Inactive and Temperature-Sensitive Folding Mutants Generated by Tryptophan Substitutions in the Membrane-Bound D-Lactate Dehydrogenase of *Escherichia coli*†

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ABSTRACT: A combination of site-specific mutagenesis and ^{19}F nuclear magnetic resonance has been used to investigate the structural properties of D-lactate dehydrogenase, a membrane-associated enzyme of *Escherichia coli*. The protein (65 000 Da) has been labeled with 5-fluorotryptophan for ^{19}F nuclear magnetic resonance studies. Tryptophan has been substituted for individual phenylalanine, tyrosine, isoleucine, and leucine residues at various positions throughout the enzyme molecule, and the fluorinated native and substituted tryptophan residues have been used as probes of the local environment. All 24 mutants thus generated are expressed in *E. coli*. Ten are fully active and purifiable following the usual procedure, while 14 either are inactive or produce low levels of activity. The amount of active enzyme produced from the low-yield mutants is dependent on the temperature at which synthesis is carried out, with more active enzyme produced at 18 °C than at 27, 35, or 42 °C. Cells grown at 27 °C and then incubated at 42 °C retain 90–100% of their activity. All of the expressed protein from the inactive mutants is Triton-insoluble, aggregated, and not readily purifiable; the inactive mutant protein appears to be improperly folded. Most of the expressed D-lactate dehydrogenase from the partially active mutants is also Triton-insoluble; a small fraction, however, is soluble in Triton and can be purified to yield active enzyme. All the purified enzymes from these low-yield mutants of D-lactate dehydrogenase have essentially normal V_{max}s, and all but two have normal K_ms. Once purified, the low-yield mutant enzymes are stable at 42 °C. Folding is temperature-sensitive, but once folded, the protein is thermally stable. Thus, in these mutants the tryptophan substitution produces temperature-sensitive folding mutants. The ^{19}F nuclear magnetic resonance spectra of a majority of these temperature-sensitive folding mutant proteins show abnormalities compared to the spectra of wild type or fully active mutants of D-lactate dehydrogenase, indicating that even though these mutants are functional, their structure is perturbed by the tryptophan substitution.

An intriguing development in the field of protein folding has been the study of temperature-sensitive folding mutants (Goldenberg & King, 1981; Sugihara & Baldwin, 1988). The most extensively studied have been those of the tail-spike protein of phage P22 (Goldenberg & King, 1981; Haase-Pettingell & King, 1988; Yu & King, 1988; Sturtevant et al., 1989). Temperature-sensitive folding mutants are characterized by being synthesized in a functional form at permissive temperatures but not at higher, nonpermissive temperatures. However, once synthesized and matured at the permissive temperature, the protein is functional and stable at the nonpermissive temperature, indicating that it is the folding of the protein that is sensitive to temperature. Even though the temperature-sensitive folding mutants are distinguishable from the wild-type protein in their electrophoretic behavior (Yu & King, 1988), the three-dimensional structure of such mutants is believed to be equivalent to that of the wild-type protein, on the basis of physiological studies and Raman spectroscopy (Goldenberg & King, 1981; Sargent et al., 1988; Thomas et al., 1990).

In making mutants of the membrane-associated respiratory enzyme D-lactate dehydrogenase (D-LDH)[1] of *Escherichia coli* containing an additional tryptophan residue, we have generated what appear to be temperature-sensitive folding mutants. We have been studying D-LDH, using a combination of site-specific mutagenesis and ^{19}F nuclear magnetic resonance spectroscopy (NMR) to investigate its structure and interactions when it is labeled with fluorinated amino acids (Rule et al., 1985, 1987a,b; Ho et al., 1989; Peersen et al., 1990; Truong et al., 1991). 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) and requires lipid or detergent for full activity (Tanaka et al., 1976; Fung et al., 1979; Kovatchev et al., 1981). The wild-type

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[1] Abbreviations: D-LDH, D-lactate dehydrogenase; FAD, flavin adenine dinucleotide; lysoPC, egg lysophosphatidylcholine; MTT, 3-(3,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PMS, phenazine methosulfate; TFA, trifluoroacetic acid; Trp, tryptophan, 5F-Trp, 5-fluorotryptophan; NMR, nuclear magnetic resonance. 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., F340W = the mutant in which Trp is substituted for Phe at position 340.

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native protein contains five Trps (Rule et al., 1985; Ho et al., 1988) and the ^{19}F NMR spectrum of the 5F-Trp-labeled protein shows five peaks (Rule et al., 1987b). In order to gain information about additional areas of the enzyme molecule, Trp has been inserted in place of Phe, Tyr, Ile, and Leu at 24 different positions throughout the enzyme. Of the resulting mutants labeled with 5F-Trp, 10 show normal activity and structure (Peersen et al., 1990; unpublished results). In this paper, we discuss how in the remaining 14 mutants the Trp substitutions produce seven inactive and improperly folded mutants and seven temperature-sensitive folding mutants. These temperature-sensitive folding mutants are thermally stable and active, yet their structures as determined by ^{19}F NMR vary from that of the wild-type D-LDH.

Materials and Methods

Site-directed mutagenesis and expression of 5F-Trp-labeled mutant D-LDHs have been carried out as described previously (Rule et al., 1987a,b). Attempts to purify the D-LDH mutant protein followed the usual purification procedure (Rule et al., 1985, 1987b; Peersen et al., 1990). The purification of the 5F-Trp-labeled low-yield D-LDH mutants described in this work required 12 L of culture from high-density fermentors (Labline Instruments) to yield 1–2 samples (~40 mg) for NMR spectroscopy. SDS–polyacrylamide gel electrophoresis (Laemmli, 1970) was carried out on sonicated cells and on fractions from the subsequent purification of D-LDH.

The Michaelis–Menten parameters, K_m and V_{max}, of the purified 5F-Trp-labeled substitution mutants were determined by Lineweaver–Burk plots using the phenazine methosulfate (PMS) 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 a standard.

For studies of temperature sensitivity, the genes for wild-type and mutant D-LDHs were carried in a pBR327-derived plasmid in *E. coli* HB101 [pBR327dld; for construction, see Rule et al. (1987b)], i.e., not in a temperature-inducible expression system. Cultures (20 mL) were grown to stationary phase in Luria broth in 125-mL Erlenmeyer flasks in a shaking water bath. The cells were centrifuged and resuspended in 2 mL of 0.01 M potassium phosphate at pH 7.2. After determination of the absorbance at 550 nm, the cells were sonicated for 1 min and then assayed for D-LDH activity.

NMR Sample Preparation of Isolated Mutant Proteins. D-LDH-containing fractions were prepared for NMR studies as described previously (Rule et al., 1987b; Peersen et al., 1990). LysoPC (20 mg; Sigma) was dissolved with 0.5–1 mM purified D-LDH in 400 μL of D_2O buffer (10 mM potassium phosphate, 0.2 mM EDTA, pH 7.2), and the particulates were removed by centrifugation before the ^{19}F NMR spectra were acquired. The effect of substrate was tested by adding 40 μL of 1 M D-lactate 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 saturates the 5F-Trp-labeled enzyme.

The NMR measurements were performed on Bruker WH-300 and AM-300 spectrometers operating at 282.4 MHz. The ^{19}F NMR spectra of isolated D-LDH mutant proteins labeled with 5F-Trp were obtained with an 8-KHz spectral width and 4K data points in a 5-mm ^{19}F probe using a 4.4-μs 60° pulse and a relaxation delay of 2 s. Free induction decays (10 000–25 000) were accumulated and Fourier transformed with 15-Hz line broadening, and the resulting spectra were corrected for phase and baseline distortions. Chemical shifts are expressed relative to trifluoroacetic acid (TFA).

Results

Expression of Mutant Protein. Twenty-four D-LDH mutations have been generated by Trp substitution and placed in the temperature-inducible expression vector for D-LDH (Rule et al., 1985). After induction, SDS–PAGE of the sonicated cells shows that a 65-kDa polypeptide, which is absent in the absence of plasmid and comigrates with purified D-LDH, is expressed in all the mutants (Figure 1A,B; F300W and F315W not shown).

Of the 24 mutants, however, 14 have very little or no enzymatic activity after induction at 42 °C (Table I). Attempts to isolate protein from these negative mutants have been made, following the usual procedure for D-LDH purification (Figure 2; Rule et al., 1987b). After sonication of the cells and ammonium sulfate treatment, the precipitated D-LDH is solubilized in a buffer containing Triton X-100. While wild type (Figure 3) and active D-LDH mutants are solubilized by Triton X-100 so that 80–95% of the 65-kDa polypeptide is found in the supernatant and only very little is found in the pellet, the reverse is true for the negative mutants. The behavior of the negative mutants falls into two classes. In the case of the inactive mutants I99W, I152W, L203W, F263W, F279W, F326W, and F490W, the 65-kDa peptide is found only in the Triton pellet, with no detectable D-LDH activity in either the pellet or the supernatant (results not shown). For the low-yield mutants, mutants L110W, F176W, I193W, Y309W (Figure 3), F412W, L456W, and F544W, the 65-kDa peptide is found mostly in the pellet, though a small fraction partitions into the supernatant. The supernatant, but not the Triton pellet, has measurable D-LDH enzymatic activity.

Attempts to solubilize the 65-kDa polypeptide found in the Triton-insoluble fraction from both sets of mutants have been made by using a variety of detergents and denaturants. The polypeptide is not solubilized by 2 M guanidine hydrochloride or 5 M urea, but it is solubilized from the pellet by 1% SDS. The 65-kDa polypeptide constitutes 50% of the protein in the SDS extract but less than 20% in the remaining pellet (estimated by the intensity of the bands in the stained protein gel). The solubilized fraction does not contain flavin, as evidenced by the absence of absorption at 450 nm, and has no detectable enzymatic activity. Attempts to reconstitute the Triton-insoluble fraction with FAD have so far been unsuccessful.

The 65-kDa fraction that is solubilized by Triton X-100 from the low-yield mutants has been purified by the usual protocol and yielded active enzyme containing FAD. Since the Triton-soluble fraction represents at most 10% of the total protein expressed, the active form of these D-LDH mutants can be purified only in low yields. The low yield of mutant F176W led us to change its classification from an active mutant (Peersen et al., 1990) to that of a low-yield mutant.

Kinetics. The kinetic parameters of wild type, active, and low-yield mutant D-LDHs are given in Table II. The mutants have K_ms that are within 50% of the K_m for 5F-Trp-labeled wild-type D-LDH, except for the Y309W and L456W mutants, in which the K_m is higher. The V_{max} values, in general, are of the same order of magnitude as that of wild type.

Synthesis of Active Enzyme at Varying Temperatures. The temperature dependence of the synthesis of enzymatically active wild-type and mutant D-LDHs was measured in *E. coli* HB101 cells containing the *dld* gene inserted into a pBR327 plasmid without the P_L promoter (Rule et al., 1985). For wild type and active mutants, the expressed D-LDH activity is decreased at higher temperatures (Table I), probably due to

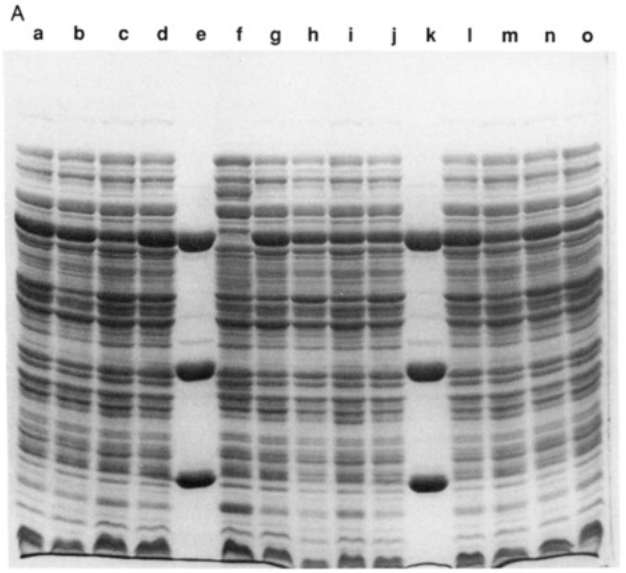

FIGURE 1: (A) SDS–polyacrylamide gel of cells with plasmids containing the mutant D-LDHs (a) F12W, (b) F39W, (c) I99W, and (d) L110W; (e) degraded purified wild-type D-LDH; (f) cells with no plasmid; (g) cells with plasmids containing wild-type D-LDH; cells with plasmids containing the mutant D-LDHs (h) I152W, (i) F176W, and (j) I193W; (k) degraded purified wild-type D-LDH; cells with plasmids containing the mutant D-LDHs (l) L203W, (m) Y243W, (n) F263W, and (o) F279W. The degraded wild-type D-LDH was generated by storage at 4 °C for several months (Rule et al., 1985). (B) SDS–polyacrylamide gel of cells with plasmids containing the mutant D-LDHs (a) Y309W, (b) F326W, (c) F340W, and (d) F361W; (e) degraded purified wild-type D-LDH; (f) cells with no plasmid; (g) cells with plasmids containing wild-type D-LDH; cells with plasmids containing the mutant D-LDHs (h) Y388W, (i) F412W, and (j) F435W; (k) degraded purified wild-type D-LDH; cells with plasmids containing the mutant D-LDHs (l) L456W, (m) F490W, (n) L517W, and (o) F544W.

the lower copy number of the plasmid: for cells grown at 35 °C, the activity is 40–60% of that of cells grown at 27 °C; for cells grown at 42 °C, the activity has decreased to 10–20%. For cells grown at 18 °C, a greater amount of D-LDH activity is expressed than for cells grown at 27 °C. In contrast, the activity of the low-yield mutants, which is comparable to that of wild type or active mutants when grown at 18 and 27 °C, is more dramatically affected by the higher growth temperatures. The D-LDH activity expressed in cells containing the low-yield mutants decreases to less than 35% for cells grown at 35 °C and is barely measurable for cells grown at 42 °C.

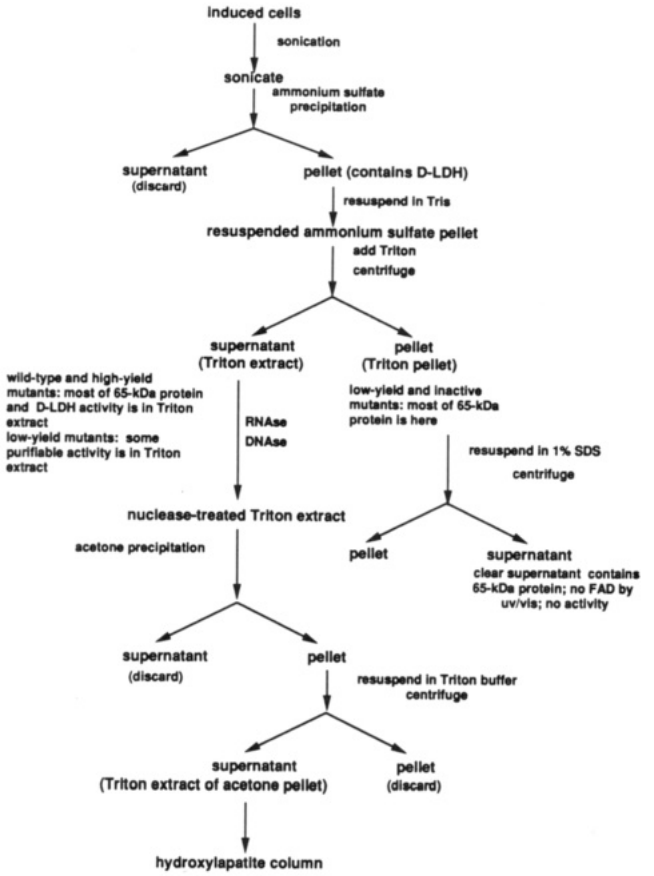

FIGURE 2: An outline of the isolation procedure for D-LDH.

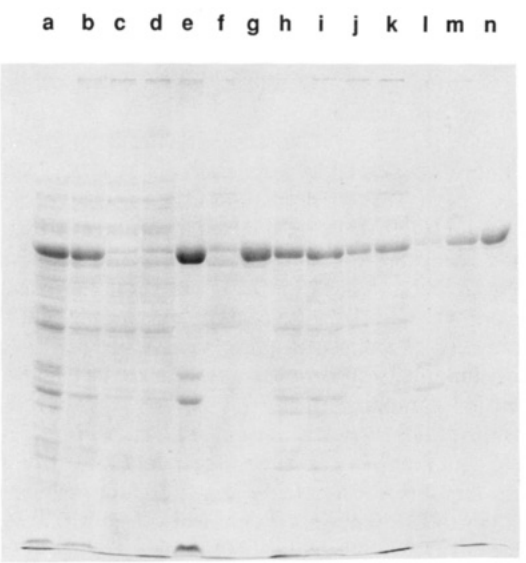

FIGURE 3: SDS–polyacrylamide gel of fractions from the purification of D-LDH. Y309W mutant: (a) sonicate, (b) ammonium sulfate pellet, (c) Triton extract, (d) nuclease-treated Triton extract, (e) Triton pellet, (f) resuspended pellet from acetone step. Wild-type D-LDH: (g and n) purified enzyme, (h) sonicate, (i) ammonium sulfate pellet, (j) Triton extract, (k) nuclease-treated Triton extract, (l) Triton pellet, (m) resuspended pellet from acetone step (see Figure 2).

The inactive mutants grown at 27 °C have very little or no activity, and have essentially no activity when grown at the higher temperatures.

When cells are grown at 27 °C and then placed for 1 h at 42 °C, however, they retain essentially all of their activity (Table I). Some of the inactive mutants appear to lose more activity after the heat treatment than the low-yield, active, and wild-type D-LDH; however, there is a greater error in the

Table I: Yield of D-LDH Activity from the Temperature-Inducible Expression Vector and Temperature Dependence of the Synthesis of D-LDH Activity Expressed in HB101 Cells

D-LDH	W3110trpA33/ pGA2 or pGA2a[a] yield[b]	HB101/pBR327dld[a] growth temperature: 27 °C act.[c]	27 °C rel act.[d]	18 °C rel act.[d]	35 °C rel act.[d]	42 °C rel act.[d]	HB101/pBR327dld[a] grown at 27 °C, treated at 42 °C rel act.[d]
			wild-type and active mutants				
wild type	6.4	1.8	1.0	1.4	0.7	0.2	1.0
F12W	2.2	2.1	1.0	1.3	0.4	0.1	0.9
F39W	2.5	1.2	1.0	3.8	0.6	0.3	0.9
Y243W	1.5	1.9	1.0	2.8	0.5	0.1	1.0
F300W	2.9	1.7	1.0	2.8	0.6	0.1	0.9
Y315W	3.9	1.7	1.0	2.7	0.6	0.1	0.9
F340W	3.1	2.1	1.0	1.5	0.5	0.2	1.0
F361W	4.6	1.9	1.0	2.2	0.5	0.1	0.9
Y388W	1.1	1.9	1.0	2.2	0.4	0.1	1.0
F435W	1.1	0.9	1.0	2.0	0.4	0.2	1.0
L517W	1.0	1.3	1.0	1.2	0.4	0.1	1.0
			low-yield mutants				
L110W	0.9	1.3	1.0	2.5	0.2	0.02	1.0
F176W	0.7	1.3	1.0	3.2	0.3	0.04	0.9
I193W	0.3	0.5	1.0	1.2	0.1	0.04	0.9
Y309W	0.4	1.9	1.0	1.2	0.3	0.02	1.0
F412W	0.4	1.7	1.0	1.1	0.2	0.02	1.0
L456W	0.4	0.8	1.0	1.0	0.3	0.02	1.0
F544W	0.7	1.4	1.0	3.1	0.1	0.04	0.9
			inactive mutants				
I99W	<0.1	0		0	0	0	
I152W	<0.1	0		0	0	0	
L203W	<0.1	0.8	1.0	3	0.1	0	0.9
F263W	<0.1	0.2	1.0	4	0	0	1.1
F279W	<0.1	0.3	1.0	2	0.2	0.02	0.7
F326W	<0.1	0.6	1.0	1	0.2	0.02	1.0
F490W	<0.1	0.4	1.0	6	0	0	0.8

[a] pGA2 was used for wild type, and pGA2a was used for mutants. Both pGA2 and pGA2a contain the temperature-inducible P_L promoter; pBR327dld does not contain P_L. See Rule et al. (1987b) for the construction of plasmids. The W3110trpA33 were grown in the presence of 5F-Trp after induction; the HB101 cells were grown without 5F-Trp (Peersen et al., 1990). [b] D-LDH activity in temperature-inducible expression vector. Yield per fermentor (~3 L), 6 h after induction at 42 °C in the presence of 5F-Trp, followed by freezing, storage at −80 °C, and sonication. Units: μmol of MTT reduced·min^{-1}·mL^{-1} × 10^{-5}. [c] Units: μmol of MTT reduced·min^{-1}·mL^{-1}·A_{550}^{-1} × 10^{-1}. The normalized activity of HB101 cells without the *dld*-containing plasmid was subtracted from the activities. [d] Relative activity, expressed as a fraction of the activity at 27 °C.

measurement of the relative activities of the inactive mutants due to their very low levels of enzymatic activity.

^{19}F NMR Studies of the Low-Yield Mutants. Once purified, the low-yield D-LDH mutant proteins are able to withstand the conditions for NMR measurements (12–36 h at 42 °C, a temperature at which very little D-LDH activity is expressed in vivo) and retain activity. The purified low-yield mutants display distinct resonances in their ^{19}F NMR spectra (Figure 4). The line widths of the resonances are similar to those of the wild-type spectrum, indicating that the motional properties of F-Trps in the mutants are similar to those of the five F-Trps in the native enzyme. A sixth resonance arising from the substituted F-Trp would be expected in the spectra of these mutants; however, the spectra of all low-yield mutants are perturbed. The substitution at Phe 176 displaces the resonance of the native Trp 59; it is clear from a comparison of the spectrum with that of the wild-type D-LDH that the Trp 176 resonance occurs in the vicinity of −46 ppm. Assignment of the substituted Trp is not as straightforward for the other low-yield mutants. The substitution at Leu 110 perturbs Trps 407 and 567 such that their resonances are not clearly separated, but this may be caused by interference from the resonance arising from Trp 110.

Eight major resonances are seen in the spectrum for F544W. The peak at ~−49.5 ppm may arise from sample degradation or contamination. SDS–PAGE of the NMR sample, however, shows that ≥95% of the sample is D-LDH and that only minor proteolytic degradation has occurred (Rule et al., 1985). Substitution at Phe 544 affects the resonances of Trp 407 and/or Trp 567 such that peak assignments cannot be readily made. At least one Trp, at position 407, 544, or 567, appears to be in more than one environment.

For most low-yield mutants, the native Trp 469 is perturbed. L456W shows only a small peak in the −45.5 to −48 ppm region where native Trp 469 usually appears. The addition of the substrate D-lactate, to which Trp 469 is sensitive in native and high-yield D-LDH mutants (Rule et al., 1987b; Peersen et al., 1990), causes this peak to shift and broaden, but the bulk of the intensity from Trp 469 appears to be under the peaks between −48 and −51 ppm (Figures 4 and 5). In Y309W, there is no discernible resonance in the −45.5 to −48 ppm region, even after addition of D-lactate (results not shown). Apparently, substitutions at the nearby Leu 456 and at Tyr 309 perturb the resonance arising from Trp 469 such that it occurs between −48.5 and −50.5 ppm in the same region as that where Trp 407 and 567 resonate; addition of substrate does not help differentiate the resonances (Figure 5; results not shown).

In the spectrum of the F412W mutant, the resonance arising from Trp 469 is also disturbed, when compared to that of wild-type D-LDH. The resonance from Trp 469 is broad and ill-defined; D-lactate has no effect on the spectrum (results not shown). A further abnormality in the spectrum of the F412W mutant is that the peaks corresponding to the native Trps constitute a minority of the total signal. Most of the ^{19}F NMR signal arises from a peak at −48.8 ppm (Figure 4) that does not change in intensity over the time course of the acquisition of the spectrum. The SDS–PAGE of the sample was normal.

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

residue	predicted structure[a]	yield of active form	K_m[b]	V_{max}[c]
wild type		high	1.5	13
F12W	amphipathic helix	high	1.8	11
F39W	β-turn	high	1.5	9.9
I99W	hydrophobic sheet	none		
L110W	amphipathic sheet	low	1.9	6.4
I152W	hydrophobic sheet	none		
F176W	amphipathic sheet	low	2.4	12
I193W	amphipathic sheet	low	2.3	7
L203W	amphipathic sheet	none		
Y243W	amphipathic sheet	high	1.8	11
F263W	hydrophobic sheet	none		
F279W	hydrophobic sheet	none		
F300W	amphipathic helix	high	2.5	9.2
Y309W	amphipathic helix	low	4.7	10
Y315W	amphipathic helix	high	1.4	7.9
F326W	hydrophobic sheet	none		
F340W	hydrophobic sheet	high	2.9	25
F361W	amphipathic helix	high	1.8	12
Y388W	amphipathic helix	high	2.1	19
F412W	hydrophobic sheet	low	2.0	6.2
F435W	hydrophobic helix	high	1.6	2.5
L456W	amphipathic helix	low	9	2.6
F490W	hydrophobic sheet	none		
L517W	amphipathic helix	high	2.4	17
F544W	amphipathic helix	low	1.2	3.6

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

The large peak may arise from denatured enzyme generated during the last steps of preparation of the mutant enzyme or during storage of the sample. Indeed, the abnormal peak has a chemical shift similar to that of D-LDH denatured in guanidine hydrochloride or urea (results not shown).

Substitutions at Leu 110, Ile 193, and Phe 544 also affect the resonance from Trp 469, but less dramatically. The resonance arising from Trp 469 appears in its usual position but is less intense (Figure 4). In these three cases, an additional resonance appears down-field. Addition of D-lactate to the I193W mutant results in one broad peak (Figure 5), similar to the resonance arising from Trp 469 in the presence of substrate in wild-type D-LDH (Rule et al., 1987b). Analogous results are obtained for L110W and F544W (results not shown). Thus, in the L110W, I193W, and F544W mutants, the resonance arising from Trp 469 is split in the absence of substrate; addition of substrate collapses the splitting. It appears that Trp 469 is in two different environments as a result of the substitution and that the exchange rate between the two environments is less than 100 s^{-1}. On this basis, Trp 193 must give rise to the peak at −48 ppm, and Trp 544 must appear somewhere between −48.5 and −49.5 ppm, along with Trps 407 and 567.

To further test the thermal stability of the low-yield mutants, the ^{19}F NMR spectrum of mutant I193W was taken at several temperatures from 27 to 47 °C. Aside from the expected changes in line widths, due to increased mobility of the amino acid residues at higher temperatures, no other changes in the spectra were seen (results not shown), indicating that the structure of this mutant enzyme is neither temperature-dependent nor thermally labile in this range.

Discussion

All 24 Trp substitution mutations are expressed in nearly equivalent amounts on induction, but not all of them result in an active enzyme. Ten produce active enzyme in yields close to that of wild-type D-LDH (Peersen et al., 1990; unpublished results), keeping in mind that the gene for wild-type D-LDH is carried on the pGA2 plasmid, which produces twice as much as the pGA2a plasmid on which the mutant genes are carried (Rule et al., 1987b). Seven result in a very low yield of fully active enzyme, and seven have little or no measurable activity, after induction at 42 °C. In contrast to wild type and the fully active mutants, most of the protein expressed from the low-yield mutants is in an inactive form that is not solubilized by Triton X-100. The same behavior is seen for all of the protein from the inactive mutants.

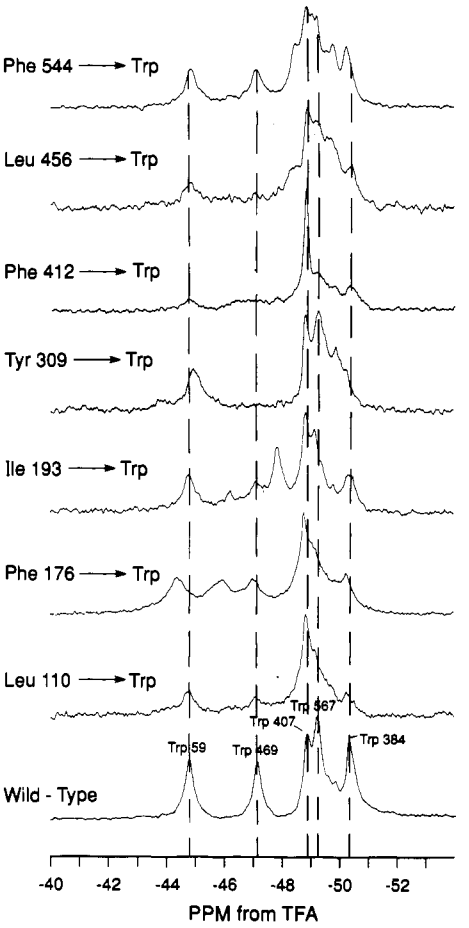

FIGURE 4: 282.4-MHz ^{19}F NMR spectra of 5F-Trp-labeled wild-type D-LDH and seven low-yield Trp substitution mutants. The enzyme concentrations are ~1 mM in 100 mM lysoPC/10 mM phosphate/0.2 mM EDTA at pH 7.2 and 42 °C.

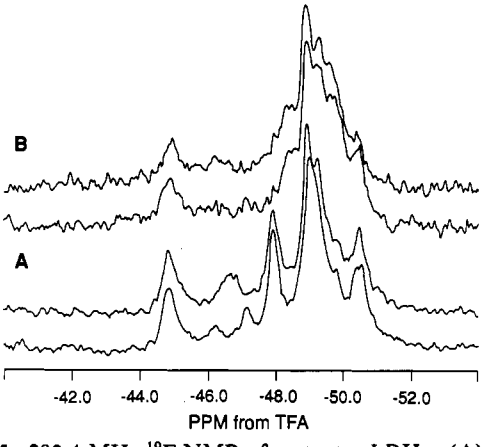

FIGURE 5: 282.4-MHz ^{19}F NMR of mutant D-LDHs: (A) I193W and (B) L456W D-LDH mutants in the absence (lower spectra) or presence (upper spectra) of D-lactate (100 mM final concentration).

The low yield of purified active enzyme from the low-yield mutants raises the possibility that native wild-type D-LDH has been produced from the chromosomal gene or from a "leaky" mutation, due to uncertainty in the codon translation (Toth et al., 1988; Schimmel, 1989). Such is not the case, however, since the ^{19}F NMR spectra of the low-yield mutant proteins are different from that of the wild-type D-LDH (see Figure 4).

The synthesis of protein having D-LDH enzymatic activity in cells that carry DNA coding for low-yield mutants is temperature-sensitive, since less D-LDH *activity* is detected upon growth at higher temperatures than is observed for wild type and active mutants. But, once synthesized, the mutant enzymes are active and appear not to denature at 42 °C. Thus, to use the terminology of Sadler and Novick (1965), the low-yield mutants are not thermolabile but are temperature-sensitive synthesis mutants. In fact, the polypeptide is expressed at 42 °C in amounts equivalent to the wild type, but only a small fraction of the protein is active, indicating that the defect introduced by the Trp substitution is a defect in the maturation of the protein. These low-yield mutants appear to belong to the class of temperature-sensitive folding mutants (Goldenberg & King, 1981; Sugihara & Baldwin, 1988). Our system for the production of D-LDH uses the P_L promoter, which requires heat shock for induction. These results suggest that if a system were used that did not need high temperatures, a greater yield of the low-yield mutants would be achieved and the inactive mutants other than I99W and I152W might yield active protein (Haase-Pettingell & King, 1988).

The ^{19}F NMR spectra of the purified temperature-sensitive folding mutants are recognizable as spectra of a mutant D-LDH (Rule et al., 1987b; Peersen et al., 1990); however, the positions of resonances arising from the native Trps in the ^{19}F NMR spectra of the purified temperature-sensitive folding mutants are often altered from their respective positions in the spectrum of wild-type D-LDH, more than in the spectra of high-yield mutant D-LDHs (Peersen et al., 1990). Thus, the structure of the enzyme is perturbed more in the temperature-sensitive folding mutants than it is in the high-yield mutants. The structure of the protein presumably loosens in order to accommodate the bulkier Trp residue, as has been observed with other substitutions in several other systems (Alber et al., 1988; Alber, 1989). In some of the temperature-sensitive folding mutants, D-LDH appears to assume more than one conformation in response to the Trp substitution. Most often, the native Trp 469, which is sensitive to the oxidation of the flavin cofactor (Rule et al., 1987b), is disturbed. This suggests that in these temperature-sensitive folding D-LDH mutants, the catalysis of the reaction might be perturbed, and an alteration of the K_m of the enzyme be observed. Indeed, for the Y309W and L456W mutants, where the Trp 469 resonance is the most perturbed (in that no resonance is observed in the −45.5 to −48 ppm region), the K_m values of the mutant D-LDH are higher than normal.

The P22 tail-spike protein temperature-sensitive folding mutants are functional and are reported to have structures equivalent to that of the wild-type protein (Sargent et al., 1988; Thomas et al., 1990). The temperature-sensitive folding mutants of D-LDH, though active, have perturbed structures. However, the structure of the tail-spike mutants was determined by Raman spectroscopy, which gives a measure of the overall α-helix and β-sheet content. Thus, the temperature-sensitive folding tail-spike mutants have the same secondary structure content as the wild type, and differences in the local environment of the mutation sites may not be detected. The ^{19}F NMR measurements used with D-LDH are very sensitive to small changes in the local environment of the F-Trp residues, and thus differences in the spectra of wild-type and mutant D-LDH reflect local changes at the site of the five native Trps but may not imply an overall change in the secondary or tertiary structure.

Most of the expressed protein from the temperature-sensitive folding mutants is found in the Triton-insoluble fraction; the same holds true for all of the protein from the inactive mutants. This is in contrast to the properties of wild-type and active mutant D-LDHs, where only a small fraction of the expressed protein is not extractable by Triton X-100. Partitioning between a soluble form and an insoluble form, found in inclusion bodies, is typical of the behavior of native and mutated proteins that are overexpressed in *E. coli* (Krueger et al., 1990). A similar behavior is observed for the tail-spike protein which forms native, active protein and also accumulates in aggregated, nonnative states. The distribution between native and aggregated forms depends on the maturation temperature and on whether the protein is wild type or a temperature-sensitive folding mutant (Haase-Pettingell & King, 1988). The case of D-LDH is more complicated, probably due to its nature as a membrane-associated protein. In the induced cells, wild-type and mutant D-LDHs are all found in inclusion bodies (Rule et al., 1985; unpublished results); during purification they partition between Triton-soluble and Triton-insoluble, as opposed to water-soluble and water-insoluble fractions. Apparently, the inclusion bodies contain both Triton-soluble and Triton-insoluble forms of D-LDH. The Triton-insoluble form is enzymatically inactive, while the Triton-soluble form is active. Correspondingly, most of the expressed protein from the wild-type D-LDH and the active mutants is Triton-soluble, and most of the expressed polypeptide from temperature-sensitive folding mutants is Triton-insoluble.

The lack of enzymatic activity and the absence of flavin in the Triton pellets, which contain most of the expressed D-LDH of the temperature-sensitive folding mutants, suggest that the protein is in an unfolded or partially folded or misfolded state; more recent evidence suggests that overexpressed proteins in a nonnative state are in a partially folded state (Jaenicke, 1991). The difficulty in solubilizing the Triton pellets indicates that the polypeptides are aggregated. Thus, it appears that the inclusion bodies in induced cells contain both properly folded and partially folded D-LDH in varying amounts, ranging from a preponderance of properly folded, active enzyme for the wild-type D-LDH and high-yield, active mutants to all improperly folded polypeptide for the inactive mutants I99W and I152W.

It has been shown that there is competition between proper folding and aggregation during the synthesis of several proteins (Brems, 1990; Jaenicke, 1991). This problem is more acute for a multidomain protein (Jaenicke, 1984). It has been suggested that D-LDH has three distinct regions (Peersen et al., 1990); functionally at least, it requires a substrate-binding site, a cofactor-binding site, and a membrane-binding region. Furthermore, in overexpressed proteins native to *E. coli*, there is a competition between folding and intermolecular aggregation (Krueger et al., 1990). At the high levels of polypeptide synthesis during induction, more intermolecular collisions are likely to occur, leading to aggregation and precipitation of the protein in intermediate stages of folding. For D-LDH, the aggregation reaction(s) would have to occur before binding of the flavin cofactor, or cause its loss. If the temperature-sensitive folding D-LDH mutants result in a polypeptide that folds more slowly than the wild-type protein, the competing

aggregation would predominate and lead to a larger amount of insoluble, nonpurifiable protein. This problem would be exacerbated at higher temperatures, due to increased intermolecular collisions. Alternatively, the folding intermediates of the temperature-sensitive folding mutant D-LDHs may be less stable or more prone to aggregation than those of the wild-type and active mutant D-LDHs.

Since all of these inactive mutants are aggregated and Triton-insoluble, none of them correspond to a mutation in the substrate-binding site or active site of D-LDH that destroys the substrate binding or catalytic activity of the enzyme, without affecting its overall structure. Such a mutant would be expected to display the same physical properties as wild-type D-LDH and, thus, be extractable by Triton X-100 and purifiable following our usual procedures.

The inactive mutants appear to fall into two classes: one group expresses D-LDH activity in low amounts at low temperatures (L203W, F263W, F279W, F326W, F490W), and the other (I99W, I152W) shows no activity at any temperature. The distinction between temperature-sensitive (low yield) and inactive mutants is based functionally on whether any purifiable protein was obtained; this distinction may be misleading. The first group of inactive mutants could actually be strict temperature-sensitive folding mutants that do not fold at all at 42 °C, while the low-yield mutants represent temperature-sensitive folding mutants for which a small fraction folds properly at 42 °C; however, the present system does not allow us to test this possibility.

To date, attempts to refold the Triton-insoluble form of D-LDH in the presence of the flavin cofactor have been unsuccessful despite the use of a variety of detergents and denaturants, including conditions that do reconstitute the purified wild-type D-LDH apoenzyme with FAD (unpublished results). This is in contrast to what has been observed with other aggregated, temperature-sensitive folding mutants or other aggregated mutant proteins found in inclusion bodies, where the protein that partitions into the insoluble fraction is able to refold into a proper, active conformation (Seckler et al., 1989; Krueger et al., 1990). Since, in our experience, it is difficult to reconstitute the purified D-LDH apoenzyme with FAD once it has been precipitated, the lack of success with the Triton-insoluble fractions is hardly surprising. D-LDH is probably a multiple-domain protein; thus, refolding after aggregation has occurred would be expected to be more difficult to achieve than for a single-domain protein (Jaenicke, 1984).

In the P22 tail-spike protein, the temperature-sensitive folding mutations are located in the central 350 residues of the 666-residue protein (Villafane & King, 1988). The majority of the amino acid residues replaced are hydrophilic, and none are aromatic, which is a significant underrepresentation of hydrophobic and aromatic residues. The local sequences resemble those reported for β-turns. In contrast, the mutation sites of the temperature-sensitive folding mutants of D-LDH are distributed throughout the gene, the sites were chosen to be hydrophobic, and four out of seven of the replaced amino acid residues are aromatic. The local structure (Ho et al., 1988) of the region of the substitution site is predicted to be an amphipathic sheet (3), amphipathic helix (3), and hydrophobic sheet (1). The difference in the properties of the substitution sites between tail-spike protein and D-LDH are probably due not only to the difference in structure but also partly to the very small sample size of the D-LDH temperature-sensitive folding mutants and to the different methods used to generate the mutations (random mutagenesis vs site-specific mutagenesis to insert a Trp). It is perhaps also interesting to note that mutations of amino acid residues predicted to be in amphipathic helices have a preponderance of fully active protein while those predicted to be in hydrophobic sheets make up most of the totally inactive mutants.

Why are the Trp substitutions disruptive? The contribution of a given amino acid and a particular site to the folding and stability of proteins has been studied using a variety of methods (Ho & Russu, 1985; Ackers & Smith, 1985; Goldenberg, 1988; Alber, 1989). The contribution of Trps to the folding and stability of proteins is equivocal. Replacing a native amino acid located in the protein core with the more hydrophobic Trp (Tanford, 1962) might be expected to increase the internal hydrophobic interactions that stabilize the protein (Alber, 1989; Matsumura et al., 1988; Yutani et al., 1987) and, thus, increase the stability of the protein. Nevertheless, single amino acid replacements by a Trp are believed to be destabilizing (Yutani et al., 1987; Matsumura et al., 1988; Goldenberg, 1988; Sandberg, 1989; Lim & Sauer, 1989; Kellis et al., 1989), due to the larger volume of the Trp residue [at least 34 Å^3 greater than any other amino acid (Zamyatnin, 1972)]. Previous work showed that many of the Trp substitutions that result in active D-LDH are at positions near to or at the surface of the enzyme (Peersen et al., 1990), where changes in volume are more readily tolerated (Yu & King, 1988; Reidhaar-Olson & Sauer, 1988; Alber, 1989; Bowie et al., 1990). The positions of substitutions that produce the inactive and low-yield mutants may be located in the core of the protein or of a folding intermediate. Alternatively, the Trp substitution may decrease the rate of folding of the polypeptide by retarding an important interaction due to steric hindrance, or it may increase the competing aggregation reaction by forming a patch with greater hydrophobicity.

If the larger volume of the Trp substitution is a destabilizing factor, one might expect that the use of 5F-Trp would result in a smaller amount of active protein for the temperature-sensitive folding mutants than for the wild-type and active mutants. Incorporation of 5F-Trp into temperature-sensitive folding mutants, active mutants, or wild-type D-LDH yields activities half of those obtained with unlabeled Trp (normalized to the cell culture density), so that no additional disruption by the F-Trp is observed (results not shown).

Thus, the Trp substitutions that we have generated in D-LDH produce three classes of mutants. The fully active mutants, which are synthesized in yields of active protein close to that of the wild type, tolerate the Trp substitution. A majority of these Trp substitution sites are located at the surface of the enzyme, and the structure of the protein is minimally disturbed by the Trp substitution. In the inactive mutants, the Trp substitution prevents proper folding of the enzyme and all of the synthesized protein aggregates, has no enzymatic activity, and is apparently unfolded or misfolded. Finally, Trp substitutions also produce temperature-sensitive folding mutants; the maturation of these mutant proteins is temperature-dependent, but the final folded protein is thermally stable at 42 °C. In these mutants, most of the protein synthesized at 42 °C is aggregated, Triton-insoluble, and misfolded; however, a small fraction of the protein synthesized folds properly, and is active and stable at 42 °C. The Trp substitution apparently not only interferes with the folding of the polypeptide chain but also perturbs the final structure of the enzyme, even though the enzyme is functional.

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