; Peersen et al., 1990). The F-Phe- and F-Tyr-labeled wild-type D-LDHs were prepared in the same manner as 5F-Trp-labeled enzyme with the following exceptions: The strains used were *E. coli* KA197 (*thi*-1, *phe*A97, *rel*Al, λ^-, *spo*T_1) supplied by Dr. Barbara J. Bachmann (EGSC, New Haven, Connecticut) for F-Phe labeling; and JB17019, from this laboratory, a general aromatic negative strain, for F-Tyr labeling. Instead of casamino acids, the growth medium contained a mixture of amino acids: alanine, 2 g; arginine, 2.4 g; asparagine, 4 g; aspartic acid, 1 g; cysteine, 0.5 g; glutamic acid, 3 g; histidine, 0.62 g; isoleucine, 2.5 g; leucine, 2.5 g; lysine, 2.5 g; methionine, 1 g; phenylalanine, 1 g; proline, 1 g; serine, 0.5 g; threonine, 2 g; tryptophan,

0.4 g; tyrosine, 1 g; valine, 2.5 g/L. The amino acid to be labeled (i.e., tyrosine or phenylalanine) was used at 42 mg/L. After induction, 37 mg/L of the labeled amino acid (*m*-fluorotyrosine, *o*-, *p*-, or *m*-fluorophenylalanine, all from Sigma) were added.

The Michaelis-Menten parameters, K_m and V_{max}, of the purified 5F-Trp-labeled substitution mutants and F-Phe- and F-Tyr-labeled wild-type D-LDHs were determined by Lineweaver-Burk plots using the phenazine methosulfate and 3-(3,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay system (Pratt et al., 1979) at 25 °C. Substrate concentrations varied from 0.02 to 10.0 mM D-lactate, and the protein concentrations were determined by the Bradford assay (Bradford, 1976), with bovine serum albumin as the standard.

The D-LDH-containing fractions were prepared for NMR as described previously (Rule et al., 1987b; Peersen et al., 1990). The orientation and conformation of the D-LDH molecule in egg lysophosphatidylcholine (lysoPC, Sigma) is similar to its orientation when interacting with a phospholipid bilayer, as determined by ^{19}F-NMR spectroscopy (Truong et al., 1991a). The lysoPC (20 mg) was dissolved with 0.5–1 mM purified D-LDH in 400 μL D_2O buffer (10 mM potassium phosphate, 0.2 mM EDTA, pH 7.2), and the particulates were removed by centrifugation before the sample was placed into a 5-mm NMR tube. The effect of substrate was tested by adding 40 μL of 1 M D-lactate (lithium salt) in D_2O buffer to a 400-μL sample containing lysoPC and purging with nitrogen to minimize reoxidation of D-LDH. Under these conditions, D-lactate is in 100–200-fold excess and would be expected to saturate the 5F-Trp-labeled enzyme.

In the SIIS experiment, a 400-μL sample of D-LDH in D_2O buffer was placed in a Centricon-30 filter cone and exchanged against 1 mL H_2O buffer four times, with a final dilution estimated at 100–200-fold. The concentrated solution was then brought up to 400 μL with the same H_2O buffer, with 5% D_2O buffer for the NMR lock signal.

The nitroxide spin-labeled fatty acid, 8-doxyl palmitic acid, was incorporated into the lysoPC by mixing 4 mg of 8-doxyl palmitic acid (in $CHCl_3$) with 20 mg lysoPC and adding methanol until the solution cleared. The solvent was then evaporated with nitrogen gas, and the lipid was dried under vacuum for 12–24 h. A sample of D-LDH (400 μL) was added directly to the dried film to solubilize the lipid; the sample was centrifuged before being placed in a 5-mm NMR tube. For some labeled D-LDH mutants in scarce supply, the enzyme was first mixed with lysoPC. Following acquisition of the NMR spectrum, the sample was transferred to a tube containing dried spin-labeled fatty acid, incubated at 37 °C until all the solid had dissolved, and transferred to a new 5-mm NMR tube for acquisition of the spectrum of mutant D-LDH in the presence of spin-labeled fatty acid. Samples of wild-type D-LDH and D-LDH mutants studied previously were treated in the same way and gave spectra identical to those obtained by the usual method.

The ^{19}F-NMR measurements were performed with a 5-mm ^{19}F probe on Bruker WH-300 and AM-300 spectrometers operating at 282.4 MHz. The ^{19}F-NMR spectra were obtained with an 8-kHz spectral width and 4K data points. Free induction decays (10,000–25,000) were accumulated and Fourier transformed with 15-Hz line-broadening, and the resulting spectra were corrected for phase and baseline distortions. The ^{19}F-NMR chemical shifts are expressed relative to trifluoroacetic acid (TFA). The ^{19}F-NMR spectra of 5F-Trp-, *m*-F-Phe- and *o*-F-Phe-labeled D-LDHs were acquired with a 4.4-μs 60° pulse and a relaxation delay of 4 s, and those of D-LDH labeled with *p*-F-Phe with a 6.6-μs 90° pulse and an 8-s relaxation delay to ensure full relaxation of the resonances. The spectra of *m*-F-Phe-labeled D-LDH were also obtained under the latter conditions; there were no differences in the areas of the spectra. For the 5F-Trp-labeled D-LDHs, the spectra were normalized by setting the area of the native Trp 59 peak to be equal. For the F-Phe-labeled D-LDHs, the areas of the spectra in the presence of a nitroxide spin-labeled fatty acid were normalized by setting the area of an unaffected region of the spectrum equal to that of the corresponding region in the control spectrum. The areas of other unaffected regions were compared as an additional control.

The samples of *m*-F-Tyr-labeled D-LDH contained 2 mM *m*-F-Phe as an internal standard. The spin-lattice relaxation rates (T_1^{-1}) of the different resonances were determined in the absence and presence of 8-doxyl palmitic acid using a saturation-recovery pulse sequence. Acquisitions were taken with a 90°-pulse and a 4-s relaxation delay, under which conditions all resonances from the F-Tyr-labeled D-LDH are fully relaxed. With the same acquisition parameters, the resonance arising from the internal standard is fully relaxed in the presence of the spin-labeled fatty acid ($T_1 < 0.2$ s), but not in its absence ($T_1 \approx 2.4$ s). To correct for the difference in relaxation rates, the spectrum of the internal standard was also acquired with a 90° pulse and an 8-s delay. The areas of F-Tyr-labeled D-LDH resonances were compared by normalizing to the peak height of the internal standard, after an appropriate correction for the difference in relaxation rates.

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References

Alber, T. (1989). Mutational effects on protein stability. *Annu. Rev. Biochem. 58*, 765–798.

Arseniev, A.S., Utkin, Yu.N., Pashkov, V.S., Tsetlin, V.I., Ivanov, V.T., Bystrov, V.F., & Ovchinnikov, Yu.A. (1981). ^{19}F-NMR determination of intramolecular distances in spin- and fluorine-labeled proteins. *FEBS Lett. 136*, 269–274.

Barnes, E.M., Jr. & Kaback, H.R. (1971). Mechanisms of active transport in isolated membrane vesicles. I. The site of energy coupling between D-lactic dehydrogenase and β-galactoside transport in *Escherichia coli* membrane vesicles. *J. Biol. Chem. 246*, 5518–5522.

Bradford, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem. 72*, 248–254.

Chothia, C. (1975). Structural invariants in protein folding. *Nature 254*, 304–308.

Cronan, J.E., Gennis, R.B., & Malloy, S.R. (1987). Cytoplasmic membrane. In *Escherichia coli and Salmonella typhimurium: Cellular and Molecular Biology* (Neidhardt, F.C., Ingraham, J.L., Low, K.B., Magasanik, B., & Umbarger, H.E., Eds.), pp. 31–55. American Society for Microbiology, Washington, D.C.

Fasman, G.D. (1989). The development of prediction of protein structure. In *Prediction of Protein Structure and the Principles of Protein Conformation* (Fasman, G.D., Ed.), pp. 193–316. Plenum Press, New York.

Fung, L.W.-M., Pratt, E.A., & Ho, C. (1979). Biophysical and biochemical studies on the interaction of a membrane-bound enzyme, D-lactate dehydrogenase of *Escherichia coli*, with phospholipids. *Biochemistry 18*, 317–324.

Futai, M. (1973). Membrane D-lactate dehydrogenase from *Escherichia coli*. Purification and properties. *Biochemistry 12*, 2468–2474.

Futai, M. (1974). Reconstitution of transport dependent on D-lactate or glycerol 3-phosphate in membrane vesicles of *Escherichia coli* deficient in the corresponding dehydrogenases. *Biochemistry 13*, 2327–2333.

Ho, C., Pratt, E.A., & Rule, G.S. (1989). Membrane-bound D-lactate dehydrogenase of *Escherichia coli*: A model for protein interactions in membranes. *Biochim. Biophys. Acta 988*, 173–184.

Ho, C., Rule, G.S., Pratt, E.A., Wu, C.-S., & Yang, J.T. (1988). Structural and dynamic studies of membrane-bound D-lactate dehydrogenase of *Escherichia coli*. In *Structure and Expression*, Vol. 1: *From Proteins to Ribosomes* (Sarma, M.H. & Sarma, R.H., Eds.), pp. 35–48. Adenine Press, Guilderland, New York.

Kohn, L.D. & Kaback, H.R. (1973). Mechanisms of active transport in isolated bacterial membrane vesicles. XV. Purification and properties of the membrane-bound D-lactate dehydrogenase from *Escherichia coli*. *J. Biol. Chem. 248*, 7012–7017.

Kovatchev, S., Vaz, W.L.C., & Eibl, H. (1981). Lipid dependence of the membrane-bound D-lactate dehydrogenase of *Escherichia coli*. *J. Biol. Chem. 256*, 10369–10374.

Matsumura, M., Becktel, W.J., & Matthews, B.W. (1988). Hydrophobic stabilization in T4 lysozyme determined directly by multiple substitutions of Ile 3. *Nature 334*, 406–410.

Peersen, O., Pratt, E.A., Truong, H.-T.N., Ho, C., & Rule, G.S. (1990). Site-specific incorporation of 5-fluorotryptophan as a probe of the structure and function of the membrane-bound D-lactate dehydrogenase of *Escherichia coli*: A ^{19}F nuclear magnetic resonance study. *Biochemistry 29*, 3256–3262.

Pratt, E.A., Fung, L.W.-M., Flowers, J.A., & Ho, C. (1979). Membrane-bound D-lactate dehydrogenase of *Escherichia coli*: Purification and properties. *Biochemistry 18*, 312–316.

Reeves, J.P., Hong, J.-S., & Kaback, H.R. (1973). Reconstitution of D-lactate dependent transport in membrane vesicles from a D-lactate dehydrogenase mutant of *Escherichia coli*. *Proc. Natl. Acad. Sci. USA 70*, 1917–1921.

Rule, G.S., Pratt, E.A., Chin, C.C.Q., Wold, F., & Ho, C. (1985). Overproduction and nucleotide sequence of the respiratory D-lactate dehydrogenase of *Escherichia coli*. *J. Bacteriol. 161*, 1059–1068.

Rule, G.S., Pratt, E.A., & Ho, C. (1987a). NMR, biochemical, and molecular genetics studies of the membrane-bound D-lactate dehydrogenase of *Escherichia coli*. In *Membrane Proteins* (Goheen, S.C., Ed.), pp. 3–20. Bio-Rad Laboratories, Richmond, California.

Rule, G.S., Pratt, E.A., Simplaceanu, V., & Ho, C. (1987b). Nuclear magnetic resonance and molecular genetic studies of the membrane-bound D-lactate dehydrogenase of *Escherichia coli*. *Biochemistry 26*, 549–556.

Short, S.A., Kaback, H.R., & Kohn, L.D. (1974). D-lactate dehydrogenase binding in *Escherichia coli dld*$^-$ membrane vesicles reconstituted for active transport. *Proc. Natl. Acad. Sci. USA 71*, 1461–1465.

Steck, T.L. & Yu, J. (1973). Selective solubilization of proteins from red blood cell membranes by protein perturbants. *J. Supramol. Struct. 1*, 220–248.

Tanaka, Y., Anraku, Y., & Futai, M. (1976). *Escherichia coli* membrane D-lactate dehydrogenase. Isolation of the enzyme in aggregated form and its activation by Triton X-100 and phospholipids. *J. Biochem. 80*, 821–830.

Truong, H.-T.N., Pratt, E.A., & Ho, C. (1991a). 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. *Biochemistry 30*, 3893–3898.

Truong, H.-T.N., Pratt, E.A., Rule, G.S., Hsue, P.Y.N., & Ho, C. (1991b). Inactive and temperature-sensitive folding mutants generated by tryptophan substitutions in the membrane-bound D-lactate dehydrogenase of *Escherichia coli*. *Biochemistry 30*, 10722–10729.