A ^{19}F-NMR Study of the Equilibrium Unfolding of Membrane-Associated D-Lactate Dehydrogenase of *Escherichia coli*†

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Received August 15, 1996; Revised Manuscript Received November 1, 1996⊗

ABSTRACT: Partially folded protein intermediates have been observed by ^{19}F-NMR spectroscopy during the equilibrium unfolding of the membrane-associated D-lactate dehydrogenase (D-LDH) of *Escherichia coli* by a denaturant, guanidine hydrochloride (Gdn.HCl). The results from ^{19}F-NMR and circular dichroism spectroscopic studies suggest that the intermediates observed at low Gdn.HCl concentrations (<3.5 M) exhibit features similar to "molten globules" that contain considerable amounts of secondary and tertiary structure. The results of ^{19}F-NMR studies on 5F-Trp-labeled D-LDH, such as the chemical shift changes, nuclear Overhauser effect, and solvent-induced isotopic shift effect, show that different regions of D-LDH unfold nonuniformly in Gdn.HCl in the presence of lysophosphatidylcholine. The polypeptide appears to unfold in a general order from the carboxyl end to the amino end, in agreement with previous findings from our laboratory that the carboxyl-terminal region of D-LDH is largely exposed to the solvent while the amino-terminal region is buried in the protein core. The structure of the partially unfolded intermediate forms of D-LDH is stabilized in the presence of lipid-like detergents, such as lysophosphatidylcholine.

The membrane-associated D-lactate dehydrogenase (D-LDH)[1] of *Escherichia coli* oxidizes D-lactate to pyruvate and is one of the primary dehydrogenases in the bacterial respiratory chain (Cronan et al., 1987). This 65-kDa protein contains a noncovalently bound cofactor, flavin adenine dinucleotide (FAD), and requires lipids or detergents for its purification and full enzyme activity (Futai, 1973; Kohn & Kaback, 1973; Tanaka et al., 1976; Fung et al., 1979; Pratt et al., 1979; Kovatchev et al., 1981). The three-dimensional molecular structure of D-LDH has yet to be determined by X-ray crystallography or nuclear magnetic resonance (NMR) spectroscopy. Previous studies in our laboratory using a combination of genetic engineering, biochemical, and ^{19}F-NMR techniques have led to the proposal of a three-domain structural model for D-LDH that includes a substrate-binding domain, a cofactor-binding domain, and a membrane-binding domain (Rule et al., 1987; Ho et al., 1989; Peersen et al., 1990; Sun et al., 1993). In this study, we have carried out ^{19}F-NMR spectroscopic investigations of the properties of the partially unfolded intermediate forms of D-LDH during equilibrium unfolding by guanidine hydrochloride (Gdn.HCl), in order to better understand (i) the stability of different regions in D-LDH and (ii) the interaction of the partially unfolded protein intermediates with detergent micelles.

Intermediate structures that occur during protein unfolding have been studied by a variety of techniques, such as differential scanning calorimetry, optical absorption, fluorescence, circular dichroism (CD), and NMR spectroscopies (Eftink, 1995). Many of these partially unfolded intermediates, sometimes called "molten globule" or "compact denatured states", may be important for the protein folding process and during translocation of proteins across membranes (Dobson, 1992; Ptitsyn, 1992). Two recent studies have used ^{19}F-NMR spectroscopy to investigate the unfolding processes of 6F-Trp-labeled rat intestinal fatty acid binding protein (15.4 kDa) and dihydrofolate reductase of *E. coli* (17.7 kDa) (Ropson & Frieden, 1992; Hoeltzli & Frieden, 1994). ^{19}F-NMR spectroscopic studies of ^{19}F-labeled proteins benefit from the high sensitivity of the ^{19}F nuclear spin, the lack of a natural background signal, and minimal perturbation of the protein structure (Ho et al., 1985; Gerig, 1994).

The interactions between partially unfolded protein intermediates and surfactants are not well understood, although their importance in protein folding and membrane translocation has become more evident. Lipids or detergents have been used in many cases to assist protein refolding by preventing aggregation of partially folded or misfolded intermediates (Zardeneta & Horowitz, 1994; Wetlaufer & Xie, 1995). In addition, membrane binding can induce denaturation of cytochrome *c* by stabilizing an unfolding intermediate (Muga et al., 1991), and the molten globules of many proteins, such as α-lactalbumin and acidic fibroblastic growth factor, can induce fusion of lipid vesicles (Kim & Kim, 1986; March & Middaugh, 1995). In this study, we show that the stability of the partially unfolded intermediates of D-LDH depends on the interaction with micelles formed by specific types of detergents.

† This work was supported by a research grant from the National Institutes of Health (GM-26874).

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⊗ Abstract published in *Advance ACS Abstracts,* December 15, 1996.

[1] Abbreviations: ANS, 8-anilino-1-naphthalenesulfonic acid; CD, circular dichroism; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; CMC, critical micelle concentration; CSA, chemical shift anisotropy; C_{16}TAB, hexadecyltrimethylammonium bromide; D-LDH, D-lactate dehydrogenase; FAD, flavin adenine dinucleotide; 5F-Trp, 5-fluorotryptophan; Gdn.HCl, guanidine hydrochloride; lysoPC, lysophosphatidylcholine; Mega-10, decanoyl-*N*-methylglucamide; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; SIIS, solvent-induced isotopic shift; TFA, trifluoroacetic acid; Trp, tryptophan.

S0006-2960(96)02061-2 CCC: $12.00

MATERIALS AND METHODS

Materials. Gdn.HCl, *n*-tetradecyl-*N*,*N*-dimethyl-3-ammonio-1-propanesulfonate (Zwittergent 3-14), octyl glucoside (*n*-octyl β-D-glucopyranoside), and decanoyl-*N*-methylglucamide (Mega-10) were purchased from Calbiochem. 5-Fluorotryptophan (5F-Trp), lysophosphatidylcholine (lysoPC), t-octylphenoxy-polyethoxyethanol (Triton X-100), hexadecyltrimethylammonium bromide (C_{16}TAB), and 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) were purchased from Sigma. 8-Anilino-1-naphthalenesulfonic acid (ANS) was purchased from Aldrich.

Protein Preparation and Characterization. 5F-Trp-labeled wild-type and "Trp-knock-out" mutant D-LDHs were produced and purified as previously described (Rule et al., 1987). D-LDH concentrations were determined by measuring the absorption of FAD at 450 nm under denaturing conditions in 6 M Gdn.HCl, pH 4.0. D-LDH activity was determined by an assay system using phenazine methosulfate (PMS) and 3-(3,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) as previously described (Pratt et al., 1979).

NMR Experiments. Gdn.HCl solutions were prepared in 10 mM potassium phosphate buffer and adjusted to pH 7.2 with HCl. Samples for NMR experiments were prepared by mixing 0.1 mL of 1 mM D-LDH with 0.3 mL of Gdn.HCl solutions before transfer to a 5-mm NMR tube. The NMR samples normally contain 0.25 mM D-LDH and 25 mM lysoPC in 10 mM potassium phosphate buffer, pH 7.2, in H_2O, unless otherwise specified. The NMR experiments on D-LDH unfolded by Gdn.HCl were carried out at 24 °C after the equilibrium states had been reached. The 282.4-MHz ^{19}F-NMR spectra of 4K data size were acquired using a Bruker AM-300 spectrometer (7.0 T), with a relaxation delay of 4 s and typically 8000−16 000 scans. An external standard of trifluoroacetic acid (TFA) in a coaxially-placed capillary was used as reference. Each free induction decay signal was treated with 5-Hz Lorentzian line-broadening before being Fourier transformed into an NMR spectrum.

In the nuclear Overhauser effect (NOE) experiments, a 1.5-s ^{1}H presaturating pulse with a field strength of approximately 1.3 kHz was applied prior to the ^{19}F 90° acquisition pulse, and the total delay between acquisitions was kept at 4 s. The length of the ^{1}H presaturating pulse was chosen to be longer than the longest T_1 (spin−lattice relaxation time) of the 5F-Trps from D-LDH (0.8 s at 1.75 M Gdn.HCl and 0.7 s at 5.25 M Gdn.HCl), while minimizing sample heating at high salt concentrations. The ^{19}F-NMR spectra acquired in the NOE experiments were fitted manually by simulated peaks. The NOE values were then calculated according to $(A - A_0)/A_0$, where A is the intensity of the ^{19}F resonance peaks upon irradiating the protons and A_0 is the intensity without irradiation.

In the solvent exchange experiments on partially unfolded D-LDH in D_2O, the protein samples were exchanged extensively with D_2O buffer, and the Gdn.HCl solutions were prepared directly in D_2O. Due to the proton content in the Gdn.HCl salt crystals, the deuterium content was 90% in the NMR sample in 1.75 M Gdn.HCl, and 70% in the NMR sample in 5.25 M Gdn.HCl, as confirmed from ^{1}H-NMR measurements.

Optical Experiments. The circular dichroism (CD) and fluorescence experiments were carried out at room temperature (20−24 °C) in the laboratory of Dr. Carl Frieden at Washington University. The CD spectra of D-LDH were acquired using a Jasco J-600 spectrometer. The samples were prepared by adding 4 μL of 0.2 mM D-LDH with 40 mM lysoPC to 0.2-mL solutions of varying concentrations of Gdn.HCl in 10 mM potassium phosphate at pH 7.2. The spectrometer was purged with N_2 gas for 5 min before each measurement. The fluorescence emission spectra of D-LDH were acquired using a Photon Technology International (PTI) fluorimeter with an excitation wavelength at 290 nm. The samples were prepared by diluting 1 μL of 0.2 mM D-LDH with 40 mM lysoPC in 1-mL solutions of varying concentrations of Gdn.HCl in 10 mM potassium phosphate at pH 7.2.

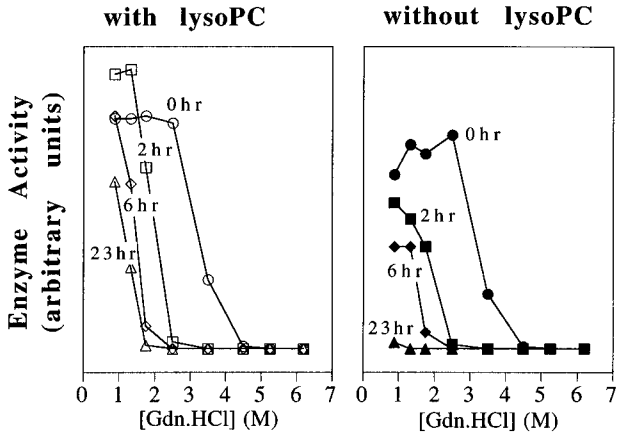

FIGURE 1: Denaturation of D-LDH by varying concentrations of Gdn.HCl in the presence (open symbols) and absence (filled symbols) of lysoPC, as monitored by enzyme activity assayed at 0 h (circles), 2 h (squares), 6 h (diamonds), and 23 h (triangles) after sample preparation at room temperature.

RESULTS

Initial Unfolding Stage. The rates at which D-LDH unfolds in the presence of varying concentrations of Gdn.HCl have been measured by monitoring the changes in enzyme activity at room temperature (Figure 1). The results show that D-LDH in the presence of 4.5 M or higher concentrations of Gdn.HCl has no activity immediately (<1 min) after sample preparation. D-LDH at 3.5 and 2.5 M Gdn.HCl retains 30−100% of the enzyme activity immediately after sample preparation, but the activity disappears completely after 1 h. The enzyme activity of D-LDH at 1.75 M or lower concentrations of Gdn.HCl lasts much longer, especially in the presence of lysoPC (Figure 1). Thus, lysoPC not only enhances the enzyme activity (Fung et al., 1979), but also appears to protect the native D-LDH from denaturation. The enzyme activity results have been used as references to set the sample equilibration time before carrying out the NMR, CD, and fluorescence experiments.

The ^{19}F-NMR spectrum of 1 mM 5F-Trp-labeled wild-type D-LDH in the presence of 100 mM lysoPC contains five peaks from the five native tryptophan residues (Figure 2A). In the presence of 0.5 M Gdn.HCl, D-LDH undergoes a small conformational change, as shown by the upfield shift of the W59 peak (0.07 ppm) and the downfield shifts of the W407 (0.18 ppm) and W384 (0.15 ppm) peaks (Figure 2B). The native protein appears to be stable for at least 24 h when in solutions containing up to 1 M Gdn.HCl. At higher denaturant concentrations, several sharp resonance peaks corresponding to a partially unfolded intermediate begin to appear in the ^{19}F-NMR spectra. At Gdn.HCl concentrations

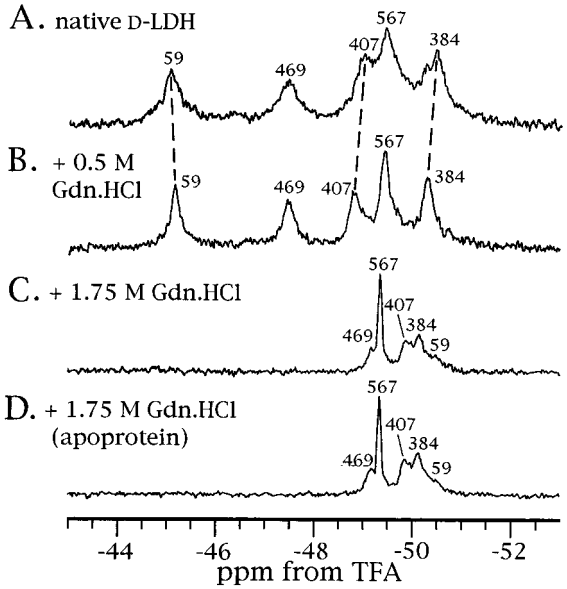

FIGURE 2: 282.4-MHz ^{19}F-NMR spectra of 5F-Trp-labeled D-LDH: (A) 1 mM D-LDH with 100 mM lysoPC in buffer only; (B) 1 mM D-LDH with 100 mM lysoPC in 0.5 M Gdn.HCl; (C) 0.25 mM D-LDH with 25 mM lysoPC in 1.75 M Gdn.HCl; and (D) 0.25 mM apoprotein of D-LDH (after filtering out FAD) in 1.75 M Gdn.HCl with 25 mM lysoPC. The samples were in 10 mM potassium phosphate buffer, pH 7.2, in H_2O at 24 °C.

up to 1.75 M, the intensities of the five 5F-Trp peaks from the native D-LDH decrease with time at the same rate (results not shown), suggesting that a global structural change is involved during the initial transition between the native D-LDH and the partially unfolded intermediate, when the range of chemical shift dispersion for the 5F-Trps decreases from 6 to 1.5 ppm. The ^{19}F-NMR spectrum of D-LDH at 1.75 M Gdn.HCl shows that the W59, W407, and W469 peaks from the unfolding intermediate are shifted upfield 5.4, 0.9, and 1.7 ppm, respectively, compared to those from the native protein (Figure 2A,C).

The cofactor, FAD, is released from the denatured protein during the initial unfolding. The optical absorption spectrum of D-LDH at 1.75 M Gdn.HCl shows that an absorption peak from the FAD cofactor is shifted from 454 nm, the protein-bound form, to 450 nm, the free FAD form. The released FAD molecule can be removed from the protein solution at 1.75 M Gdn.HCl by using a Centricon-30 membrane concentrator with a molecular weight cutoff of 30 kDa. The ^{19}F-NMR spectrum of the remaining solution of the apoprotein is identical to that of the unfiltered sample (Figure 2C,D), suggesting that FAD has indeed been separated from the protein molecule. Results from enzyme activity assays of D-LDH at 1.75 M Gdn.HCl suggest that the addition of excess amounts of FAD can slow down the denaturation process (results not shown). Thus, the initial denaturation of D-LDH may involve the release of the FAD cofactor and the subsequent structural unfolding of the apoprotein.

Equilibrium Unfolding. The ^{19}F-NMR spectra of 5F-Trp-labeled D-LDH at different equilibrium unfolding stages in the presence of varying concentrations of Gdn.HCl are shown in Figure 3. The resonance peaks in these spectra have been assigned to the five native 5F-Trp residues using the "Trp-knockout" mutants, in which each individual native Trp was replaced by a phenylalanine or tyrosine by site specific mutagenesis (Rule et al., 1987). More specifically, the assignments of the native 5F-Trp peaks of D-LDH at 1.75

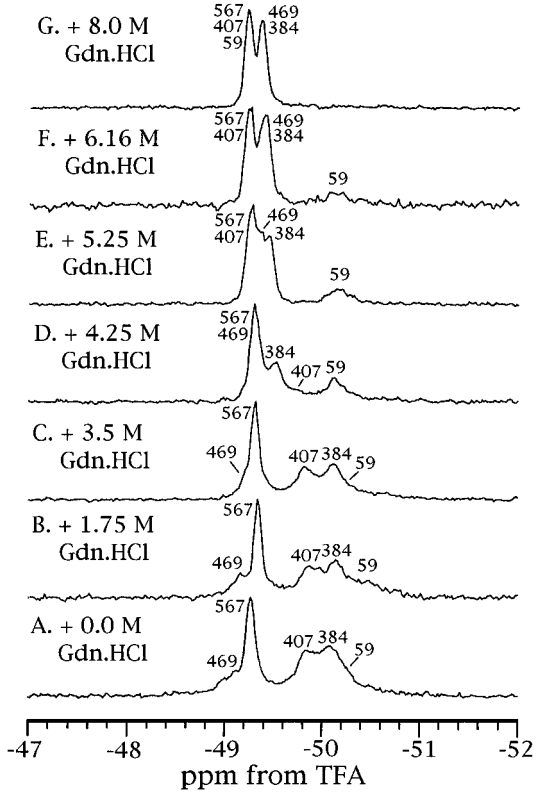

FIGURE 3: 282.4-MHz ^{19}F-NMR spectra of 0.25 mM 5F-Trp-labeled D-LDH in the presence of 25 mM lysoPC in (A) buffer only (exchanged from 1.75 M Gdn.HCl), (B) 1.75 M Gdn.HCl, (C) 3.5 M Gdn.HCl, (D) 4.25 M Gdn.HCl, (E) 5.25 M Gdn.HCl, (F) 6.16 M Gdn.HCl, and (G) 8.0 M Gdn.HCl. The samples were in 10 mM potassium phosphate buffer, pH 7.2, in H_2O at 24 °C.

and 5.25 M Gdn.HCl were carried out using the five 5F-Trp-labeled mutants, W59→Y, W384→Y, W407→F, W469→F, and W567→F. The identities of most of the 5F-Trp peaks in the other ^{19}F-NMR spectra can be readily deduced from these results. Only in a few cases were some of these "Trp-knockout" mutants used separately to confirm the assignments or to resolve ambiguities caused by overlapping peaks.

The ^{19}F-NMR spectra in Figure 3B,C show that D-LDH is partially unfolded, but relatively well structured at low denaturant concentrations (1.75 and 3.5 M Gdn.HCl). The spectrum shown in Figure 3A was obtained using a sample prepared from D-LDH denatured at 1.75 M Gdn.HCl for 24 h and then exchanged back to 10 mM potassium phosphate buffer at pH 7.2. The results show that the structure of this unfolding intermediate does not depend on the presence of Gdn.HCl (Figure 3A−C) and that the initial unfolding step is not readily reversible. The ^{19}F-NMR spectrum of D-LDH unfolded by 6 M urea in the presence of lysoPC (results not shown) is very similar to that of D-LDH unfolded by 3.5 M Gdn.HCl, indicating that the unfolding process should be similar whether the denaturant is urea or Gdn.HCl.

At higher denaturant concentrations (>3.5 M Gdn.HCl), D-LDH undergoes several further unfolding steps (Figure 3C−G), which are reversible by exchanging back to lower Gdn.HCl concentrations. The W384 and W407 peaks are shifted downfield when the Gdn.HCl concentration is increased from 3.5 to 5.25 M (Figure 4A), indicating protein structural changes during these unfolding transitions. The relative integrated area of the 5F-Trp peaks in the downfield region (> −49.6 ppm) as a percentage of the total integrated

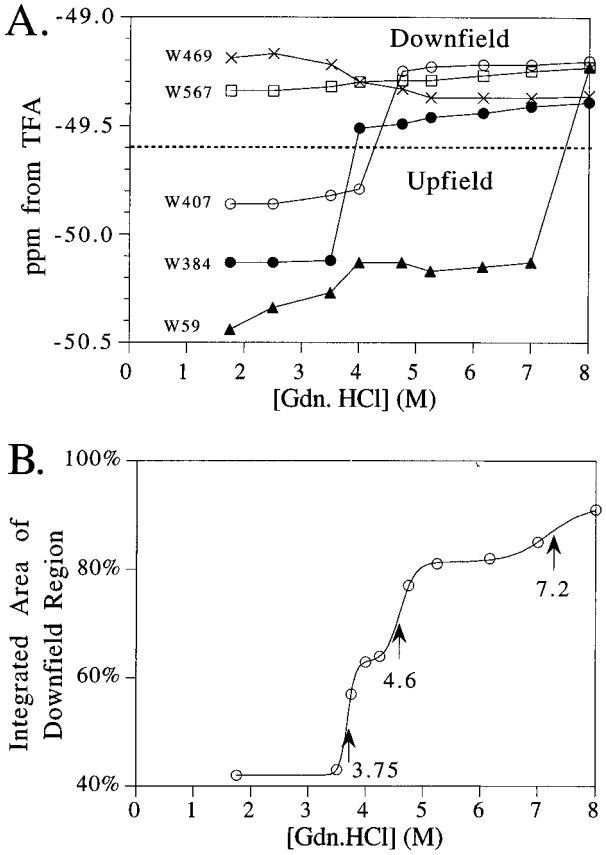

FIGURE 4: (A) The ^{19}F chemical shifts of 5F-Trps from the unfolding intermediates of D-LDH, and (B) the integrated area of the 5F-Trp peaks in the region downfield from −49.6 ppm, as a function of Gdn.HCl concentration. The arrows in (B) indicate unfolding transitions at 3.75, 4.6, and 7.2 M Gdn.HCl. Data were obtained from the ^{19}F-NMR spectra shown in Figure 3.

area also shows two distinct unfolding transitions: at 3.75 M Gdn.HCl for W384 and at 4.6 M Gdn.HCl for W407 (Figure 4A,B). There is some indication from the chemical shifts that W469 may also take part in these transitions (Figure 4A). The remaining W59 peak in the upfield region becomes broadened (suggesting a slow exchange process), and its intensity starts to decrease at higher Gdn.HCl concentrations, until finally the W59 peak is moved downfield at 8.0 M Gdn.HCl (Figure 3E−G). The integrated peak area of the downfield region at 8.0 M Gdn.HCl does not reach 100% of the total area (Figure 4B). This is because part of the shoulder regions of the W384 and W469 peaks, and possibly a small portion of the W59 peak, are located upfield of the dividing line at −49.6 ppm and left out in the integration.

Trp Mobility and Exposure to Solvent. The results of nonselective ^{1}H/^{19}F heteronuclear Overhauser effect (NOE) measurements of 5F-Trps from the native state of D-LDH, and the intermediate forms at 1.75 and 5.25 M Gdn.HCl, are shown in Table 1. ^{1}H/^{19}F NOEs are normally between 0.5 (extreme line-narrowing condition) and −1.0 (extremely immobile condition), with positive or small negative values indicating higher mobility in the local environment of the ^{19}F nuclei. The estimated errors of the NOEs were derived from the upper bounds of the uncertainty in the integrated areas of the 5F-Trp peaks. The data in Table 1 show that the ^{1}H/^{19}F NOEs from the 5F-Trps become smaller and less negative (indicating increased mobility) as the protein unfolds with increasing Gdn.HCl concentrations. The different

Table 1: Nonselective ^{1}H/^{19}F NOE and Effective Correlation Time τ_e of 5F-Trps in D-LDH[a]

D-LDH		native	+1.75 M Gdn.HCl	+5.25 M Gdn.HCl
NOE	W59	−0.75 ± 0.06	−0.67 ± 0.06	−0.43 ± 0.05
	W384	−0.50 ± 0.06	−0.56 ± 0.05	−0.28 ± 0.05
	W407	−0.79 ± 0.06	−0.53 ± 0.05	−0.38 ± 0.06
	W469	−0.79 ± 0.06	−0.57 ± 0.10	−0.33 ± 0.04
	W567	−0.63 ± 0.06	−0.23 ± 0.04	−0.17 ± 0.04
τ_e (ns)	W59	2.2 ± 0.3	1.9 ± 0.2	1.3 ± 0.1
	W384	1.4 ± 0.1	1.6 ± 0.1	1.0 ± 0.1
	W407	2.5 ± 0.4	1.5 ± 0.1	1.2 ± 0.1
	W469	2.5 ± 0.4	1.6 ± 0.3	1.1 ± 0.1
	W567	1.8 ± 0.2	0.9 ± 0.1	0.8 ± 0.1
av τ_e (ns)		2.1	1.5	1.1

[a] Data obtained for 5F-Trp-labeled D-LDH in the presence of 25 mM lysoPC and varying concentrations of Gdn.HCl in 10 mM potassium phosphate buffer, pH 7.2, in H_2O, at 24 °C and 7.0 T magnetic field. The τ_e values were calculated using a simplified model (for details, see text).

NOEs of the 5F-Trps may reflect variations in the mobility of different regions in the protein under the same denaturing condition. For example, after the initial unfolding from the native protein to the partially unfolded intermediate at 1.75 M Gdn.HCl, the W567 residue becomes extremely mobile compared to the other 5F-Trps, suggesting extensive unfolding of the carboxyl-terminal region. The NOE and the mobility for the W407 residue at 1.75 M Gdn.HCl suggest that its environment is similar to those of the W59, W384, and W469 residues, even though its resonance peak is close to W567 in the downfield region (Figure 4A). The NOEs for W59 indicate that it is generally less mobile compared to the other 5F-Trps, and is probably located in the protein core region (Rule et al., 1987).

The ^{19}F-NMR spectra of the unfolding intermediates of D-LDH in H_2O and D_2O are compared in Figure 5. The resonances of 5F-Trps and other fluorinated amino acid residues exposed to the aqueous solution exhibit a solvent-induced isotopic shift (SIIS) effect when the solvent is changed from H_2O to D_2O (Hagen et al., 1979; Rule et al., 1987; Shen et al., 1989). For the native state of D-LDH, it was found that the W384 and W567 residues are exposed to solvent, while the W59, W407, and W469 residues are buried inside the protein (Rule et al., 1987; Peersen et al., 1990). Our results show that, for the D-LDH intermediate at 1.75 M Gdn.HCl, only the W567 residue is notably exposed to the solvent (Figure 5A), while for the unfolding intermediate at 5.25 M Gdn.HCl, all except the W59 residue are exposed to the solvent (Figure 5B).

Effect of Detergents. The presence of lipids or detergents, such as lysoPC, is required for the solubilization of the native D-LDH into its monomeric state (Tanaka et al., 1976; Rule et al., 1987). The ^{19}F-NMR spectrum of D-LDH at 3.5 M Gdn.HCl with no detergent (Figure 6A) shows that the W59 resonance at upfield is extremely broad, possibly due to less-defined structure or protein aggregation, and the rest of the 5F-Trps at downfield are in the unfolded state as determined from NOE measurements. In the NMR experiments, the samples of D-LDH at 2.5 M or lower concentrations of Gdn.HCl without detergent tend to solidify due to aggregation of the denatured protein, in contrast to under other conditions.

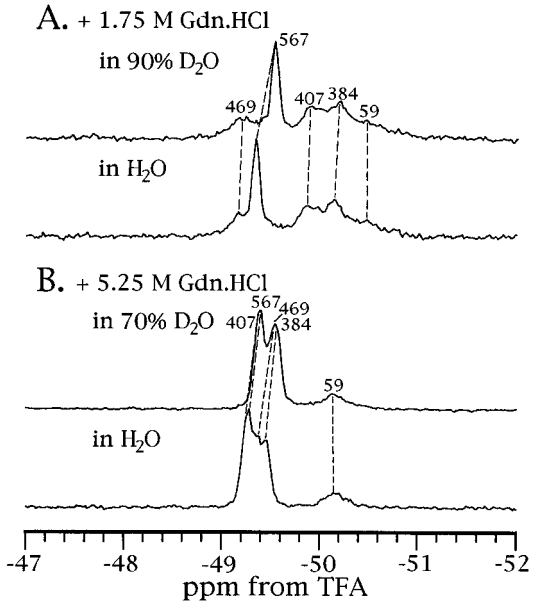

FIGURE 5: 282.4-MHz ^{19}F-NMR spectra of 0.25 mM 5F-Trp-labeled D-LDH in (A) 1.75 M Gdn.HCl in 90% D_2O (top) and 100% H_2O (bottom) and (B) 5.25 M Gdn.HCl in 70% D_2O (top) and 100% H_2O (bottom), showing solvent-induced isotopic shift effects. The D_2O contents were confirmed from the corresponding ^{1}H-NMR spectra. The samples were in 10 mM potassium phosphate buffer, pH 7.2, in the presence of 25 mM lysoPC at 24 °C.

A variety of detergents, including the cationic detergent C_{16}TAB (50 mM), zwitterionic detergents lysoPC (25 mM), Zwittergent 3-14 (55 mM), and CHAPS (45 mM), and nonionic detergents Mega-10 (90 mM), octyl glucoside (130 mM), and Triton X-100 (2.5%), have been added to D-LDH samples in 3.5 M Gdn.HCl to study the effects of different types of detergents on the structure of the unfolding intermediate (Figure 6B−H). The concentrations of the detergents used are higher than the critical micelle concentrations (CMC) (Jones et al., 1987). Anionic detergents were not used because of the precipitation problem with Gdn.HCl. The mobility of the 5F-Trps was determined by ^{1}H/^{19}F NOE measurements, and residues with smaller NOEs (between −0.2 to −0.4) are considered to be in the unfolded state.

The ^{19}F-NMR spectra of D-LDH in the presence of CHAPS (zwitterionic) or Triton X-100 (nonionic) (Figure 6B,C) are very similar to the spectrum without any detergents (Figure 6A). Results of area integration show that the area of the broadened W59 peak is one-fifth of the total area, i.e., corresponds to one Trp. Close examination reveals that the W59 peak may consist of a doublet separated by 80−100 Hz, indicating two local conformations (see insets in Figure 6A−C). D-LDH in the presence of the nonionic detergents octyl glucoside or Mega-10 is partially structured, with W59 at around −50.4 ppm, W384, W407, and W469 poorly resolved, and W567 in an unfolded state at −49.3 ppm (Figure 6D,E). D-LDH in the presence of C_{16}TAB (cationic), or two zwitterionic detergents, lysoPC and Zwittergent 3-14, is the most structured, with W567 in an unfolded state at −49.3 ppm, and the rest of the 5F-Trps peaks largely resolved (Figures 6F−H).

Optical Spectroscopic Results. The far-UV circular dichroism spectra of unlabeled D-LDH in the presence of lysoPC in different concentrations of Gdn.HCl are shown in Figure 7. The structural perturbation from the incorporation of the 5F-Trp is minimal as shown by the CD spectra of 5F-Trp-

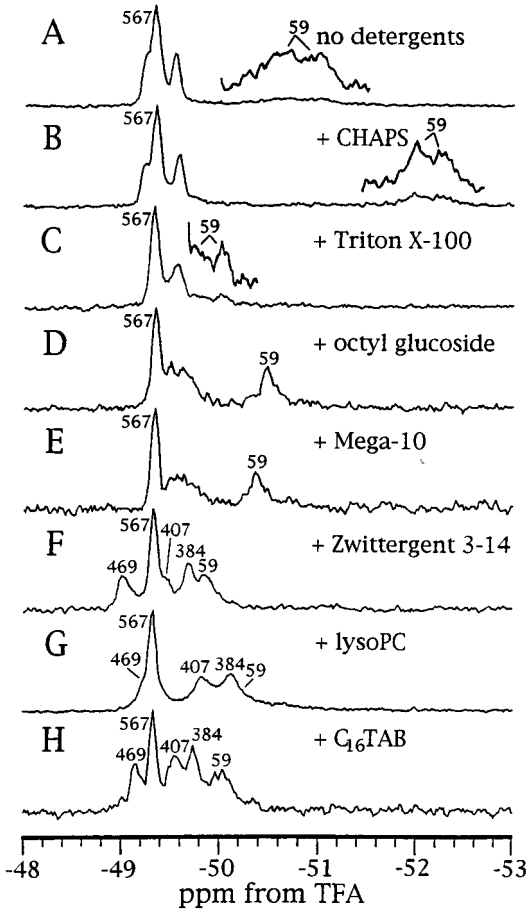

FIGURE 6: 282.4-MHz ^{19}F-NMR spectra of 0.25 mM 5F-Trp-labeled D-LDH in 3.5 M Gdn.HCl, 10 mM potassium phosphate buffer, pH 7.2, in H_2O at 24 °C, in the presence of (A) no detergent, (B) 45 mM CHAPS, (C) 2.5% Triton X-100, (D) 130 mM octyl glucoside, (E) 90 mM Mega-10, (F) 55 mM Zwittergent 3-14, (G) 25 mM lysoPC, and (H) 50 mM C_{16}TAB. The insets are 6× (A), and 4× (B and C) expanded plots of the W59 peaks.

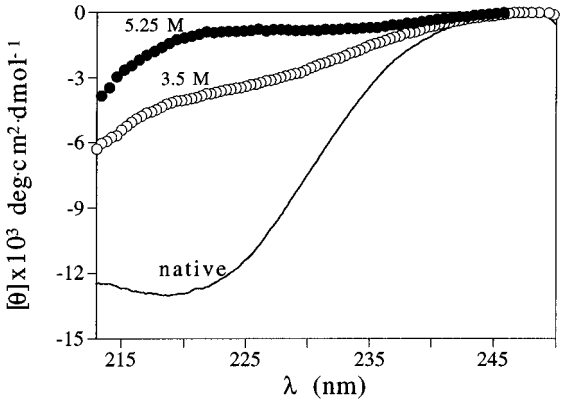

FIGURE 7: Circular dichroism spectra of the native D-LDH (solid line) and partially unfolded D-LDH at 3.5 M Gdn.HCl (open circles) and at 5.25 M Gdn.HCl (filled circles), in 10 mM potassium phosphate buffer, pH 7.2, at room temperature.

labeled and unlabeled D-LDH (Rule et al., 1987). It was estimated from the elliptical absorption at 219 nm that about 30% of the secondary structure of D-LDH remains at 3.5 M Gdn.HCl after the initial unfolding step. The CD spectrum of D-LDH at 5.25 M Gdn.HCl shows that a second large decrease in the amount of secondary structure occurs concomitantly with the unfolding transitions at 3.75 to 4.6 M Gdn.HCl as observed by ^{19}F-NMR spectroscopy. The residual secondary structure observed at 5.25 M Gdn.HCl

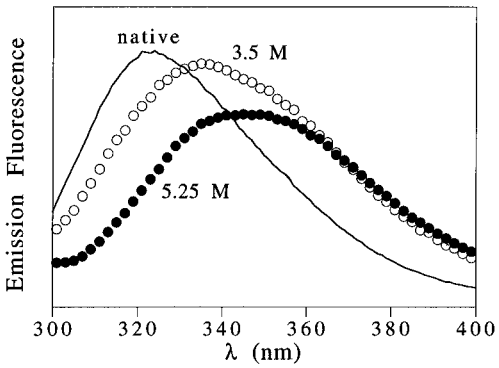

FIGURE 8: Fluorescence emission spectra (excited at 290 nm) of the native D-LDH (solid line) and partially unfolded D-LDH at 3.5 M Gdn.HCl (open circles) and at 5.25 M Gdn.HCl (filled circles), in 10 mM potassium phosphate buffer, pH 7.2, at room temperature.

may correspond to the more stable amino-terminal region containing the W59 residue.

The fluorescence spectra of unlabeled D-LDH during equilibrium unfolding in the presence of lysoPC show that the fluorescence emission peak from the five native Trp residues becomes red-shifted with increasing Gdn.HCl concentration (Figure 8), probably caused by increased solvent exposure of the Trp residues as the protein unfolds. However, the technique of fluorescence spectroscopy is not sensitive enough to reveal the multiple transition steps during the equilibrium unfolding of D-LDH. The fluorescence probe, 1-anilino-8-naphthalenesulfonic acid (ANS), can be used to study exposed hydrophobic regions during protein unfolding (Semisotnov et al., 1987). Unfortunately, in the case of the equilibrium unfolding of D-LDH in the presence of lysoPC, the experimental results are complicated by the interactions between the ANS probe and the detergent micelles (results not shown).

DISCUSSION

In a number of CD and fluorescence studies of protein unfolding processes, single tryptophan mutants had to be generated to characterize different regions in such proteins as L-lactate dehydrogenase (Smith et al., 1991), phosphoglycerate kinase (Sherman et al., 1995), and colicin E1 channel peptide (Steer & Merrill, 1995). In contrast, our investigation of the unfolding of 5F-Trp-labeled D-LDH and other similar ^{19}F-NMR spectroscopic studies have the advantage of monitoring more than one Trp residue at once and minimizing perturbation to the native protein structure.

Intermediate forms of rat intestinal fatty acid binding protein during the equilibrium unfolding by urea have been inferred from the different transition rates of the two Trps and the missing intensity of W82 in the ^{19}F-NMR spectrum (Ropson & Frieden, 1992; Frieden et al., 1993). Our results in this study show the distinctive CD and ^{19}F-NMR spectra of at least two partially unfolded intermediates of D-LDH during the equilibrium unfolding by Gdn.HCl. The ^{19}F-NMR results of the changes in the 5F-Trp mobility, solvent exposure, and chemical shifts are consistent with each other and suggest that different regions of D-LDH unfold nonuniformly in a general order from the carboxyl end to the amino end. The first unfolding stage of D-LDH starts with a small conformational change and the release of the FAD cofactor, followed by extensive structure changes resulting in the complete unfolding of the carboxyl-terminal region (W567). In the second unfolding stage, the middle region of the protein (W384, W407, and W469) unfolds in at least two separate unfolding steps at 3.75 and 4.6 M Gdn.HCl. Finally, the amino-terminal region (W59) unfolds as the Gdn.HCl concentration is increased up to 8.0 M. This hard-to-unfold region may contain the "initiation sites" for protein folding. These results are in agreement with previous findings that the carboxyl-terminal region of D-LDH is largely exposed to the solvent while the amino-terminal region is buried in the protein core (Rule et al., 1987; Peersen et al., 1990).

In the ^{19}F-NMR spectrum of D-LDH at 8.0 M Gdn.HCl (Figure 3G), the resonance peaks from the unfolded 5F-Trp residues are clustered around −49.37 ppm, the resonance position of free 5F-tryptophan in aqueous solution. The chemical shifts of these 5F-Trp resonances differ by up to 0.2 ppm, even though the protein should have almost completely unfolded as suggested by CD and fluorescence measurements and there is no residual structure maintained by disulfide bridges (Dowd et al., 1995). Similar patterns of multiple resonance peaks have been observed for 6F-Trp-labeled rat intestinal fatty acid binding protein and dihydrofolate reductase (Ropson & Frieden, 1992; Hoeltzli & Frieden, 1994) and are believed to be caused by sequence-specific chemical shift variations (Wishart et al., 1995). In the case of D-LDH at 8.0 M Gdn.HCl, the W384 and W469 residues from the upfield peak at −49.37 ppm have more adjacent charged amino acid residues (within a seven-residue segment) and lower hydrophobicity index (Ho et al., 1988) than the W59, W407, and W567 residues from the downfield peak at −49.23 ppm. This rule appears to be applicable to the two 6F-Trp-labeled proteins mentioned above as well.

The ^{19}F-NMR dynamic study of the transverse relaxation time T_2 (determined from linewidth) of the 5F-Trps is complicated by the large ^{19}F chemical shift anisotropy (CSA) and possible chemical exchange effects (Hull & Sykes, 1975). In this study, we have focused on the ^{1}H/^{19}F NOE, which takes less time to obtain and is easier to analyze than the spin−lattice relaxation time T_1. At high magnetic fields, ^{19}F CSA could play an important role in addition to the nuclear dipolar relaxation, especially when internal molecular motions are involved. Although our present data are not sufficient for a detailed analysis taking into account these effects, we can nonetheless make semiquantitative estimates of the mobility of the 5F-Trps. Using a simplified spectral density function

$$J(\omega) = \tau_e/[1 + (\omega\tau_e)^2] \quad (1)$$

and neglecting the ^{19}F CSA, the ^{1}H/^{19}F NOE can be calculated according to Solomon (1955):

$$NOE = (\gamma_H/\gamma_F)[6J(\omega_H + \omega_F) - J(\omega_H - \omega_F)]/[6J(\omega_H + \omega_F) + 3J(\omega_F) + J(\omega_H - \omega_F)] \quad (2)$$

where τ_e is the effective correlation time for the overall motions of 5F-Trp, and γ_H and γ_F are the gyromagnetic ratios and ω_H and ω_F are the Larmor frequencies of the ^{1}H and ^{19}F nuclei. Thus, the effective correlation time τ_e for the 5F-Trp residues can be evaluated from the NOEs determined at a magnetic field of 7.0 T (Table 1). The estimated τ_e values in Table 1 indicate increased Trp mobility in the partially unfolded intermediate of D-LDH compared to the native protein, and with increasing Gdn.HCl concentrations. The

difference between the estimated τ_e values of the 5F-Trps and the correlation time of the overall rotational motion for the entire D-LDH molecule, on the order of 60 ns (Ho et al., 1989), suggests that a more realistic model should take into account the contributions from ^{19}F CSA and various internal molecular motions.

The effects of different detergents on stabilizing the D-LDH unfolding intermediates vary. Most of the detergents used in our experiments have a long hydrophobic polyethylene chain, except for CHAPS and Triton X-100 which do not stabilize the unfolding intermediates. CHAPS, in particular, contains a cyclopentaphenanthrene ring with both a hydrophobic and a hydrophilic face, and is believed to interact with proteins by forming a monolayer instead of a micelle (Bailyes et al., 1987). In addition, it was observed that octyl glucoside when used at a concentration below the CMC has no effect on stabilizing the D-LDH intermediate, further underlining the importance of the micelle formation. Our results also show that the unfolding intermediates of D-LDH in the presence of the detergents containing charged head groups, such as C_{16}TAB, lysoPC, and Zwittergent 3-14, have a better defined structure than those in the presence of nonionic detergents octyl glucoside and Mega-10. It is conceivable that the electrostatic interaction between the charged detergent head groups and the amino acid residues in protein could further stabilize the unfolding intermediates. A previous proteolytic digestion study on the native D-LDH in association with lipid vesicles has shown that the protein conformation is dependent on the type of head groups of the phospholipids (Truong et al., 1991a). Thus, we believe that both the micelle formation and the detergent head groups are important for stabilizing the unfolding intermediates of D-LDH.

Previous ^{19}F-NMR studies have suggested that each D-LDH molecule binds 40−60 lysoPC molecules in a relatively small membrane-binding region in the linkage area between the substrate and FAD binding domains (Rule et al., 1987; Peersen et al., 1990; Sun et al., 1993). The same membrane-binding region in the D-LDH unfolding intermediates is likely to be involved in interacting with the detergent micelles. The disruption of this hinge region at high denaturant concentrations (>3.5 M Gdn.HCl) may be responsible for the instability of the unfolding intermediate and the instantaneous denaturation of the native D-LDH (Figures 1 and 3). Further experiments using mutant D-LDHs with additional Trp probes in this region will be carried out to study the role of the membrane-binding region in the protein unfolding process.

The ^{19}F-NMR spectrum of a 5F-Trp-labeled mutant D-LDH, T79→W (in which the amino acid residue threonine-79 is replaced by a tryptophan), is completely different from that of the wild-type protein, but very similar to that of the partially unfolded intermediate of wild-type D-LDH at 3.5 M Gdn.HCl (Figure 9). This mutant protein was produced in very low yield, and was inactive due to the absence of the cofactor FAD. Yet, unlike other low yield, temperature-sensitive folding mutants of D-LDH (Truong et al., 1991b), mutant T79→W can be solubilized and further purified following the protocol for the correctly folded wild-type D-LDH. This suggests that the apparently misfolded mutant may nonetheless have a partially-preserved native-like structure. Future studies of the relationship among the unfolding intermediates of D-LDH and the partially folded mutants, such as T79→W, may lead to a better understanding of the protein folding processes in the *E. coli* cell.

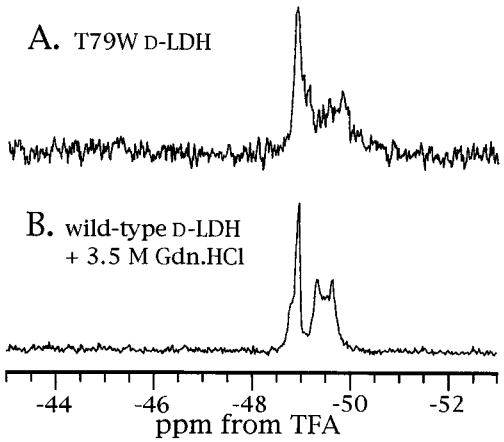

FIGURE 9: 282.4-MHz ^{19}F-NMR spectra of 5F-Trp-labeled D-LDH: (A) mutant T79W with 100 mM lysoPC, and (B) 0.25 mM wild-type D-LDH with 25 mM lysoPC in 3.5 M Gdn.HCl. The samples were in 10 mM potassium phosphate buffer, pH 7.2, in D_2O at 42 °C.

In summary, we have obtained several interesting results about the structure and dynamics of the intermediate forms of D-LDH during equilibrium unfolding by Gdn.HCl using ^{19}F-NMR spectroscopy. Partially unfolded intermediates have been observed at low denaturant concentrations. The ^{19}F-NMR results of chemical shifts, solvent exposure, and dynamic studies suggest that different regions in D-LDH unfold nonuniformly in a general order from the carboxyl end to the amino end. The protein unfolding intermediates appear to be stabilized by micelles formed by lipid-like detergents, such as lysoPC. Thus, ^{19}F-NMR spectroscopy is a very useful tool in identifying protein unfolding intermediates, as well as in the studies of their structure and the interaction with surfactants.

ACKNOWLEDGMENT

We are grateful to Dr. Carl Frieden for providing us the opportunity to obtain the circular dichroism and fluorescence spectra in his laboratory in Washington University. We thank Dr. Susan R. Dowd for helpful discussions.

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BI9620619