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†

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ABSTRACT: The structure and function of the membrane-bound D-lactate dehydrogenase of *Escherichia coli* have been investigated by fluorine-19 nuclear magnetic resonance spectroscopy of 5-fluorotryptophan-labeled enzyme in conjunction with oligonucleotide-directed, site-specific mutagenesis. 5-Fluorotryptophan has been substituted for nine phenylalanine, tyrosine, and leucine residues in the enzyme molecule without loss of activity. The ^{19}F signals from these additional tryptophan residues have been used as markers for sensitivity to substrate, exposure to aqueous solvent, and proximity to a lipid-bound spin-label. The nuclear magnetic resonance data show that two mutational sites, at amino acid residues 340 and 361, are near the lipid environment used to stabilize the enzyme. There are a number of amino acid residues on the carboxyl side of this region that are strongly sensitive to the aqueous solvent. The environment of the wild-type tryptophan residue at position 469 changes as a result of two of the substitution mutations, suggesting some amino acid residue–residue interactions. Secondary structure prediction methods indicate a possible binding site for the flavin adenine dinucleotide cofactor in the carboxyl end of the enzyme molecule. These results suggest that the membrane-bound D-lactate dehydrogenase 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. This type of three-domain structure may be of general significance for understanding the structure of membrane-bound proteins which do not traverse the lipid bilayer of membranes.

Membrane-bound D-lactate dehydrogenase (D-LDH)[1] from *Escherichia coli* is an enzyme with a molecular weight of 65 000 which, on the basis of immunological evidence, is located on the cytoplasmic face of the cell membrane (Futai & Tanaka, 1975; Short et al., 1975). D-LDH converts D-lactate to pyruvate with a concomitant reduction of the flavin adenine dinucleotide (FAD) cofactor and donates the resulting electron to the respiratory electron-transport chain where it may be used to provide energy for active transport (Barnes & Kaback, 1971; Futai, 1973; Kohn & Kaback, 1973). The addition of purified enzyme can restore transport of sugars and amino acids across D-LDH-deficient *E. coli* membrane vesicles (Futai, 1974; Short et al., 1974). Purified D-LDH requires a lipid-like environment to prevent aggregation, and various lipids and detergents enhance its stability and activity (Tanaka et al., 1976; Fung et al., 1979; Kovatchev et al., 1981; Rule et al., 1987a). The D-LDH gene has been cloned and sequenced (Young et al., 1982; Campbell et al., 1984; Rule et al., 1985), and the inferred 571-residue peptide sequence has been confirmed by amino acid analysis and amino-terminal sequence analysis (Rule et al., 1985).

The amino acid sequence of D-LDH provides few structural clues about the enzyme molecule (Ho et al., 1988), and crystallographic data are not available. D-LDH has no large hydrophobic sequences; a secondary-structure analysis does not predict any likely transmembrane helices, suggesting that D-LDH does not span the lipid bilayer (Ho et al., 1988). However, the requirement for a lipid environment to solubilize D-LDH indicates that the enzyme is not an electrostatically bound peripheral membrane protein.

In an effort to gain information about the structure and function of D-LDH, we have incorporated 5-fluorotryptophan (5F-Trp) into the enzyme molecule and studied it with ^{19}F nuclear magnetic resonance (NMR). There are five tryptophan residues in D-LDH (Campbell et al., 1984; Rule et al., 1985). When 5F-Trp is incorporated into D-LDH, the ^{19}F NMR spectrum contains five distinct resonances, which have been assigned to specific tryptophan residues by site-specific mutagenesis (Rule et al., 1987a). The labeled D-LDH retains activity (Pratt et al., 1983), and the resulting ^{19}F resonances can then be used as indicators of localized sensitivity to substrate, exposure to aqueous solvent, and proximity to lipid interaction sites on the enzyme molecule. Previous work from this laboratory has shown that only one wild-type resonance,

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[1] Abbreviations: D-LDH, D-lactate dehydrogenase; 5F-Trp, 5-fluorotryptophan; NMR, nuclear magnetic resonance; DMPC, dimyristoylphosphatidylcholine; FAD, flavin adenine dinucleotide; NAD, nicotinamide adenine dinucleotide; NOE, nuclear Overhauser effect; lysoPC, L-α-lysophosphatidylcholine; SIIS, solvent-induced isotopic shift; PMS, phenazine methosulfate; MTT, 3-(3,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; TFA, trifluoroacetic acid.

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from Trp 469, is sensitive to the addition of substrate, while two resonances, from Trps 384 and 567, are sensitive to the exchange of H_2O for D_2O in the buffer, indicating exposure to solvent (Rule et al., 1987a). The use of a lipophilic paramagnetic broadening agent has determined that none of the five wild-type Trps is within 10 Å of the lysophosphatidylcholine (lysoPC) lipid phase used to solubilize the enzyme.

Determination of the topology of D-LDH is necessary for a deeper understanding of the function of the enzyme. Our initial studies with the five native Trps failed to define the membrane-binding region of D-LDH. Consequently, we sought to incorporate additional Trps with the minimal goal of defining the membrane-binding domain. We mutated 22 hydrophobic residues, which are predictd to be located in either hydrophobic areas or amphipathic structures and which are spaced throughout the D-LDH molecule (Rule et al., 1987b; Ho et al., 1988). Of the 22 mutants, 9 are overexpressed in *E. coli* without significant loss of D-LDH activity. The ^{19}F resonances from these new 5F-Trps are characterized in the same manner as the wild-type tryptophans. Thus, the new 5F-Trps become reporters or probes of structural and functional roles in areas of the enzyme molecule not accessible with the wild-type Trps.

Materials and Methods

Site-Directed Mutagenesis. The oligonucleotide-directed, site-specific mutagenesis was performed by the procedure of Kunkel (1985) as described in our previous work (Rule et al., 1987a). The oligonucleotides were either synthesized manually by the phosphoramidite method on a solid support in our laboratory or purchased from the DNA Synthesis Facility at the University of Pittsburgh. To rule out the presence of more than one mutation site in each mutagenized gene, we have sequenced the entire D-LDH coding region of each mutant and found no other alterations in the gene.

Expression of 5F-Trp-Labeled D-LDH. The gene coding for D-LDH has been placed under the control of the phage λ P_L promoter, allowing us to heat induce D-LDH expression in *E. coli* strain W3110*trp*A33, a tryptophan auxotroph. Cells were induced in a 3-L high-density fermentor (LAB-Line/S.M.S., Model 29500) by raising the temperature over a period of 1 h. 5F-Trp was added, and the cells were allowed to grow for an additional 6 h after the start of induction, producing 75–100 mg of D-LDH. The enzyme was purified [for a complete protocol, see Rule et al. (1985 and 1987a)], exchanged into a D_2O buffer containing 0.2 mM EDTA and 10 mM potassium phosphate at pH 7.2, concentrated to ~1 mM enzyme with Amicon ultrafiltration cones, and frozen at −80 °C as 400-μL samples.

Kinetic Studies. The Michaelis–Menten parameters K_m and V_{max} of the Trp-substitution mutations were determined by Lineweaver–Burk plots with the PMS–MTT assay system (Pratt et al., 1979) at 20 °C. Substrate concentrations varied from 10 μM to 0.5 mM D-lactate, and the protein concentrations were determined according to the Bradford (1976) assay with bovine serum albumin as a standard.

Reconstitution of Oxidase Activity. Reconstitution of oxidase activity of D-LDH-deficient membrane vesicles was measured as previously described (Pratt et al., 1983).

NMR Sample Preparation. Twenty milligrams of lysoPC (purchased from Sigma) was solubilized with ~1 mM purified D-LDH in 400 μL of D_2O buffer, and particulates were removed by centrifugation before the ^{19}F NMR spectra were acquired.

Substrate sensitivity was tested by adding 44 μL of 1 M D-lactate in D_2O buffer to a 400-μL sample with lysoPC and purging with nitrogen to minimize reoxidation of D-LDH. Under these conditions, D-lactate (100 mM final concentration) saturates the 5F-Trp-labeled enzyme.

The accessibility of the ^{19}F nuclei to aqueous solvent was examined by exchanging D-LDH in D_2O buffer with H_2O buffer in Amicon Centricon 30 microconcentrators until the samples were ~96% H_2O and then adding 20 mg of lysoPC as in the control samples.

A spin-labeled fatty acid, 8-doxylpalmitic acid, was incorporated into the lysoPC by mixing 4 mg of 8-doxylpalmitic acid solubilized in $CHCl_3$ with 20 mg of 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 400-μL D-LDH sample was added directly to the dried film, solubilizing the lipid; the sample was centrifuged before being placed into a 5-mm NMR tube.

Phospholipid vesicles were prepared by adding buffer to solid dimyristoylphosphatidylcholine (DMPC, purchased from Avanti) 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 solution of perdeuterated DMPC (purchased from Avanti) was dried under nitrogen and then under vacuum overnight to obtain a powder residue before the addition of buffer and sonication. The phospholipid vesicles were stored at 22 °C and were resonicated to clarity immediately before the start of the NMR experiment. Typically, equal volumes of phospholipid vesicle suspension and purified D-LDH were mixed to yield ~1 mM D-LDH and 60 mM DMPC.

NMR Measurements. The ^{19}F NMR spectra were obtained at 42 °C with Bruker WH-300 and AM-300 spectrometers operating at 282.4 MHz. All spectra were taken with an 8-kHz spectral width and 4K data points in a high-resolution 5-mm Bruker ^{19}F probe equipped with a ^1H-decoupling coil. The control spectra were acquired with a 6.6-μs 90° pulse and a 4-s delay between pulses to accommodate ^1H–^{19}F NOE experiments (Hull & Sykes, 1974; Rule et al., 1987a). All other spectra were collected with a 4.4-μs 60° pulse and a 2-s delay for relaxation. The ^{19}F chemical shift is relative to trifluoroacetic acid (TFA) measured at the same temperature. The negative sign in the ^{19}F chemical shift indicates that the resonance is upfield from that of TFA. A total of 10000–15000 free induction decays were accumulated and then were Fourier transformed with 20-Hz line broadening and corrected for phase and base-line distortions.

Results

Kinetic Studies. The functional integrity of the nine active Trp-substitution mutants has been tested by determining the K_m and V_{max} values of 5F-Trp-labeled enzyme. As shown in Table I, most of the mutants that show measurable activity have values within about 50% of the K_m and V_{max} values of the wild-type enzyme, indicating essentially normal behavior. Also, reconstitution studies with the nine active mutants show a linear relationship between enzyme bound and oxidase activity restored to D-LDH-deficient membrane vesicles (Figure 1). Thus, the nine active mutant enzymes are capable of binding to membrane vesicles and coupling to the respiratory chain in a normal manner.

NMR. The ^{19}F NMR spectra of the active Trp-substitution mutants studied are shown in Figure 2. All of these spectra show an expected sixth peak, which is resolved from the resonances of the native Trps. In addition, the line widths of the additional Trps are similar to the wild-type line width. This indicates that the motional properties of the new Trps are very similar to the native Trps (Ho et al., 1989). In several cases,

Table I: Kinetic Parameters of 5F-Trp-Labeled D-LDH and Trp-Substitution Mutations

residue	predicted structure[a]	K_m[b]	V_{max}[c]
wild type		1.6	11
Phe 12	amphipathic helix	2.1	14
Phe 39	β-turn	0.6	5.7
Ile 99	hydrophobic sheet		negative
Leu 110	amphipathic sheet		negative
Ile 152	hydrophobic sheet		negative
Phe 176	amphipathic sheet	0.9	2.4
Ile 193	amphipathic sheet		negative
Leu 203	amphipathic sheet		negative
Tyr 243	amphipathic sheet	1.5	10
Phe 263	hydrophobic sheet		negative
Phe 279	hydrophobic sheet		negative
Tyr 309	amphipathic helix		negative
Phe 326	hydrophobic sheet		negative
Phe 340	hydrophobic sheet	0.9	3.8
Phe 361	amphipathic helix	2.3	9.8
Tyr 388	amphipathic helix	1.5	8.5
Phe 412	hydrophobic sheet		negative
Phe 435	hydrophobic helix	1.1	2.8
Leu 456	amphipathic helix		negative
Phe 490	hydrophobic sheet		negative
Leu 517	amphipathic helix	1.8	5.2
Phe 544	amphipathic helix		negative

[a] Predicted secondary structure from Ho et al. (1988). [b] Units: [D-lactate] × 10^{-4} M. [c] Units: mol of MTT min^{-1} (mg of protein)$^{-1}$ × 10^{-5}. Based on total protein content as determined by Bradford protein assay.

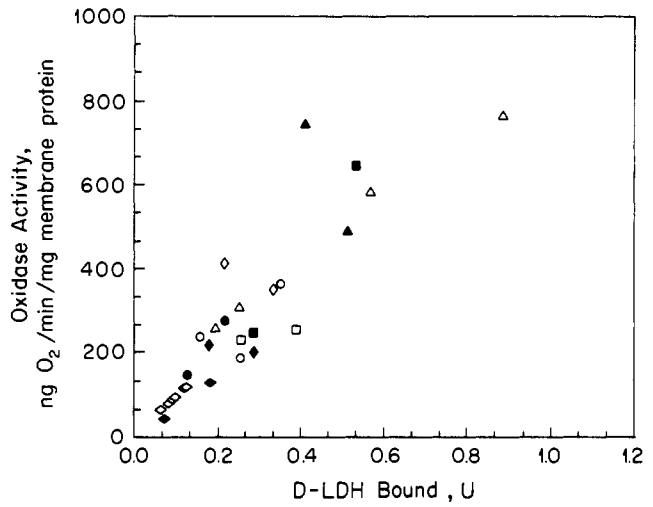

FIGURE 1: Reconstitution of oxidase activity in D-LDH-deficient membrane vesicles with mutant D-LDHs: (○) wild-type D-LDH; (●) Phe 12 → Trp; (□) Phe 39 → Trp; (open flattened diamond) Phe 176 → Trp; (■) Tyr 243 → Trp; (△) Phe 340 → Trp; (▲) Phe 361 → Trp; (closed flattened diamond) Tyr 388 → Trp; (♦) Phe 435 → Trp; (◇) Leu 517 → Trp.

the Trp substitution results in perturbations of the wild-type resonances. Most noticeably, the Tyr 388 mutation affects the nearby Trp 384; two substitutions, at Phe 39 and Leu 517, cause shifts of the native Trp 469 resonance; and the Phe 176 substitution affects the resonances from Trps 59 and 567.

In addition to the expected number of resonance lines, the spectra also contain a number of minor resonances. The Phe 176 and the Tyr 243 substitutions show the presence of a shoulder on the main resonance line from the additional Trp (Figure 2). Given the intensity of these shoulders, it is likely that the substituted Trp is found in two environments and that the exchange rate between the two environments is slower than 100 s^{-1}. This suggests that in the case of these two mutants, where a symmetrical side chain (Phe/Tyr) was replaced with Trp, more than one conformation of the indole ring is acceptable in the folded form of the enzyme. Additional minor

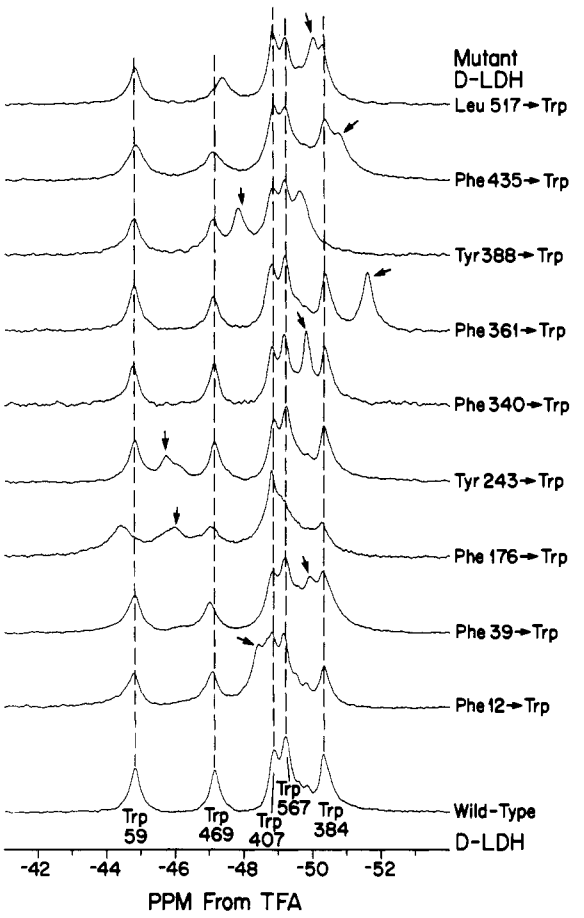

FIGURE 2: 282.4-MHz ^{19}F NMR spectra of 5F-Trp-labeled wild-type D-LDH and nine active Trp-substitution mutants. The enzyme concentrations are ~1 mM in 100 mM lysoPC, 10 mM phosphate, and 0.2 mM EDTA at pH 7.2 and 42 °C. The arrows indicate the resonances from the Trp-substitution mutants.

resonances are found occasionally at −49.5 and −49.8 ppm. These lines probably arise from contaminating proteins or degradation products in the sample, since the intensity of these lines varies from preparation to preparation. In particular, the resonance at −49.8 ppm increases in intensity during NMR acquisitions that last for more than 36 h.

Substrate Sensitivity. In the ^{19}F NMR spectra of several D-LDH mutants, the resonance arising from the Trp substitution is shifted or broadened upon addition of D-lactate. In the wild-type enzyme, only the resonance from Trp 469 is affected by D-lactate, exhibiting a 0.3-ppm downfield shift and broadening from 90 to 240 Hz. Minor changes due to substrate addition are seen in the ^{19}F resonances from substitutions at Phe 340 and Phe 435 (results not shown). Addition of D-lactate causes the resonances from Trp substitutions at Phe 39 and Tyr 243 to show large shifts of 1.0 and 0.6 ppm, respectively, as well as some broadening (Figure 3). In the case of the Tyr 243 substitution, where the additional Trp normally gives rise to a broad line with an apparent shoulder, addition of substrate results in the resonance shifting and merging with the Trp 59 resonance (Figure 3), so that its true line shape can no longer be discerned. When the spectra are fitted by computer, the total intensity of this "merged" peak is accounted for reasonably well by two Lorentzian lines, suggesting that the resonance arising from the Tyr 243 substitution no longer has a shoulder. This may indicate that in the presence of substrate the Trp residue at position 243 is in only one conformation.

Spin-Labeled Fatty Acid. A spin-labeled hydrophobic probe, 8-doxylpalmitic acid, has been used to determine the

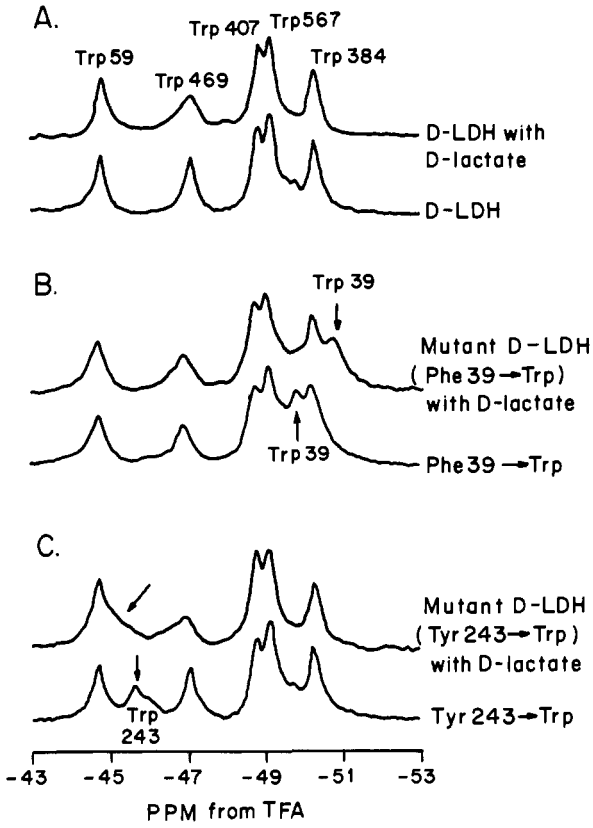

FIGURE 3: 282.4-MHz ^{19}F NMR spectra of 5F-Trp-labeled wild-type D-LDH and two Trp substitutions (at Phe 39 and Tyr 243) that are sensitive to substrate. Note the broadening of the wild-type Trp 469 resonance (panel A) when D-lactate is added (100 mM final concentration). In panels B and C, the arrows indicate the resonances from the new Trp residues. The D-LDH concentration was ~1 mM in 100 mM lysoPC, 10 mM phosphate, and 0.2 mM EDTA at pH 7.2 and 42 °C.

proximity of ^{19}F-labeled Trp residues to the lipid environment. The doxylpalmitic acid is mixed with the lysoPC, forming micelles containing both species which then interact with D-LDH. The paramagnetic electron on the doxyl group causes line broadening of 100 Hz or more when a ^{19}F nucleus is within 10 Å of the spin-label and a smaller broadening of 15–100 Hz for nuclei that are 10–15 Å away (Arseniev et al., 1981; Rule, 1986). The normal line widths in the spectra are 80–100 Hz, making the paramagnetic doxyl group a convenient marker for those amino acid residues that are within ~15 Å of the lipid and, therefore, are likely to be part of the lipid-binding region of D-LDH. Two mutations, at Phe 340 and Phe 361, show loss (i.e., severe line broadening) of the ^{19}F resonance in the presence of spin-label (Figure 4), while the resonance from Tyr 243 → Trp broadens somewhat (results not shown).

Solvent Exposure. The resonance frequency of the ^{19}F nucleus of 5F-Trp shows a solvent-induced isotopic shift (SIIS), resonating 0.23 ppm (65 Hz) further downfield in H_2O than in D_2O (Rule et al., 1987a). This is analogous to the 0.25 ppm SIIS of fluorotyrosine, which has been used to demonstrate that the two tyrosine residues in phage M13 coat protein are not exposed to an aqueous environment (Hagen et al., 1979). By comparison of the ^{19}F NMR spectra of D-LDH in H_2O and D_2O buffer, an index of aqueous exposure can be calculated for each ^{19}F nucleus. Given the broad resonance lines in these spectra and the somewhat poor signal to noise ratio, it is difficult to determine chemical shifts with an accuracy of greater than 10 Hz. This difficulty is further compounded by overlapping resonance lines and the presence of additional, low-intensity lines that arise from contaminants.

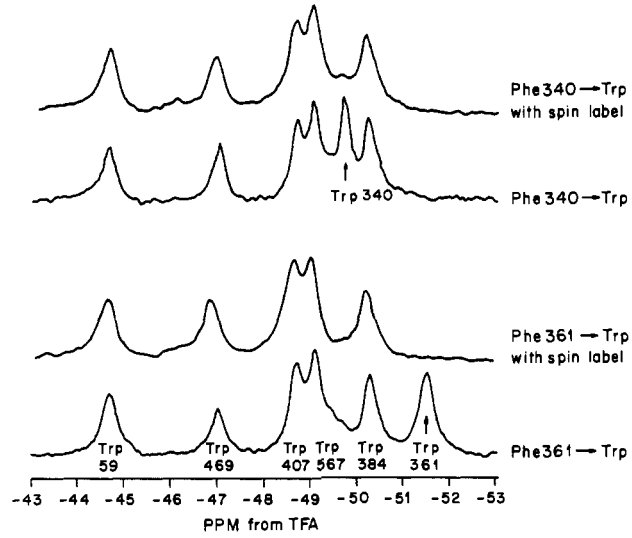

FIGURE 4: 282.4-MHz ^{19}F NMR spectra of ~1 mM mutant D-LDHs (Phe 340 → Trp and Phe 361 → Trp) showing sensitivity to 28 mM 8-doxylpalmitic acid in 100 mM lysoPC, 10 mM phosphate, and 0.2 mM EDTA at pH 7.2 and 42 °C. The arrow indicates the resonances that are severely broadened by the spin-label.

Table II: Aqueous Exposure Indices from Solvent-Induced Isotopic Shifts

mutation	index[a]	exposure	wild type	index[a]	exposure
Phe 12 → Trp	0.0	buried	Trp 59	0.0	buried
Phe 39 → Trp	0.0	buried	Trp 384	1.0	exposed
Phe 176 → Trp	0.9	exposed	Trp 407	0.4	partially exposed
Tyr 243 → Trp	0.3	partially exposed	Trp 469	0.0	buried
Phe 340 → Trp	0.0	buried	Trp 567	0.7	partially exposed
Phe 361 → Trp	0.0	buried			
Tyr 388 → Trp	0.5	partially exposed			
Phe 435 → Trp	0.4	partially exposed			
Leu 517 → Trp	0.3	partially exposed			

[a] Relative to an index of 1.0, where 1.0 corresponds to the shift of 0.23 ppm obtained for free 5F-Trp in solution, when its environment is changed from D_2O to H_2O.

Consequently, we do not report accurate exposure indices, but simply note whether a Trp is not exposed ($\Delta\delta$ < 10 Hz), partially exposed (10 Hz < $\Delta\delta$ < 40 Hz), or fully exposed ($\Delta\delta$ > 40 Hz). For those Trp residues that are partially exposed, we cannot distinguish between static or dynamic contributions to the degree of exposure. We also recognize that our determination of solvent exposure may only be distantly related to the accessible surface area of the protein molecule. This complication arises because of the possibility that deuterium–hydrogen exchange can occur at sites that are buried in the protein molecule. However, given the slow exchange rate of buried hydrogens, hydrogen exchange is not likely to affect the interpretation of our data for the majority (if not all) of the exposed Trps.

As shown in Table II, the Trp resonances from five of the nine mutations show noticeable aqueous exposure. The substitution at Tyr 243 behaves in an interesting manner; the line shape of the normal line changes when the solvent is changed. This indicates that the two (or more) conformations of this residue have different degrees of solvent exposure. New data from the mutations prompted a reevaluation of the solvent sensitivity of the wild-type Trp 407. This amino acid residue had been characterized as being not exposed (Rule et al.,

1987a), but it is actually partially exposed.

Nuclear Overhauser Effect. The ^1H–^{19}F nuclear Overhauser effect (NOE) is a sensitive test of the internal motion of ^{19}F-labeled amino acid residues in the protein molecule (Hull & Sykes, 1974). In the native protein, the five Trp residues have NOEs ranging from −0.75 to −0.90 (Rule, 1986; Ho et al., 1988, 1989), and this range is also observed for six of the nine substitutions (Phe 176 was not investigated), while the Phe 340 and Phe 361 substitutions have NOE values in the range of −0.5 to −0.6. The observed NOEs of the Phe 340 and Phe 361 substitutions are not altered when the experiments are performed with perdeuterated lipid (results not shown).

Discussion

Effects of Mutations in D-*LDH.* The goal of this Trp substitution study is to locate structural and functional regions of the D-LDH, such as the membrane-binding region and the amino acid residues sensitive to catalysis and reduction of FAD. Implicit in such an analysis is the assumption that the mutations do not greatly perturb the structure of the native enzyme molecule. A total of 9 of 22 mutants fit this criterion by exhibiting essentially normal kinetics and oxidase activity as well as retaining an essentially wild-type ^{19}F NMR spectrum. A number of Trp substitutions alter the chemical shifts of the native Trps (such as Phe 39, Phe 176, Tyr 388, Leu 517). These changes are small, and thus, it can be concluded that the structure of all the active mutants is very similar to that of the wild-type enzyme.

However, some of the mutations appear to affect the dynamics of D-LDH. In the wild-type enzyme, the resonance from Trp 469 is broadened in the presence of substrate. Previous NOE data suggest that in the native structure the Trp 469 residue is rigid and that the broadening seen when D-lactate is added is due to exchange broadening of the resonance as a result of increased motional freedom of Trp 469 (Ho et al., 1989). Broadening of the resonance from Trp 469 is also seen with substitutions at Phe 435 and Leu 517 in the *absence* of substrate. This line broadening is very similar to that observed for Trp 469 when substrate is added to the wild-type enzyme (see Figure 3). In the case of the Trp substitutions, one possibility is that they cause small changes in the structure of D-LDH, increasing the conformational space available to Trp 469 to produce an effect similar to that of adding substrate. It is interesting to note that none of the nine active mutations prevent the substrate-induced line broadening at Trp 469, suggesting that this aspect of protein dynamics may be important to the function of D-LDH. Thus, mutations that appear to have no gross effect on the structure of the protein molecule may affect the dynamics of the mutant protein near the point of mutation. In the case of D-LDH, these effects do not appear to alter the function significantly. In the discussion that follows, it should be clear that the experimental data are obtained on mutant proteins and that the conclusions reached only strictly apply to each mutant in particular. However, all nine positive mutants appear to be good structural probes for the function of D-LDH, and thus the application of data obtained with these mutant enzymes to native D-LDH is justified.

The other 13 mutants show little or no activity, yet all of the 22 Trp-substitution mutants appear to be expressed, as determined by SDS–polyacrylamide gel electrophoresis of lysates from induced cells (results not shown). Why are some of the mutants inactive? Of the positive mutants, eight are aromatic replacements and one is an aliphatic replacement. In contrast, the inactive mutants arise from the replacement of essentially equal numbers of aliphatic and aromatic residues. Furthermore, of the nine active mutants, the new Trp residues of five are somewhat exposed to the solvent (Phe 176, Tyr 243, Tyr 388, Phe 435, and Leu 517) and of two are near the lipid (Phe 340 and Phe 361). These observations indicate that the inactivation of the enzyme by Trp insertion is not due to an alteration in the free energy of the folded protein which arises from the Trp substitution alone. The free energies of transfer of an amino acid from the solvent to the internal hydrophobic core of the protein would actually favor folding of the Trp substitution. Given the fact that most of the active substitutions are found on the surface of the protein, it is reasonable to infer that inactivation by Trp substitution results from the introduction of packing strain into the enzyme. The bulky Trp side chain is 34–38 Å^3 larger than Tyr or Phe and 61 Å^3 larger than Leu or Ile (Zamyatnin, 1972). If the replacement site is in the interior of the protein molecule, it would be very difficult to accommodate the increase in molecular volume without the disruption of a large number of contacts between amino acid residues at the site of the replacement. Such a disruption would lead to a large loss of van der Waals energy, which could be sufficient to destabilize the protein. Similar conclusions on the contributions of hydrophobicity and volume to the stability of mutant proteins were reached by Lim and Sauer (1989) and Sandberg and Terwilliger (1989).

An alternative explanation is that the Trp replacement affects the folding pathway of the enzyme such that the native state is no longer accessible due to kinetic barriers. At this time, it is difficult to distinguish between these two explanations. However, structural studies on the inactive enzymes, which are currently underway, could contribute to our understanding of this manner.

Topology of D-*LDH.* The nine Trp substitutions combined with the five native Trps have greatly increased our ability to investigate the structure and function of various regions of D-LDH by ^{19}F NMR spectroscopy. The results obtained for all nine active mutations plus the five native Trps are summarized in Figure 5. Some features of the topology of D-LDH are evident in Figure 5.

(*A*) *Membrane Interactions.* D-LDH binds to membranes and micelles in a nonelectrostatic manner and thus must have a hydrophobic region on the surface of the enzyme molecule which interacts with lipids. The data from the substitution mutants indicate that such a region is found near positions 340 and 361. These two mutations show high sensitivity to the spin-labeled fatty acid, indicating close proximity to the hydrophobic region of the enzyme molecule which binds to micelles. The NOE data also support the idea that the substitutions at amino acid residues 340 and 361 are in an environment different from that of the rest of the molecule. They both have significantly smaller negative NOEs than the other Trp residues in the protein, indicating either that they are being relaxed by something which is undergoing rapid motion such as lipids or that the amino acid residues themselves are rotating rapidly, which is possible inside a lipid environment. The relaxation most likely does not arise from the protons of the acyl chains of the lipid. This conclusion is based on our experiments where D-LDH is bound to sonicated unilamellar phospholipid vesicles composed of DMPC. In this system, the NOE of amino acid residues 340 and 361 is also smaller in magnitude than that of the other Trp residues. Changing the lipid to perdeuterated DMPC has no effect on the NOEs of amino acid residues at positions 340 and 361 (results not shown).

On the amino side of the hydrophobic region, the substitution at Tyr 243 is slightly spin-label sensitive and also ex-

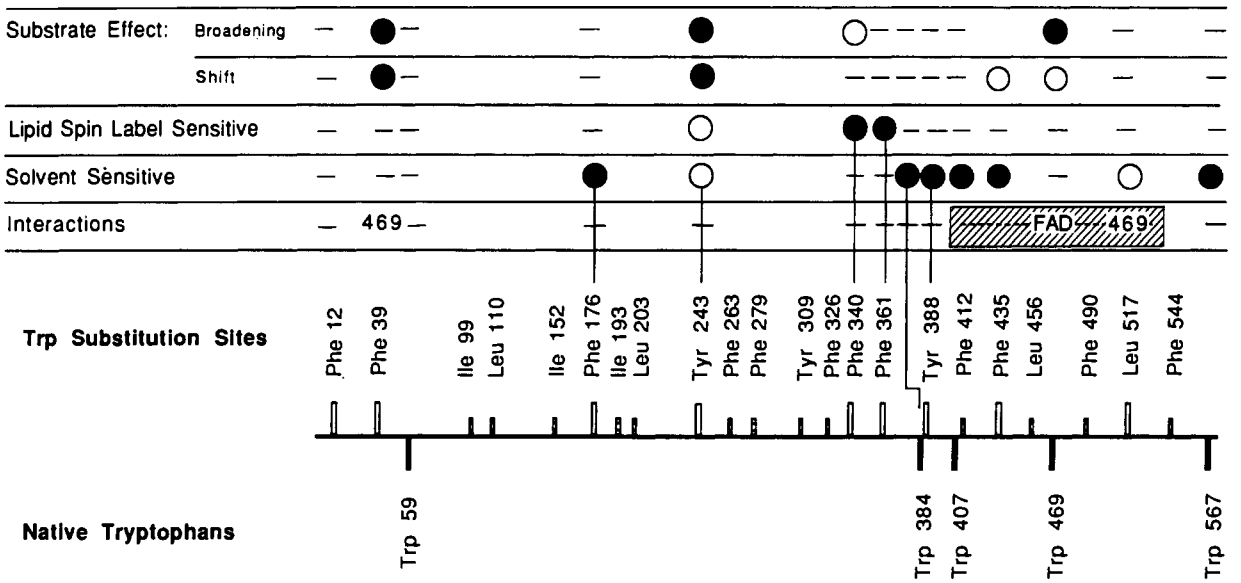

FIGURE 5: Linear map of D-LDH summarizing the results of the Trp-substitution experiments. The possible FAD-binding site is indicated. In general, open circles indicate observed, but small, effects, and the filled circles indicate major effects. Substrate broadening: (○) 20–50%; (●) >50%. Substrate shift: (○) <0.2 ppm; (●) >0.2 ppm. Spin-label sensitivity: (○) slight broadening; (●) loss of resonance from spectrum. Solvent sensitivity: (○) exposure index ≤0.3; (●) index >0.3. A dash (—) indicates that no effect was seen. The shaded area indicates the putative FAD-binding site.

hibits slight solvent sensitivity. On the basis of the amount of spin-label-induced broadening, Tyr 243 is 10–15 Å away from the nitroxide spin-label. Since this amino acid residue is in a somewhat aqueous environment, it is probably near the hydrophobic/aqueous interface.

On the carboxyl side of the hydrophobic region, there are four amino acid residues, at positions 384, 388, 407, and 435, which show no spin-label sensitivity whatsoever and which are also exposed to the aqueous solvent. Keeping in mind that an effect of the spin-label does not require contact with the nitroxide moiety while the SIIS experiment requires direct interaction with the H_2O or D_2O molecules, this implies that there is a major environmental difference between the spin-label-sensitive stretch and the amino acid sequences that surround it. Our previous data indicate that this region of D-LDH is also susceptible to proteolytic cleavage, with the most exposed sites being Phe 356, Ala 365, and Lys 368 (Rule et al., 1985), and that Trp 384 is fairly mobile (Rule et al., 1987a). This strongly suggests that between amino acid residues 361 and 384 there is an exposed link in which the polypeptide chain moves away from the hydrophobic binding structure and enters an aqueous environment.

(B) Catalytic Domain. Five amino acid residues are sensitive to the presence of the substrate. Assignment of these substitution mutations to the catalytic site in D-LDH is not yet possible. The major obstacle is that the substrate effects seen in the NMR spectra can be due to conformational changes in the enzyme molecule or due to the change in oxidation state of the FAD, and at this point these effects cannot be discriminated from each other without the use of inhibitors or nitroxide-labeled substrates.

The number of amino acid residues affected by the addition of D-lactate is an indication of the degree of substrate-induced conformational change in D-LDH. Of the four completely internal residues (Trp 59, Trp 469, and substitutions at Phe 12 and Phe 39), as determined by accessibility to solvent and lipid, only two (Trp 469 and the substitution at Phe 39) are markedly affected by the presence of substrate. Of the six partially exposed residues (Trp 407, Trp 567, and the substitutions at Tyr 243, Tyr 388, Phe 435, and Leu 517), only two (Tyr 243 and Phe 435) are perturbed by the addition of substrate. Thus, for the most part, substrate-induced changes in D-LDH are restricted to certain regions of the enzyme molecule. This conclusion is supported by some observations on interresidue effects. Substitutions at Phe 39 and Leu 517 affect the environment of Trp 469, yet the substrate effects are only seen with Trp 469 and the substitution at Phe 39; they are not propagated to the substitution at Leu 517. In the absence of long-range conformational effects in D-LDH, we propose that all substrate-sensitive residues are in close proximity to the active site, either at the substrate-binding site or at the FAD-binding region.

The amino acid residues that are sensitive to substrate, Trp 469 and substitutions at Phe 39, Tyr 243, Phe 340, and Phe 435, are in topologically different environments. Phe 39 and Trp 469 are located in the interior of D-LDH, Tyr 243 is exposed to the solvent and in proximity to the lipid phase, Phe 340 interacts with the lipid phase, and Phe 435 is partially exposed to the solvent. The substitution at Tyr 243 is sensitive to both the addition of substrate and the presence of spin-label in the lipid phase, a combination that indicates it may be sensitive to the electron-transfer pathway in D-LDH. The Phe 39 substitution is extremely sensitive to D-lactate addition, shifting by 1 ppm, which may indicate ring-current effects from the reduced flavin. We have found that the substitution of Trp for Phe 39 or Leu 517 causes a change in the ^{19}F chemical shift of the wild-type Trp 469 resonance (Figure 2). In both cases, the resonances arising from the substitutions occur at approximately −49.9 ppm from TFA. These findings suggest that amino acid residues 39 and 517 are in very similar environments and that they are both spatially close to Trp 469.

Relationship between D-LDH and Other Dehydrogenases. It is useful to discuss the relationship of the topology of D-LDH, a membrane-bound dehydrogenase, to that of soluble dehydrogenases. D-LDH could have evolved separately from other dehydrogenases, or it could be an adaptive version of the cytoplasmic dehydrogenases. In the first case, it would be difficult to obtain complete structural information about the 571-residue D-LDH unless the enzyme can be crystallized for an X-ray crystallographic investigation, which is proving to be difficult. However, the second possibility allows one to use the information known about the structure of other de-

hydrogenases to model D-LDH and then test predictions made from this model.

Studies of several cytoplasmic dehydrogenases have shown two common features among these enzymes. A two-domain structure, containing a substrate-binding domain and a cofactor-binding domain, folds together such that the active site of the enzyme molecule is formed in a cleft between them (Rossmann et al., 1975). Among the dehydrogenases, there is little sequence and structure homology in the substrate-binding domain, but the cofactor-binding domain contains a well-conserved structure, the core of which is a twisted four-strand β-sheet, with the cofactor bound at its carboxyl end (Rossmann et al., 1975; Birktoft & Banaszak, 1984). This model binding structure, used to bind nicotinamide adenine dinucleotide (NAD), also exists in FAD-containing enzymes, such as glutathione reductase (Schulz et al., 1982).

In the case of D-LDH, there is evidence for a cofactor-binding domain in the carboxy-terminal 200 residues of the enzyme molecule. A secondary-structure prediction indicates structural homology to a nucleotide-binding sequence from amino acid residues 410–530 (Ho et al., 1988). The first part of this proposed FAD-binding site is homologous to the $\beta\alpha\beta\alpha\beta$ sequence of an NAD-binding site, while the second half has a predicted $\beta\beta\beta\alpha\beta$ motif, similar to the FAD-binding site of glutathione reductase (Schulz et al., 1982). The participation of this region of the enzyme molecule in the catalytic site is supported by our NMR data from Phe 435 and Trp 469. If the FAD binds at the carboxyl-terminal end, then the substrate-binding domain could be contained in the amino-terminus region. It is interesting to note that amino acid residues 10–240 of D-LDH show some amino acid sequence homology (16%) to phosphoenolpyruvate carboxylase (Katagiri et al., 1985), an enzyme whose substrate is similar to D-lactate. The participation of the amino-terminal region in the active site is supported by our NMR data from Phe 39 and Tyr 243. Thus, D-LDH may resemble soluble dehydrogenases in that the substrate-binding and catalytic domains fold together to form an active site. In addition, D-LDH appears to contain a small segment that is responsible for membrane attachment and coupling to lipophilic electron carriers. This architecture for a membrane protein may also be present in a number of other membrane-bound dehydrogenases, such as *E. coli* NADH dehydrogenase (Ho et al., 1989).

In conclusion, the combination of 5-fluorotryptophan labeling and ^{19}F NMR spectroscopy has proven useful as a probe for determining an overall picture of discrete structural and functional regions within D-LDH. The ability of these mutant enzymes to bind to membranes and couple to the respiratory chain, as shown by normal oxidase activity, indicates that these new Trp residues can potentially be used as spectroscopic probes to investigate the coupling of D-LDH to the respiratory chain.

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