not only in relation to the Cu(II) site but also in connection with the catalytic mechanism of dopamine β-monooxygenase, which has yet to be determined in detail.

ACKNOWLEDGMENTS

We are indebted to Professor T. Nagatsu of Nagoya University for advice on enzyme purification.

Registry No. Cu, 7440-50-8; CN^-, 57-12-5; N_3^-, 14343-69-2; dopamine β-monooxygenase, 9013-38-1; water, 7732-18-5.

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Characterization of Tryptophan Environments in Glutamate Dehydrogenases from Temperature-Dependent Phosphorescence

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Received December 3, 1986; Revised Manuscript Received March 24, 1987

ABSTRACT: Tryptophan room temperature phosphorescence in solution was detected in glutamic dehydrogenase from bovine liver and *Escherichia coli* with lifetimes of 1.2 and 0.65 s, respectively. Although these enzymes possess three and five tryptophanyl residues per polypeptide chain, respectively, the temperature dependence of the phosphorescence quantum yield estimates that the room temperature emission is due, in either case, to a single residue. Long triplet-state lifetimes and very small rates of O_2 quenching indicate that these tryptophanyl side chains are embedded in a highly inflexible internal region of the macromolecule. Aided by sequence homology with dehydrogenases of known structure and theoretical predictions of secondary structure [Wootton, J. C. (1974) *Nature (London) 252*, 542–546; Brett, M., Chambers, G. K., Holder, A. A., Fincham, J. R. S., & Wootton, J. C. (1976) *J. Mol. Biol. 106*, 1–22], the phosphorescing tryptophans have been tentatively placed in the catalytic coenzyme binding domain of each enzyme. The particular sensitivity of the triplet-state lifetime in probing local changes in conformation provides a strong indication that within the time window of phosphorescence measurements the six subunits in the hexameric enzymes are equivalent. Furthermore, while in the bovine enzyme this parameter is markedly affected by the interaction with ligands which have a funtional role, the constancy of the phosphorescence lifetime at various degrees of polymerization suggests that the association process is not accompanied by important conformational changes in the macromolecule.

It has recently been shown that the phosphorescence lifetime and quantum yield of tryptophan are strongly affected by the fluidity of the surrounding matrix (Strambini & Gonnelli, 1985). As a result of this dependence, phosphorescence is not detectable in fluid solutions. In globular proteins, this emission has been rarely reported in aqueous solution at room temperature, being confined invariably to tryptophan residues

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0006-2960/87/0426-4968$01.50/0

buried within highly inflexible regions of the macromolecule. To date, the most notable examples of such residues are Trp-314 of liver alcohol dehydrogenase and Trp-109 of alkaline phosphatase (Saviotti & Galley, 1974). In both cases, the indole side chain is trapped within an extended β-pleated sheet interconnected by α-helical rods which in the former enzyme constitute the coenzyme binding domain. Such arrangement of secondary structure is known to provide for rigid inflexible cores in globular proteins (Gregory & Lumry, 1985).

Glutamic dehydrogenase, GDH, is a class of enzymes which catalyze the oxidative deamination of L-glutamate into 2-oxoglutarate. Bovine and bacterial GDHs, which find themselves at the opposite ends of the evolutionary scale, differ markedly in their specificity for the coenzyme as well as in the extent to which the catalytic function is modulated by effectors. Indeed, the activity of the mammalian enzyme is regulated by a host of ligands including the coenzyme, and the peculiarities of the kinetic and regulatory properties of this enzyme have made it a model for the study of allosteric behavior.

The active unit in bovine and *Escherichia coli* GDHs comprises six identical polypeptide chains. Knowledge of the three-dimensional structure of this important class of proteins is still lacking, due to the difficulty of growing appropriate crystals for X-ray analysis. The information available to date relies on spectroscopic techniques and theoretical predictions. Employing the latter approach and the sequence homology with dehydrogenases of known structure, Wootton (1974) identified in bovine GDH two sequences each containing a tryptophan residue with a secondary structure typical of the NAD binding domain. Were the rigidity of such domains comparable to that observed for alcohol dehydrogenase, then this structure predication anticipates that long-lived room temperature phosphorescence might be detected in this enzyme as well.

In the present paper, we show that bovine GDH is indeed intensely phosphorescent in aqueous solution at room temperature. This unusual emission is not confined to the mammalian enzyme but has similar features in the protein of bacterial origin. Room temperature phosphorescence from these enzymes has been characterized in terms of the number of emitting residues per polypeptide chain and their most likely location in the macromolecule. In view of the sensitivity of triplet-state lifetimes in monitoring even subtle conformational changes (Strambini & Gonnelli, 1986), we point out that this as yet unexplored emission will provide us with a natural probe to investigate the dynamic/structural aspects in these macromolecules, which considering the great differentiation in allosteric behavior may give us a possible relationship to function.

Materials and Methods

Beef liver glutamic dehydrogenase and liver alcohol dehydrogenase from horse were obtained as crystalline suspensions in ammonium sulfate from Boehringer, Mannheim. Prior to use, bovine GDH was thoroughly dialyzed at 4 °C against 0.1 M potassium phosphate, pH 7.3, containing 10 μM ethylenediaminetetraacetic acid (EDTA) and then centrifuged for 20 min (at 15000 rpm) to remove any precipitate. Enzyme concentrations were measured spectrophotometrically by using $E_{280} = 0.97$ mg^{-1} mL cm^{-1} (Olson & Anfinsen, 1952). The enzyme activity was determined from the absorption at 340 nm by following the oxidation of NADH with α-ketoglutarate and ammonia at 25 °C. The reaction was carried out in 0.1 M imidazole buffer at pH 7.9 in the presence of 13 mM α-ketoglutarate, 210 mM NH_4Cl, 0.1 mM NADH, 0.9 mM EDTA, and 2 mM ADP. Under these conditions, the specific activity was 140 ± 5 units/mg of protein as specified by the supplier.

GDH from *E. coli* K_{12} was purified according to the method of Veronese et al. (1975) from the cell paste supplied by the Centre for Applied Microbiology and Research, Salisbury, U. K. The enzyme concentrations was determined spectrophotometrically by using $E_{280} = 0.95$ mg^{-1} mL cm^{-1} (Veronese et al., 1975). The catalytic activity was determined in 0.1 M tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl) buffer, pH 8, 0.1 M NH_4Cl, 2.5 mM α- ketoglutarate, and 0.1 mM NADPH at 22 °C. Under these conditions, the specific activity was 240 ± 5 units/mg of protein which is in good agreement with Veronese et al. (1975).

KI, NaCl, β-mercaptoethanol, and spectroscopic-grade propylene glycol were from Merck, Darmstadt, FRG. Very pure L-tryptophan, from Fluka, Buchs, FRG, was twice recrystallized from water/ethanol before use.

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 of repeated application of moderate vacuum followed by an inlet of very pure nitrogen (0.1 ppm in O_2, SIO, Florence, Italy) 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).

Various O_2 concentrations at 20 °C were introduced by equilibrating the thermostated solution for about 15 min with known partial pressures of O_2. Partial pressures were determined from the overhead pressure (digital pressure meter OG 713 and OG 973, Officine Galileo, Florence, Italy) and the composition of appositely prepared O_2/N_2 gas mixture (SIO, Florence). Final concentrations of O_2 were calculated by using Henry's law and the solubility of O_2 in water at 20 °C (Handbook of Chemistry and Physics, 41st ed.). Measurements of enzyme activity before degassing and after phosphorescence measurements showed no deterioration of the sample.

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, in both the excitation

and emission beams. The excitation wavelength was 300 nm while the emission was centered in the O–O vibronic band (412 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 vertically polarized exciting beam. The correction factor G is the ratio of the vertically to horizontally polarized emission intensities obtained with horizontal excitation.

In determining the thermal quenching of the phosphorescence, the intensity measured over a temperature range of 150 K is subject to variations which, besides fluctuation in the exciting lamp output, are due to (1) a change in the concentration of the chromophore following density variation and (2) changes in the refractive index and multiple internal reflections appearing with stresses or cracks in the glass. In 1:1 propylene glycol buffer, these factors were found to have negligible effects at temperatures above 220 K, 30–40 K warmer than the glass transition temperature. Since tryptophan fluorescence spectra and yields remain the same at temperatures below 220 K, this emission was used as an internal reference signal. Accordingly, in these experiments, the phosphorescence intensity at warm temperatures was normalized by the exciting light intensity whereas below 220 K it was corrected by the corresponding fluorescence intensity. When this procedure was carefully tested with liver alcohol dehydrogenase in which one of the two tryptophans is fully quenched at higher temperatures, the agreement with expectation was within 2–3%.

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.

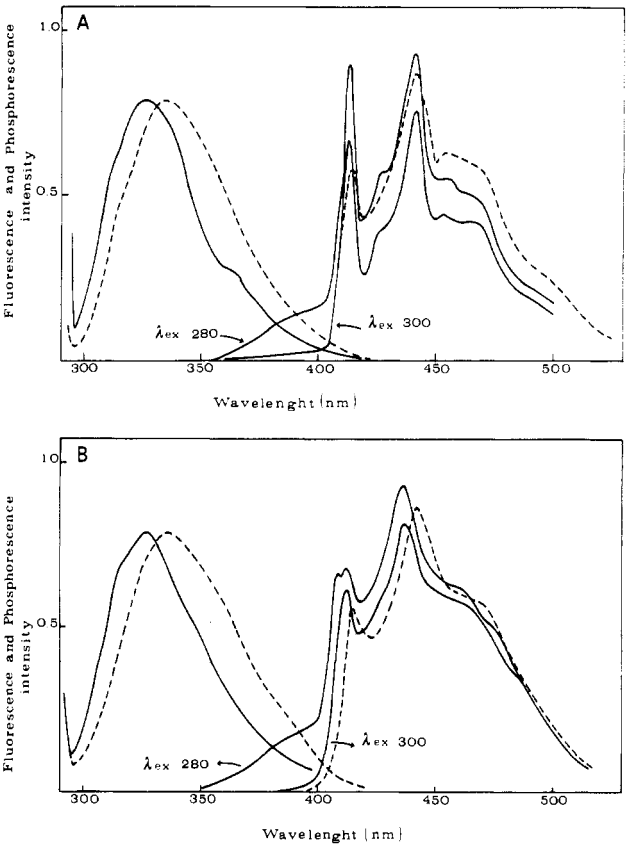

FIGURE 1: Fluorescence (λ_{ex} = 295 nm) and phosphorescence spectra (λ_{ex} = 280 and 300 nm) in 50:50 (v/v) propylene glycol/phosphate buffer, 0.1 M, pH 7.3 at 140 K (—), and in phosphate buffer at 293 K (- - -) (λ_{ex} = 295 nm). (A) Bovine GDH at a concentration of 2 mg/mL. (B) *E. coli* GDH at a concentration of 1 mg/mL. The relative magnitudes of the fluorescence to the phosphorescence intensity maxima are roughly 25:1 and 100:1 for bovine and *E. coli* GDH, respectively.

RESULTS

Low-Temperature Emission. Fluorescence and phosphorescence spectra obtained for bovine and bacterial GDH in a propylene glycol buffer glass are displayed in Figure 1. When both tyrosine and tryptophan are excited, the emission is dominated by the latter residue as commonly found for most proteins. The tyrosine contribution is clearly distinguishable in the phosphorescence spectrum which in comparison to free nonionized tyrosine is rather structureless and red-shifted by 7–8 nm. The tyrosine phosphorescence intensity at 380 nm decays in a nonexponential fashion, 80–90% of it having a lifetime of 1.4–1.6 s and a smaller component with a lifetime of about 2.4 s. Such spectral and decay characteristics resemble the emission from the tyrosinate anion or tyrosine perturbed by the proximity to disulfide bridges (Longworth, 1971). However, neither of these hypotheses seems to apply. Disulfide bridges have never been detected in any GDH, and tyrosinate involvement may be excluded because upon excitation at 297 nm where this species still absorbs the tyrosine component disappears entirely. A plausible interpretation of tyrosine phosphorescence is that most of the emitting residues are shielded away from the solvent, forming intramolecular complexes with amide linkages or positively charged side chains (Longworth, 1971).

While the tyrosine emission is practically identical in both sources of the enzyme, the phosphorescence from tryptophan reveals substantial differences between them. The well-resolved vibronic bonds in the phosphorescence spectrum of the indole nucleus quite often enable one to distinguish between the contribution from residues placed in environments of different polarity (Purkey & Galley, 1970). The relatively broad O–O vibronic band in the spectrum of GDH from *E. coli* with peaks at 409 and 412.5 nm manifests a degree of heterogeneity in the chemical environment of the five tryptophans (Valle et al., 1984) in each subunit. The 412.5-nm component is typical of a nonpolar solvation site whereas the 409-nm component reflects a more polar site even if not so polar as the aqueous solvent (Galley, 1976). Heterogeneity in solvation is present also in absorption spectrum; in fact, exciting on the red edge, at 305 nm, the O–O phosphorescence band narrows about a maximum at 412.5 nm, the blue component disappearing entirely.

Heterogeneity in the phosphorescence spectrum of tryptophan is not apparent in bovine GDH. The O–O vibronic band centered at 412.5 nm is relatively narrow, and its width does not depend on the excitation wavelength. Spectral data then place the three residues (Julliard & Smith, 1979) of each polypeptide in nonpolar sites of the macromolecule with similar dielectric constants.

The polar nature of the solvation site has long been utilized as an indicator of the degree of exposure of tryptophan to the aqueous solvent (Galley, 1976). On the basis of this parameter, spectral data suggest that only in the bacterial enzyme are there tryptophan residues partially exposed to the aqueous phase. Another criterion used to estimate the proximity of this aromatic residue to the solvent–protein interface is base on the perturbation which may be induced to the phosphorescence by heavy atoms which, due to their charged na-

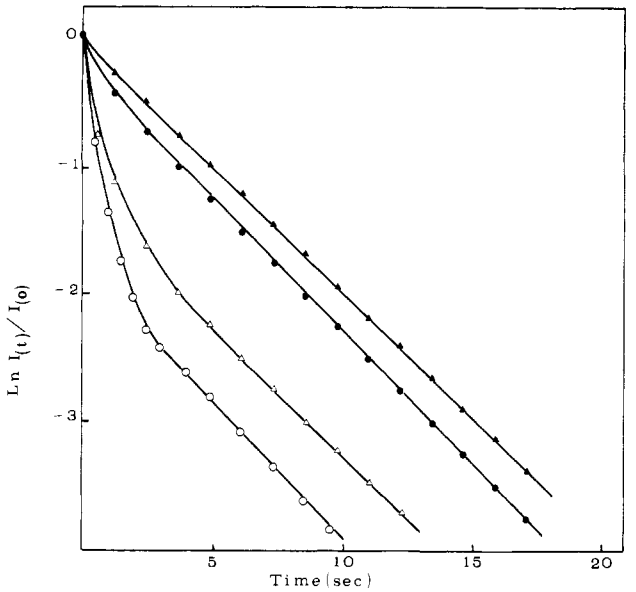

FIGURE 2: Effect of heavy-atom perturbation in the decay of the tryptophan phosphorescence emission at 465 nm upon excitation at 290 nm in a 50:50 (v/v) propylene glycol/phosphate buffer glass at 140 K. (△) Bovine GDH in the presence of 1 M KI and (▲) 1 M KCl; (○) *E. coli* GDH in the presence and (●) absence of iodide.

ture, are relegated to the aqueous medium. Studies with iodide ions show that when the aromatic ring is within a few angstroms of van der Waals contact of the heavy atom there is an increase up to 2–3 times in phosphorescence quantum yield followed by a shortening of the triplet-state lifetime from 6 s to a fraction of a second (McGlynn et al., 1969; Lee, 1985).

The effect of 1 M KI on the tryptophan lifetime of these enzymes is shown in Figure 2. In both cases, the presence of a short-lived component in the decay provides direct evidence that some tryptophan residues are subjected to the heavy-atom perturbation. On the other hand, a substantial portion of the emission with unchanged lifetime confirms that in both macromolecules some residues are sufficiently buried to be out of range of the perturbation. While these results with GDH from *E. coli* might have been anticipated from the heterogeneity in the phosphorescence spectrum, with the bovine enzyme they reveal that, given the distance dependence of the heavy-atom effect (Lee, 1985), at least one tryptophan is not further than 3–5 Å from the protein–water interface.

Thermal Quenching of Tryptophan Phosphorescence. A fair estimate of the number of tryptophan residues in a protein which contribute to the phosphorescence at room temperature may be obtained from the fraction of the lifetime-normalized intensity which persists on going from a glassy matrix at cold temperature to a fluid solution at higher temperatures (Domanus et al., 1980). The variation in phosphorescence intensity (P) with temperature, normalized by the lifetime (τ')) of the longest-lived component, is shown for both enzymes in Figure 3. With both enzymes, the phosphorescence begins to be quenched at temperatures 5–10 °C warmer than for free tryptophan, an indication that the environment of the quenched residues is more rigid than the bulk glycol–water solvent. as the temperature is increased P/τ' decreases monotonically, approaching a constant value at −30 to −20 °C above which no further quenching is detected up to room temperature. For bovine GDH, the fraction of the normalized phosphorescence intensity which persists at room temperature is 0.36 ± 0.03. When it is recalled that this macromolecule possesses three tryptophans per subunit (Julliard & Smith, 1979), the most direct interpretation of this value is that the long-lived phos-

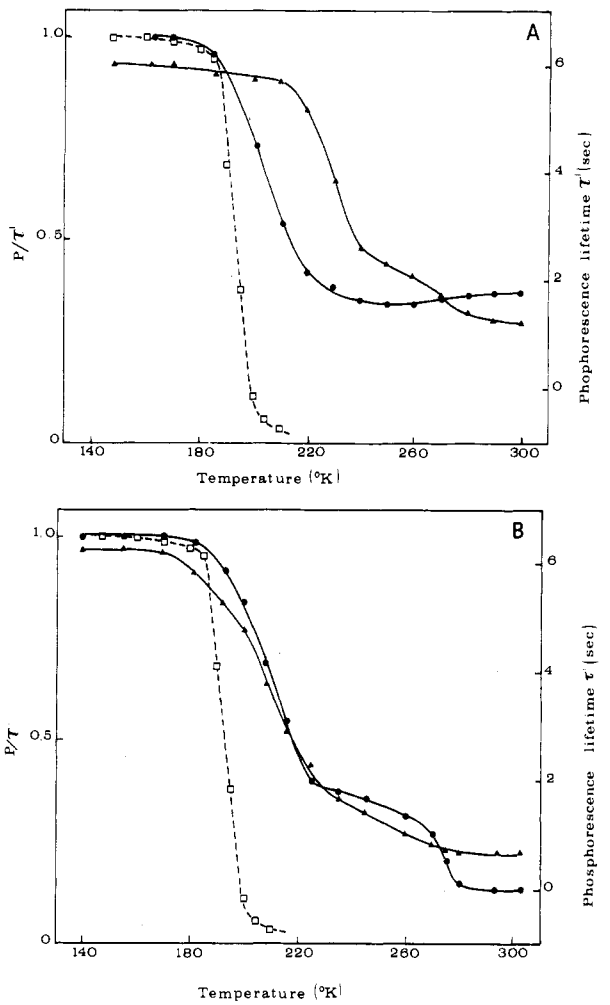

FIGURE 3: Phosphorescence thermal quenching profiles (●). (A) Bovine GDH (2 mg/mL) and (B) *E. coli* GDH (1 mg/mL) in 50:50 propylene glycol/phosphate buffer, 0.1 M, pH 7.3. The phosphorescence intensity, P (λ_{ex} = 290 nm, λ_{em} = 465 nm) was normalized by the longest component in the decay, τ' (▲). The loss in phosphorescence intensity of a 2×10^{-5} M solution of tryptophan (□) is also included.

phorescence comes from a single residue. Less intense is the room-temperature phosphorescence, with bacterial enzyme being only about 0.15 of the cold temperature limit. This amount is smaller than 0.2, the mininum value estimated for a polypeptide chain with five residues. Values smaller than predicted are not unusual (Domanus et al., 1980) and are typical of residues whose intersystem crossing quantum yield is somewhat lower than the value at cold temperature averaged over all residues. In globular proteins, lower yields are often the result of energy migration among adjacent tryptophans, a process which does take place in this protein as the limiting anisotropy of the phosphorescence is about 30–40° lower than the maximum value (see below). Alternative interpretations cannot be ruled out, however, in particular, the possibility that not all subunits are equivalent or that only a fraction of macromolecules is in a conformational state giving rise to room temperature phosphorescence.

Phosphorescence Anisotropies and Hydrodynamic Volumes. Macromolecular associations, which are mostly entropy driven, are known to become labile at low temperature, and dissociation into subunits is not uncommon with polymeric enzymes (Franks, 1985). To check whether the phosphorescence properties reported above relate to the macromolecule in its hexameric form, the hydrodynamic volume of each GDH was estimated at low temperature from measurements of the

Table I: Rotational Correlation Times (θ) Determined for the Two GDHs in 50:50 (v/v) Propylene Glycol/Phosphate Buffer (0.1 M, pH 7.3) at Three Different Temperatures[a]

	T (K)	θ (s)	θ/θ_0	mol wt
GDH from bovine liver	187	17.5	2.8	337 000
	191	4.7	2.9	352 000
	193	3.1	2.9	346 000
				av 345 000
GDH from *E. coli*	187	14.6	2.3	281 000
	191	4.1	2.6	307 000
	193	2.6	2.4	290 000
				av 293 000

[a] The values of θ were obtained from the phosphorescence anisotropy, A, and triplet-state lifetime, τ ($\theta = A\tau/A_0 - A$). θ_0 refers to the rotational correlation time of liver alcohol dehydrogenase under the same conditions. Enzyme concentrations were 2 mg/mL. Excitation was at 300 nm and emission at 413 nm.

phosphorescence anisotropy (Strambini & Galley, 1976, 1980).

The rotational correlation time, θ, of a chromophore can be determined in a steady-state experiment from the loss of anisotropy, A, of its emission, whenever the lifetime, τ, of the excited state is of comparable magnitude ($A_0/A = 1 + \tau/\theta$). The phosphorescence anisotropy at infinite viscosity, A_0, was found to be 0.14 ± 0.01 and 0.10 ± 0.01 for the bovine and the bacterial enzyme, respectively. A value of A_0 lower than 0.14 obtained for a protein in rigid matrices is invariably an indication that energy transfer takes place among tryptophan residues in the same or adjacent subunits of the macromolecule.

The rotational correlation times (θ) evaluated from the anisotropy at different temperatures are reported in Table I. In order to relate θ to the hydrodynamic volume, V, of the macromolecule, knowledge is required of the solvent viscosity ($\theta = V\eta/kT$). Since sufficiently accurate values of η are not available at these temperatures, an alternative route is to determine η from θ with a macromolecule of known hydrodynamic volume. Using liver alcohol dehydrogenase from horse with $V = 87\,000$ cm^3 mol^{-1} as standard (Strambini & Gonnelli, 1985), we obtain an average V equal to 252 000 ± 4400 and 214 000 ± 8000 cm^3 mol^{-1} for bovine and *E. coli* GDHs, respectively. These molar volumes when stripped of the hydration shell one water molecule thick (4 Å) correspond to molecular weights of 345 000 ± 16 000 and 293 000 ± 11 000 if a partial specific volume of 0.73 cm^3 g^{-1} is employed. The agreement with the known molecular weights, 336 000 and 27 000 (Sund et al., 1977; Veronese et al., 1975), of the hexameric units of these enzymes is remarkably good.

For GDH from *E. coli*, which is stable as a hexamer (Sakamoto et al., 1975), this result might have been anticipated if independent rotational motions of the indole side chains within a subunit and of subunits relative to one another were substantially slower than the rotational diffusion of the entire macromolecule. A different conclusion might have been expected for the bovine enzyme which at room temperature in buffer at the present concentration of 2 mg/mL associates to yield linear chains of hexamers (Eisenberg et al., 1976). The results of anisotropy measurements imply, therefore, that the most stable form in glycol–water at low temperature is the hexameric unit. This finding is not entirely unexpected as a depolymerizing effect of the organic solvent was also reported by light-scattering measurements in ethylene glycol buffer (Gauper et al., 1974).

Room Temperature Fluorescence and Iodide Quenching. Fluorescence spectral relaxation and quenching can provide evidence on the location and solvent accessibility of tryptophan residues in a protein which is either complementary or parallel to that obtained from the phosphorescence emission. The fluorescence spectra of the two enzymes excited at 295 nm (Figure 1) are practically identical. At room temperature, the spectrum is broader in comparison to cold temperature, and its maximum at 335 nm is about 10 nm red shifted. Overall the spectral shift is less pronounced than would be anticipated for residues exposed to the solvent. The modest extent of the spectral relaxation implies, therefore, that only a small fraction of tryptophan residues is either exposed to the aqueous medium or located in sufficiently flexible regions of the macromolecule.

Table II: Quenching Constants and Fractions of Maximally Accessible Fluorescence of L-Tryptophan, Bovine GDH, and GDH from *E. coli*

	f_a(eff)[a,b]	K_q(eff)[a,c] (M^{-1})
L-Trp	0.97	9.8
bovine GDH (4 mg/mL)	0.29	3.4
bovine GDH (0.1 mg/mL)	0.31	2.6
GDH from *E. coli* (0.1 mg/mL)	0.5	3.5

[a] Determined from Lehrer's plot, $F_0/(F_3 - F)$ vs. $1/[I^-]$ (Lehrer, 1971), in phosphate buffer (0.1 M, pH 7.3) with 10^{-2} M β-mercaptoethanol. The ionic strength was kept constant (0.45 M) by addition of NaCl. λ_{ex} = 290 nm, λ_{em} = 330 nm. The temperature is 20 °C. [b] f_a(eff) = fraction of maximally accessible fluorescence. [c] K_q(eff) = quenching constant of accessible fluorophores.

A criterion often employed to evaluate the degree of solvent accessibility of tryptophan in protein is based on the ability of iodide ions to quench its fluorescence (Lehrer, 1971). Quenching experiments were carried out with both GDHs, and the quenching constants (K_q) were obtained from the change in fluorescence intensity as a function of the iodide ion concentration. In all cases, when the data were analyzed according to the Stern–Volmer relationship, the plots were not linear, tending to plateau at a high concentration of iodide, implying that some residues are unable to interact with it. This was confirmed by Lehrer's plots of the data obtained at 20 °C, the results of which are shown in Table II.

For the bacterial enzyme, we observe that only half of the intensity is quenched by iodide and its effectiveness, expressed by a value of K_q equal to 3.5 M^{-1}, is about 35% of that for free tryptophan. With bovine GDH, the fluorescence quenched by iodide is about a third of the total, and this amount is only slightly reduced in the polymerized form. The value of K_q for the latter is 3.4 M^{-1} compared to 2.6 M^{-1} for the hexameric unit. In both proteins, the quenched component of the fluorescence has a spectrum only 2–3 nm red shifted with respect to the total intensity. Were the residues accessible to iodide quenching directly in contact with the aqueous medium, a greater red shift in their spectrum would be anticipated.

Overall fluorescence quenching data strengthen and add further details to the assignments made by phosphorescence alone. With the bovine enzyme, in particular, spectra and quenching effectiveness confirm that no residue is directly exposed to the solvent. Of the three tryptophans, only one is sufficiently close to the surface of the macromolecule to be reached by the quencher (consistent with the heavy-atom effect on phosphorescence). The other two residues are more deeply buried, and according to phosphorescence, only one of them is in a highly rigid structure. This picture is essentially unaltered by the polymerization process in spite of the fact that one residue, supposed to be on the hexamer–hexamer contact region (Witzemann et al., 1974), may be expected to undergo structural and/or dynamic changes in its environment as a result of it.

Room Temperature Phosphorescence. The phosphorescence spectra obtained with these proteins in phosphate buffer at 20 °C are reported in Figure 1. There is practically no dif-

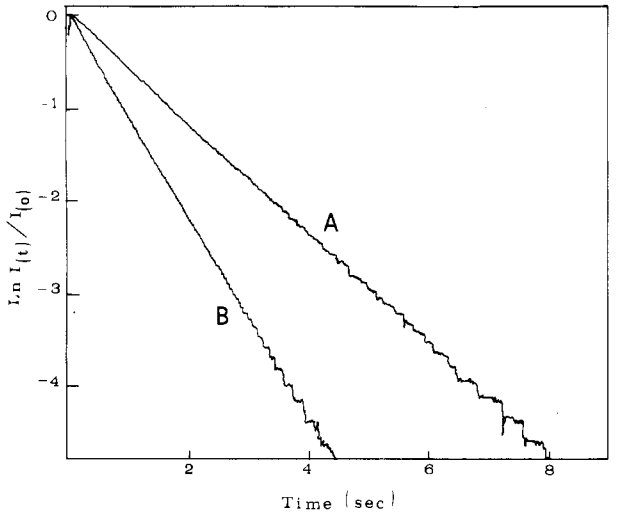

FIGURE 4: Decay of phosphorescence intensity with time (λ_{ex} = 290 nm; λ_{em} = 440 nm) at 20 °C in phosphate buffer, 0.1 M, pH 7.3. (A) Bovine GDH; (B) *E. coli* GDH.

ference between the two GDHs, the spectrum also being similar to that reported for other proteins at room temperature. The O–O vibronic band at 414 nm is 1.5–2 nm to the red of the cold temperature emission, a modest shift when compared to the 8–9 nm caused by solvent relaxation of free tryptophan in the same medium. This implies that the chromophore's site either has a low dielectric constant and/or is too rigid to allow for complete dipolar reorientation during the triplet-state lifetime.

The decay in time of phosphorescence intensity is shown in Figure 4 for both enzymes to obey a strictly exponential law. The lifetime, τ, in phosphate buffer, pH 7.3 at 20 °C, is 0.65 ± 0.03 and 1.2 ± 0.5 s for bacterial and bovine GDH, respectively. With the latter macromolecule which is known to polymerize in buffer, τ, in the range from 0.05 to 10 mg/mL, was found to be independent of protein concentration.

The observation that the phosphorescence decays monoexponentially has important implications for oligomeric proteins. With the bacterial enzyme, if we assume that each subunit is phosphorescent, then, given the sensitivity of the triplet-state lifetimes of indole to its environment, a single phosphorescence lifetime is a test of equivalence among the subunits of the hexamer. For bovine GDH, the concentration independence of τ extends such equivalence of the subunits even to polymerized forms.

The intrinsic phosphorescence lifetimes in these macromolecules are large when compared to 15–20 μs for tryptophan in aqueous solution (Bent & Hayon, 1975). Using the empirical relationship found between the triplet lifetime of indole and microviscosity (Strambini & Gonnelli, 1985), we obtain effective viscosities at the sites of the chromophores of 6.4×10^5 and 1.9×10^5 P for bovine and bacterial GDH, respectively, values which attest to the exceptional rigidity of the embedding structures.

An independent assessment of the dynamic makeup of globular proteins has often been sought in the ability of small molecules, O_2 in particular, to penetrate and diffuse within the macromolecule, leading to quenching of the emission from the indole side chains (Lakowicz & Weber, 1973; Saviotti & Galley, 1974; Eftink & Ghiron, 1977; Calhoun et al., 1983). The shortening of the phosphorescence lifetime upon increasing O_2 concentrations was measured for both GDHs in phosphate buffer, 0.1 M, pH 7.3. The rates of quenching $(1/\tau - 1/\tau_0)$ obey a linear relationship dependent on the concentration of O_2, the intercept going through the origin. At 20 °C, the bimolecular rate constants derived from a least-squares fitting of these data are $(4.5 \pm 0.5) \times 10^7$ and $(4.8 \pm 0.3) \times 10^6$ M^{-1} s^{-1} for bovine and bacterial enzymes, respectively. These values, when compared to 4×10^9 M^{-1} s^{-1} obtained with model indole compounds in water (Bent & Hayon, 1975), can only be interpreted in terms of hindered diffusion through a rather inflexible region of the protein matrix. Among the proteins studied so far, these values of k_{O2} are among the smallest ever found, having a magnitude comparable only to 1×10^6 M^{-1} s^{-1} for alkaline phosphatase and 3.7×10^7 M^{-1} s^{-1} for liver alcohol dehydrogenase (Strambini, 1987). It is important to point out that in these proteins the poorly quenched residues are in each case enclosed within a structure formed by an extended β-pleated sheet.

Discussion

In a glassy matrix, the phosphorescence from tryptophan residues in proteins is normally an intense emission whose quantum yield, spectrum, and lifetime, in the absence of specific perturbing effects experienced in proximity to sulfur atoms or heavy atoms, do not differ markedly from one macromolecule to another or at different sites of the globular structure. However, because the excited triplet state is strongly affected by the viscosity of its immediate environment (Strambini & Gonnelli, 1985), the decrease in phosphorescence lifetime and quantum yield observed in warming up glassy matrices into fluid solutions is, barring diffusion-mediated quenching reactions, a direct measure of the increased flexibility of the protein/solvent structure surrounding the chromophore. When two or more tryptophans are present in a macromolecule, the thermal quenching profile of the phosphorescence intensity then provides a means to distinguish different classes of residues according to the dynamic makeup of their local environment (Domanus et al., 1980).

With bovine GDH, the results of thermal quenching indicate that at room temperature the phosphorescence is due to only one of the three tryptophan residues. Of the other two, one is fairly close to the surface of the macromolecule within range of the heavy-atom effect of iodide (fluorescence quenching; phosphorescence enhancement); the other is buried in the inner folds of the structure in a rather fluid region (negligible phosphorescence yield). The picture obtained for the five tryptophans in the bacterial enzyme is fairly similar. One of them is embedded in a particularly rigid structure so as to yield long-lived phosphorescence. Two or three are close to the solvent interface, some of which may be partly exposed to it. The remaining are deep inside the macromolecule but again in rather flexible regions.

Long-lived phosphorescence in fluid solutions at room temperature, with lifetimes about 1 s, has been reported for only two proteins, namely, liver alcohol dehydrogenase (τ = 0.4 s) and alkaline phosphatase (τ = 1.6 s) (Saviotti & Galley, 1974; Strambini, 1987). In both enzymes, a single tryptophan per subunit is phosphorescent (Domanus et al., 1980), and this is located in a structurally inflexible region of the macromolecule enclosed by an extended β-pleated sheet whose strands are interconnected by α-helical rods. In the dehydrogenase, this arrangement constitutes the coenzyme binding domain.

In all dehydrogenases, whose X-ray structure has been determined, the NAD binding domain retains a typical β-strand arrangement (Branden, 1980). This pattern of secondary structure according to evidence from H exchange (Gregory & Lumry, 1985) and neutron scattering (Kossiakoff, 1985) yields highly inflexible cores in the macromolecule. The unusually high rigidity, therefore, found at the sites of the

phosphorescing tryptophans of GDHs strongly suggests that such residues in these enzymes, like for alcohol dehydrogenase, reside in the coenzyme binding domain. By the use of secondary structure prediction and sequence homology (Wootton, 1974; Brett, 1986), two sequences have been identified in bovine and *Neurospora* GDHs, having the characteristics of the coenzyme binding domain. According to these assignments, Trp-72 in bovine GDH is part of the βD strand of domain 1 (active site, residues 9–128) whereas Trp-281 is in a large loop of domain 3 (regulatory site, residues 245–356) between strand βB and an α-helical rod replacing strand βC. The putative substrate binding domain (residues 128–245) contains the third residue, namely, Trp-178. According to this structure prediction then, the most likely candidate for room temperature phosphorescence is Trp-72 in the "catalytic" coenzyme binding domain. Trp-281 is in a large loop not followed by a β-strand, and it has, therefore, greater opportunity of finding itself in a more flexible region of the macromolecule removed from the tight core formed by interlocking β-strands. In favor of this assignment are the data regarding the quenching of the room temperature phosphorescence following coenzyme binding at the catalytic site. It is known that upon NADH binding the fluorescence of bovine GDH is partially quenched by Förster's type energy transfer to the nicotinamide ring, some residues transferring much more efficiently than other (Brochon et al., 1976, 1977). The corresponding quenching of the phosphorescence (unpublished data) shows that the tryptophan involved is one which transfers very efficiently to the coenzyme, implying, therefore, a close proximity between the two chromophores.

For the bacterial enzyme, there are no structural predictions, and identification of the tryptophan phosphorescing at room temperature is a more uncertain task. Advantage may be taken, however, of the strong sequence homology with NADH-dependent *Neurospora* GDH (Valle et al., 1984) in order to assume the same secondary structure prediction (Wootton, 1974). Taking *Neurospora* as a guideline, Trp-36, Trp-67, and Trp-80 would share the catalytic coenzyme binding domain being located in strands βB, βC, and the loop between βE and βD. Trp-311 is in the βE strand of the "regulatory" domain. Finally, Trp-394 is in the COOH-terminal part of the polypeptide, a region with no salient structural features, the predictions also being highly variable among different sources. Since for the bacterial enzyme there is no experimental evidence of a regulatory coenzyme binding domain, we might anticipate, from the cnsiderations made earlier, that the tryptophan candidated to phosphoresce at room temperature is one of the three in the catalytic domain. Because juxtaposition of β-strands causes the three residues in this domain to be sufficiently close in space for energy transfer to occur among themselves, this assignment accounts also for both the low limiting phosphorescence anisotropy and also the finding that the phosphorescence yield at room temperature is less than one-fifth the minimum value predicted for noninteracting residues.

Room temperature phosphorescence when reporting on structural/dynamic features of catalytic centers of the macromolecule can be uniquely sensitive to the functional state of the enzyme (Strambini & Gonnelli, 1986). A question much debated and still unsettled with bovine GDH is whether the polymerized form of the enzyme, the one supposed to be present in vivo, has catalytic properties differing from the hexameric unit (Zeiri et al., 1978; Cohen et al., 1979). The observation of a monoexponential decay in the phosphorescence with a constant lifetime throughout the concentration range from 0.05 up to 10 mg/mL, at the extremes of which we have entirely monomeric and polymeric forms, respectively (Eisenberg et al., 1976), implies that the structure of the region about the probe is not affected by hexamer–hexamer association. The insensitivity of τ to the polymerization process contrasts profoundly with the large effects which are instead observed in response to the binding of substrates, coenzymes, and allosteric effectors (unpublished results).

In conclusion, the detection of room temperature phosphorescence from GDH provides us with an additional noninvasive probe, located in a strategic part of the macromolecule, which may help us to understand the mechanism of action of allosteric effectors and address questions such as subunit interactions and cooperative behavior.

Registry No. Trp, 73-22-3; bovine GDH, 9029-12-3; *E. coli* GDH, 9029-11-2.

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A Large Reservoir of Sulfate and Sulfonate Resides within Plasma Cells from *Ascidia ceratodes*, Revealed by X-ray Absorption Near-Edge Structure Spectroscopy[⊥,†]

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Received September 10, 1986; Revised Manuscript Received January 16, 1987

ABSTRACT: The study of sulfur within the plasma cells of *Ascidia ceratodes* [Carlson, R. M. K. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 2217–2221; Frank, P., Carlson, R. M. K., & Hodgson, K. O. (1986) *Inorg. Chem. 25*, 470–478; Hedman, B., Frank, P., Penner-Hahn, J. E., Roe, A. L., Hodgson, K. O., Carlson, R. M. K., Brown, G., Cerino, J., Hettel, R., Troxel, T., Winick, H., & Yang, J. (1986) *Nucl. Instrum. Methods Phys. Res., Sect. A 246*, 797–800] has been extended with X-ray absorption near-edge structure (XANES) spectroscopy. An intense absorption feature at 2482.4 eV and a second feature at 2473.7 eV indicate a large endogenous sulfate concentration, as well as smaller though significant amounts of thiol or thioether sulfur, respectively. A strong shoulder was observed at 2481.7 eV on the low-energy side of the sulfate absorption edge, deriving from a novel type of sulfur having a slightly lower oxidation state than sulfate sulfur. The line width of the primary transition on the sulfur edge of a vanadium(III) sulfate solution was found to be broadened relative to that of sodium sulfate, possibly deriving from the formation of the VSO_4^+ complex ion [Britton, H. T. S., & Welford, G. (1940) *J. Chem. Soc.*, 761–764; Duffy, J. A., & Macdonald, W. J. D. (1970) *J. Chem. Soc.*, 977–980; Kimura, T., Morinaga, M., & Nakano, J. (1972) *Nippon Kagaku Zaishi*, 664–667]. Similar broadening appears to characterize the oxidized sulfur types in vanadocytes. A very good linear correlation between oxidation state and peak position (in electronvolts) was found for a series of related sulfur compounds. This correlation was used to determine a 5+ oxidation state for the additional sulfur type at 2481.7 eV. Construction of a cellular sulfur minus vanadium(III) sulfate difference spectrum, along with comparison with spectra of known compounds, identified the novel sulfur(V) as an aliphatic sulfonic acid analogous to cysteic acid. The overall sulfonic acid concentration is comparable to that of sulfate in plasma cells and appears to be unprecedented in marine organisms.

The biological chemistry of ascidians (mostly sessile, strictly marine urochordate filter feeders) has consistently displayed highly unusual attributes (Stroeker, 1980; Krishnan, 1975; Smith & Dehnel, 1971; Swinehart et al., 1974; Bruening et al., 1985; Frank et al., 1986). For *Ascidia ceratodes*, significant attention has focused on the inorganic biochemistry of vanadocytes (~60% of the plasma cell population (Biggs & Swinehart, 1979)). Within a related series of organisms, these cells are known to contain within vacuoles (vanadophores) ~1.4 M vanadium(III) (Swinehart et al., 1974; Carlson, 1975; Danskin, 1978; Tullius et al., 1980; Frank et al., 1986[1]) associated with considerable sulfur (Carlson, 1975; Bell et al., 1982; Rowley, 1982; Pirie & Bell, 1984; Frank et al., 1986) and tunichrome (Bruening et al., 1985) (~1 M): a highly modified tripodal trimer of 3,5-dihydroxytyrosine. Small amounts of vanadyl ion are also known to be present

⊥ Dedicated to the memory of Dr. Donald P. Abbott.

† This work was supported by National Science Foundation Grant CHE-8512129. Measurements were made at the Stanford Synchrotron Radiation Laboratory, which is supported by the U.S. Department of Energy, Office of Basic Energy Sciences, and the National Institutes of Health, Division of Research Resources.

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[1] In this work, the A_0 from the EPR spectrum of vanadyl ion within vanadocytes from *Ascidia nigra*, as presented in Dingley et al. (1981), was calculated and reported by us to be 1.06×10^{-2} cm^{-1}. However, the correct value is 1.01×10^{-2} cm^{-1}, indicative that a weak carboxylate-like complex of vanadyl ion predominates in this organism (Reeder & Rieger, 1971; Chasteen, 1981). The width of the $(-^7/_2)_\parallel$ line in the frozen-solution EPR spectrum arising from these cells is, therefore, not a direct measure of pH (Frank et al., 1986). We note, however, that vanadyl carboxylate complexes are stable to very low pH values (Kustin & Pizer, 1970; Reeder & Rieger, 1971) and appear to have been detected by EPR in vanadocytes from *Ascidia mentula* (Bell et al., 1982).

0006-2960/87/0426-4975$01.50/0