Time-Resolved Protein Phosphorescence in the Stopped-Flow: Denaturation of Horse Liver Alcohol Dehydrogenase by Urea and Guanidine Hydrochloride

Margherita Gonnelli and Giovanni B. Strambini*

CNR—Istituto di Biofisica, Via S. Lorenzo 26, 56127 Pisa, Italy

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ABSTRACT: This study reports the implementation of room temperature protein phosphorescence in the stopped-flow technique. Time-resolved Trp phosphorescence can now be detected following rapid mixing of protein solutions with a time resolution of 10 ms and a sensitivity in terms of chromophore concentration down to 0.1 μM. Calibration tests with monomeric and multimeric proteins proved that in all cases the delayed emission is not affected by artefacts that could arise from either enrichment of trace impurities along the flow lines or deformation of the macromolecules by the shear stress of laminar flow. To illustrate the potential of Trp phosphorescence in the stopped-flow to detect the time evolution of protein conformation the interaction of urea and guanidine hydrochloride (GdnHCl) with the native structure of horse liver alcohol dehydrogenase (LADH) has been re-examined under conditions of rapid denaturation. Remarkable differences in the action of the two denaturing agents has been confirmed by the phosphorescence lifetime (τ_P) of the internal Trp residue (W314). Whereas in urea, up to 8 M, τ_P is not minimally perturbed, in GdnHCl it decreases sharply and progressively from 800 ms down to 23 ms in 6 M solutions. Such reduction of τ_P implies that in the region of W314 the polypeptide structure has become highly loose and flexible prior to the major unfolding transition. Therefore, denaturation of LADH in GdnHCl, as opposed to urea, proceeds from a partly unfolded intermediate conformation of the protein. Other characteristics of this intermediate state are a partial loss of tertiary structure, as revealed by the circular dichroism of the aromatics, and an almost complete inhibition of the catalytic activity. Control experiments with equimolar NaCl demonstrate that τ_P, the tertiary structure and the catalytic activity are affected to a much smaller extent and that, therefore, salt effects do not account for the difference between urea and GdnHCl. Finally, measurements of the unfolding reaction emphazise that the kinetics of LADH denaturation are heterogeneous with both denaturing agents. From the constancy of τ_P during the course of the reaction it is concluded that the multiphasic behavior is a manifestation of multiple unfolding pathways owing to a plurality of stable LADH conformations.

Much of what is known about the thermodynamic stability of globular proteins (at physiological conditions) has been learned from unfolding these macromolecules with large concentrations of urea or GdnHCl[1] and then extrapolating the free energy change, ΔG_{N-D}, to zero denaturant concentration ($\Delta G°_{N-D}$). Although the procedure is widely adopted, concerns have been raised that $\Delta G°_{N-D}$ may not represent the free energy change governing the stability of the folded state under native conditions. Indeed, $\Delta G°_{N-D}$ may not necessarily be a property of the protein as its magnitude often depends on the denaturant (D) employed (Pace, 1975; Ahmad & Bigelow, 1982; Ropson et al., 1990; Monera et al., 1993; Yao & Bolen, 1995) or on whether extrapolation to zero denaturant concentration is carried out according to the solvent exchange model (linear dependence on [D]) or the binding model (logarithmic dependence on [D]) (Pace, 1975; Ahmad, 1993).

An important but as yet little characterized aspect of protein denaturation is the extent to which these denaturing agents interact with and affect the native fold. Strong interactions with the native state may lead to a gradual but progressive deterioration of its structure or to the formation of one or more stable intermediate forms. In either case the reaction cannot be modeled in terms of a fixed two-state process and therefore a main assumption in the derivation of $\Delta G°_{N-D}$ is violated. Several lines of evidence suggest that urea and even more GdnHCl affect the native protein structure. From transfer studies of small molecules emerges that both urea and GdnHCl interact with every group of every amino acid (Shellman & Gassner, 1996). Specific interactions with the denaturant have been documented in the crystal structure of α-chymotrypsin in which both urea and GdnHCl are seen to complex with superficial as well as internal residues (Hibbard & Tuluisky, 1978). Makhatadze and Privalov (1992), in their thermodynamic study of three proteins, conclude that the number of binding sites available to GdnHCl and urea merely doubles on unfolding, implying that their interaction with denaturants should be considerable also with the native state. Some evidence suggests that the interaction is stronger with or is specific of GdnHCl. For example, with RNase T1 the $GdnH^+$ cation was shown to bind and actually raise by 2 kcal mol^{-1} the stability toward unfolding (Mayr & Schmid, 1993). Likewise, GdnHCl but not urea was found to increase the flexibility of the native fold, as deduced from enhanced H-exchange rates in RNase A (Mayo & Baldwin, 1993) and Trp phosphorescence

* To whom correspondence should be addressed.

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[1] Abbreviations: AP, alkaline phophatase; AZ, apoazurin; RNase T1, ribonuclease T1; LADH, horse liver alcohol dehydrogenase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GdnHCl, guanidine hydrochloride; W, tryptophan; τ_P, phosphorescence lifetime; N, native state; U, denatured state; D, denaturing agent; RTP, room-temperature phosphorescence; CD, circular dichroism.

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lifetime on LADH (Strambini & Gonnelli,1986). In GdnHCl the formation of partially unfolded intermediates prior to crossing the main thermodynamic barrier to the unfolding state has been reported for the denaturation of barstar (Nath et al., 1996), ribonuclease A (Kiefhaber et al., 1995), and other proteins (Eaton et al., 1997). For proteins such as disulfide isomerase and barstar the interaction with GdnHCl is sufficiently strong to populate stable intermediates under equilibrium conditions (Swaminathan et al., 1996; Morjana et al., 1993; Pace et al., 1990).

The lack of an appropriate probe is often the reason for the nonobservation of intermediate species and for the general adoption of the two-state model in protein denaturation studies. In this respect it should be pointed out that Trp phosphorescence can represent a uniquely sensitive monitor of protein conformation that is particularly suited for probing protein structure in the vicinity of the native fold. The sensitivity of room temperature phosphorescence (RTP) is based on the dramatic dependence of the RTP lifetime (τ_P) on the local flexibility of the protein matrix (Strambini & Gonnelli, 1995), τ_P varying by as much as 4−5 orders of magnitude from internal rigid cores of the macromolecule to mobile superficial regions (Gonnelli & Strambini, 1995). Variations in τ_P have, indeed, been instrumental for unveiling even subtle changes in protein conformation induced by binding of substrates/allosteric effectors (Cioni & Strambini, 1989), change in chemical composition (buffer, pH, ionic strength, and cosolvent) (Strambini & Gonnelli, 1990) or physical state of the medium (Strambini & Gabellieri, 1996; Strambini & Gonnelli, 1988), exposure to denaturing agents (Strambini & Gonnelli, 1986; Subramaniam et al., 1995) or to extreme conditions of temperature and pressure (Cioni & Strambini, 1994, 1996).

In phosphorescence measurements the need of thorough removal of O_2 from the solution has, until now, imposed lengthy sample preparations (several minutes) and as such the technique of RTP has been applied only to conditions for which protein systems are either stable in time or evolve sufficiently slowly. Here we report the implementation of protein RTP in the stopped-flow apparatus, and to illustrate the potentiality of time-resolved RTP to follow the time evolution of protein conformation we re-examine the interaction of urea and GdnHCl with the folded state of LADH up to denaturant concentrations of 8 and 6 M, respectively, conditions in which denaturation proceeds rapidly. The results confirm the profound differences between the two classical denaturants and demonstrate that whereas urea does not detectably perturb the native state of LADH, GdnHCl is remarkably effective in producing progressively looser and partly unfolded conformation in times shorter than the 10 ms resolution of the apparatus.

MATERIALS AND METHODS

Materials. The proteins LADH and GAPDH from *Bacillus stearothermophilus* were supplied by Boehringer (Mannheim, Germany). Alkaline phosphatase from *Escherichia coli* was from Sigma (St. Louis, MO), and ribonuclease T1 (RNase T1) was from Calbiochem Corp. (San Diego, CA). Copper-free azurin from *Pseudomonas aeruginosa* was a gift of Finazzi-Agrò, University of Rome (Tor Vergata, Italy). To remove NAD^+ from GAPDH the enzyme was treated with activated charcoal as reported before (Strambini & Gabellieri, 1989). All protein samples were dialyzed against 0.01M Tris-HCl, pH 8, except for Ribonuclease T1 where the buffer was 0.01 M sodium cacodylate, pH 5.5. Ultrapure GdnHCl and urea were purchased from U.S.B. Corp. (Cleveland, OH), and their concentration was determined from the density of the solution using the equation of Kawahara and Tanford (1966). Fresh stocks of denaturant solutions were prepared daily. Water, doubly distilled over quartz, was further purified by a Milli-Q Plus system (Millipore Corp., Bedford, MA). All glassware used for sample preparation was conditioned in advance by standing 24 h in 10% suprapure HCl (Merck, Darmstadt).

Enzymatic Activity. The turnover number of LADH for the oxidation of ethanol by NAD^+ was measured at 20 °C in the presence of 1.8 M GdnHCl or NaCl, by the method of Dalziel (1957).

Conventional Spectroscopic Measurements. Steady-state protein fluorescence and phosphorescence spectra and yields and phosphorescence decays under pulsed excitation were measured with a home-made apparatus (Strambini, 1983; Strambini & Gonnelli, 1995). The exciting light, whether provided by a continuous xenon lamp for steady-state work or a flash-pumped frequency-doubled dye laser for time-resolved phosphorescence emission, had a constant wavelength of 295 nm. In all luminescence studies the concentration of protein samples was adjusted to be between 1 and 3 μM utilizing the molar absorptivity. In phosphorescence measurements O_2 was thoroughly removed from the sample by a procedure described before (Strambini et al., 1989).

Circular dichroism measurements were carried out with a Jasco J-500A spectropolarimeter. For the aromatic region of the spectrum (260−310 nm), the cell path length was 10 mm and the LADH concentration was typically 1 mg/mL whereas for the peptide region the cell path length was 0.5 mm and the protein concentration was 0.4 mg/mL. Multiple sweeps were averaged to improve the signal to noise ratio. The kinetics of unfolding of LADH in 2.3 M GdnHCl were followed by measuring the molar ellipticity at 222 nm. The dead time of manual mixing was about 15 s.

Protein fluorescence and phosphorescence intensity (see next section) kinetic profiles were obtained with the Applied Photophysics SX-17MV stopped-flow apparatus. Stock LADH solutions (3 μM in 10 mM Tris-HCl, pH 8) were diluted in a 1/5, v/v, mixing ratio with GdnHCl or urea (in 10 mM Tris-HCl, pH 8) of the desired concentration. With fluorescence the excitation was set at 295 nm and the emission was selected by a 320 nm long-pass filter. Control experiments in the absence of denaturant showed that the fluorescence intensity decreased by about 7−8% in 10 min. This drop was attributed to sample photochemistry, and all traces were corrected accordingly. All spectroscopic data refer to 20 °C.

Protein Phosphorescence in the Stopped-Flow. the apparatus is a modified version of Applied Photophysics model SX-17MV stopped-flow system designed for continuous fluorescence and absorbance measurements. An outline of the various components is given in Figure 1. Pulsed excitation at 295 nm is provided by a frequency-doubled flash-pumped dye laser (UV500M, Candela). The pulse duration is 1 μs and the light energy per pulse ranges between 1 and 10 mJ. The laser light, directed along the long axis of the optical cell, is focused within the 1×2 mm^2 cell cross section. Ambient light is blocked from the optical cell

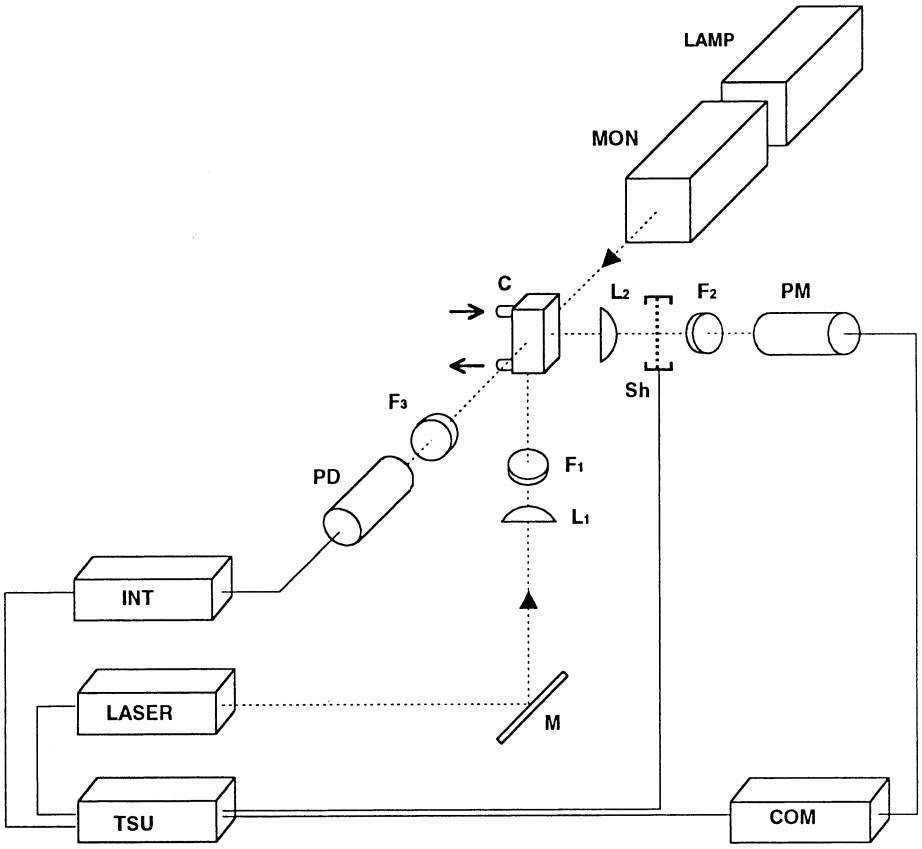

FIGURE 1: Outline of the optical components of the stopped-flow apparatus modified for protein phosphorescence detection. LAM, MON, COM, PM, and C refer to, respectively, continuous lamp, monochromator, computer, photomultipler, and optical cell of the standard stopped-flow system (Applied Photophysics, model SX.17MV/S/2). PD is a UV sensitive photodiode (Centromics, model OSD 100-7); F_1 is a UV-transmitting filter (Schott, UG5); F_2 represents a set of two interchangeable filters, one for fluorescence emission (320 nm long-pass), one for phosphorescence emission (410 nm long-pass plus the interference filter DT-Blau, Balzer), F_3 is a 310 nm long-pass filter; L_1 and L_2 are one-inch diameter quartz lenses of 7 and 2 cm focal length, respectively; Sh is a one-inch aperture electronic shutter (Uniblitz, Vincent Associates); LASER is a flash-pumped frequency-doubled Rhodamine 6G dye laser (UV500M, Candela); TSU is a home-made trigger synchronizing unit (Strambini et al., 1997); INT is a charging capacitor/voltage display circuit.

by an UV-transmitting filter (Schott UG5). Phosphorescence, collected by a condenser lens at right angle from the excitation, is selected by a filter combination (420 nm long-pass plus the interference filter DT-Blau, Balzers) with a transmission window, 420−480 nm, centered around the maximum of the Trp phosphorescence spectrum. The photomultiplier is protected from the intense light pulse of prompt fluorescence by an electronic shutter that permits the delayed emission to be detected 8−10 ms after the excitation pulse. The photocurrent is amplified, and the signal is recorded, stored, and analyzed by the standard Applied Photophysics signal detection and analysis unit. Phosphorescence decays were analyzed in terms of a sum of discrete exponential components by the local fitting software.

To correct for the variability in phosphorescence intensity due to laser instability, changes in protein concentration, sample photochemistry, and inner filter artefacts, the fluorescence intensity measured in the same excitation pulse was used as an internal standard with which to normalize all phosphorescence signals. Pulsed protein fluorescence is selected by a 310 nm long-pass filter and monitored at right angles from the excitation by an UV-sensitive photodiode (OSD 100-7, Centronics) whose photocurrent is integrated in a charging capacitor circuit.

Rapid mixing of solutions is synchronized with laser excitation, shutter opening, fluorescence integration, and computer acquisition of phosphorescence decays by means of an appositedly constructed trigger synchronization unit whose electronic circuitry are described elsewhere (Strambini et al., 1997). This unit takes over the normal microprocessor control of mixing and signal acquisition events. Using a variable delay (0−8 s) from the time of mixing plus repeated excitation/acquisition cycles on the same sample mixture at a frequency of 0.1 Hz the unit provides a train of pulses that permit to follow the time evolution of protein phosphorescence starting from about 10 ms after initiation of the reaction.

Bimolecular quenching by impurities represents a great obstacle in the way of monitoring the intrinsic phosphorescence lifetime of Trp in proteins at ambient temperature since, in fluid media, even submicromolar quencher concentrations can drastically affect the decay kinetics. An ubiquitous quencher is molecular O_2, but many other compounds, such as oxidizing agents in general and a wide variety of organic compounds can be just as effective. To avoid quenching by contaminants it is paramount to employ highly purified water, reagents, and buffering salts of top grade and to use carefully conditioned glassware and sample cuvettes (Strambini & Gonnelli, 1995). In the stopped-flow apparatus, a major concern is the amount of impurities that can be released form the different kinds of materials employed in the construction of the flow system (loading/driving syringes, valves, plastic tubing, optical cell, and the connection between the various parts) or that can enter the

latter through microleaks at junctions. Impurity quenching was cut down to negligible levels after some of the sealing elements were replaced (Teflon washers, O-rings) and all of the connections were more firmly tightened, in addition to prolonged (3 days) conditioning of the entire flow system with dilute HCl (1%).

O_2 removal from samples and flow lines is accomplished in three separate steps. First, the flow lines and optical cell are made O_2 free by filling them with a 1 mM dithionite solution (10 mM Tris-HCl, pH 7) prepared in a N_2 atmosphere. Subsequently, the buffers that are used to rinse away the dithionite are deoxygenated *in situ* from two appositely constructed loading syringes mounted directly on the loading ports of the solution mixing unit. Through a vacuum tight T-connection the loading syringes are connected alternatively to a supply of ultrapure N_2 (<0.01 ppm) or to moderate vacuum. The rinsing buffers, 1−1.5 mL each, are deoxygenated by repetitive inlet of N_2 and its subsequent removal (Strambini et al., 1989). Magnetic stirring of the solutions ensures effective O_2 exchange and complete removal in about 10 min. Subsequent to deoxygenation, rinsing buffers are introduced into driving syringes, flow lines, and the optical cell. The last step is the deoxygenation of the samples which is carried out by the same procedure outlined above for the rinsing buffers. To avoid O_2 enrichment of the solution, all sections of the mixing unit are enclosed in a N_2 atmosphere and the thermal bath submerging flow lines and optical cell is made O_2 free with a 5 mM dithionite solution (50 mM Tris-HCl, pH 7).

RESULTS

Detection of Protein Phosphorescence in the Stopped-Flow. To test the performance of the stopped-flow for monitoring Trp phosphorescence, a set of 5 proteins was chosen in order to take into account differences that may arise from molecular size, number of subunits in the oligomer and the magnitude of RTP lifetimes. The proteins are monomeric RNase T1 (τ_P = 32 ms) and apoazurin (τ_P = 620 ms); dimeric LADH (τ_1 = 200 ms, τ_2 = 980 ms, α_1 = 0.20) and alkaline phosphatase (τ_P = 1.95 s); and tetrameric GAPDH (τ_1 = 45 ms, τ_2 = 116 ms, α_1 = 0.15). In each case only a single Trp residue per subunit exhibits RTP. RNase T1 and azurin are single Trp proteins whereas the only phosphorescing Trp at ambient temperature was shown to be W314 for LADH (Saviotti & Galley, 1974), W109 for alkaline phosphatase (Sun et al., 1997), and W84 for GAPDH (Gabellieri et al., 1996)

Protein stocks were rapidly mixed in the stopped-flow apparatus with the same buffer in a 1/5 (v/v) protein/buffer ratio to a final protein concentration of about 1 μM in phosphorescing Trp residues. Representative decays of the phosphorescence emission, following single pulse excitation, are reported in Figure 2. An analysis of the decay curves confirms that for each protein the phosphorescence lifetime obtained in the stopped-flow is, within experimental error (typically around 5%), identical to that measured with standard equipment. Also significant for LADH and GAPDH, where the decay is distinctly nonexponential, both amplitudes and lifetime components are in accord with the usual determination. In view of the extreme sensitivity of RTP both to quenching by impurities and to even subtle alterations of protein conformation, such good agreement

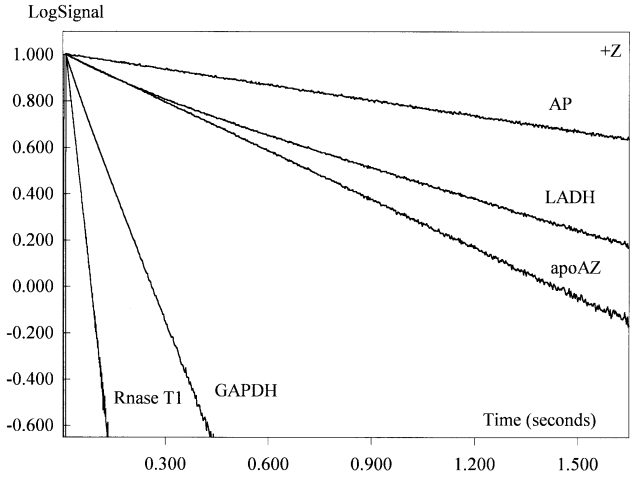

FIGURE 2: Representative phosphorescence decays of various proteins obtained with single excitation pulse subsequent to rapid mixing of the solution with buffer in the stopped-flow. The proteins are apoazurin (AZ), ribonuclease T1 (RNaseT1), liver alcohol dehydrogenase (LADH), alkaline phosphatase (AP), and glyceraldehyde 3-phophate dehydrogenase (GAPDH). The buffer is 10 mM Tris-HCl, pH 8, except for RNase T1 for which it is 10 mM sodium cacodylate, pH 5.5. The final protein concentration was typically 1 μM for AZ and RNase T1, 0.5 μM for LADH and AP, and 0.3μM for GAPDH.

with standard methods is remarkable and leads to the conclusions that (1) the deoxygenation procedure is satisfactory; moreover, the solutions do not become contaminated along the flow circuit by either O_2 or other quenching compounds that might leak at junctions or be released from various materials, and that (2) potential deformations of protein structure that might be caused by the shear stress of laminar flow, particularly on large, asymmetric, macromolecules, do not persist beyond the 10 ms dead time of the apparatus.

The sensitivity of phosphorescence detection in the stopped-flow is determined by the efficiency with which the emitted light is collected and the level of unavoidable spurious emission from the optical components. Relative to a standard phosphorimeter (Strambini, 1983), in the stopped-flow both the collection efficiency and the intensity of unwanted background emission are larger. However, although the signal to noise ratio of phosphorescence signals is better in the stopped-flow, relative to a standard phosphorimeter, the phosphorescence/background intensity ratio is worse. This spurious background lasts for up to about 20 ms and can amount from 5% to 10% of the initial protein phosphorescence signal. Naturally, the spurious signal can be made negligible upon increasing the protein concentration or, alternatively, it can be subtracted from the overall signal by employing suitable blanks. The same air-saturated protein sample which obviously has equal scattering characteristics as the sample, but does not phosphoresce, was employed as a blank. Another advantage of using the same protein as a blank is that any variability in excitation intensity between blank and sample will be reflected in the integrated fluorescence intensity monitored by the reference photodiode. In general, by applying fluorescence normalization to the phosphorescence decays and subtracting spurious background, reliable phosphorescence decay kinetics can be measured in a single-pulse mode down to a chromophore concentration of about 0.1 μM.

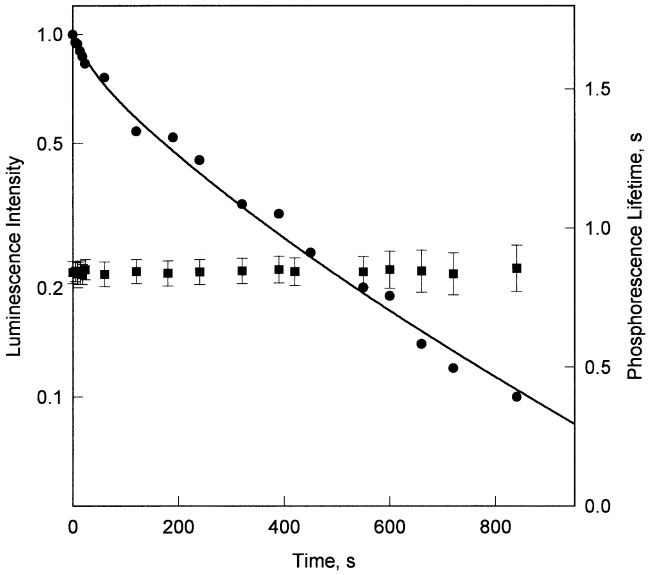

FIGURE 3: Denaturation kinetics of LADH in 8M urea followed by the relative change in fluorescence intensity $[(F_t - F_\infty)/(F_0 - F_\infty)]$ (—), the normalized phosphorescence intensity P_0 (●), and the average phosphorescence lifetime ($\tau_{av} = \alpha_1\tau_1 + \alpha_2\tau_2$) (■). Buffer and final protein concentration are as in Figure 2.

LADH Denaturation by Urea. In concentrated urea solutions LADH undergoes irreversible denaturation. The reaction is accompanied by a parallel and total loss of enzymatic activity, secondary structure, and quenching of RTP (Strambini & Gonnelli, 1986). Also, in the process the fluorescence quantum yield is reduced to about 30% and the fluorescence spectrum is red-shifted to wavelength typical of solvent-exposed Trp residues ($\lambda_{max} = 351$ nm).

Prior to the implementation of RTP in the stopped-flow, the phosphorescence properties could be measured only after several minutes from the initiation of the reaction. Consequently, changes in fluorescence and phosphorescence could only be compared after an initial lag period of several minutes and the measurements had to be circumscribed to urea concentrations ≤6.5 M for which the rate of denaturation is rather slow (Strambini & Gonnelli, 1986). Here we report the time course of the decrease in fluorescence (F) and phosphorescence intensity (P_0) of LADH in 8 M urea, at 20 °C. P_0 refers to the initial phosphorescence intensity of individual phosphorescence decays, obtained from a train of excitation pulses suitably delayed from the time of mixing protein with denaturant. The relative changes of fluorescence intensity, $[(F_t - F_\infty)/(F_0 - F_\infty)]$, and of P_0 are shown in Figure 3. The results confirm the expected reduction of F and the complete quenching of P_0. Further, they show that the fluorescence and phosphorescence kinetic profiles are superimposable throughout. Thus, fluorescence and phosphorescence changes occur concomitantly and, therefore, are governed by the same rate-limiting step of the reaction.

The phosphorescence lifetime of LADH is not affected up to 8 M urea. Further, as shown in Figure 3, τ_P remains remarkably constant also throughout the course of denaturation where the phosphorescence intensity drops to zero. Such constancy of τ_P confirms the observation of the earlier report (Strambini & Gonnelli, 1986) that during the course of denaturation there is a single phosphorescing species, one for which the environment of the phosphorescent probe (W314) is wholly native-like. A constant τ_P implies that the decrease in P_0 during unfolding is due exclusively to the formation of LADH species that are not detectably phosphorescent, conformations of the polypeptide in which the structure about W314 is very fluid-like. The concomitant red-shift of the fluorescence spectrum indicates that the structural rearrangement responsible for phosphorescence quenching involves the exposure of W314 to the aqueous solvent.

Table 1: Room Temperature Phosphorescence Lifetimes[a] of LADH in the Presence of Increasing Concentrations of GdnHCl

[GdnHCl] (M)	α_1 (%)	τ_1 (ms)	τ_2 (ms)	τ_{av} (ms)	χ^2
0.0	0.20	200	980	844	1.18
0.1	0.39	170	624	447	1.03
0.2	0.40	146	500	358	0.95
0.3	0.45	140	365	264	1.15
0.5	0.50	120	240	180	1.25
1.0	0.50	92	200	146	1.20
1.4	0.52	67	157	110	1.33
1.8	0.73	60	140	82	1.28
2.3	0.67	53	127	77	1.30
2.8	0.66	46	105	66	1.45
3.5	0.50	40	82	61	1.44
4.5	0.26	36	51	40	2.5
5.5	1.00	30 (35)[b]			1.3
6.0	1.00	22 (27)[b]			1.5

[a] These data are the averages of three independent experiments. Reproducibility is typically better than 5%. [b] Indicated in brackets are the lifetimes corrected for the rate of denaturation according to $1/\tau = (1/\tau_{obs} - k_d)$, where τ_{obs} is the lifetime actually measured and k_d is the unfolding rate constant obtained from the decrease in fluorescence intensity.

An important feature of the kinetic trace of Figure 3, not manifest in previous slow mixing experiments (Strambini & Gonnelli, 1986), is the clearly nonexponential dependence on time. Multiphasic unfolding kinetics are not uncommon, and such behavior is usually taken as evidence of multistep sequential reactions with the buildup of kinetic intermediates having spectroscopic properties that differ from both native and denatured state. In LADH denaturation by urea, the lack of phosphorescent intermediates suggests that the heterogeneous kinetic profile should rather be ascribed to a distribution of unfolding rates, that is, to a multiplicity of independent unfolding pathways.

Denaturation of LADH by GdnHCl. In contrast to urea, the addition of GdnHCl to LADH dramatically reduces its RTP lifetime. At all denaturant concentrations, the change in τ_P is rapid and occurs entirely during the first 10 ms after mixing. Dilutions with buffer, demonstrate that the reduction of τ_p is as promptly reversed. The amplitudes and the lifetime components describing the phosphorescence decay kinetics at various concentrations of GdnHCl in terms of one or two exponential terms are collected in Table 1. The average lifetime, $\tau_{av} = \alpha_1\tau_1 + \alpha_2\tau_2$, is displayed in Figure 4. GdnHCl decreases the magnitude of both lifetime components and at the same time increases the fraction of short-lived species. Above 4.5 M, lifetime heterogeneity is no longer evident and the decay is well represented by a monoexponential function. Also, at these higher concentrations the rate of unfolding is sufficiently large to actually contribute to the phosphorescence decay. Under these conditions the intrinsic lifetime, τ_P, is given by $1/\tau_P = (1/\tau_{obs}) - k_d$, where τ_{obs} is the lifetime actually measured and k_d is the unfolding rate constant derived from the fluorescence quenching profile. Corrected lifetimes are reported in Table 1 and in Figure 4.

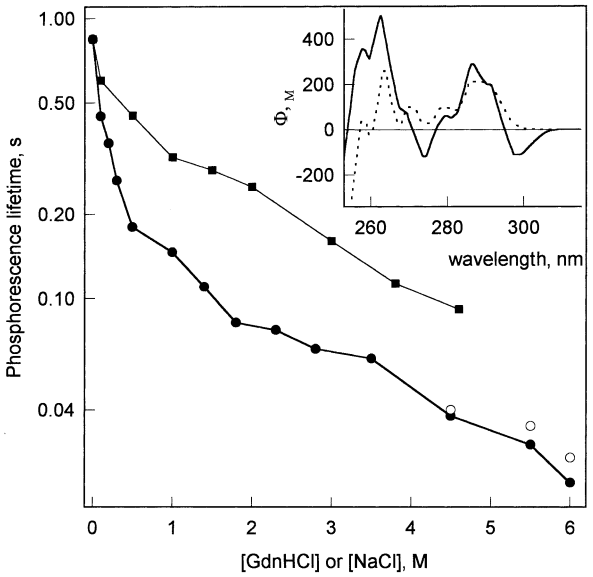

FIGURE 4: Dependence of the average phosphorescence lifetime of LADH on the concentration of GdnHCl (●) or NaCl (■). The phosphorescence decay was measured immediately after mixing the protein with denaturant. The data points indicated by empty circles refer to the lifetime corrected for the rate of denaturation (see text). Final protein concentration and buffer are as in figure 2. Inset: near-UV molar ellipticity of LADH in buffer (—) and in 1.8 M GdnHCl (···).

In the 0−6 M concentration range examined, τ_{av} decreases monotonically up to about 30 times prior to major unfolding. Since GdnHCl per se is not a quencher of Trp phosphorescence (Gonnelli & Strambini, 1995), the large reduction of τ_{av} must reflect a LADH structure remarkably loose and more flexible in the region of the chromophore. It should be noted that, inspite of the drastic reduction of the phosphorescence lifetime, this state of LADH exhibits a fluorescence yield and spectrum (up to 2 M GdnHCl, where it can be measured) that are native-like. Hence, the interaction with GdnHCl does not lead to the exposure of the chromophore to the aqueous solvent, as that would quench its fluorescence and red-shift the spectrum (λ_{max} from 338 to 350 nm).

To discriminate between a specific action of the denaturant on the conformation of LADH and effects deriving from the increase in ionic strength of the solution, or alternatively, from binding of Cl^-, phosphorescence measurements were also conducted in equimolar solutions of NaCl. The results, shown in Figure 4, demonstrate that although salt effects are important in the reduction of τ_{av} they account for only part of the lifetime shortening observed in GdnHCl.

In Figure 4 we note that a considerable reduction of τ_{av} in GdnHCl occurs already in the subdenaturational concentration range (≤1.8 M). Near- and far-UV circular dichroism (CD) spectra, in 1.8 M GdnHCl, point out that spectral perturbations, with significant attenuation of the ellipticity, are observed exclusively in the aromatic region (inset, Figure 4). By contrast, in 1.8 M NaCl, the CD signal of the aromatic is slightly increased. This loss in ellipticity of the aromatics attests to a decreased asymmetry of the chromophores' environment, a finding that is consistent with partial loss of tertiary structure. As an additional indirect monitor of protein conformation the catalytic activity of LADH was measured in the presence of 1.8 M NaCl or GdnHCl. At 20 °C, the turnover number for the oxidation of ethanol by NAD^+ dropped to 54% in NaCl as compared

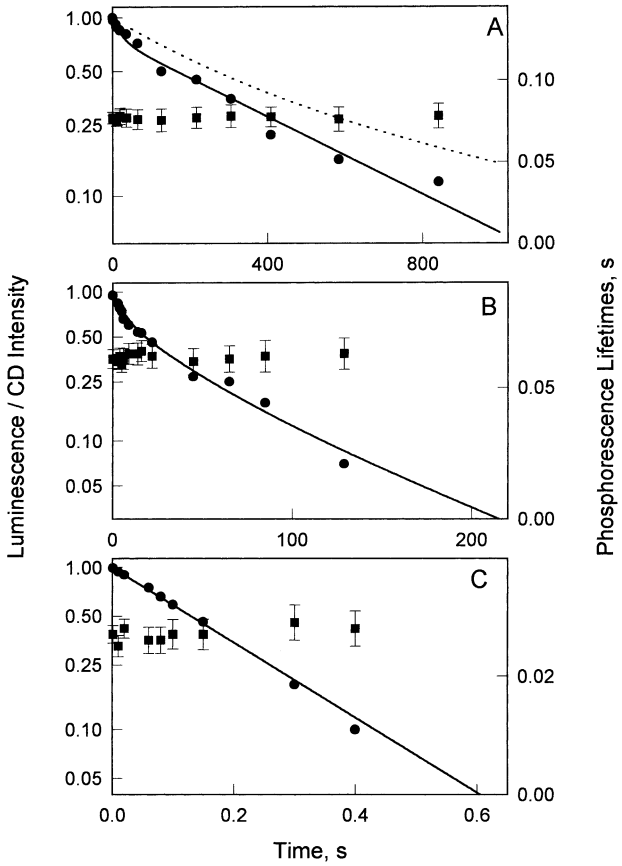

FIGURE 5: Denaturation kinetics of LADH at 3 GdnHCl concentrations: 2.3 M (A), 3.5 M (B), and 5.5 M (C); (—) relative change in fluorescence intensity, $[(F_t - F_\infty)/(F_0 - F_\infty)]$; (●) normalized phosphorescence intensity, P_0; (···) relative change in molar ellipticity at 222 nm, $[(\Theta_t - \Theta_\infty)/(\Theta_0 - \Theta_t)]$; (■) average phosphorescence lifetime. The final protein concentration and the buffer are as in Figure 2.

to 3−4% in GdnHCl, indicating again enhanced structural perturbations by the denaturing agent.

The kinetics of LADH denaturation by GdnHCl, at 20 °C, were followed at three denaturant concentrations (2.3, 3.5, and 5.5 M) by monitoring fluorescence and phosphorescence intensities, phosphorescence lifetime and, for the 2.3 M sample, also the molar ellipticity at 222 nm (θ_{222}). The kinetic traces are reported in Figure 5. Some features of these profiles are common to urea denaturation. The phosphorescence lifetime, measured up to a residual phosphorescence intensity of 10−15%, is largely invariant during the course of denaturation. On full denaturation, the fluorescence intensity decreases to about 30% whereas the phosphorescence is completely quenched. At each concentration, $(F_t - F_\infty)/(F_0 - F_\infty)$ and P_0 profiles are practically superimposable. Also, at the lower GdnHCl concentrations the profiles are distinctly multiphasic as with urea denaturation. However, they become less heterogeneous on increasing the denaturant concentration until at 5.5 M the profile is monophasic as would be expected for a first-order reaction. As for the decrease in luminescence intensities, the change in molar ellipticity at 2.3 M GdnHCl is multiphasic. Note, however, that the drop in θ_{222} is more gradual than that of luminescence intensity and therefore demonstrates that in GdnHCl, as opposed to urea (Strambini & Gonnelli, 1986), the undoing of the α/β secondary structure is not in step with the changes in Trp luminescence.

DISCUSSION

Time-Resolved Protein Phosphorescence in the Stopped-Flow. This paper reports the successful implementation of protein RTP in the stopped-flow technique. The method allows for the determination of phosphorescence yields and lifetimes following rapid mixing of protein solutions with a time resolution of 10 ms. With the five proteins examined the agreement in phosphorescence lifetimes obtained with the traditional method is satisfactory and suggests that no artefacts are introduced in the measurement by either the mixing circuit or the rapid flows.

The scope of applicability of the technique for studying the time evolution of protein structure is limited by the dead time of the apparatus and its sensitivity in terms of chromophore's concentration. Satisfactory signal to noise ratios in phosphorescence decays of proteins on single pulse excitation can be obtained down to chromophore's concentrations of about 0.1 μM. The 10 ms time resolution of the apparatus poses a lower limit both on the time range of the rate processes that can be studied and on the magnitude of the phosphorescence lifetimes that can be measured. The latter circumscribes the variety of proteins and experimental conditions that can be examined as Trp residues with lifetime shorter than 10 ms are not detected. Among these are Trp residues exposed to the solvent or located in very flexible superficial loops of the polypeptide (Gonnelli & Strambini, 1995). On the other hand, the vast majority of internal Trp residues are detectable in the stopped-flow as they have been found to exhibit RTP lifetimes that are generally larger than 10 ms. Of course, borderline cases may also be brought into the measurable range by either lowering the temperature or by the addition of stabilizing cosolvents, such as glycerol. In conclusion, protein RTP in the stopped-flow will be exclusive of Trp residues that are substantially buried within the folds of the polypeptide or become internalized as a result of profound structural isomerization, such as refolding from denaturing condition.

Naturally, a phosphorescing Trp also exhibits a strong fluorescence signal and therefore the two emissions provide independent monitor of the same local protein structure. Of course, thanks to the large difference in their lifetime, they report on dynamic processes in totally complementary time scales. It may be instructive to compare the potentiality of phosphorescence for characterizing the evolution of protein structure to that of the better known, and routinely used, fluorescence. Until recently, fluorescence measurements in the stopped-flow dealt with variations in yields and more rarely spectra. However, lately time-resolved fluorescence techniques have advanced to the point of allowing lifetime measurements in the stopped-flow with a time resolution (acquisition time) of 10 ms (Jones et al., 1995). Thus, now both techniques can provide time-resolved excited-state lifetimes, and from these other structural parameters such as rotational correlation times (from time-resolved anisotropy) and second-order quenchers diffusion rate constants. An advantage of these parameters is that they are microscopic quantities and as such can also reveal possible structural heterogeneity in the ensemble of macromolecules, information not readily available with this time resolution.

Important differences between fluorescence and phosphorescence are a direct consequence of the 6−9 orders of magnitude difference in excited-state lifetime. For example, quenching experiments in fluorescence typically require molar quencher concentrations and even so, compact cores of the structure may not be reached by a quencher molecule in the aqueous phase during the ns time interval of fluorescence. On the other hand, nonperturbing micromolar quencher concentrations are often sufficient to affect phosphorescence. Indeed, the delayed emission is so prone to impurity quenching that, as compared to fluorescence, great precautions must be taken and laborious sample preparations are needed to avoid artefacts. Another consequence of the different lifetime is the interpretation of emission heterogeneity in single Trp proteins in terms of distinct conformations of the macromolecule. Since heterogeneity manifests itself when the various molecular forms do not interconvert during the excited state lifetime, in the case of fluorescence heterogeneity implies that interconversion is slow on the nanosecond time scale as opposed to the tens or hundreds of milliseconds scale in the case of phosphorescence. Consequently, heterogeneous decays are very likely to be observed in fluorescence, where indeed it seems the norm, but not in phosphorescence as structural averaging is expected to be quite effective in the long millisecond−second time scale. Conversely, a heterogeneous phosphorescence decay is unequivocal evidence of the existence of multiple conformations of the macromolecule that, being separated by a large activation barrier, are likely to be relatively stable. Another major difference between the two emissions is owed to the forbidden character of the phosphorescence electronic transition which is responsible for its long lifetime and for its peculiar sensitivity to the dynamic structure of the polypeptide (Strambini & Gonnelli, 1995). For example, during protein unfolding τ_P can vary up to 4 orders of magnitude (from 10^3 to 0.1 ms) against a factor that is typically 2−3 for τ_F. Likewise, there are numerous examples of considerable modulations of τ_P in proteins in response to specific interactions with ligands (Cioni & Strambini, 1989) or changes in conditions of the medium (Strambini & Gonnelli, 1988, 1990; Strambini & Gabellieri, 1996) that are not accompanied by alterations of the fluorescence properties. Such sensitivity of τ_P to protein flexibility points out that RTP is best suited for characterizing the conformational state of the macromolecule in the vicinity of the native fold rather than more extensive loosening of the globular structure leading to the exposure of the indole nucleus to the solvent in that these would result in complete quenching of phosphorescence. As a rule, these more drastic structural alterations are conveniently followed by changes in the fluorescence properties. Finally, with multitryptophan proteins, most if not all residues contribute to the fluorescence signal and therefore data analysis is invariably complex. In contrast, fewer of them exhibit RTP and the natural discrimination between internal and surface residues helps in their identification and in the interpretation of phosphorescence data. Indeed, in several proteins RTP was found to originate from a single, often readily identifiable chromophore.

LADH Denaturation by Urea and GdnHCl. The difference in structural sensitivity between fluorescence and RTP and the ability of the latter to unveil perturbations of the protein native fold is illustrated in the denaturation of LADH by urea and GdnHCl. LADH is a dimer with two tryptophan residues per subunit. W15 is solvent exposed and nonphosphorescent, whereas W314 is buried at the subunit interface

region (Eklund & Bränden, 1983) and exhibits a long-lived RTP ($\tau_P \cong$ 800ms). Either unfolding of the polypeptide or subunit dissociation would expose W314 to the solvent and quench its phosphorescence whereas its fluorescence would become red shifted and partially quenched. These changes in Trp luminescence are observed on denaturation of LADH by both urea and GdnHCl. However, while the fluorescence properties of LADH are unchanged prior to the unfolding transition, the phosphorescence lifetime points out substantial differences in the structure of the "native" state in the presence of urea or GdnHCl. Rather strikingly, the phosphorescence lifetime of W314 is not minimally affected by urea, even in 8 M solutions where denaturation proceeds rapidly. By contrast, GdnHCl, causes a drastic reduction of τ_P prior to unfolding, a change that is consistent with a progressive loosening of the large β-sheet enveloping the triplet probe, the alteration taking place without subunit dissociation.

GdnHCl is generally a more effective protein denaturant than urea, and this is also found with LADH. It has often been postulated that the greater effectiveness of GdnHCl is owed to the concomitant large increase in ionic strength of the solution (Santoro & Bolen, 1992; Yao & Bolen, 1995; Ropson et al., 1990; Smith & Scholtz, 1996; Hagihara et al., 1993). Control experiments with equimolar NaCl confirm that either salt effects or weak binding of Cl^- (Maret & Zeppezauer, 1986) do contribute to the loosening of the interface region. However, as shown in Figure 4, the decrease of τ_P is only one-half in NaCl as compared to GdnHCl. Further, a specific structure destabilizing role of $GdnH^+$ is also inferred from its effects on the tertiary structure (CD of the aromatics) and on the enzymatic activity, an influence that is not observed with NaCl. Some of the characteristics of this GdnHCl-induced early intermediate state of LADH are as follows: (1) It is formed rapidly ($<$10 ms) and reversibly. (2) It is stable in time; τ_P is invariant for hours below 1.9 M GdnHCl and remains constant throughout the course of denaturation, at higher concentrations. (3) It does not represent a well-defined intermediate state of LADH as its structure is progressively more flexible at larger GdnHCl concentrations. (4) The CD spectrum at [GdnHCl] = 1.8 M (Figure 4) indicates that whereas the secondary structure is wholly native-like tertiary contacts about the aromatic amino acids residues are fewer, a finding that is in accord with the greater flexibility reported by τ_P. (5) Its enzymatic activity is drastically reduced. (6) Its Trp fluorescence is native-like, and therefore there is no evidence that W314 comes in contact with the aqueous solvent as would happen upon subunit dissociation. Altogether, the GdnHCl-induced intermediate is a dimeric conformation of LADH structurally similar to the native fold but considerably less rigid and compact. Similar conclusions about the effects of GdnHCl on globular proteins were also drawn from other studies. Enhanced structural fluctuations in the presence of GdnHCl have been observed in RNase A by H-exchange (Mayo & Baldwin, 1993) and NMR (Kiefhaber et al., 1995). Swelling of α-lactalbumin was found in predenaturation GdnHCl concentrations (Kuwajima et al., 1976), and a continuously expanding intermediate was observed for the heat shock protein (Palleros et al., 1993). Motional dynamics of buried Trp revealed the presence of partially structured forms during GdnHCL denaturation of barstar (Swaminathan et al., 1996; Nath et al., 1996).

Such distinction between the behavior of urea and GdnHCl, also pointed out in a previous report (Strambini & Gonnelli, 1986), is also important in the choice of the model, weak binding (Tanford, 1970; Shellman, 1975) or solvent exchange (Pace, 1975; Shellman, 1978), to be adopted for extrapolating denaturational free energy changes to zero denaturant concentration. In a binding model the number of binding sites was estimated on three different proteins to merely double on unfolding of the polypeptide (Makhatadze & Privalov, 1992). This presumes that a large number of denaturant molecules bind to and affect the native structure. The inertness of urea with LADH is remarkable in this regard, especially after observing that large alterations in flexibility of the region of W314 are elicited by apparently milder perturbations such as binding of substrates and analogues (Strambini & Gonnelli, 1990), the addition of organic cosolvents (Gonnelli & Strambini, 1993) or even changing buffering salts (Gonnelli and Strambini, unpublished data). Apparently, then, urea interacts rather weakly if at all with the native state of LADH and the variation of ΔG_{N-D} with [D] should be attributed entirely to the specific stabilization of the denatured state by urea. Accordingly, LADH denaturation by urea should be best treated in terms of the solvent exchange rather than the binding model. By contrast, GdnHCl clearly affects the native state of LADH and a binding model would therefore seem to be more appropriate in this case. A pertinent case of the latter appears to be GdnHCl denaturation of metmyoglobin where a binding model was shown to fit better the nonlinear dependence of ΔG_{N-D} and to provide an estimate of $\Delta G°_{N-D}$ in good agreement with that obtained with urea (Gupta et al., 1996). Still, other examples indicate that GdnHCl denaturation of proteins may even be a complex multistep reaction. This and other studies (Swaminathan et al., 1996; Morjana et al., 1993) have pointed out that the interaction with GdnHCl may be so strong or be so specific as to populate stable intermediate species before major unfolding of the polypeptide is detected. In these cases there are serious concerns on the thermodynamic interpretation of denaturation profiles in terms of a two-state reaction, native and denatured.

While the findings reported above confirm and extend previous observations made by slow mixing experiments at lower denaturant concentration (Strambini & Gonnelli, 1986), the ability to follow the initial stage of the reaction has revealed the heterogeneous nature of the unfolding kinetics. Up 4 M GdnHCl and 8 M urea both fluorescence and phosphorescence intensity profiles exhibit a distinctly multiphasic behavior. Complex kinetic profiles are not compatible with a simple, first-order, two-state reaction. They are generally ascribed to consecutive multistep reactions with the buildup of kinetic intermediates having spectroscopic properties that differ from both native and denatured states (Kim & Baldwin, 1990). In principle, heterogeneous kinetics may also originate from multiple unfolding pathways having distinct activation free energy barriers. Discrimination between these two possibilities requires knowledge of the distribution in molecular forms, and of their spectroscopic properties, during the course of the reaction. Such information is generally not available from the usual spectroscopic monitors of denaturation, UV-absorbance, CD, luminescence spectra, and intensities as they provide quantities that are ensemble averages. The phosphorescence lifetime, instead,

is a microscopic quantity and can therefore reveal whether or not the phosphorescence intensity originates from an homogeneous fraction of macromolecules. The invariance of τ_p during the course of the reaction implies that there are no intermediate states with phosphorescence properties distinct from the initial and final state (in the case of GdnHCl, the initial state is the one formed rapidly and prior to unfolding). Thus, as far as the phosphorescence probe is concerned, throughout the reaction there are only two kinds of LADH molecules: wholly phosphorescent or totally quenched. Note also that, the coincidence between fluorescence and phosphorescence intensity profiles shows that even the decay in fluorescence intensity is governed by the same all or none transition. In the absence of intermediate forms, a sequential multistep reaction is ruled out, and a multiphasic luminescence intensity profile can only be accounted for by independent, unfolding pathways with distinct activation barriers. This is possible only if the LADH population is conformationally heterogeneous in the time scale of the reaction. Evidence of long-term conformational heterogeneity in several proteins has been rapidly accumulating in the last decade, particularly from high-pressure subunit dissociation studies of oligomeric proteins (Weber, 1992). It is interesting to note that the heterogeneity in the fluorescence and phosphorescence denaturation profile (Figure 5) and that in the phosphorescence decay kinetics (Table 1) both vanish at large GdnHCl concentrations (above 4 M). A plausible explanation for this finding may be that the dynamic interaction between GdnHCl and LADH enhances the rate of conformer interconversion to such an extent that in the time scales of unfolding and phosphorescence decay the ensemble of macromolecules appears essentially homogeneous (Weber et al., 1996). This is, to our knowledge, the first time that a multiphasic denaturation profile is ascribed to a multiplicity of conformational states of the native fold, and it is quite possible that LADH is not a peculiar isolated case.

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