Phosphorescence Properties and Protein Structure Surrounding Tryptophan Residues in Yeast, Pig, and Rabbit Glyceraldehyde-3-phosphate Dehydrogenase

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ABSTRACT: An investigation of the phosphorescence emission properties of tryptophan (Trp) was carried out in glyceraldehyde-3-phosphate dehydrogenase from yeast and from pig and rabbit muscle. Aided by the external heavy-atom effect of iodide, the dependence on excitation wavelength, and thermal quenching profiles, it was established that the 0,0 vibronic band peaked at 406 nm in the pig and rabbit proteins is made up of overlapping contributions from two Trp residues. In contrast to a previous report [Davis, J. M., & Maki, A. H. (1984) *Biochemistry 23*, 6249–6256], this implies that even in the muscle enzymes all three aromatic side chains are phosphorescent. Further, when the nature of the local environment of each residue is compared to the crystallographic structure of lobster GPDH, it leads to a complete new assignment of the individual phosphorescence spectra. With each protein, a single Trp, identified as Trp-310, was found to display long-lived phosphorescence at room temperature. The decay of this emission gives evidence of conformational homogeneity among the subunits of the tetrameric molecule.

Glyceraldehyde-3-phosphate dehydrogenase (GPDH) catalyzes the oxidation and phosphorylation of D-glyceraldehyde 3-phosphate to 1,3-diphosphoglycerate during the glycolytic conversion of glucose to pyruvic acid. It is an oligomeric protein composed of four chemically identical subunits whose sequence has been determined for several species. The primary structure of GPDH is strongly conserved, and, judging from the ease with which enzymes from phylogenetically distant sources are able to form hybrid tetramers, a high degree of conservation is predicted also for the tertiary and quaternary structure (Harris & Waters, 1976). Crystallographic structures are available for the muscle enzyme of lobster (Buehner et al., 1974; Murthy et al., 1980) and for *Bacillus stearothermophilus* (Biesecker et al., 1977; Leslie & Wonacott, 1983).

Davis and Maki (1982, 1984) have recently reported a well-resolved phosphorescence spectrum of tryptophan in GPDH from yeast, porcine, and rabbit muscle. The three Trp residues (Trp-84, Trp-193, and Trp-310) give rise to three distinct phosphorescence spectra in yeast and two in the muscle enzymes. Mostly on the basis of the optically detected magnetic resonance signal of the separate 0,0 vibrational bands, the authors assign each spectrum to particular Trp residues and conclude that in the muscle enzymes one residue is not phosphorescent.

Inspection of the crystallographic structure of lobster GPDH shows that Trp-310 is deeply buried within the subunit, sandwiched in by an extended seven-strand β-sheet and four α-helical rods (Buehner et al., 1974; Moras et al., 1975), an assembly that invariably gives rise to highly inflexible cores in polypeptide structures (Kossiakof, 1985; Lumry & Gregory, 1986; Strambini, 1987; Strambini & Gabellieri, 1987). Given the dependence of the phosphorescence lifetime of tryptophan on the fluidity of the matrix surrounding the chromophore (Strambini & Gonnelli, 1985), one may anticipate that Trp-310, like similar residues in alkaline phosphatase, horse liver alcohol dehydrogenase (Saviotti & Galley, 1974), and glutamate dehydrogenase (Strambini et al., 1987), is phosphorescent in aqueous solution at room temperature.

In this work, we characterize the phosphorescence properties of Trp residues in yeast, pig, and rabbit GPDH. Aided by the external heavy-atom effect, the dependence on the excitation wavelength, and the thermal quenching profile, we establish that for each protein all three residues are phosphorescent in the glassy state. The nature of the local environment of each residue when compared to the crystallographic structure leads to a complete new assignment of the individual phosphorescence spectra. One tryptophan displays long-lived phosphorescence at room temperature, and the decay of this emission gives evidence of conformational homogeneity among the subunits of the tetrameric molecule.

Materials and Methods

GPDH from yeast and rabbit muscle were obtained as crystalline suspensions in an ammonium sulfate solution from Boehringer (Mannheim). Porcine muscle GPDH was purchased as a crystalline suspension in 2.6 M ammonium sulfate solution from Sigma (St. Louis, MO). Before the experiments were done the proteins were extensively dialyzed in 20 mM potassium phosphate, pH 7.2, containing 5 mM EDTA. Water doubly distilled over quartz was used throughout. To remove NAD^+ from porcine and rabbit GPDHs, these enzymes were treated with charcoal (Norit A from Serva) as described by Henis and Levitzki (1977). Apo-GPDHs so obtained had an A_{280}/A_{260} value of 1.75–1.85. Enzyme concentrations were measured spectrophotometrically by using $E^{0.1\%}_{280nm} = 0.894$ cm^{-2} mg^{-1} for yeast GPDH (Kirschner et al., 1971) and $E^{0.1\%}_{280nm} = 0.83$ cm^{-2} mg^{-1} for apo-GPDH from muscle (Fox & Dandliker, 1956). The specific activities measured spectrophotometrically using 3-phosphoglycerate and NADH (Kirschner & Voight, 1968) were as specified by the suppliers. KI, NaCl, and spectroscopic-grade glycerol were from Merck (Darmstadt).

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 as described in a previous report (Strambini et al., 1987). In oxygen quenching experiments, controlled quantities of the gas were introduced by equilibrating the degased sample for about 15 min with known partial pressures of O_2. Partial pressures were determined from the overhead pressure and the composition of a purposely prepared O_2/N_2 gas mixture (SIO, Florence). Oxygen concentrations were determined by using

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Henry's law and the solubility of O_2 in water at 20 °C (Handbook of Chemistry and Physics, 41st ed.).

Phosphorescence Measurements. Phosphorescence spectra were obtained with a conventionally designed homemade instrument (Strambini, 1983). The excitation wavelengths were selected by a 250-mm grating monochromator (Jarrel-Ash) employing a band-pass of 10 nm. 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, if not otherwise specified by a double-shutter arrangement, permitting the emission to be detected 2 ms after the excitation cutoff. The decaying signal was digitized by an Applescope system (HR-14, RC Electronics) and then transferred to an Apple II computer for averaging and subsequent exponential decay analysis by a least-squares method.

Anisotropy measurements were carried out by inserting linear polarizers (Polaroid type HNPB) in both the excitation and emission beams. The excitation wavelength was 300 nm, while the emission was at 420 nm for yeast and at 406 nm for rabbit GPDH. The anisotropy (A) was calculated in the usual way from the equation:

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

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

In thermal quenching and anisotropy measurements, the temperature was regulated by a flow of cold nitrogen through a quartz dewar and a temperature controller (Oxford DTC2), achieving an accuracy of ±0.2 K.

Results

Low-Temperature Emission. Phosphorescence spectera obtained for GPDH from yeast and from the muscle enzymes of rabbit and pig in glycerol/phosphate buffer glass are displayed in Figure 1. As previously reported by Davis and Maki (1984) in ethylene glycol buffer, the phosphorescence spectra of the muscle enzymes are practically identical but distinct from that of yeast GPDH. In the latter, we can distinguish three 0,0 vibronic bands, with maxima at 408.5, 415, and 420 nm, the separation in energy reflecting markedly different tryptophan environments for the three residues in the polypeptide. In the muscle enzymes, the 0,0 vibronic band in the phosphorescence spectrum shows only two components centered at 406 and 415 nm, respectively, the shoulder at 419 nm representing a higher order vibrational band of the progression starting at 406 nm. The heterogeneity in solvation is reflected in both emission and absorption spectra. Exciting on the red edge of the absorption spectrum, at 308 nm, the contributions of the 409- and 415-nm bands in yeast and of the 406-nm band in muscle GPDH are considerably smaller relative to the remaining components.

The 0,0 vibronic band in the phosphorescence spectrum of Trp in nonpolar solvents is characterized by peak wavelengths of 412 and 413 nm. Any shift to the blue and to the red of these wavelengths does indicate stabilizing and unstabilizing polar interactions, respectively, between the ground-state indole nucleus and its surrounding matrix. While maxima in the range of 406–409 nm could arise from its exposure to the aqueous solvent, shifts to the red necessarily imply anisotropic polar interactions of buried chromophores. From this, it follows that only the 406- and 409-nm bands could conceivably be due to solvent-exposed tryptophan side chains; all the other chromophores must be buried within the folds of the polypeptide.

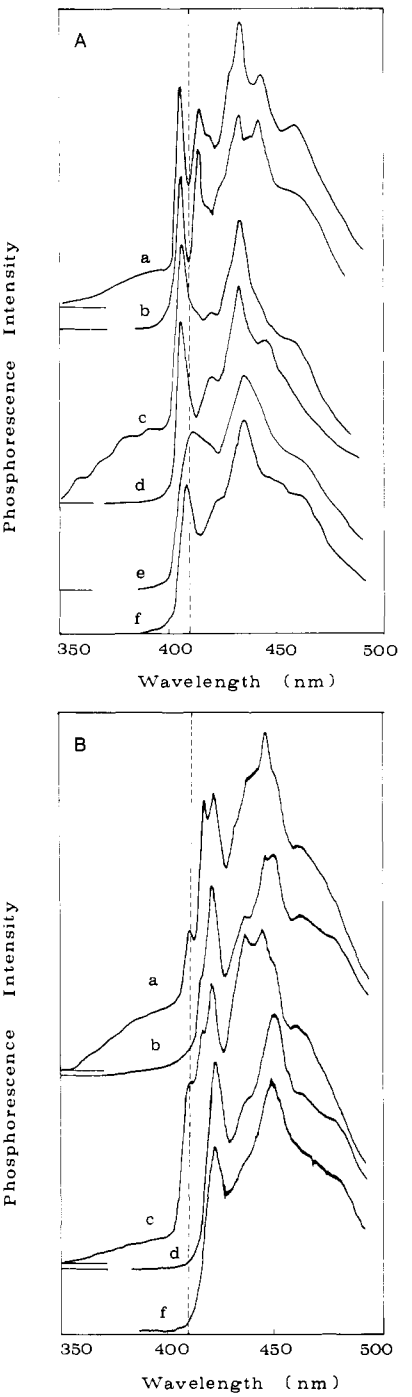

FIGURE 1: Phosphorescence spectra of (A) GPDHs from pig and rabbit muscle and (B) GPDH from yeast in glycerol/phosphate buffer (60:40 v/v) at various temperatures and excitation wavelengths. (a) λ_{ex} = 280 nm, T = 140 K; (b) λ_{ex} = 308 nm, T = 140 K; (c) λ_{ex} = 280 nm, T = 140 K, in the presence of 1 M KI; (d) λ_{ex} = 280 nm, T = 220 K; (e) λ_{ex} = 280 nm, T = 220 K, in the presence of 1 M KI; (f) λ_{ex} = 295 nm, T = 20 °C. Protein concentrations were typically 2 mg/mL. The spectra for the two muscle enzymes are practically identical.

Heavy-Atom Perturbation of the Phosphorescence Emission. Another criterion used to estimate the proximity of tryptophanyl side chains to the solvent–protein interface is based on the perturbation that may be induced to the phosphorescence by heavy atoms that, due to their charged nature, are relegated to the aqueous medium. Studies with iodide show

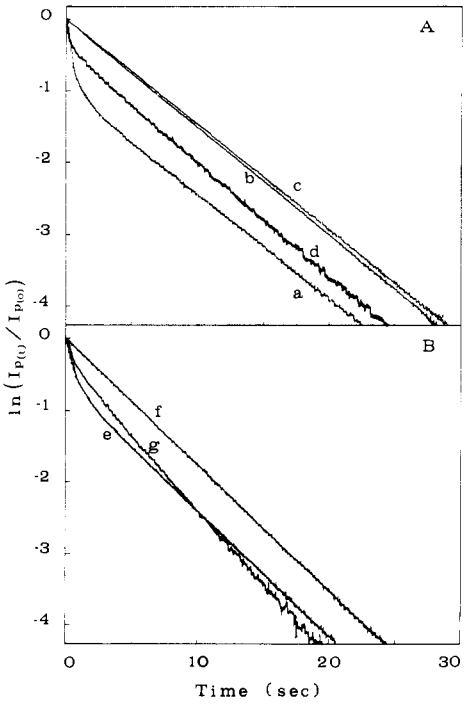

FIGURE 2: Heavy-atom (KI) and temperature-induced heterogeneity in the decay of Trp phopshorescence emission from GPDH in glycerol/phosphate buffer (60:40 v/v) at low temperature. (A) GPDH from rabbit and pig muscle: (a) λ_{ex} = 295 nm,, λ_{em} = 406 nm, 1 M KI, and T = 140 K; (b) λ_{ex} = 308 nm, λ_{em} = 406 and 440 nm, 1 M KI, and T = 140 K; (c) control, λ_{ex} = 295 nm, λ_{em} = 440 nm, 1 M KCl, and T = 140 K; (d) λ_{ex} = 295 nm, λ_{em} = 440 nm, and T = 195 K. (B) GPDH from yeast: (e) λ_{ex} = 295 nm, λ_{em} = 440 nm, 1 M KI, and T = 140 K; (f) control λ_{ex} = 295 nm, λ_{em} = 440 nm, 1 M KCl, and T = 140 K; (g) λ_{ex} = 295 nm, λ_{em} = 440 nm, and T = 195 K.

that when the aromatic ring is within a fews angstroms (3–5 Å) of van der Waals contact with the heavy atom there is an increase of up to 2–3 times in phophorescence quantum yield followed by a shortening of the triplet-state lifetime from 6 s to a fraction of a second (Mc Glynn et al., 1969; Lee, 1985). The effect of 1 M KI, relative to 1 M KCl, on the phosphorescence spectrum and the triplet-state lifetime is shown in Figures 1 and 2, respectively. In the yeast protein, the intensity of the 409-nm band is clearly enhanced by KI. The heavy-atom perturbation is also evident on the decay of the phosphorescence intensity, which changes from monoexponential with a lifetime of 5.6 ± 0.1 s to multiexponential with short-lived components accounting for 35% of the total steady-state intensity. From the extent of the perturbation, it would appear that only the tryptophan emitting at 409 nm is subject to the interaction with iodide. The rest of the emission with unchanged lifetime confirms that the other two residues are buried deeper than 5 Å from the protein–water interface.

In the muscle enzymes, the heavy-atom effect is particularly revealing. Upon excitation at 280 nm, the 406-nm band is considerably enhanced by iodide, the other component at 415 nm becoming almost entirely submerged as a result of it (Figure 1A). Not all the emission at 406 nm is, however, affected by the perturbation. At this wavelength, the short-lived component accounts for no more than 70% of the total intensity, the remaining 30% having the unperturbed value of the lifetime (6.9 ± 0.1 s). Upon excitation at 308 nm, the phosphorescence spectrum is very similar to that in the absence of KI, and more importantly the emission at 406 nm now decays exponentially with a lifetime equal to 6.8 s (Figure 2A). This finding demonstrates unequivocally that the 406-nm band

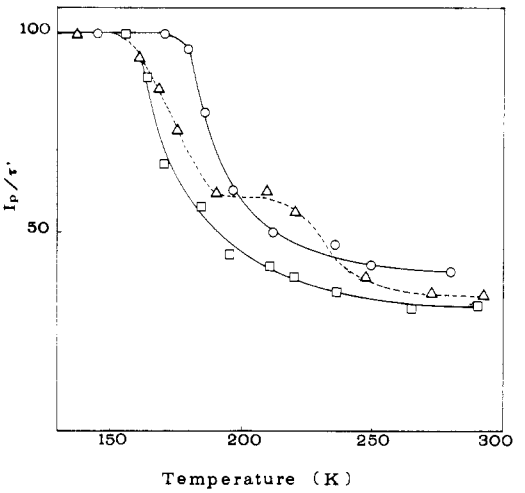

FIGURE 3: Phosphorescence thermal quenching profiles of yeast GPDH (○), rabbit muscle GPDH (□) and porcine muscle GPDH (△). The phosphorescence intensity, I_p, was normalized by τ', the longest component in the decay (λ_{ex} = 290 nm, λ_{em} = 440 nm). Other conditions as in Figure 1.

is made up of contributions from two distinct tryptophan residues: one, probably solvent exposed or near the protein surface; the other, which absorbs further to the red, sufficiently buried to be out of range of the heavy-atom perturbation. In summary, the excitation at 308 nm selects only internal residues (406 or 415 nm) and results therefore in a decrease in intensity of the 406-nm peak relative to the 415-nm peak. The composite nature of the emission at 406 nm, corroborated also by temperature-dependent studies, contrasts with the conclusion from ODMR experiments that only two tryptophan residues are phosphorescing in muscle GPDHs.

Thermal Quenching of Tryptophan Phosphorescence. As the temperature of the solution is raised above the glass transition temperature, the increased fluidity of the solvent allows the protein to gradually aquire its natural flexibility. Because radiationless transitions from the triplet state of indole are particularly sensitive to the fluidity of its microenvironment, the increase in temperature is invariably accompanied by a shortening of the lifetime, τ. For some residues, this effect may be so dramatic that as a consequence their contribution to the total phophorescence emission becomes too small for detection. In Figure 3, we show the decrease in phosphorescence intensity (P) normalized by the lifetimes of the longest component (τ') as the temperature of the solution is raised from the glassy state up to room temperature. For the yeast enzyme, P/τ' drops by about 60% in the range from 180 to 220 K and after that remains constant all the way to room temperature. Over the temperature interval for which thermal quenching is observed, the phosphorescence decays in a distinctly nonexponential fashion (Figure 2). However, short-lived components in the decay are no longer detectable above 220 K. Parallel to the reduction in P/τ', the composite spectrum collapses into a single 0,0 vibronic band centered at 420 nm. This peak wavelength will eventually relax to 422 nm on approaching room temperature. Thus, the yeast enzyme is phosphorescent in fluid solution, and because the 409- and 415-nm bands are quenched at relatively low temperatures, its emission arises from a single Trp residue (420-nm band). According to P/τ', this residue contributes about 40% to the overall phosphorescence intensity in the glassy state, its quantum yield being only a few percent larger than the average value.

For pig and rabbit GPDHs, the similarity noted in their phosphorescence spectrum is extended once again to the triplet

lifetime and the thermal quenching pattern (Figure 3). In the glassy state, 70–75% of the intensity decays with a lifetime of 7.0 ± 0.1 s while for the remaining portion, τ is about 5–6 s. The drop in P/τ' occurs entirely in the 160–235 K temperature interval, the decay becoming strictly monoexponential and P/τ' constant thereafter. From the changes in the phosphorescence spectrum that accompany the decrease in intensity, we note that the 145-nm band is quenched over the range 170–210 K about 25 K colder in comparison to the other thermally quenched portion that contributes to the 406-nm band. As for yeast, the room temperature phosphorescence of rabbit and pig GPDHs is due to a single Trp residue. According to P/τ', this tryptophan is responsible for about 31% and 35% of the total phosphorescence intensity in the glassy state (λ_{ex} = 290 nm) again a quantum yield that is roughly the average value.

To further demonstrate the composite nature of the 406-nm band, one can exploit the different mobility between the solvation sites of the two chromophores. Relaxation of a fluid polar environment about the triplet state causes a distinct red shift (8–10 nm) in the spectrum. However, because concomitant to the shift there is strong quenching of the emission, the relaxing component generally becomes submerged by the nonrelaxing one, and the shift is not observed. Should the relaxing component be accessible to the heavy-atom effect, then its much greater phosphorescence yield might allow observation of the red shift. Figure 1A shows that at 220 K in the presence of 1 M KI, the 406-nm band does indeed becomes broader to the red, thus signaling that the emission from the mobile site has shifted to longer wavelengths. At still warmer temperatures, when the contribution from this site is no longer detectable, the 0,0 vibrational band sharpens again around 407 nm.

Phosphorescence Anisotropies and Hydrodynamic Volumes. Entropy-driven molecular association is known to became labile at low temperature. To verify the state of association of the subunits of GPDH in this solvent system at low temperature and relate the spectroscopic observations to molecular structure, the hydrodynamic volume of each enzyme was estimated from measurements of the 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. When excited at 300 nm and monitored over the 0,0 vibrational bands, the phosphorescence anisotropy at infinite viscosity, A_∞, was found to be −0.149 ± 0.002 for all three proteins. Because this magnitude is the maximum value obtained with dilute solutions of tryptophan, we note that unlike with some other multi-tryptophan polypeptides in GPDH there is particularly no singlet–singlet or triplet–triplet energy transfer among aromatic residues.

The rotational correlation times (θ) evaluated from the anisotropy at three 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 the viscosity of the solvent is not known 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 69 600 cm^3 mol^{-1} for yeast and 82 900 cm^3 mol^{-1} for rabbit GPDHs, respectively. These molar volumes, when stripped of the hydration shell one water molecule thick (4 Å), correspond to molecular weights of 57 000 and 69 800, respectively, if a partial specific volume of 0.73 cm^3 g^{-1} is employed. In each case, these weights are considerably smaller than the 144 000 molecular weight of the tetramer. The simplest interpretation of these results is that under these experimental conditions GPDH is present mostly in the dimeric state. The alternative view that each subunit in the tetramer enjoys a large degree of independent rotational freedom is not compatible with a well-defined quaternary structure and the large dissociation free energies for monomer formation. On the other hand, the notion that the tetrameric macromolecule is a dimer of dimers and that the association between dimers is labile at low temperature is experimentally well documented (Harries & Waters, 1976).

Table I: Rotational Correlation Time (θ) Determined for Yeast and Rabbit GPDHs in 60:40 Glycerol/Phosphate Buffer (20 mM, pH 7.2, Containing 5 mM EDTA) at Three Different Temperatures

	T (K)	θ (s)[a]	θ/θ_0	V (×10^{-3})[b]	mol wt (av)
GPDH from yeast	188.5	25.6	0.80	69.6	
	194.5	2.8	0.91	79.2	
	197.3	1.1	0.69	60.0	57 000
GPDH from rabbit	188.5	25.9	0.81	70.5	
	194.5	3.5	1.04	90.5	
	197.3	1.6	1.01	87.8	69 800

[a] The values of θ were obtained from the phosphorescence anisotropy, A, and the triplet-state lifetime, τ [$\theta = A^*/(A_0 - A)$]. θ_0 refers to the rotational correlation time of liver alcohol dehydrogenase from horse under the same conditions. Enzyme concentrations were 2 mg/mL. Excitation was at 300 nm, and emission was at 420 nm for yeast and at 406 nm for rabbit GPDH. [b] Hydrodynamic volumes (V_h) were obtained by assuming the macromolecule to be a spherical rotor (θ v $V_h\eta/kT$) and V_h = 87 000 cm^3 mol^{-1} for liver alcohol dehydrogenase.

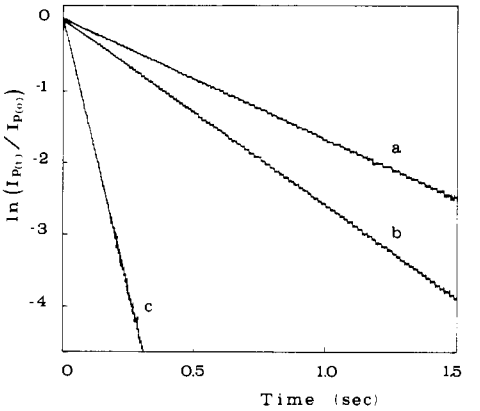

FIGURE 4: Phosphorescence decay at 20 °C in phosphate (20 mM)/EDTA (5 mM), pH 7.2, λ_{ex} = 295 nm, λ_{em} = 440 nm. (a) Rabbit muscle GPDH; (b) porcine muscle GPDH; (c) yeast GPDH.

Room Temperature Phosphorescence. The phosphorescence spectrum of each GPDH in phosphate buffer at 20 °C is shown in Figure 1. Relative to the glassy state, there is a modest 1–2-nm shift in the 0,0 vibrational bands to longer wavelength, the small change implying that the polar chromophore's site is too rigid to allow for complete dipolar reorientation during the triplet-state lifetime.

The decay in the time of the phosphorescence intensity is shown in Figure 4 to obey for each enzyme a strictly exponential law. The observation that the phosphorescence decays monoexponentially implies that all chromophores are equivalent in the dynamic properties of their environment, when averaged over a time window on the order of τ. For oligomeric proteins, such equivalence means either that all subunits posses very similar configurations or that asymmetric substates interconvert rapidly compared to τ. The intrinsic phosphorescence lifetimes at 20 °C are 395 ± 10, 600 ± 12, and 66 ms

Table II: Emission and Crystallographic Data Relative to the Three Trp Residues in GPDH Juxtaposed According to the Assignment Proposed in the Work

		Trp-84	Trp-193	Trp-310
wavelength of 0,0 vibrational band	yeast	409	415	420
	porcine	406	415	406
	rabbit	406	415	406
selective excitation at 308 nm	yeast		yes	yes
	porcine		yes	yes
	rabbit		yes	yes
heavy-atom perturbation by I^-	yeast	yes		
	porcine	yes		
	rabbit	yes		
room temp phosphorescence	yeast			yes
	porcine			yes
	rabbit			yes
nature of the environment		polar, proximity to solvent, flexible	polar, buried, flexible	highly polar, buried, rigid
crystallographic structure (lobster)				
location		surface	subunit interface	deep interior
surrounding secondary structure		random-coil	random-coil, short β-sheet	nine-strand β-sheet, α-helical rods
anticipated flexibility		highly flexible	flexible	very rigid

for porcine, rabbit, and yeast GPDH, respectively, values that are large when compared to the 15–20 μs for tryptophan in aqueous solution (Bent & Hayon, 1975). According to the empirical relationship between the triplet lifetime of indole and microviscosity (Strambini & Gonnelli, 1985), we estimate local effective viscosities at the sites of the phosphorescing residues of 8.1×10^4, 1.69×10^5, and 3.4×10^3 P, respectively. These values of τ attest therefore to a considerable rigidity of the embedding structures; similar values were reported for Trp residues within large β-sheets of horse liver alcohol dehydrogenase, alkaline phophatase (Strambini, 1987; Strambini & Gabellieri, 1987), and glutamic dehydrogenase (Strambini et al., 1987).

An independent assessment of the dynamic makeup of the internal region of the macromolecule hosting the phosphorescing residue may be sought on the ability of O_2 to migrate to this site and quench its emission (Lakowicz & Weber, 1973; Saviotti & Galley, 1974; Strambini, 1987). The shortening of the phosphorescence lifetime upon increasing O_2 concentration was measured for all three GPDHs in phophate buffer, 50 mM, pH 7.2. The rates of quenching $(1/\tau - 1/\tau_0)$ obey a linear dependence on the O_2 concentration, and the bimolecular rate constants derived from a least-squares fitting are $(3.6 \pm 0.3) \times 10^7$, $(3.4 \pm 0.3) \times 10^7$, and $(2.8 \pm 0.2) \times 10^7$ M^{-1} s^{-1} for porcine, rabbit, and yeast GPDHs, respectively. These values, when compared to 4×10^9 M^{-1} s^{-1} obtained with model indole compounds in water (Bent & Hayon, 1975), attest to highly hindered diffusion through inflexible regions of the protein matrix. These values of K_{O_2} are among the smallest ever reported with proteins (Strambini et al., 1987) and practically identical with that found for Trp-314 enveloped within the β-sheet of the coenzyme domain of LADH (Strambini, 1987).

Discussion

The phosphorescence emission from yeast GPDH represents, among polypeptides with more then two Trp residues, perhaps the best known example in which the heterogeneity in the chemical nature of the chromophores' environment gives rise to well-separated 0,0 vibronic bands (409, 415, and 420 nm) in the spectrum. For the muscle enzymes of pig and rabbit, only two 0,0 vibrational bands (406 and 415 nm) can be distinguished. Selective excitation on the red edge of the absorption spectrum shows, however, that the 406-nm band is made up of two overlapping contributions, one of which absorbs very little at this wavelength. The ability to resolve the individual emission of all three tryptophan residues in the macromolecule allows us to determine the following for each chromophore: (1) the polarity of the local environment; (2) the degree of solvent exposure or its proximity to the aqueous phase: (3) the flexibility of the surrounding protein structure. When such detailed information on the nature of the environment is combined with the crystallographic structure (lobster muscle), the assignment of individual spectra to particular Trp residues becomes rather straightforward. In this use of crystallographic data, one should be remined that according to hydrodynamic volumes the emission properties at low temperature refer in all likelihood to the dimeric and not the tetrameric state of the proteins.

In Table II, together with relevant elements of the crystallographic structure we summarize the emission properties of each 0,0 vibrational band that form the basis for the proposed assignment.

GPDH from Yeast. The 409-nm band is subject to the external heavy-atom perturbation and is thermally quenched at low temperature. These are attributes of chromophores in proximity to the aqueous phase (within 3–5 Å) either partially exposed to the solvent or in very flexible segments of the polypeptide. Only Trp-84 located in a random-coil region on the outer surface of the subunit facing the solvent can fit this description. The 415-nm band originates from a residue in a buried (deeper then 5 Å) polar site, and judging from the low temperature at which it is thermally quenched, this tryptophan experiences a rather fluid surrounding. Trp-193 in the lobster enzyme is in the S-loop region buried at the interface between "red" and "blue" subunits. This part of the macromolecule is largely random coil, an organization of the polypeptide that in general enjoys considerable flexibility. Changes in the conformation of GPDH upon NAD^+ binding are in fact confined mostly to this region of the subunit (Murthy et al., 1980). We attribute the 415-nm component to Trp-193 and identify with Trp-310 the remaining chromophore (420 nm) phosphorescing at room temperature. The latter residue is placed roughly at the center of the subunit embedded within a seven-strand β-sheet interwined by long α-helical rods. There is ample evidence that such an assembly of secondary structure gives rise to exceptionally rigid cores in proteins (Kossiakof, 1985; Lumry & Gregory, 1986; Strambini, 1987; Strambini & Gabellieri, 1987). Clearly, only Trp-310 can fulfill the requirement of an inflexible surrounding that is a requisite for the long phosphorescence lifetime and the small permeability to O_2.

GPDH from Pig and Rabbit Muscle. Practically the same considerations advanced for the yeast enzyme apply here. The

406-nm band was shown to be of composite nature. The component selected upon red-edge excitation is not affected by the external heavy atom and phosphoresces at room temperature. Again, judging from the unrelaxed nature of the spectrum, the long triplet-state lifetime, and the slow migration of O_2 to this site, the room temperature phosphorescence can only be assigned to Trp-310. The other contribution to the 406-nm band is affected by iodide and is thermally quenched at low temperature. Because it mimicks in every respect the behavior of the 409-nm band in yeast, by the same token we assign this emission to Trp-84. The remaining 415-nm band, which is common to all three proteins, again belongs to Trp-193.

It should be pointed out that the assignment proposed above disagrees with the one based on the optically detected magnetic resonance (ODMR) signals associated with the 0,0 vibrational bands (Davis & Maki, 1982, 1984). With the muscle enzymes, the parameters E and D are apparently unable to discern two different environments for the tryptophans phosphorescing at 406 nm. This led the authors to conclude that the band was due entirely to a single residue and as a consequence that only two of the three tryptophans are effectively phosphorescing in pig and rabbit GPDHs. Further, the starting point in the assignment proposed by these authors is based on the interpretation of the external heavy-atom perturbation induced by CH_3Hg^{II} attached to Cys-281. The external heavy-atom effect of Hg(II) on tryptophan was shown by Monsigny et al. (1978) to elicit an enhancement (by a few times) of the phosphorescence quantum yield and a reduction in the triplet-state lifetime from 6.5 down to about half a second. From the proximity of Cys-281 to Trp-310 and the short-range nature of the heavy-atom effect, one might expect a selective perturbation of this chromophore and thus an increase of its contribution to the emission relative to the other two Trp residues. The phosphorescence spectra reported for CH_3Hg^{II}–GPDH samples of rabbit and pig are almost completely dominated by the 406-nm band, the 416-nm band being barely discernible. The dominance of the 406-nm component over the other can be interpreted as a selective enhancement of the 406-nm emission or as a quenching of the 416-nm component by a peculiar, as yet unknown, interaction with Hg. While our assignment is consistent with the first interpretation, the authors adopt the second one. They also detect a short-lived species (15 ms) in the overall emission having a poorly defined tryptophan-like spectrum which being red-shifted relative to 406 nm they identify with the perturbed 416-nm component. On this basis, the 416-nm band was assigned to Trp-310.

The finding of long-lived phosphorescence from Trp-310 in buffer provides us with an intrinsic probe to the dynamic structure of the catalytic domain. The unique sensitivity of τ (Strambini & Gonnelli, 1986; Strambini & Gabellieri, 1984) to discriminate between conformational states of the subunits of the tetramer will be particularly suitable in addressing questions regarding the structural equivalence of the subunits and changes in conformation induced by ligand binding. The crystallographic structure of GPDH from lobster and *B. stearothermophilus* shows that the tetramer is asymmetric when NAD^+ is bound. Whether the apoenzyme in lobster is symmetrical is not clear (Leslie & Wonacott, 1983). The strong negative cooperativity displayed by the muscle enzymes toward NAD^+ binding is by most attributed to ligand-induced conformational changes in an otherwise symmetrical molecule (Koshland et al., 1966; Stallcup & Koshland, 1973) even if some chemical modification studies are consistent with preexisting asymmetry in the tetramer (Malhorta & Bernard, 1973). On the other hand, the positive cooperativity in yeast GPDH seems instead to arise from a slow isomerization between inactive (T form) and active (R form) conformational states (Kiskhener et al., 1971; Kirschener, 1971), the T form representing in the absence of NAD^+ 98% of macromolecules. The monoexponential nature of the phosphorescence decay in solutions of yeast, pig, and rabbit GPDHs, reported above, is consistent with equivalence in the τ-averaged structure of the subunits in the tetramer. Thus, any asymmetric state of the oligomer either does not show up in τ or is retained only for time intervals shorter than τ. The preponderance of the T conformer in yeast precluses detection of the R form. Preliminary studies show that NAD^+ binding has remarkable effects on the flexibility of the catalytic domain; the nature of the change and the heterogeneity in subunit structure at various degrees of saturation are now under investigation.

The search for molecular details responsible for the allosteric behavior in these enzymes has focused particularly on the S-loop region of the polypeptide (Murthy et al., 1980; Leslie & Wonacott, 1983). From a comparison of the emission properties of yeast and muscle GPDHs, enzymes which display opposite cooperative behavior, it is evident that important differences between them exist also in terms of the charge arrangement (cf. 420 nm in yeast vs 406 nm in muscle GPDH) and in the flexibility (almost 2 orders of magnitude) about Trp-310 in the catalytic domain. Of course, whether or not these differences are correlated to allosterism is an open question.

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Detection, Resolution, and Nomenclature of Multiple Ubiquitin Carboxyl-Terminal Esterases from Bovine Calf Thymus†

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ABSTRACT: In vivo, ubiquitin exists both free and conjugated through its carboxyl terminus to the α- and ϵ-amino groups of a wide variety of cellular proteins. Ubiquitin carboxyl-terminal hydrolytic activity is likely a necessary step in the regeneration of the ubiquitin cofactor from ubiquitin–protein conjugates. In addition, this type of activity is required to generate the active, monomeric ubiquitin from the only known gene products: the polyprotein precursor and various ubiquitin fusion proteins. Thus, this activity is of vital importance to systems that utilize ubiquitin as a cofactor. A generic substrate, ubiquitin ethyl ester, was previously developed [Wilkinson, K. D., Cox, M. J., Mayer, A. N., & Frey, T. (1986) *Biochemistry 25*, 6644-6649] and utilized here to monitor the fractionation of these activities from calf thymus. By use of a rapid HPLC assay, four distinct, ubiquitin-specific esterases were identified and separated. A previously undescribed activity has been resolved and characterized, in addition to the bovine homologue of ubiquitin carboxyl-terminal hydrolase purified from rabbit reticulocytes. Two other activities resemble deconjugating activities previously detected in crude extracts but not previously purified. These activities appear to form a family of mechanistically related hydrolases. All four activities are inhibited by iodoacetamide, indicating the presence of an essential thiol group, and are inhibited to various extents by manganese. All have specific ubiquitin binding sites as judged by the low observed K_m values (0.6-30 μM). The carboxyl-terminal aldehyde of ubiquitin is a potent inhibitor of these enzyme activities, with K_i values approximately 1000-fold lower than the respective K_m values. The use of ubiquitin ethyl ester provides a rapid, quantitative assay for purification of these enzyme activities and for the comparison of the known and postulated differences between the members of this important group of enzymes. A systematic nomenclature for these enzyme activities and their substrates is proposed in order to facilitate comparison of studies by various laboratories. This nomenclature clearly indicates the nature of the reactions catalyzed and the substrates used to detect the various activities and should avoid the idiosyncrasies of the multiple nomenclatures which have arisen previously. These results should greatly facilitate the purification and characterization of the proteins and the genes of this important family of enzymes from a single mammalian source.

Ubiquitin is a 76 amino acid polypeptide found in all eukaryotic organisms studied. It exists as the free monomer and covalently attached to a wide variety of cellular proteins. The dynamic nature of the cellular ubiquitin–protein conjugate pool has been demonstrated in vivo by microinjection (Carlson et al., 1987; Carlson & Rechsteiner, 1987) and immunochemical techniques (Haas & Bright, 1985). These studies have demonstrated that the reversible ubiquitination of proteins is a controlled phenomenon that responds to external stimuli such as heat shock and nutritional status. The enzymes which catalyze the conjugation of ubiquitin have been partially characterized (Hershko et al., 1983; Haas et al., 1982). In contrast, the enzymes which proteolytically process the poly-

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