Phosphorescence Anisotropy of Liver Alcohol Dehydrogenase in the Crystalline State. Apparent Glasslike Rigidity of the Coenzyme-Binding Domain

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ABSTRACT: Phosphorescence anisotropy from internal tryptophan (Trp) residues in proteins which are in the crystalline state may provide an experimental approach suitable to study the flexibility of rather rigid segments of protein structure. The phosphorescence anisotropy of Trp-314 in liver alcohol dehydrogenase, which is enclosed within the β-sheet forming the coenzyme-binding domain, was measured with the protein free in solution and in the crystalline state. In contrast to the free protein, where the rotational correlation time reflects the tumbling rate of the whole macromolecule, there is effectively no loss in anisotropy in the crystalline state. At room temperature, the triplet lifetime of 0.5 s implies that the rotational correlation time of the indole side chain must be larger than 1 s. Anisotropy data show that fluctuations of the indole ring about the average position can only be of limited amplitude (cone of semiangle less than 15°) and that the resistance opposed by the β-sheet to out-of-plane rotational motions is equivalent to a viscosity larger than 2.5×10^8 P, a value which confirms the particular rigidity anticipated for such an assembly of secondary structure.

The interest aroused in recent years by the dynamical properties of protein structures has focused on discovering the variety of structural fluctuations these macromolecules are capable of as well as the role these motions may play with respect to biological function. Current knowledge on protein flexibility has been assembled from a number of experimental approaches: flash photolysis (Austin et al., 1975), hydrogen-exchange kinetics (Englander & Kallenbach, 1983), NMR spectroscopy (Wagner, 1983), X-ray diffraction (Ringe & Petsko, 1985), neutron scattering (Kossiakoff, 1985), and fluorescence emission (Yguerabide et al., 1970; Munro et al., 1979; Lackowicz & Weber, 1973; Rholam et al., 1984) to mention the principal ones. The emerging picture emphasizes the wide range of structural fluctuations, in terms of frequency and amplitude, that different regions of the macromolecule may be undergoing.

In luminescence studies, flexibility in protein structure has been inferred from the barrier offered by the protein matrix either to the diffusion of small molecules (O_2, acrylamide, NO) toward buried tryptophan residues or to the rotational freedom of this aromatic chromophore within the macromolecule. The accessibility of internal tryptophan residues to quenching by oxygen molecules during their fluorescence lifetime has been interpreted to mean that in general proteins undergo structural fluctuations on the nanosecond time scale. According to this parameter, there are small differences among proteins or sites in the same protein, and the diffusion of O_2 is slowed down by at most a factor of 10 in comparison to migration in the aqueous solvent (Lackowicz & Weber, 1973; Hagaman & Eftink, 1984).

A much greater differentiation in the environment of these residues is revealed by the rotational mobility of this chromophore. Indeed, time-dependent fluorescence anisotropy shows that while some tryptophan side chains exhibit rapid rotational motions of limited angular range in the subnanosecond time scale, others are completely immobile during the excited-state lifetime (Munro et al., 1979). For emission anisotropy to report on much slower structural fluctuations, that is, more rigid segments of the macromolecule, the emission must be long-lived and the macromolecule held in such a way as to prevent free tumbling motions. Since slow rotational motions can be detected by means of the phosphorescence anisotropy of tryptophan (Trp)[1] residues (Strambini & Galley, 1976, 1980), we propose to study unflexible regions of the protein structure utilizing this emission with macromolecules which have been immobilized in the crystal lattice. In this work, an investigation was carried out with liver alcohol dehydrogenase which in O_2-free solution at room temperature possesses a long-lived phosphorescence, the emission coming exclusively from Trp-314 (Saviotti & Galley, 1974). This residue is embedded in the coenzyme-binding domain which in all dehydrogenases of known structure is formed by a typical six-stranded β-pleated sheet interlocked by α-helical rods (Bränden et al., 1975; Bränden, 1980). Such assemblies of secondary structure have been shown by H exchange (Gregory & Lumry, 1985; Kossiakoff, 1985) to constitute probably the most inflexible segments of protein structures. Indeed, determination of the local fluidity, at the site of Trp-314, based on the sensitivity of the triplet-state lifetime of indole on microviscosity estimates that its environment is over a million times more viscous than the aqueous solvent (Strambini & Gonnelli, 1985, 1986). The present results of phosphorescence anisotropy measurements on this enzyme do confirm the exceptional rigidity of this domain and point out that appreciable rotational motion of the indole side chain does not occur on a second time scale.

Materials and Methods

Liver alcohol dehydrogenase from horse (LADH) was obtained as a crystalline suspension from Boehringer (Mannheim). MPD and TES were from Aldrich (Steinheim); MPD was distilled under a nitrogen atmosphere prior to use. Very pure L-tryptophan, from Fluka (Buchs), was twice recrystallized from water/ethanol. Spectroscopic-grade glycerol was from Merck (Darmstadt).

The enzyme was dialyzed for at least 24 h against 0.03 M pyrophosphate buffer, pH 8.6. Any remaining insoluble

[1] Abbreviations: LADH, liver alcohol dehydrogenase from horse; MPD, 2-methyl-2,4-pentanediol; TES, *N*-[tris(hydroxymethyl)-methyl]-2-aminoethanesulfonic acid; Trp, tryptophan.

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precipitate was removed by centrifugation. The activity of LADH preparations, as measured by the method of Dalziel (1957), ranged between 130% and 145%. Active-site concentrations were also determined by the spectrophotometric titration of LADH coenzyme-binding sites with NAD^+ in the presence of excess pyrazole (Theorell & Yonetani, 1963). On the basis of a molar extinction coefficient $E_{280} = 3.53 \times 10^4$ M^{-1} cm^{-1}, the coenzyme-binding capacity was typically 95% or better.

Crystals of LADH were grown as described by Eklund and Bränden (1979). Two milliliters of the enzyme solution (concentration of 8–10 mg mL^{-1}) was dialyzed at 4 °C against 0.05 M TES/NH_3 buffer, pH 7.5, to which MPD was slowly added. Large crystals began to appear when the MPD concentration reached 6–9% by volume; the final concentration of 30% was reached in 3 weeks time. Subsequently, crystals were sedimented by gentle centrifugation and twice washed with TES/MPD (70/30 v/v) to remove crystalline debris and denatured molecules.

Sample Preparation for Phosphorescence Measurements. To obtain reproducible phosphorescence data in fluid solutions, it is of paramount importance to remove thoroughly all dissolved oxygen. Satisfactory deoxygenation was achieved by placing about 0.5 mL of the protein solution in the short arm of an L-shaped quartz cell provided with a small stirrer where gas exchange and equilibration are to take place. The solution is finally transferred to the thin arm (4-mm i.d.) for emission studies. The short arm is connected to a vacuum line for gas exchange by means of a vacuum-tight steel cap (Swagelock pat. D-316) which, upon detachment from the line, avoids air leakage. Satisfactory removal of O_2 from the solution was obtained in about 10 min by repeated application of moderate vacuum followed by an inlet of very pure nitrogen nitrogen (0.1 ppm in O_2; SIO, Florence) at a pressure of 3 atm and gentle stirring. A check on the thoroughness of deoxygenation is provided by the dependence of the phosphorescence lifetime on the amount of excitation absorbed by the sample (Strambini, 1983).

Enzyme concentrations were 2×10^{-6} M for LADH dissolved in 50/30/20 (v/v) glycerol/MPD/TES and 2.5×10^{-5} M for the crystalline suspension in the same solvent. Determination of LADH concentration in equilibrium with the crystals showed that the fraction of free protein is typically 1–2%.

Spectroscopic Measurements. Fluorescence and phosphorescence spectra were obtained with a conventionally designed instrument (Strambini, 1983). The excitation was selected by a 250-mm grating monochromator (Jarrel-Ash) employing a band-pass of 2 nm for fluorescence and 10 nm for phosphorescence. The emission was dispersed by a 250-mm grating monochromator (Jobin-Yvon H25) and detected with an EMI 9635 QB photomultiplier. Phosphorescence decays were monitored at 440 nm by a double-shutter arrangement permitting the emission to be detected 2 ms after the excitation cutoff. The decaying signal was stored and on occurrence averaged in a Varian C-1024 time-averaging computer and successively transferred to an Apple II computer for exponential decay analysis by a least-squares method.

Anisotropy measurements were carried out by inserting linear polarizers, Polaroid type HNP'B, in both the excitation and emission beams. The excitation wavelength was 300 nm while the emission was centered in the O–O vibronic band (413 nm). The anisotropy (A) was calculated in the usual way from the formula:

$$A = \frac{I_\parallel - GI_\perp}{I_\parallel + 2GI_\perp}$$

where $I_\parallel$ and $I_\perp$ are the emission intensities polarized parallel and perpendicular, respectively, to the vertically polarized exciting beam. The correction factor (G) is the ratio of the vertically to horizontally polarized emission intensities obtained with horizontal excitation.

With crystals, which do not randomize their orientation in the time of the experiment, care must be exercised, particularly at the warmest temperatures, to avoid selective photobleaching. We have verified that prolonged vertical excitation, for example, reduces both the overall intensity and the apparent anisotropy. Time-dependent anisotropy measurements were performed with the same apparatus by operating the excitation shutter such as to provide light pulses 50–100 ms in width. Compensation for weaker phosphorescence intensities with pulsed excitation relative to steady-state conditions was done by averaging a proportionally larger number of decays. The dead time between excitation and emission was 3 ms. Also, since the decays of the emission polarized parallel and perpendicular to the plane of the polarized excitation were not recorded simultaneously, corrections for variations in lamp output were done with the corresponding integrated fluorescence signal.

The temperature in these experiments was regulated by the flow of cold nitrogen through a quartz Dewar and a temperature controller (Oxford DTC2) achieving an accuracy of ±0.2 K.

Results and Discussion

When dealing with proteins in the crystalline state, the question is often raised as to whether the constraints imposed on the macromolecule by the process of crystallization are so severe as to alter the conformational state(s) prevalent in solution. By and large, this question has been answered in the negative sense (Rupley, 1969; Ringe & Petsko, 1985; Fersht, 1985). LADH has been shown to retain catalytic activity in the crystal (Bignetti et al., 1979) although the kinetics of coenzyme binding are considerably slower (Theorell et al., 1966).

Room temperature fluorescence and phosphorescence spectra of crystalline LADH show no alteration in the state of the tryptophan residues when compared to the enzyme free in solution. An emission parameter particularly sensitive to perturbations in the conformational state of the NAD-binding domain is the phosphorescence lifetime of Trp-314 (Strambini & Gonnelli, 1986). Figure 1 compares the triplet decay kinetics of LADH in the crystalline state with that of the enzyme free in the same solvent over a wide range of temperatures. The agreement in lifetime between the two states of the enzyme is remarkably good, implying that the structural/dynamical features of this region of the macromolecule have not been influenced by crystallization.

The steady-state anisotropy of the phosphorescence from L-tryptophan, Trp-314, of LADH in solution and in the crystal is displayed in Figure 2. The measurements span a temperature range which goes from the glass-transition temperature of the solvent mixture up to room temperature. As the glass softens into a fluid solution, the increased tumbling rate of the chromophore results in the loss of the anisotropy first of L-tryptophan followed by LADH in solution. Comparative studies with other globular proteins show that the rotational correlation time obtained by approximating the macromolecule to a rigid sphere is that anticipated from the hydrodynamic volume of the hydrated macromolecule (Strambini & Galley,

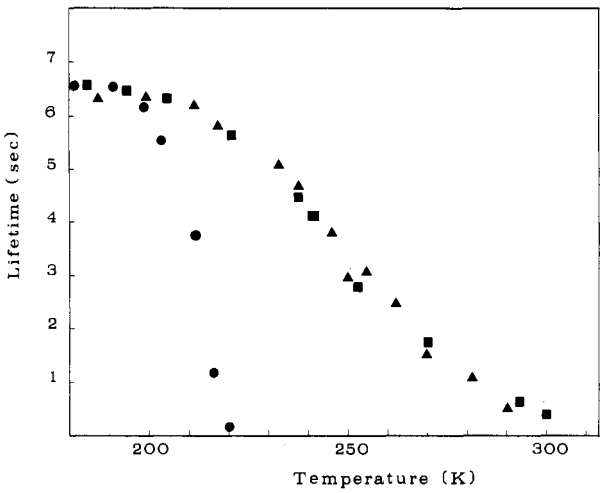

FIGURE 1: Phosphorescence lifetime of (●) L-tryptophan and (■) Trp-314 of LADH (2×10^{-6} M) free in solution and (▲) in the crystalline state (2.5×10^{-5} M). The solvent was 50/30/20 (v/v) glycerol/MPD/TES buffer, 50 mM, pH 7.5. λ_{ex} = 303 nm and λ_{em} = 440 nm.

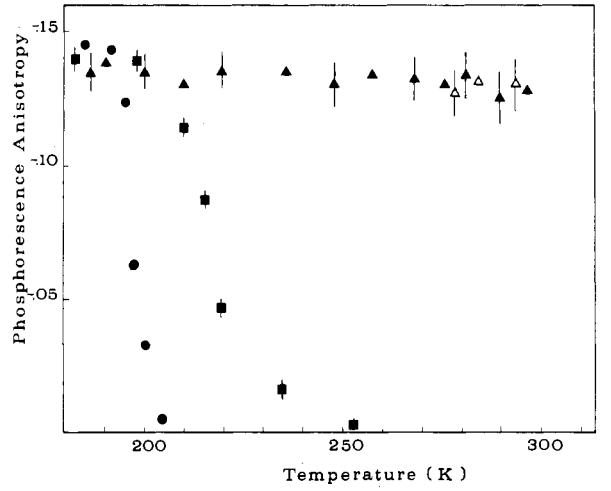

FIGURE 2: Temperature dependence of the phosphorescence anisotropy of tryptophan (measured on the O–O vibronic band upon excitation at 303 nm) in a 50/30/20 (v/v) glycerol/MPD/TES, 50 mM, pH 7.5, solvent mixture. (●) L-Tryptophan; (■) Trp-314 of LADH free in solution (2×10^{-6} M); (▲) Trp-314 of LADH in the crystalline state. Above 0 °C, anisotropy data were also obtained with crystalline suspensions in 30/70 (v/v) MPD/TES (△).

1976, 1980). In contrast to the behavior in solution, the anisotropy of LADH in the crystalline state remains within experimental error unchanged up to 20 °C, the highest temperature at which crystals are sufficiently stable. Anisotropy data from the crystalline suspension are in general less reproducible and more so at the higher temperatures. Furthermore, the average value is about 10% less than the maximum value observed with the free protein at low temperature. Both effects are thought to be due to the depolarizing effect of the inhomogeneous sample as an equivalent drop in anisotropy was found also with the corresponding fluorescence emission. In any case, for a given sample no change in anisotropy could be observed across the entire temperature range if no stirring of the solution intervened between the two measurements. Time-dependent anisotropy measurements in which the emission was monitored 5 ms after the excitation were carried out at temperatures of −20, 0, and 20 °C. The decay of polarized phosphorescence at 20 °C is shown in Figure 3. The results confirm that during the decay of the emission there is no evidence of changes in anisotropy with

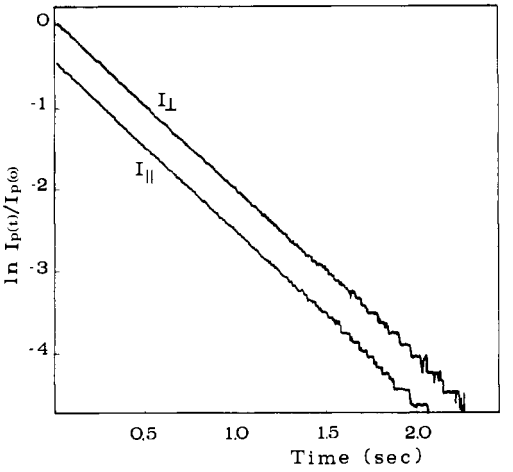

FIGURE 3: Decay of the phosphorescence of Trp-314 in LADH crystals polarized perpendicular ($I_\perp$) and parallel ($I_\parallel$) to the plane of the polarized excitation at 20 °C. The crystals were suspended in 50/30/20 (v/v) glycerol/MPD/TES. λ_{ex} = 303 nm, λ_{em} = 413 nm. Excitation was provided by light pulses 0.05 s in width and the emission detected after a dead time of 3 ms. Relative intensities of the two components were obtained from the G factor and the integrated fluorescence signal which corrects for lamp output.

time, its value being consistent with that observed with continuous excitation.

Anisotropy data in the crystalline state demonstrate unequivocally that even at 20 °C the indole nucleus of Trp-314 does not change appreciably its average orientation in a time of 0.5 s, the triplet-state lifetime. Consequently, its rotational correlation time, θ, must be greater than 1 s. If indole is treated as a sphere with an effective hydrodynamic volume V = 100 cm^3 mol^{-1} (Mantulin & Weber, 1977), then θ = 1 s implies ($\theta = V\eta/kT$) that the barrier to rotation imposed by the protein matrix is equivalent to a viscosity of 2.5×10^8 P. Incidentally, when the same criterion is applied to the entire LADH molecule with V = 87 000 cm^3 mol^{-1} (Strambini & Galley, 1980), we obtain that the effective barrier to rotational diffusion imposed by the crystal lattice contacts is equivalent to about 3×10^5 P, a large value if one considers that a good portion of the volume in the crystal i occupied by mother liquor. While we are not aware of any measurement of the resistance to reorientation of a macromolecule in a crystal lattice, this kind of information may turn out useful for assessing the strength of crystal lattice contacts and/or free volume effects. With LADH which undergoes a major conformational change upon binding of the coenzyme (Eklund & Bränden, 1979), these interactions may play an important role in determining the catalytic activity in the crystalline state.

An additional point of interest concerns the type of rotational motions which depolarize the phosphorescence of indole (as opposed to its fluorescence). Because the triplet- to ground-state transition dipole moment is almost perpendicular to the plane of the aromatic ring (Konev, 1967), only out-of-plane rotations, i.e., rotations about single bonds C$^\gamma$–C$^\beta$ and C$^\beta$–C$^\alpha$, or bending motions involving these atoms and the βF strands are effective in depolarizing the phosphorescence. Since bending motions can only reach limited amplitude, unless melting of the β-sheet occurs, this leaves to rotations about the bond C$^\gamma$–C$^\beta$ the dominant depolarizing effect. Anisotropy data on crystals confirm that the distribution in orientations which the indole ring may attain about this single bond during the phosphorescence lifetime is indeed a narrow one. Even allowing for a 10% uncertainty in the absolute value of anisotropy due to rapid fluctuations, the wobbling-in-cone model (Kinosita et al., 1982) would predict diffusion restricted to a

cone of semiangle less than 15°. Recent NMR data on several proteins demonstrated that while the aromatic ring of some buried phenylalanines and tyrosines undergoes 180° ring flips at rates of $10–10^4$ s^{-1}, this type of motion has not been detected for tryptophan residues (Fersht, 1985). The reason for this is thought to lie on the much larger volume swept by a revolution about $C^{\gamma}–C^{\beta}$ in the case of indole which is bigger and asymmetric about the rotational axis.

Flexibility in protein structures is inferred from a number of phenomena, most of which yield an "effective" microviscosity of a particular region of the macromolecule. It has been pointed out before in the literature that the term microviscosity is an improper one as it implies a property of general applicability. This point may be further stressed if one compares the effective viscosity of LADH in the neighborhood of Trp-314 as obtained by three independent methods: O_2 quenching of the luminescence, the intrinsic triplet-state lifetime, and phosphorescence anisotropy data. Oxygen diffusion to this site of the protein is slowed down as compared to the aqueous medium by a factor of about 10, according to fluorescence (Hagaman & Eftink, 1984), or 200, according to phosphorescence (Strambini, 1987). The reduction is taken to indicate the greater viscosity experienced by O_2 in diffusing to this region of the macromolecule. By this criterion, the β-structure enclosing Trp-314 is rather fluidlike (or has holes), offering but a modest barrier to the penetration of O_2. On the other hand, the empirical relationship between the triplet-state lifetime of indole and the local viscosity (as determined from the rotational diffusion of the chromophore in fluid solutions) (Strambini & Gonnelli, 1985) implies that a lifetime of 0.5 s corresponds to a local viscosity around 10^5 P. Still, this value would be too small by at least 3 orders of magnitude in order to account for the above anisotropy data. The different appraisals of the flexibility of the NAD-binding domain of LADH inevitably reflect the different kinds of motions probed by the three approaches. The discrepancy between quenching data and triplet-state lifetimes, observed also with alkaline phosphatase, has been discussed elsewhere (Strambini, 1987). It is evident from their respective activation energies that the effects on O_2 diffusion and triplet-state lifetime are mediated by different structural fluctuations. Indeed, the two phenomena may not even report on the same segments of the macromolecule. Quenching interactions extend beyond collisional radii and thus may not require the quencher to penetrate through the β-sheet enveloping Trp-314. The difference, instead, between intrinsic lifetime and anisotropy data probably lies in the fact that lifetimes are affected by even small-amplitude structural fluctuations (the type manifested in the crystallographic B values) whereas rotational fredom of the aromatic chromophore requires a concerted breaking of an unusually large number of hydrogen bonds. Indeed, according to Eklund et al. (1976), Trp-314 is enclosed in an unusual position between two pleated sheet strands in a tightly packed hydrophobic area in the subunit–subunit interface. The melting of a β-structure of such proportions may thus be prohibitive in free energy terms.

In conclusion, phosphorescence anisotropy measurements in the crystalline phase confirm the rigidity of the β-sheet in the coenzyme-binding domain of LADH anticipated from the long phosphorescence lifetime of Trp-314. They point out the high degree of rigidity that certain regions of protein structure may attain and for which the ever increasing potentialities of computational simulations will have to account. Finally, we anticipate that this approach may be extended to much more flexible regions of the protein structure, recalling that dichroism in triplet–triplet absorption of tryptophan can substitute phosphorescence anisotropy in the millisecond–microsecond time scale.

Registry No. LADH, 9031-72-5; Trp, 73-22-3; NAD, 53-84-9.

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