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Nuclear Magnetic Resonance and Molecular Genetic Studies of the Membrane-Bound D-Lactate Dehydrogenase of *Escherichia coli*†

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ABSTRACT: In this study we demonstrate the potential of combining fluorine-19 nuclear magnetic resonance (NMR) spectroscopy with molecular genetics. We are using the membrane-bound enzyme D-lactate dehydrogenase of *Escherichia coli* as a model system to characterize interactions between proteins and lipids. We have labeled D-lactate dehydrogenase with 4-, 5-, and 6-fluorotryptophans and obtained high-resolution fluorine-19 NMR spectra showing five resonances, in agreement with the five tryptophan residues expected from the DNA sequence. The five ^{19}F resonances in the spectra have been assigned to the specific tryptophan residues in the primary sequence of D-lactate dehydrogenase by site-directed oligonucleotide mutagenesis of the cloned gene. We observe large differences in the relative fluorine-19 chemical shifts of each tryptophan residue when labeled by different isomers of fluorotryptophan. We have determined by NMR methods that two tryptophans are exposed to the solvent and that none of the tryptophan residues are within 10 Å of the lipid phase. On the basis of ^{19}F NMR spectroscopy of the labeled tryptophan residues, the conformation of D-lactate dehydrogenase is similar in aqueous solution and in the presence of a variety of lipids and detergents. This result indicates that the presence of lipids or detergents is not required to maintain the tertiary structure of this membrane-bound enzyme. In contrast, Triton X-100 induces a change to an abnormal conformation of the enzyme as judged from both NMR spectroscopy and the effect of temperature on the maximal velocity of the enzyme in the presence of this detergent.

The biochemical function of a large number of membrane enzymes is well understood. However, the body of information describing structural properties of membrane enzymes is quite sparse. Consequently, it is difficult to describe structure–function relationships for this type of enzyme. To obtain information about biological membranes, our laboratory has been using ^{19}F nuclear magnetic resonance (NMR)[1] techniques to investigate the structure and dynamics of membrane lipids and proteins. For a recent review, see Ho et al. (1985).

As a model system in which to study protein–lipid interactions, we have chosen the membrane-bound enzyme D-lactate dehydrogenase (D-LDH) of *Escherichia coli*. This flavin-containing enzyme has a molecular weight of 65 000 and 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 (Barnes & Kaback, 1971; Futai, 1973; Kohn & Kaback, 1973). These activities can be reconstituted by the addition of purified enzyme to D-LDH-deficient membrane vesicles (Futai, 1974; Short et al., 1974). The activity of D-LDH is enhanced by a wide variety of lipids and detergents (Fung et al., 1979; Kovatchev et al., 1981). Thus, it is possible to study the properties of D-LDH in the presence of defined, synthetic lipids and detergents.

The gene encoding D-LDH has been cloned (Young et al., 1982), and the nucleotide sequence of the D-LDH gene has also been determined (Campbell et al., 1984; Rule et al., 1985). The availability of the cloned gene has allowed us to combine the techniques of molecular genetics and ^{19}F NMR to investigate protein–lipid interactions in this model system. We have been able to overproduce D-LDH by 300-fold (Rule et al., 1985) and thus obtain sufficient material for the NMR studies described in this paper. In addition, it has also been possible to use site-directed oligonucleotide mutagenesis to assign the ^{19}F resonances. This is accomplished by generating mutant

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[1] Abbreviations: NMR, nuclear magnetic resonance; D-LDH, D-lactate dehydrogenase; F-Trp, fluorotryptophan; SIIS, solvent isotopic induced shift; $C_{12}E_7$, heptaethylene glycol dodecyl ether (Brij); PMS, phenazine methosulfate; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; EDTA, ethylenediaminetetraacetic acid; SDS, sodium dodecyl sulfate; DPPG, dipalmitoylphosphatidylglycerol; T_1, spin–lattice relaxation time; NOE, nuclear Overhauser effect; TFA, trifluoroacetic acid; CD, circular dichroism; FAD, flavin adenine dinucleotide.

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enzymes that have one of the labeled amino acids replaced by a different residue. The resultant NMR spectrum of the labeled mutant protein will be missing the resonance that arose from the replaced amino acid residue. Our technique is analogous to other genetic methods that have been used to assign NMR resonances in a number of systems [for example, see Jarema et al. (1981) and Ho and Russu (1981)].

Although considerable information about protein structure and dynamics has been obtained by the use of ^1H NMR, this nucleus possesses a number of disadvantages when used to study membrane proteins. For example, resonances from the protons present in the amphiphile which is required to solvate the protein may mask some of the proton resonances from the protein. Furthermore, protein–lipid complexes are usually quite large, thus resulting in broad spectral lines that lead to unresolved resonances.

Isotopic labeling of a particular amino acid with a variety of magnetic nuclei (^2H, ^{13}C, ^{15}N, and ^{19}F) has been used to simplify the NMR spectrum of a number of membrane-associated proteins [for example, see Robertson et al. (1977), Hagen et al. (1978, 1979), Ho et al. (1980), Kinsey et al. (1981), Gall et al. (1982), Dettman et al. (1984), Post et al. (1984), and Wilson and Dahlquist (1985)]. There are several advantages associated with the use of ^{19}F, including 100% natural abundance, a sensitivity close to that of protons, and a large chemical shift range. Additionally, the chemical shift tensor of fluorine is often anisotropic, and the effects of this anisotropy on the NMR relaxation mechanisms can be useful in determining the motional properties of the fluorine atom.

We have biosynthetically incorporated 4-, 5-, and 6-fluorotryptophans (F-Trp) separately into D-LDH. There are three reasons why we decided to label tryptophan residues in D-LDH. First, D-LDH contains only five tryptophans, and thus we expected to obtain a well-resolved NMR spectrum. Second, tryptophan is a hydrophobic amino acid, and thus we anticipated that it would probe regions of the protein that are involved in protein–lipid contacts. Third, previous work in our laboratory has demonstrated that the F-Trp-labeled D-LDH is catalytically active and capable of stimulating D-lactate-driven active transport in D-LDH deficient *E. coli* membrane vesicles (Pratt et al., 1983).

In this paper, we demonstrate the usefulness of F-Trp labeling for studying the effects of different lipid environments on the conformation of D-LDH. In addition, we have used the solvent isotopic induced shift (SIIS; Hagen et al., 1979) method to show that two of the five Trp residues in D-LDH are exposed to the aqueous phase. We also demonstrate, by use of a paramagnetic lipophilic probe, that none of the Trp residues are close to the lipid phase. Finally, we have also observed the effects of D-LDH on the environment of (8,8-difluoromyristoyl)lysolecithin by ^{19}F and ^{31}P NMR.

Materials and Methods

Materials. Fluorotryptophans, phospholipids, and 8-doxylstearic acid were purchased from Sigma and were used without further purification. $C_{12}E_7$ (heptaethylene glycol dodecyl ether) was manufactured by the Nikkol Chemical Co. (Japan) and was a generous gift of J. T. Yang (University of California at San Francisco). Reagents for oligonucleotide synthesis were purchased from American Bionuclear. (8,8-Difluoromyristoyl)lysolecithin was synthesized in our laboratory by S. R. Dowd. Enzymes used for recombinant DNA work were purchased from either New England Biolabs or Pharmacia. Nucleoside triphosphates were from Pharmacia.

Plasmids, Phage, and Bacterial Strains. The plasmids and strains used for the overproduction of D-LDH have been described previously (Rule et al., 1985). The M13 phage mp18 (Hu & Messing, 1982) was obtained from B. D. Karger, plasmid pBR327 (Soberon et al., 1980) from E. G. Minkley, Jr., and strain BW313 [*dut ung thi*-1 *relA spoT*1/F′*lysA*] from B. C. Hoopes, all of Carnegie-Mellon University.

Production of Fluorotryptophan-Labeled D-*LDH.* In order to obtain large quantities of highly substituted F-Trp-labeled D-LDH, we have employed a strain in which the expression of D-LDH is temperature-inducible (Rule et al., 1985). Three-liter cultures of strain W3110trp*A33*(pGA2, pRK248cI[Ts]) were grown in a high-density fermentor (Sadler et al., 1974; Lab-Line Instruments, Inc.) with M-9 medium (Miller, 1972) containing 2 times the usual amount of phosphate salts and 2% casamino acids (Difco Laboratories). Glycerol (1%) was used as a carbon source, and the Trp concentration was 3×10^{-5} M. The pH of the culture during growth was maintained at 7.0 by the addition of 2 N NaOH. The airflow into the fermentor (6 L/min) was replaced by pure oxygen after the cells had reached an optical density of 1.5 at 550 nm. The culture was grown at 32 °C until the optical density reached 2.5. At this time, the thermostat of the fermentor was raised to 42 °C. When the bath had reached 40–42 °C (after approximately 45 min), the fluorinated analogue (4-, 5-, or 6-F-Trp) was added to give a concentration of 10^{-4} M Trp. After another 30–45 min, the temperature of the culture was reduced to 39 °C. The cells were harvested when the D-LDH activity of the culture stopped increasing (approximately 5 h after the start of induction).

The F-Trp-labeled D-LDH was purified as described previously for the unlabeled enzyme through the hydroxylapatite column (Rule et al., 1985). D-LDH activity was measured as described with PMS and MTT (Pratt et al., 1979). The concentration of D-LDH was determined by measuring the absorption of the flavin cofactor under conditions that unfold the protein (6 M guanidine hydrochloride, 0.1 M sodium acetate, pH 4.0). An extinction coefficient of 11 000 M^{-1} cm^{-1} at 470 nm was used.

Preparation of NMR Samples. To remove the detergent that is added during the purification, the purified D-LDH (in 100 mM potassium phosphate buffer at pH 7.2) was precipitated twice at 0 °C with an equal volume of ice-cold acetone. The residual acetone was removed, and the samples were equilibrated with D_2O by three to four cycles of buffer exchange with Amicon filter cones (CF25, Amicon Corp.). The final buffer composition for NMR studies was 10 mM potassium phosphate and 0.2 mM EDTA at pH 7.2 (uncorrected for the deuterium isotope effect). Samples were usually in 100% deuterium oxide, except for the SIIS experiments.

When Triton X-100, Triton X-100/sodium dodecyl sulfate (SDS), and $C_{12}E_7$ were used in NMR samples, an equal volume of the detergent solution was mixed with the enzyme solution. To prepare samples containing lysolecithin, the D-LDH solution was added directly to the dry lysolecithin. When nitroxide spin-labels were incorporated into the lysolecithin solution, the lysolecithin and the spin-label were codissolved in chloroform–methanol (1:1) and dried, and the D-LDH solution was added directly to the dried film.

Sonicated phospholipid vesicles were prepared from dipalmitoylphosphatidylglycerol (DPPG) by hydrating a dried film of the lipid with the same buffer used for the protein. The resultant multilammelar vesicle preparation was sonicated at 49 °C until it became optically clear. Equal volumes of the sonicated vesicles and the D-LDH solution were then mixed.

NMR Measurements. ^{19}F NMR measurements were obtained at 282.4 MHz on a Bruker WH-300 spectrometer. The

^{19}F NMR spectra were obtained in the absence of proton decoupling. ^{19}F spin–lattice relaxation time (T_1) values were obtained by a saturation-recovery technique. The ^{19}F spins are saturated by an aperiodic sequence of 90° pulses, in which the phase of the successive pulses is incremented by 90° and the pulse separation is decremented by a factor of 2, until it reaches 0.2 ms (see the macroinstruction SP in Bruker's DISNMRP or FTQNMR manuals). We have used a sequence of nine pulses spanning a total sequence duration of 120 ms. After the spins are saturated, the recovery of M_z is monitored with a standard 90° pulse.

The homonuclear nuclear Overhauser effect (NOE) was measured by the selective inversion of the desired ^{19}F resonance by application of a DANTE pulse sequence (Bruker DISNMRP software manual). The NOE on the remaining ^{19}F resonances was measured at 100, 200, and 300 ms by the application of a nonselective 90° pulse. The ^{19}F chemical shifts are referred to a solution of trifluoroacetic acid (TFA) in D_2O.

^{31}P NMR spectra were obtained at 121.5 MHz on a Bruker WH-300 spectrometer. Broad-band proton decoupling was used. ^{31}P T_1 values were obtained by the inversion-recovery technique.

Circular Dichroism Measurements. Circular dichroism (CD) measurements were performed on a Jasco J-500A spectropolarimeter. Measurements were made at 25 °C in the same buffer used for the NMR experiments, with the addition of $C_{12}E_7$ to a concentration of 10 mM.

Mutation of D-*LDH Gene.* Synthetic oligonucleotides containing the desired mutation were either purchased from the DNA Synthesis Service of the University of Pennsylvania (Trp-59, -384, -469, -567) or synthesized manually (Trp-407) by the phosphoamidite method on a solid support (Adams et al., 1983). The mutational oligonucleotides were designed to change Trp to either Tyr or Phe in order to minimize deleterious effects on the enzyme. The oligonucleotide primers that were used to obtain the DNA sequence of the Trp mutants were manually synthesized in our laboratory.

Mutations were incorporated into the D-LDH gene by the method of Kunkel (1985). In order to avoid the possibility of inducing other mutations in the D-LDH gene during in vitro repair and to increase the genetic stability of the recombinant M13 phage, we cloned fragments (400–1000 base pairs) of the D-LDH gene into mp18. The recombinant phage were grown on strain BW313 for one cycle of growth. In vitro repair was performed as indicated by Kunkel (1985), except that the reaction time was increased to 15 h. We did not obtain any mutants with the recommended repair time of 2 h. The repaired phage were transfected into JM103, and the single-stranded template DNA was subjected to dideoxy sequencing as described (Rule et al., 1985) with [^{32}P]dATP as the radiolabeled base and gradient buffer gels for electrophoretic analysis (Biggin et al., 1983). The frequency of phage carrying the desired mutation was 5–30%. Replicative-form DNA was isolated from phage-infected cells, and the D-LDH fragment was excised from the phage DNA and ligated into the remaining part of the D-LDH gene. At this point, the mutant D-LDH gene was contained between the *Pst*I and the *Eco*R1 sites of pBR327. This plasmid was linearized with *Pst*I, and the 3.3-kilobase *Pst*I fragment from pGA2, containing the left promoter from λ, was inserted to construct a temperature-inducible expression vector for the mutant protein. The resultant vector pGA2a is somewhat different from the vector used to obtain the wild-type F-Trp-labeled protein (pGA2). Although the expression of D-LDH from pGA2a is 2-fold lower than from pGA2, adequate amounts of mutant protein could still be produced, and the DNA manipulations required to construct pGA2a are simpler than for the construction of pGA2.

Table I: Effect of F-Trp Incorporation on the Kinetic Properties of D-LDH

assayed in	K_M ($\times 10^4$)	V_{max} ($\times 10^{-4}$)[a]
H_2O		
unlabeled	4.0	0.025
Triton X-100 (0.6%)		
unlabeled	2.5	1.2
4F-Trp	2.7	1.0
5F-Trp	2.5	0.8
6F-Trp	2.9	1.2
$C_{12}E_7$ (10 mM)		
unlabeled	1.9	0.85
4F-Trp	1.9	0.87
5F-Trp	1.9	0.60
6F-Trp	2.1	0.98
egg lysolecithin (1 mM)		
unlabeled	3.9	2.0
4F-Trp	2.6	1.6
5F-Trp	3.2	1.4
6F-Trp	3.2	2.0

[a] Units are mol of MTT reduced (mol of enzyme)$^{-1}$ min^{-1}.

RESULTS

Properties of Fluorotryptophan-Labeled D-*LDH.* By use of a temperature-inducible expression vector and a high-density fermentor, we obtain approximately 100 mg of purified F-Trp-labeled D-LDH from 3 L of cell culture. The degree of substitution of the F-Trp for the normal Trp depends on the expression vector used and varies from 50% (pGA2a) to 90% (pGA2) as estimated from the amount of D-LDH produced after addition of the F-Trp and from a comparison of the NMR signal intensity between F-Trp-labeled D-LDH and a standard solution of F-Trp. The catalytic properties of the fluoro-labeled enzymes are presented in Table I. The incorporation of the F-Trp into D-LDH does not significantly change K_m, but in the case of 5F-Trp-labeled D-LDH, we observe a small decrease in V_{max}. These results agree well with the effects of F-Trp labeling on D-LDH previously reported by this laboratory for partially purified enzyme (Pratt et al., 1983). As seen previously for chromosomal D-LDH, much more D-LDH is made in the presence of 4F-Trp than with 5F- or 6F-Trp. Consequently, the specific activity of 4F-Trp-labeled D-LDH appears to be much higher in a partially purified preparation. However, when purified to homogeneity, all forms of D-LDH are now found to be essentially identical. The increased amount of D-LDH available has allowed us to obtain preparations that contain less Triton X-100 than previous preparations. With these preparations of D-LDH, we find that the enzyme is activated 40–50-fold by Triton X-100 rather than the 5-fold reported previously (Fung et al., 1979).

We have also determined the temperature stability of the fluoro-labeled enzymes in 1 mM lysolecithin at 55 °C. Both the 4F- and 6F-Trp-labeled enzymes have the same rate of temperature inactivation as the unlabeled enzyme, the activity decaying with a time constant of 10 s. Surprisingly, the 5-fluoro-labeled enzyme proves to be somewhat more stable than the unlabeled enzyme, and its activity decays with a time constant of 40 s.

In order to ascertain the effect of ^{19}F incorporation on the secondary structure of D-LDH, we have measured the CD of the unlabeled and F-Trp-labeled enzymes in the presence of $C_{12}E_7$ (Figure 1). $C_{12}E_7$ was used as the detergent because this compound has no absorption bands in the peptide region (190–250 nm). The CD of the 4F- and 5F-Trp-labeled D-L-

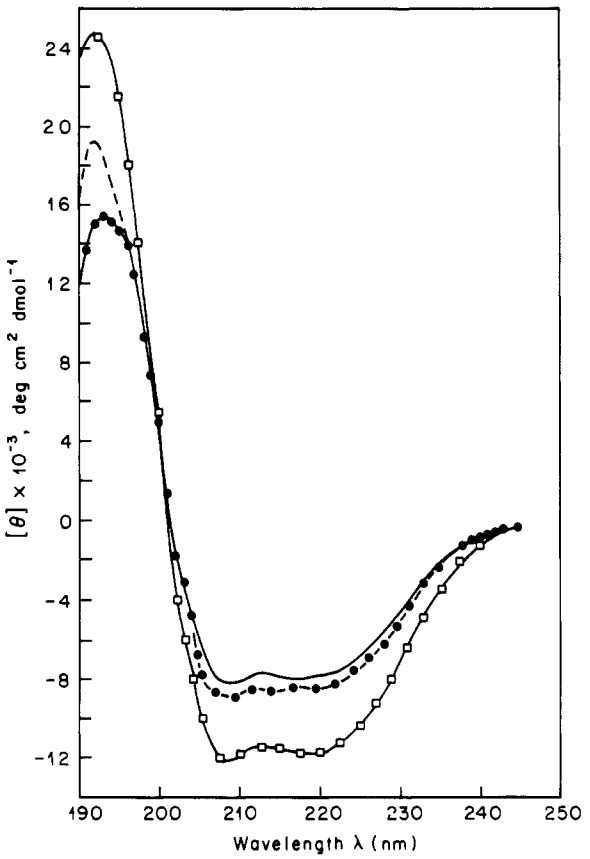

FIGURE 1: Circular dichroism of D-LDH: (—) native D-LDH; (●●) 4F-Trp; (– –) 5F-Trp; (□–□) 6F-Trp.

DH is very similar to that of the unlabeled enzyme, while the CD of the 6F-Trp-labeled D-LDH is somewhat different (see Discussion). We have also measured the CD of the FAD absorption band (400–550 nm, data not shown), and we do not detect any difference among the four forms of D-LDH.

^{19}F NMR Studies. The ^{19}F NMR spectra of 4F-, 5F-, and 6F-Trp-labeled D-LDH are presented in Figure 2. In addition, we also show the spectra for each of the five mutant enzymes. The spectrum for each of the mutant enzymes is almost the same as the spectrum of the wild-type enzyme, with the exception that one resonance is missing. In some cases we observe small changes in the ^{19}F chemical shift of the resonances in the mutant enzymes, indicating that replacement of the Trp residue by Tyr or Phe can cause a small perturbation in the protein in some situations. These chemical shift changes are small, and considering the fact that the mutant enzymes are fully active, we can state that replacement of a single Trp does not significantly affect the environment of the remaining four Trp residues. Thus, it is possible to assign each resonance to the corresponding Trp in the primary sequence.

The first impression obtained from the spectra of the three F-Trp derivatives is that, with the exception of 4F-Trp-labeled D-LDH, the five Trp residues in D-LDH give rise to five separate ^{19}F NMR resonances. (The broad resonance at −46.5 ppm for 6F-Trp-labeled D-LDH is most likely due to denatured or impure protein and is present in variable amounts for different preparations.) The fact that there are five separate resonances indicates that each Trp residue is in a unique environment. It is also striking that the pattern of the spectrum for each isomer of Trp is different. For example, in the case of 5F-Trp D-LDH, Trp-384 is the most downfield resonance, while in 4F-Trp D-LDH it is the most upfield resonance. In addition, the chemical shift range of the ^{19}F resonances is different and varies from 3 ppm (6F-Trp) to 6 ppm (4F-Trp) to 7 ppm (5F-Trp). The chemical shift range for the 5F-Trp-labeled enzyme is almost as large as the chemical shift difference between fluorophenylalanine and 5F-Trp (10 ppm). This indicates that the 5F-Trp is highly sensitive to the local environment around each Trp residue in the enzyme.

Finally, we note that the line widths of the resonances are approximately equal for each isomer of F-Trp. This indicates that each Trp residue is experiencing motions on a similar time scale. This is in contrast to what has been observed for fluorotyrosine-labeled alkaline phosphatase (Hull & Sykes, 1974) and *lac* repressor protein (Jarema et al., 1981). A detailed analysis of the dynamics of Trp residues in D-LDH will be given in a future publication.

Because of the superior spectral resolution obtained with 5F-Trp-labeled D-LDH, we will concentrate on this isomer in presenting our results.

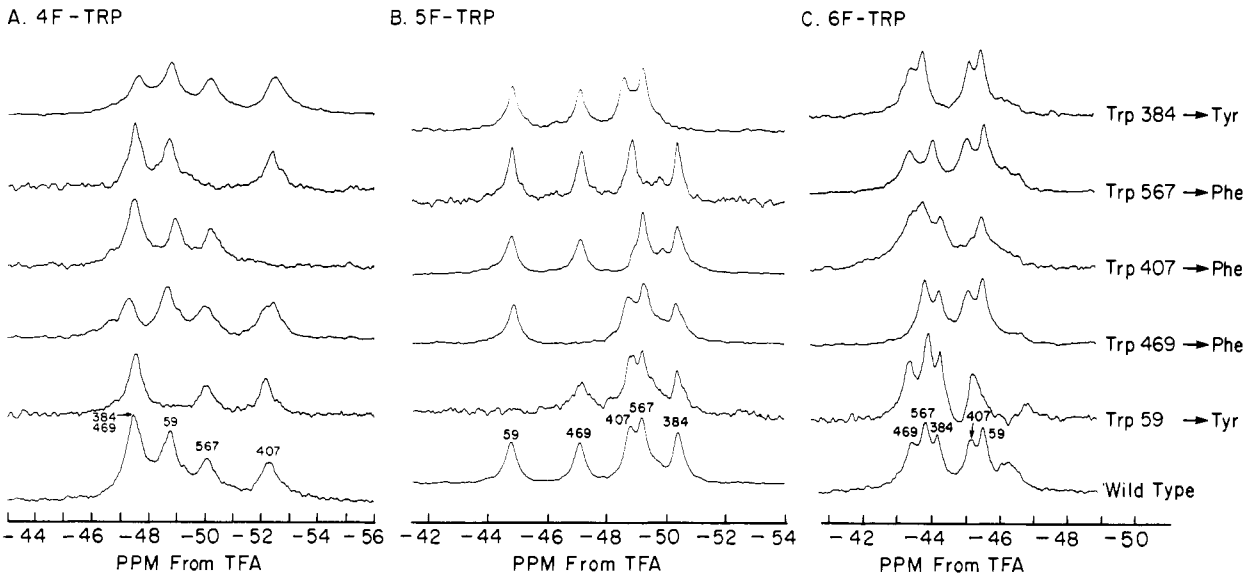

FIGURE 2: Assignment of ^{19}F resonances for D-LDH labeled with fluorotryptophan. The spectra for 4F- (panel A), 5F- (panel B), and 6F-Trp-labeled (panel C) D-LDH and the five Trp to Phe or Tyr mutations are shown. Spectra were obtained at 42 °C in 10 mM phosphate buffer containing 100 mM egg lysolecithin. Approximately 4000–20 000 free induction decays were accumulated for each spectrum. A line broadening of 20 Hz was applied to improve the signal to noise ratio in the spectra. The concentration of D-LDH was approximately 1–2 mM.

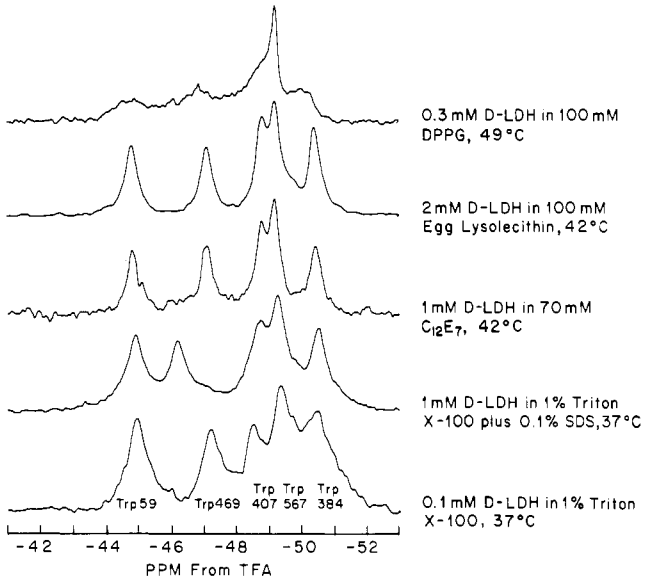

FIGURE 3: ^{19}F NMR spectra of 5F-Trp-labeled D-LDH in the presence of different lipids and detergents. In the Triton-X 100 sample, it was necessary to use a low concentration of D-LDH in order to prevent protein aggregation. A low concentration of D-LDH was used in the DPPG sample to maintain a low protein to vesicle ratio.

Protein–Lipid Interaction. (A) Effect of Lipid Environment on D-LDH. The effects of different amphiphiles on the structure of D-LDH can be determined by observing the ^{19}F spectrum of 5F-Trp-labeled D-LDH in the presence of lipids and detergents. We have incorporated the 5F-Trp-labeled D-LDH into detergent or lysolecithin micelles or into sonicated phospholipid vesicles. From Figure 3, it is apparent that the chemical shifts of two of the five resonances (Trp-59 and -567) are insensitive to the nature of the amphiphile. The resonance arising from Trp-469 undergoes a large change in chemical shift in Triton X-100 plus SDS micelles but is otherwise invariant with respect to the type of amphiphile. This may indicate a SDS-induced conformational change of the protein in the region of Trp-469 or a more specific interaction of Trp-469 with the sulfate group present in SDS. In addition, we also find that Trp-407 is affected by the presence of Triton X-100, and Trp-384 shows a small change in chemical shift in the presence of DPPG.

Variations in the line widths of the resonances in the various amphiphiles can be attributed to the size of the protein–lipid complex. Specifically, Triton X-100 forms large micelles (Robson & Dennis, 1983), and DPPG forms vesicles. These large complexes will have long correlation times and consequently large line widths. It is interesting to note that Trp-567 has a relatively narrow line width in DPPG vesicles compared to the line widths of the other four resonances. This may indicate that the carboxy-terminal domain of the protein is relatively mobile in DPPG vesicles.

We have measured the line width as a function of temperature (27–52 °C) for the five resonances of 5F-Trp-labeled D-LDH in the presence of DPPG (results not shown). We do not observe a discontinuity in the line width at the phase transition of DPPG. This indicates that the motion of the Trp residues is not sensitive to the gel to liquid-crystal phase transition.

The effect of lysolecithin concentration on the line width of 5F-Trp-labeled D-LDH is shown in Figure 4. As the lysolecithin concentration increases, the line width decreases. We observe a similar result with 4F-Trp-labeled D-LDH (data not shown). This indicates that the hydrodynamic radius of the F-Trp-labeled D-LDH is decreasing. In the absence of

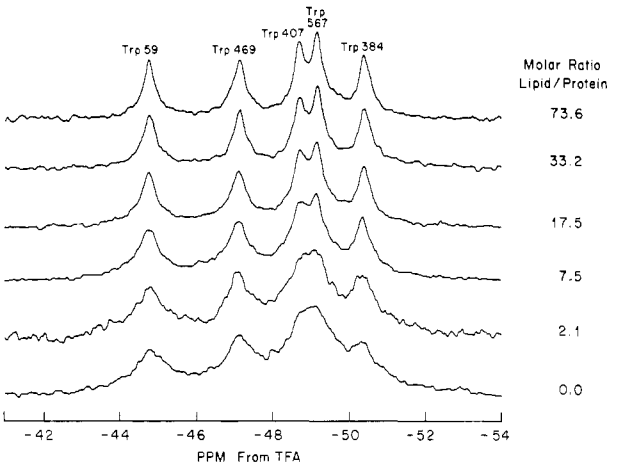

FIGURE 4: Effect of lysolecithin concentration on ^{19}F NMR spectrum of 5F-Trp-labeled D-LDH. The concentration of D-LDH was 1 mM.

detergents, D-LDH is known to form small aggregates of three to four molecules, while in excess Triton X-100 the monomeric form of D-LDH prevails (Tanaka et al., 1976). The effect of lysolecithin concentration on the line width may be due to a change in the multimer–monomer equilibrium of D-LDH. Since the concentration of lysolecithin is much greater than the critical micellar concentration in this experiment, we can assume that the protein–lipid association constant is much greater than the lipid self-association constant; otherwise, there would be no effect on the ^{19}F line widths as the lysolecithin concentration is increased. Therefore, as the lysolecithin is added to the solution of D-LDH, the lysolecithin molecules bind to the enzyme and cause the formation of D-LDH monomers. By measuring the line width as a function of the lysolecithin concentration, we observe that the decrease in line width is not linear with respect to the lysolecithin concentration. This indicates that the protein–lipid complex is in slow exchange with the aggregated protein. In addition, it is apparent that when a certain lipid to protein ratio is reached, the line width remains constant. This indicates that there are only a certain number of binding sites on the enzyme for lysolecithin. If additional lysolecithin molecules became bound to D-LDH, we would expect to see an increase in the line width as the lysolecithin–protein complex becomes larger. From the dependence of the line width on the lipid to protein ratio, we can determine that each molecule of D-LDH binds 40–60 molecules of lysolecithin.

(B) Effect of D-LDH on Lipid Phase. D-LDH may affect the structure and dynamics of the lipid molecules in the protein–lipid complexes. By incorporating D-LDH into (8,8-difluoromyristoyl)lysolecithin, we can observe effects on the acyl chain by ^{19}F NMR and effects on the head group by ^{31}P NMR. ^{19}F NMR spectra of the fluorinated lysolecithin–D-LDH complex show that the ^{19}F chemical shift and line width of the fluorolysolecithin do not change upon addition of D-LDH. In contrast, we observe changes in the ^{31}P resonance upon addition of D-LDH. Specifically, we observe an increase in line width from 7 to 23 Hz, an upfield change in ^{31}P chemical shift of 0.14 ppm, and a decrease in the ^{31}P T_1 from 2.1 to 1.4 s. These effects indicate that D-LDH decreases the mobility of the head group (Rajan et al., 1981).

Properties of D-LDH As Determined by ^{19}F NMR. The ^{19}F chemical shift has been shown to be sensitive to the deuterium content of the aqueous phase (Hagen et al., 1979). Figure 5 shows the effect of increasing the H_2O content of the buffer on the ^{19}F chemical shift of 5F-Trp and 5F-Trp-labeled D-LDH. It is apparent that Trp-384 is fully exposed to the solvent,

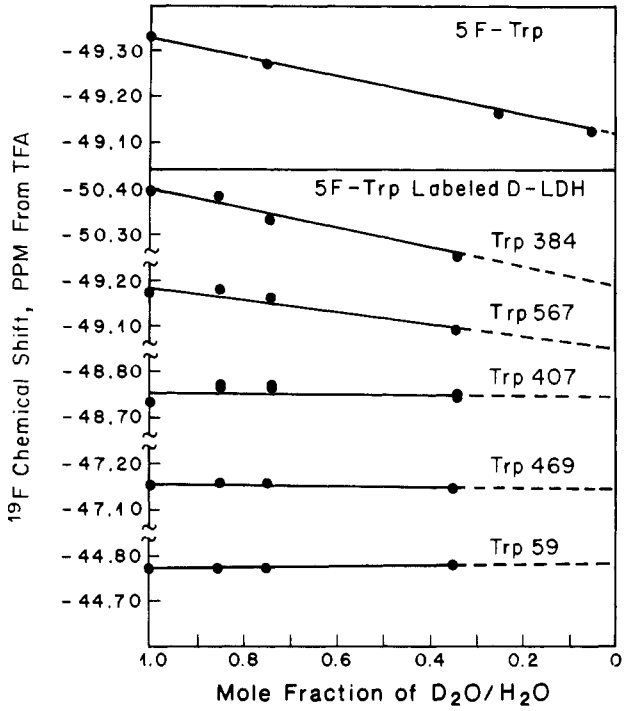

FIGURE 5: Effect of solvent composition on ^{19}F chemical shifts of Trp residues in 5F-Trp-labeled D-LDH. The chemical shifts of 10 mM 5F-Trp and 0.6 mM 5F-Trp-labeled D-LDH are shown as a function of the ratio of D_2O/H_2O in the buffer.

while Trp-567 is partially (60%) exposed. We observe similar effects with 6F-Trp-labeled D-LDH (data not shown). However, in the case of 4F-Trp, the SIIS for the free amino acid is small (<0.1 ppm), and thus it is difficult to observe a SIIS with 4F-Trp-labeled D-LDH. The conclusion that Trp-384 is fully exposed to solvent is supported by measurement of the effect of the solvent composition on the T_1. When the buffer contains H_2O instead of D_2O, the T_1 values are reduced by the greatest extent for residue 384 (data not shown). The increased relaxation rate for this resonance can be attributed to the additional proton density resulting from the neighboring water molecules.

An approach to investigating the proximity of the Trp residues to each other is to measure the homonuclear NOE between the fluorine spins. We have selectively inverted the resonances corresponding to Trp-59, Trp-384, and Trp-469 in 5F-Trp-labeled D-LDH. Due to the small chemical shift difference between the resonances for Trp-407 and Trp-567, we have not been able to apply a selective pulse to these two resonances. We have not observed any NOE between Trp-59, Trp-384, or Trp-469 and any other Trp. The interpretation of this null effect is complicated by the effects of spin diffusion, which would be expected to be large in a protein of this size (Kalk & Berendsen, 1976). One possibility is that the inter-fluorine distance (i.e., the distance between the two fluorine atoms in two F-Trp residues) is too large (say more than 5 Å) for the NOE to be observed. Another possibility is that, even if the distance is smaller than 5 Å, the additional spin–lattice relaxation caused by neighboring protons may quench the homonuclear ^{19}F–^{19}F NOE. Further work is needed to investigate the spatial relationship among these five Trp residues in D-LDH.

It is also possible to determine the proximity of the Trp residues to the lipid phase by the incorporation of a lipophilic nitroxide spin-label into the lipid phase. Under the conditions of the experiment (10 mol % doxylstearic acid in lysolecithin), we did not observe any effects of the nitroxide on the ^{19}F line

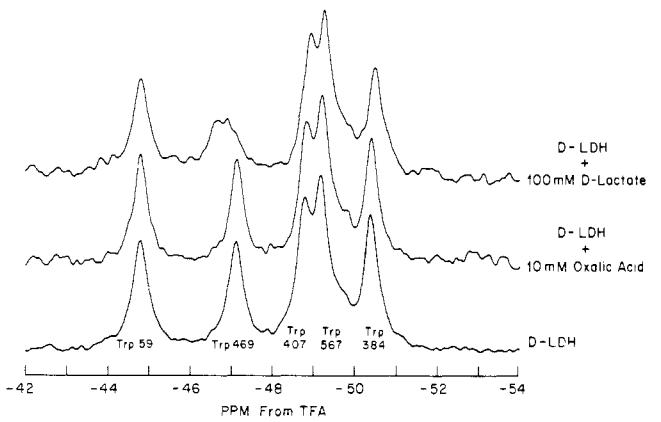

FIGURE 6: Effect of D-lactate and oxalate on ^{19}F NMR spectrum of 5F-Trp-labeled D-LDH. The concentration of D-LDH was 1 mM. The K_I for oxalic acid is in the micromolar range; thus, all of the D-LDH molecules will contain a bound oxalate. A large excess of D-lactate was used in order to ensure that the FAD cofactor remained reduced throughout the NMR experiment.

widths. This indicates that none of the Trp residues is within 10 Å of the spin-label (Arseniev et al., 1981). (One possible artifact in this experiment is the selective exclusion of the spin-label from the protein–lipid interface. We are currently synthesizing spin-labeled lysolecithin in order to address this problem.)

Finally, we have investigated the effects of the substrate on the ^{19}F spectrum of D-LDH. Upon addition of D-lactate to the enzyme, the flavin cofactor becomes reduced, and in the absence of a hydrogen acceptor, the flavin will remain reduced. Figure 6 shows that in the presence of D-lactate the ^{19}F chemical shift of the resonance from Trp-469 moves 0.3 ppm downfield and becomes broadened. When the same experiment is performed with 4F-Trp-labeled D-LDH, we observe a broadening of the first resonance corresponding to Trp-384 and Trp-469. When D-LDH containing either the Trp-469 to Phe mutation or the Trp-384 to Tyr mutation is used, we still observe an effect of the substrate on the first resonance of 4F-Trp-labeled D-LDH. This indicates that in the 4F-Trp-labeled D-LDH both Trp-384 and Trp-469 are sensitive to the presence of the substrate. In the case of 6F-Trp-labeled D-LDH, we observe an effect of D-lactate only on the resonance from Trp-469. The presence of the competitive inhibitor oxalic acid has no effect on the spectrum of 4F-, 5F-, or 6F-Trp-labeled enzyme. Since the addition of oxalic acid to D-LDH does not result in any spectral changes of the labeled D-LDH, it is likely that Trp residues 384 and 469 are sensitive to a conformation change induced in the protein upon reduction of the flavin cofactor rather than the binding of the substrate.

Discussion

The first point to consider is that of the suitability of fluorinated Trp as an NMR probe for D-LDH. There are obvious advantages that arise from the use of a fluorinated amino acid that has several isomers, such as 4F-, 5F-, and 6F-Trp. As illustrated in this study, one isomer may prove to be less perturbing than another. The kinetic parameters of D-LDH are not greatly affected by fluorination with any of the F-Trp isomers. However, the CD spectra indicate that the secondary structures of the 4F- and 5F-Trp-labeled D-LDH are similar to that of the unsubstituted enzyme, while the 6F-Trp-labeled enzyme shows perturbation of the secondary structure. (It is possible that the anomalous CD spectrum obtained for the 6F-Trp-labeled D-LDH is a result of the presence of impurities or denatured D-LDH. The existence

of such may be indicated by the broad peak at −46.5 ppm in the ^{19}F NMR spectra of 6F-Trp-labeled D-LDH.) Also the 4-fluoro and 5-fluoro isomers yield good resolution in the ^{19}F NMR spectra, with a chemical shift range of 6–7 ppm, while the spectrum from the 6-fluoro isomer has a small chemical shift range of 3 ppm. Thus, 6F-Trp does not appear to be suitable as an NMR label for D-LDH, while the 4-fluoro and 5-fluoro isomers are very promising as probes for ^{19}F NMR studies of D-LDH.

Another practical consideration is the effect of the different isomers on the production of the labeled protein. *E. coli* is more sensitive to 5F- and 6F-Trp than to 4F-Trp (Pratt & Ho, 1975). Thus, in the case of D-LDH, we find that 3–4 times as much 4F-Trp-labeled enzyme can be produced on induction. For any given protein, all of these factors must be tested, as it does not seem possible to predict in advance which isomer will work best for a given protein.

The fact that the chemical shifts of the different isomers cannot be predicted in advance, and one isomer may give well-resolved spectra while another may not, indicates that the chemical shift of the fluorine is very sensitive to the environment and should give useful information about the environment of the F-Trp residue in the protein.

The data presented in Figure 3 show that some of the Trp residues in D-LDH have different chemical shifts in the presence of different lipids and detergents, suggesting that the conformation of D-LDH is affected by the lipid environment. Such a result was obtained from ^{19}F NMR studies on the M13 coat protein (Wilson & Dahlquist, 1985). However, close inspection of the spectra presented in Figure 3 shows that the ^{19}F chemical shifts of the Trp residues in the presence of $C_{12}E_7$, lysolecithin, and DPPG are actually very similar. Figure 4 shows that the chemical shifts are also similar in aqueous solution. This implies that the conformation of D-LDH is similar in aqueous solution and in the presence of these three lipids and detergents and that this conformation is different from the conformation found in Triton X-100. Evidence for two different conformations of D-LDH is also supported by studies on the effect of temperature on the activity of D-LDH. In the case of $C_{12}E_7$, lysolecithin, or DPPG, we find that a plot of the log of V_{max} vs. $1/T$ is linear over the range of 20–55 °C. In contrast, in the presence of Triton X-100, we observe a biphasic dependence of V_{max} on temperature. There is an initial increase up to 35 °C, followed by a constant V_{max} (see Figure 7). This indicates that D-LDH, in the presence of Triton X-100, is in a more thermolabile conformation. This conclusion is also supported by studies of the temperature stability of D-LDH in the presence of Triton X-100 (Pratt et al., 1979). Thus, Triton X-100 appears to cause an aberrant, nonphysiological conformation of the protein. Other lipids and detergents are better employed to solvate the purified enzyme for studies of its properties. Using Triton X-100 for purification does not appear to affect the properties of the purified enzyme: the ^{19}F NMR spectrum in the presence of lysolecithin is identical for enzyme purified with either Triton X-100 or $C_{12}E_7$.

The data presented in Figure 4 indicate that the chemical shift, and thus the conformation of D-LDH, is invariant to the amount of lysolecithin present in the sample. Increasing the $C_{12}E_7$ concentration also shows no effect on the ^{19}F NMR spectra of 5F-Trp-labeled D-LDH (data not shown). In addition, the CD of D-LDH is not affected by varying amounts (0–10 mM) of $C_{12}E_7$ (unpublished results). These results suggest that lysolecithin and $C_{12}E_7$ simply solvate the enzyme and do not cause conformational change in D-LDH. This

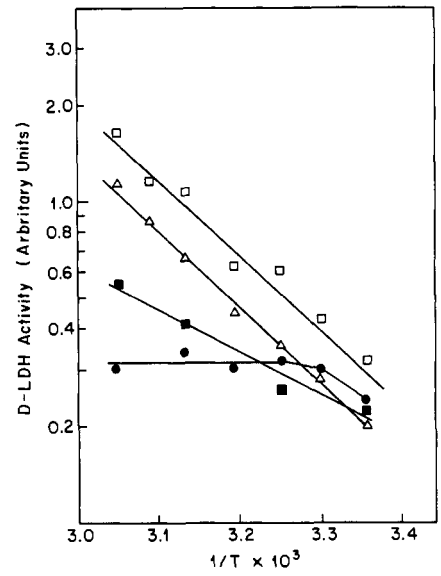

FIGURE 7: Effect of temperature on activity of D-LDH in the presence of Triton X-100 [(●) 0.6%], $C_{12}E_7$ [(■) 10 mM], egg lysolecithin [(□) 1 mM], and DPPG [(△) 1 mM].

implies that the tertiary structure of D-LDH is largely determined by self-interactions and that the lipid phase does not affect the folding of this enzyme. This conclusion is supported by the fact that active D-LDH is synthesized in vitro in the absence of lipids (Santos et al., 1982). Therefore, the effect of lipids on the activity of D-LDH cannot be ascribed to a lipid-induced conformational change in the protein structure. Furthermore, it is apparent from the NMR data that the conformation of D-LDH is not affected by the lipid phase transition of DPPG. In accord with this, we find that the kinetic properties of D-LDH in the presence of DPPG are also not affected by the phase transition (Figure 7).

The low activity of D-LDH in the delipidated state may result from steric hindrances that result from the formation of protein aggregates. For instance, the D-lactate or PMS binding sites may be occluded by adjacent molecules, or the adjacent molecules may hinder conformational changes that may be required during turnover of the enzyme. This conclusion is not without precedent. Circular dichroism studies on the membrane-bound glycerolphosphate dehydrogenase of *E. coli* by Robinson and Weiner (1980) suggest that the conformation of this enzyme is also not affected by the presence of lipid. They conclude that, in the absence of lipid, the binding site for PMS is not present and that the low activity of glycerolphosphate dehydrogenase in the delipidated state is entirely due to the inability of PMS to oxidize the reduced enzyme. Thus, the conformation of some membrane enzymes may not be affected by the presence of lipids, suggesting that some membrane enzymes can fold correctly in the absence of phospholipids.

The finding that D-LDH binds a fixed number of lysolecithin molecules places some restrictions on the nature of the protein–lipid complex. Since the aggregation number of lysolecithin is 180 (Saunders, 1966), while our results suggest that there are 40–60 mol of lysolecithin/mol of D-LDH, we can conclude that the enzyme does not insert into a lysolecithin micelle. However, the binding of lysolecithin to D-LDH may occur in a cooperative manner, such as the formation of a D-LDH–micelle intermediate structure. This speculation is supported by the fact that solutions of lysolecithins must contain micelles in order to stimulate the activity of D-LDH (Kovatchev et al., 1981). We suggest that there is a hydrophobic domain on the surface of the enzyme that binds a

sufficient number (40–60) of lysolecithin molecules to become shielded from the aqueous phase. In the absence of lipids, D-LDH molecules self-aggregate, and we propose that the intermolecular contacts between the D-LDH molecules in the aggregated state occur in this hydrophobic domain.

In addition to a hydrophobic interaction between D-LDH and phospholipids, there also appears to be an interaction between the head group and the enzyme. As suggested from the ^{31}P NMR data, D-LDH restricts the motion of the phosphate. D-LDH contains a large number of basic amino acids [see Rule et al. (1985)], and some of these may form salt bridges with the anionic head group.

At this time we can make several conclusions about the location of the Trp residues in D-LDH. Trp-384 and Trp-567 appear to be located near the surface of the enzyme. Trp residues 384 and 469 appear to be located near the FAD cofactor and are sensitive to conformational changes upon reduction of the cofactor. Since Trp-384 is also found near the surface of the enzyme, it is possible that this region of the enzyme is involved with coupling of D-lactate oxidation to other components in the respiratory chain. We can determine very little about the spatial location of the Trp residues except to say that neither Trp-59, Trp-469, nor Trp-567 is very close to the other Trp residues nor are any of the Trp close to the lipid phase.

As an approach to obtaining more information from the other regions of the D-LDH molecule, we are currently introducing Trp residues into various locations in the enzyme by site-specific mutagenesis. With this technique, we can investigate the effects of lipids on any portion of the D-LDH molecule, and we can also obtain additional information about the structure of D-LDH. Furthermore, these investigations can be extended to study the interaction between D-LDH and components of the *E. coli* respiratory chain.

In conclusion, we have demonstrated that ^{19}F NMR of fluorotryptophan-labeled protein, combined with the powerful techniques of molecular genetics, is a fruitful method for obtaining information about protein–lipid interactions.

ACKNOWLEDGMENTS

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Registry No. D-Lactate dehydrogenase, 9028-36-8; tryptophan, 73-22-3; Triton X-100, 9002-93-1; (8,8-difluoromyristoyl)lysolecithin, 105858-35-3; 4-fluoro-L-tryptophan, 106034-22-4; 5-fluoro-L-tryptophan, 16626-02-1; 6-fluoro-L-tryptophan, 19310-00-0.

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