Biophysical Chemistry, 33 (1989) 257–264
Elsevier

BIOCHE 01360

Phosphorescence properties of Trp-84 and Trp-310 in glyceraldehyde-3-phosphate dehydrogenase from *Bacillus stearothermophilus*

Edi Gabellieri and Giovanni B. Strambini

CNR, Istituto di Biofisica, via S. Lorenzo, 26, 56100 Pisa, Italy

Received 16 June 1988
Revised manuscript received 1 November 1988
Accepted 16 March 1989

Glyceraldehyde-3-phosphate dehydrogenase; Tryptophan phosphorescence; Protein dynamics

The phosphorescence spectra of Trp-84 and Trp-310 in glyceraldehyde-3-phosphate dehydrogenase from *Bacillus stearothermophilus* in an aqueous glass show distinct 0,0 vibrational bands with peaks at 406.5 and 410.5 nm. With the aid of external heavy-atom perturbation of iodide and the thermal quenching profile, it is concluded that although both chromophores are effectively buried, only one, viz., the 406.5 nm component, is embedded in a sufficiently rigid core of the protein to phosphoresce in fluid solutions at room temperature. From inspection of the crystallographic structure is it evident that only Trp-310 embedded in the β-sheet of the catalytic domain may satisfy the requirements of a long triplet-state lifetime and slow migration of O_2 to its site. This identification confirms previous analysis of the phosphorescence properties of the enzymes from yeast, pig and rabbit muscle.

1. Introduction

Glyceraldehyde-3-phosphate dehydrogenase (GPDH) is a tetrameric enzyme composed of chemically identical subunits. The amino acid sequence, which has been determined for several species, shows one of the highest degrees of homology reported [1]. High-resolution crystallographic structures of GPDH have been reported only for lobster [2–4] and *Bacillus stearothermophilus* [5,6]. Comprehensive studies of the kinetics and molecular structure have been conducted with this enzyme in particular from sources such as yeast, rabbit and pig muscle. In all of these investigations, much attention has been dedicated to uncovering the details of the molecular structure that give rise to the peculiar cooperative behaviour of NAD^+ binding, demonstrated to be positive for the yeast enzyme and negative for other forms [7–9].

Correspondence address: E. Gabellieri, CNR, Istituto di Biofisica, via S. Lorenzo, 26, 56100 Pisa, Italy.

In yeast and all eukaryotic GPDHs of known primary structure, the polypeptide possesses three tryptophan residues, the position of each being conserved in the sequence. Recently, low-temperature phosphorescence studies have shown that in yeast and muscle GPDHs from pig and rabbit the spectrum is composed of three and two well-resolved 0,0 vibrational bands, respectively [10,11]. Davis and Maki [10], on the basis of the optically detected magnetic resonance signals associated with each component of the spectrum, proposed a tentative assignment to individual tryptophan residues and concluded that Trp-84 in pig and rabbit GPDH is not detectably phosphorescent. On the other hand, Strambini and Gabellieri [11], with the aid of data on the heterogeneity in absorption, external heavy-atom effect and thermal quenching of the phosphorescence emission have also identified three distinct contributions to the phosphorescence spectrum for the enzyme from mammalian sources. Further, recently obtained experimental evidence, in conjunction with the X-ray structure of lobster enzyme, has led to a different

assignment of the 0,0 vibrational bands in the spectrum. In all three proteins, one residue, identified as Trp-310, displays long-lived phosphorescence at room temperature. In view of the sensitivity of the triplet-state lifetime to the dynamical structure of the polypeptide and, indirectly, to its conformation, room-temperature phosphorescence could provide an interesting intrinsic probe in the study of allosteric behaviour [12–14]. Of course, the interpretation of structural data obtained with the triplet probe requires knowledge of the tryptophan residue responsible for the phosphorescence at room temperature.

GPDH from *B. stearothermophilus* is structurally similar to that from lobster and lacks Trp-193 located in the S-loop region at the subunit interface. In this work, we take advantage of the lower number of chromophores and of the homology in primary, secondary and tertiary structure to compare the phosphorescence properties of this enzyme with those reported for the other species. This study shows that in *B. stearothermophilus* one tryptophan residue displays long-lived room-temperature phosphorescence and that its spectral characteristics are identical to those of the muscle proteins. Taken together the data lend further support to the previous assignment of the phosphorescence components in the spectrum of pig and rabbit GPDH and confirm that the room-temperature emission arises from Trp-310.

2. Materials and methods

B. stearothermophilus GPDH was obtained as a lyophilized powder from Sigma (St. Louis, MO). Before experiments, the protein was extensively dialyzed in 20 mM potassium phosphate buffer (pH 7.2) containing 5 mM EDTA. Water that had been doubly distilled over quartz was used thoughout. Enzyme concentration was measured spectrophotometrically employing the value $E_{280}^{0.1\%} = 0.88\ cm^{-1}\ mg^{-1}$ [15].

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 [16]. In oxygen quenching experiments, controlled quantities of the gas were introduced by equilibrating the degassed sample for about 15 min with known partial pressure of O_2. Partial pressures were determined from the overhead pressure and the composition of an appositely prepared O_2/N_2 gas mixture (SIO, Florence). Oxygen concentrations were determined using Henry's law and from the solubility of O_2 in water at 20°C (Handbook of Chemistry and Physics, 41st edn.).

2.1. Phosphorescence measurements

Phosphorescence spectra were recorded on a conventionally designed home-made instrument [17]. The excitation wavelengths were selected by a 250-mm grating monochromator (Jarrel-Ash) employing a band-pass of 10 nm. Emission was dispersed via a 250-mm grating monochromator (Jobin-Yvon H25), with detection by means of an EMI 9635 QB photomultiplier. Phosphorescence decays were monitored at 440 nm, unless specified otherwise, by a double-shutter arrangement permitting emission to be detected 2 ms after the excitation cut off. The decaying signal was digitalized 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 wavelength of excitation was 300 nm, while that of emission was 406 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 denotes the intensity ratio of the vertically to horizontally polarized emission obtained with horizontal excitation.

In thermal quenching and anisotropy measurements, the temperature was regulated by means of a stream of cold N_2 through a quartz dewar and a

temperature controller (Oxford DTC2) achieving an accuracy of ± 0.2 K.

3. Results and discussion

3.1. Low-temperature phosphorescence emission

The phosphorescence spectrum obtained for GPDH from *B. stearothermophilus* in a glycerol/buffer glass is compared in fig. 1 to that previously reported for pig and rabbit. As with the muscle enzymes the 0,0 vibrational band is split into two components which in the thermophile are centered at 406.5 and 410.5 nm, respectively. Only the blue component is coincident in all three GPDHs. The clearly evident heterogeneity in chemical nature of the chromophores' environment for emission is not apparent in the absorption spectrum. In contrast to the mammalian sources, upon excitation at 305 nm, at the red edge of the absorption spectrum, the relative contribution of the two vibrational bands is largely unaltered.

From the general relationship established between the polarity of the solvation site and the wavelength of the 0,0 vibrational band [18,19], the 406.5 nm band is representative of a site whose polarity is similar to that of the aqueous solution. Thus, while this wavelength could indicate that one chromophore is exposed to the aqueous solvent the 410.5 nm band, whose wavelength is consistent with a largely nonpolar site, implies an appreciable depth of burial for the other tryptophan residue. As shown below, however, a polar site does not necessarily imply that the indole side chain is in contact with the aqueous phase. Indeed, the indole nitrogen of Trp-310, although buried at the subunit interface, is hydrogen-bonded to Asp-293 which forms a salt bridge to Arg-194 of the P-axis-related subunit.

Heavy-atom perturbation of tryptophan by means of the iodide ion results in a greater phosphorescence yield and a shorter triplet-state lifetime. Because it is a short-range interaction (3–5 Å [20]), the heavy-atom effect provides a useful criterion by which to estimate the proximity of the indole nucleus to the protein/solvent interface.

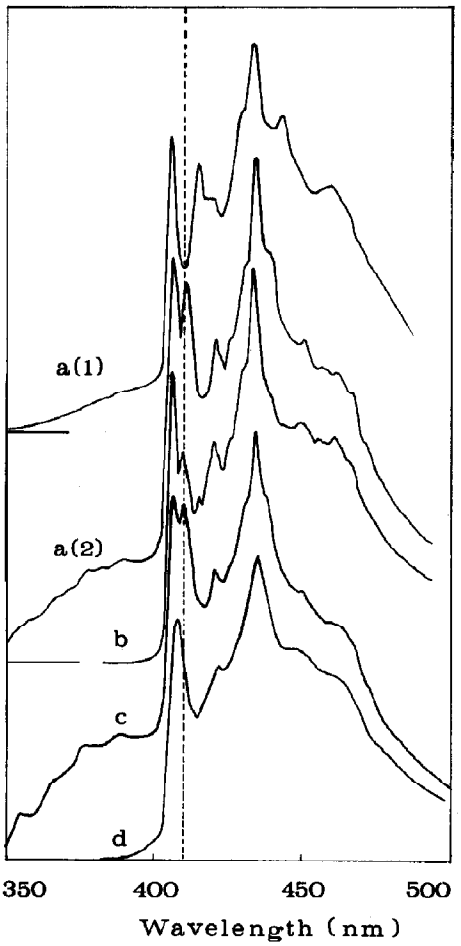

Fig. 1. Phosphorescence spectra of GPDH from *B. stearothermophilus* in glycerol/phosphate buffer (60:40, v/v) at low temperature and in buffer at room temperature. The phosphorescence spectrum of pig and rabbit GPDH, a(1), is included for comparison. a(1) and a(2), $\lambda_{ex} = 280$ nm, $T = 140$ K; b, $\lambda_{ex} = 305$ nm, $T = 140$ K; c, $\lambda_{ex} = 280$ nm, $T = 140$ K, in the presence of 1 M KI; d, $\lambda_{ex} = 295$ nm, $T = 20$ °C. Protein concentration was typically 2 mg/ml.

The phosphorescence of the bacterial protein is largely unaffected by the addition of 1 M KI (fig. 1a(2) and c) except for a minor increase of the 410.5 nm component over the other. It would seem, therefore, that both residues are beyond the range for interaction with iodide so that neither is solvent-exposed. This conclusion is corroborated by measurements of the emission decay (fig. 2). At 406 nm the phosphorescence decays monoex-

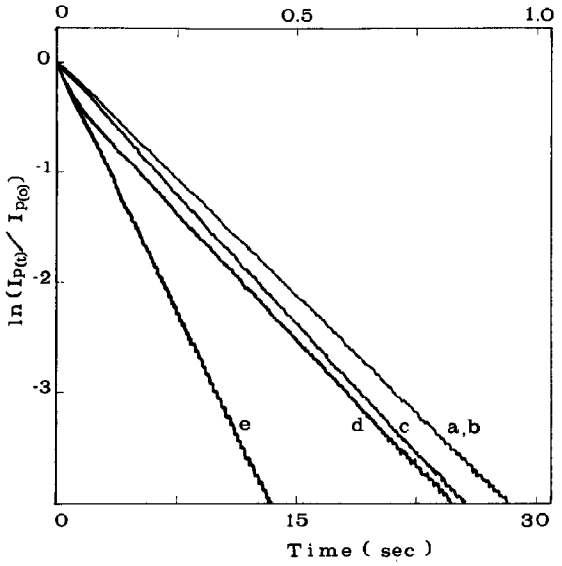

Fig. 2. Decay of phosphorescence intensity of tryptophan in *B. stearothermophilus* GPDH at 140 K, in glycerol/phosphate buffer (60:40, v/v) (curves a–d) and at 20 °C in 20 mM phosphate buffer 5 mM EDTA (pH 7.2) (curve e, upper time scale). Excitation was at 295 nm, emission being at 406 nm. a, $\lambda_{ex} = 295$ nm, $\lambda_{em} = 406$ nm; b, as for curve a, with the addition of 1 M KI; c, $\lambda_{ex} = 295$ nm, $\lambda_{em} = 440$ nm; d, as for curve c, with the addition of 1 M KI.

ponentially, the lifetime of 7.0 ± 0.1 s being unaffected by iodide. At 440 nm, where both components are monitored, the decay is not monoexponential. The fitting of the decay shows that the additional contribution has a lifetime of 5.5 ± 0.3 s. At this wavelength, a modest but detectable fraction of emission decays within even shorter times in the presence of iodide. Thus, according to the heavy-atom perturbation, both chromophores are effectively buried within the folds of the polypeptide and of the two the one emitting at 410.5 nm is nearest to the surface of the macromolecule.

In the muscle proteins the 406 nm band is the sum of two overlapping contributions, only one of which clearly interacts with iodide. The noninteracting chromophore shares the same attributes as the 406.5 nm component in *B. stearothermophilus* (peak wavelength, lifetime, deep burial) and thus appears to represent the same tryptophan residue. As described below this chromophore also shares a highly inflexible neighbouring protein structure.

3.2. Temperature dependence of phosphorescence emission

In a glassy state, unless quenching occurs at the precursor singlet level or specific interactions take place with nearby sulfur atoms (cysteine and disulfide links), all tryptophan residues in a protein phosphoresce almost equally. In fluid solutions, however, radiationless transitions to the ground state shorten the triplet-state lifetime and reduce the phosphorescence yield [12]. Consequently, information on the differing flexibility of local structure about the tryptophan residues in a protein can be gained from the phosphorescence thermal quenching profile [21].

The lifetime-normalized phosphorescence intensity of GPDH from *B. stearothermophilus* obtained upon warming the solution from the glassy state to room temperature is depicted in fig. 3. From 160 to 190 K, the overall intensity drops by about 30–35% of the initial value, thereafter remaining constant throughout the range up to 293 K. Concomitantly with thermal quenching, one observes selective disappearance of the 410.5 nm band in the spectrum. Above 190 K the spectrum is represented by a single 0,0 vibrational band

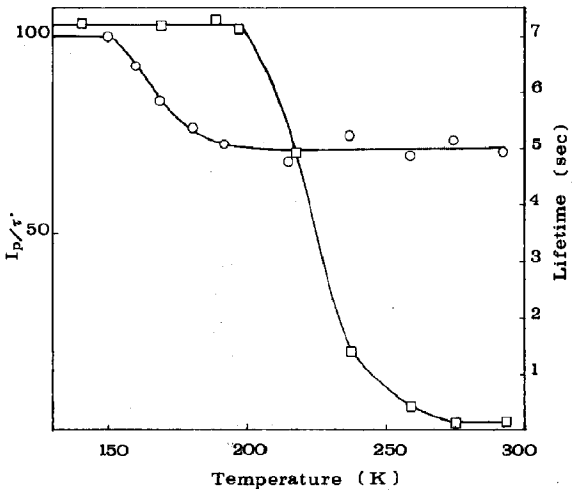

Fig. 3. Tryptophan phosphorescence thermal quenching profile, I_p/τ' (○), of GPDH from *B. stearothermophilus*. I_p denotes the steady-state phosphorescence intensity, and τ' (□) the lifetime of the slowest decaying component. $\lambda_{ex} = 290$ nm, $\lambda_{em} = 440$ nm and other conditions as in fig. 1.

with a peak at 406.5 nm that eventually relaxes to 408 nm at the highest temperatures. Thus, according to the thermal quenching profile, the residue emitting at 410.5 nm, even if buried, is in a highly fluctuating segment of the polypeptide as opposed to the other tryptophan which is trapped within a rigid agglomerate. The best examples of the inflexible structural cores in proteins are provided by the densely packed extensively hydrogen-bonded regions encountered typically with large β-sheets whose strands are interconnected by α-helical rods [14,22–24].

3.3. Phosphorescence anisotropy and hydrodynamic volume

The GPDH tetramer is known to undergo dissociation into dimers and to a lesser extent even monomers. Among the conditions which favor subunit dissociation is a decrease in temperature [1]. Thus, in order to relate the phosphorescence properties of GPDH to molecular structure, it is important to ascertain the state of association of its subunits in glycerol/buffer at low temperature. To address this question, we estimated the hydrodynamic volume of the biopolymer, V, from its rotational correlation time ($V = \theta kT/\eta$) determined from the loss of phosphorescence anisotropy assuming that the macromolecule is a spherical rotor.

Following excitation at 300 nm and emission at 406 nm the anisotropy measured at infinite viscosity was found to be -0.151 ± 0.003, the maximum value obtained with dilute solutions of tryptophan. Thus, processes such as singlet-singlet and triplet-triplet energy transfer between the two indole side chains, which generally lead to lowering of the limiting anisotropy, in all likelihood do not occur. The same conclusion on inter-tryptophan interactions was drawn from investigation of yeast, pig and rabbit GPDHs [11].

The results obtained from the decrease in anisotropy at three different temperatures together with the procedure adopted for overcoming the lack of pertinent solvent viscosity data are given in table 1. The average molecular mass of 33 kDa derived from the hydrodynamic volume is about one quarter of the value for the tetramer (144 kDa). Assuming no rapid local motions of the chromophore emitting at 406.5 nm and no torsional freedom along the subunit-subunit contact area, then the small hydrodynamic volume observed for the bacterial enzyme at low temperature is indicative of the full dissociation of GPDH into separate monomers. Recent crystallographic studies [25] point out, however, that Trp-310 is located precisely at the interface with the P-axis-related subunit, its indole ring being in van der Waals contact with Pro-205 of the P-subunit. Thus, monomer formation, unless accompanied by changes in conformation, would lead to exposure of Trp-310 to the aqueous solvent. Such accessibility to solvent was not observed in the case of heavy-atom perturbation by iodide. Further, of the two phosphorescing components only the 406.5 nm band is consistent with solvent exposure (406–409 nm range). The absence of any iodide effect and of a red shift of the 406.5 nm band corresponding to solvent relaxation, however, rules out the possibility of such a chromophore being in the proximity of the aqueous interface. With steady-state anisotropies a single average rotational correlation time is derived and it is therefore possible that relatively large errors may be

Table 1

Rotational correlation time (θ) determined for *B. stearothermophilus* GPDH in 60:40 glycerol/phosphate buffer (20 mM, pH 7.2, containing 5 mM EDTA) at three different temperatures

T (K)	θ [a] (s)	θ/θ_0 [b]	V [c] ($cm^3\ mol^{-1}$)	M_w [d]
188.5	14.10	0.46	40.1×10^3	
194.5	1.40	0.54	47.0×10^3	
197.3	0.32	0.52	45.2×10^3	
			44.0×10^3	33100

[a] The θ value was obtained from the phosphorescence anisotropy, A, and the triplet-state lifetime, τ, according to ($\theta = A\tau/A_0 - A$). Enzyme concentrations were 2 mg/ml. Excitation at 300 nm with emission at 406 nm.

[b] θ_0 refers to the rotational correlation time of horse liver alcohol dehydrogenase under the same conditions.

[c] Hydrodynamic volumes (V) were obtained assuming the macromolecule to be a spherical rotor ($V = \theta kT/\eta$) and $V = 87000\ cm^3\ mol^{-1}$ for liver alcohol dehydrogenase [12].

[d] Molecular weights were calculated from V after subtracting a 4 Å hydration shell and employing a specific volume of 0.73 $cm^3\ g^{-1}$.

introduced during evaluation of the hydrodynamic volume by the assumptions of a spherical shape (especially for prolate elipsoids) and the lack of independent motions of the indole side chain. Thus, while anisotropy data indicate that dissociation of the tetramer does occur, a phenomenon also observed with the muscle and yeast enzymes, such a process alone cannot be taken as being conclusive with respect to the exact state of association of GPDH at low temperature. Unless gross conformational changes subsequent to monomer formation lead to the burial of Trp-310, spectral and lifetime data imply that dissociation is limited to the dimer.

3.4. Room-temperature phosphorescence in buffer

The 406.5 nm spectral component displays long-lived phosphorescence in buffer at 20 °C. The peak wavelength is only 1.5–2 nm red-shifted compared to the glassy state (fig. 1) a modest value for the relaxation relative to 8–10 nm for tryptophan in polar solvents. The intensity decay of phosphorescence follows an exponential law with a lifetime of 110 ± 10 ms. Both the inability of the spectrum to relax to longer wavelength and the long triplet-state lifetime are indications of a compact inflexible protein structure about the chromophore. Indeed, from the lifetime we may estimate a local viscosity of about 1.1×10^4 P [12]. This value is intermediate between those of 3.4×10^3 P in yeast and the approx. 1×10^5 P found with the muscle enzymes.

An independent assessment of the dynamical structure in the neighborhood of the phosphorescence probe can be obtained from the ease with which small molecules such as O_2 penetrate to the internal site and quench the emission [24,26,27]. The rate of dynamic quenching of phosphorescence by O_2 was derived from the decrease in triplet-state lifetime $(1/\tau - 1/\tau_0)$ with increasing concentrations of O_2 in solution. This parameter shows a linear dependence on concentration of quencher, the slope yielding a bimolecular rate constant, k_{O_2}, of $6.5 \pm 1 \times 10^7$ M^{-1} s^{-1}. This value is small compared to that of 5×10^9 M^{-1} s^{-1} expected for a solvent-exposed residue [28] and, given the range of static interactions with O_2, implies that the chromophore is buried at an appreciable distance from the solvent interface (> 6 Å) [24] and that O_2 diffusion to the internal site is considerably hindered. Similar values for k_{O_2} have been reported for the other GPDHs as well as for tryptophan residues within the β-sheet of the coenzyme-binding domain in alcohol dehydrogenase [24], glutamate dehydrogenase [16] and other NAD^+-dependent dehydrogenases (Gabellieri and Strambini, unpublished data).

Upon inspection of the crystallographic structure of *B. stearothermophilus* GPDH, the assignment of the 0,0 vibrational bands in the phosphorescence spectrum to the correct tryptophan residues is straightforward. Trp-84 is found at the beginning of a short, three-residue, α-helical segment (αD) of the polypeptide on the surface of the macromolecule. The indole side chain is shielded from the aqueous phase by two segments of the polypeptide in a random coil. Because the flexibility of proteins is generally most pronounced in random coil segments at the periphery of the macromolecule, the site about Trp-84 is anticipated to be rather fluid. Thus, although this aromatic ring is buried (410.5 nm) and conceivably beyond the range of the heavy-atom effect, as it resides in a flexible pocket its phosphorescence is thermally quenched. In contrast, Trp-310 is located in the subunit interior. It is part of a seven-strand β-sheet (β_8) forming the catalytic domain, the tryptophanyl side chain being sandwiched in between by large α-helical rods, α_1 and α_2. Trp-310 is therefore expected to be located in a rigid core of the structure of lowest accessibility to external agents. Thus, the long phosphorescence lifetime, non-relaxed nature of the spectrum and slow migration of O_2 to the chromophore's site lead to the unique assignment of the 406.5 nm component to Trp-310.

According to the thermal quenching profile, Trp-84 (the 410.5 nm component) contributes in a glassy matrix only 30–35% to the total phosphorescence intensity. Since its triplet-state lifetime is typical of an unperturbed indole nucleus, the smaller yield implies that some degree of static quenching occurs for the precursor fluorescent state. Energy transfer to Trp-310 is not a plausible quenching mechanism because emission from the

latter is fully polarized. Another possibility is quenching by electron transfer to a vicinal scavenger group in the protein, cysteine and the histidine cation being the most efficient electron traps [29]. While the nearest cysteine, Cys-153 in the same subunit, is at least 7 Å removed from Trp-310, His-108 participates in a 2.8 Å hydrogen-bonding interaction with the indole nitrogen of Trp-84 and is the only potential electron acceptor in the immediate neighbourhood of the two chromophores. A proximal charged His^+ reduces the fluorescence yield by a factor of 4 to 6. For the neutral form hydrogen-bonding with the ring nitrogen, the precise extent of quenching is not known. By comparison with other electrophiles the reduction anticipated is by a factor of at least 2.

In bacterial GPDH as opposed to the other sources investigated, the assignment of phosphorescence bands is facilitated by the presence of only two instead of three tryptophan residues and by the possibility of direct comparison with the crystallographic structure. Structural differences between the thermophile and the lobster proteins are very minor and confined mostly to the S-loop region. Because of this, it is reasonable to expect that the structure of the lobster enzyme also faithfully represents that of yeast, pig and rabbit GPDHs, which relative to *B. stearothermophilus* have an even greater sequence homology. It follows that the gross structural features of the environment of each tryptophan residue and consequently its phosphorescence properties should be largely conserved among the four species investigated. Indeed, in all four GPDHs a single tryptophan phosphoresces in fluid solution and the identification of this emission with Trp-310 in *B. stearothermophilus* confirms the correctness of the assignment previously proposed for yeast, pig and rabbit GPDHs [11].

The cooperativity towards NAD^+ binding, namely, positive for yeast and negative for the other species investigated, requires an interaction among subunits in the tetramer. The molecular details of allosterism in GPDH are not known and until now the search has focused on the subunit interface region where the two X-ray structures available also show the greatest variability. From the differences in phosphorescence properties of Trp-310, we have previously pointed out that the arrangement of charges and the flexibility of the catalytic domain are also quite distinct between yeast and the muscle proteins of pig and rabbit. Here, we add that such structural features of the catalytic domain in GPDH from *B. stearothermophilus*, which also displays negative cooperativity, are indeed similar to those of the muscle enzymes. At present, any conclusion on these differences in structure being correlated to the type of cooperativity would be premature.

The decay behaviour of the triplet state of tryptophan provides a direct, noninvasive means of probing the dynamical structure of the surrounding polypeptide. With several enzymes this procedure has proved to be extremely sensitive to changes in conformation brought about by mild nondenaturing perturbations [13,30,31] and ligand binding (substrates, allosteric effectors) [32,33] that remain otherwise undetected with conventional spectroscopic techniques. Thus, the finding of a monoexponential decay in the GPDH tetramer is a strong indication of the equivalence in the τ-averaged structure of its subunits. Namely, if asymmetric states do exist, then they rapidly interconvert during the lifetime of the triplet state. Asymmetry among subunits introduced by partial degrees of coenzyme binding has been observed and is the subject of current investigations.

Acknowledgement

The authors are grateful to Mr. L. Favati for technical assistance.

References

1 J.I. Harris and M. Waters, in: The enzymes, ed. P.D. Boyer, (Academic Press, New York, 1976) 3rd edn., vol. 13, p. 1.
2 M. Buehner, G.C. Ford, D. Moras, K.W. Olsen and M.G. Rossman, J. Mol. Biol. 82 (1974) 563.
3 D. Moras, K.W. Olsen, M.N. Sabesan, M. Buehner, G.C. Ford and M.G. Rossmann, J. Biol. Chem. 250 (1975) 9137.
4 M.R.N. Murthy, R.M. Garavito, J.E. Johnson and M.G. Rossmann, J. Mol. Biol. 138 (1980) 859.
5 G. Biesecker, J.I. Harris, J.C. Thierry, J.E. Walker and A.J. Wonacott, Nature 266 (1977) 328.

6 A.G.W. Leslie and A.J. Wonacott, J. Mol. Biol. 165 (1983) 375.
7 A. Conway and D.E. Koshland, Jr, Biochemistry 7 (1968) 4011.
8 F. Seydoux, S. Bernhard, O. Pfenninger, M. Payne and O.P. Malhotra, Biochemistry 12 (1973) 4290.
9 R.A. Cook and D.E. Koshland, Jr, Biochemistry 9 (1970) 3337.
10 J.M. Davis and A.H. Maki, Biochemistry 23 (1984) 6249.
11 G.B. Strambini and E. Gabellieri, Biochemistry 28 (1989) 160.
12 G.B. Strambini and M. Gonnelli, Chem. Phys. Lett. 155 (1985) 196.
13 G.B. Strambini and M. Gonnelli, Biochemistry 25 (1986) 2471.
14 G.B. Strambini and E. Gabellieri, Biochemistry 26 (1987) 6527.
15 G. Branlant, B. Eiler, L. Wallen and J.-F. Biellmann, Eur. J. Biochem. 127 (1982) 519.
16 G.B. Strambini, P. Cioni and R.A. Felicioli, Biochemistry 26 (1987) 4968.
17 G.B. Strambini, Biophys. J. 43 (1983) 127.
18 W.C. Galley, in: Biochemical fluorescence: concepts, eds R.F. Chen and H. Edelhoch (Dekker, New York, 1976) vol. II, p. 409.
19 M.V. Hershberger, A.H. Maki and W.C. Galley, Biochemistry 19 (1980) 2204.
20 W.E. Lee, Ph.D. Thesis, McGill University, Montreal, Quebec (1985).
21 J. Domanus, G.B. Strambini and W.C. Galley, Photochem. Photobiol. 31 (1980) 15.
22 A.A. Kossiakoff, Annu. Rev. Biochem. 54 (1985) 1195.
23 R. Lumry and R.B. Gregory, in: The fluctuating enzyme, ed. G.R. Welch (Wiley, New York, 1986) p. 1.
24 G.B. Strambini, Biophys. J. 52 (1987) 23.
25 T. Skarzynski, P.C.E. Moody and A.J. Wonacott, J. Mol. Biol. 193 (1987) 171.
26 J. Lakowicz and W.G. Weber, Biochemistry 12 (1973) 4171.
27 M.L. Saviotti and W.C. Galley, Proc. Natl. Acad. Sci. U.S.A. 71 (1974) 4154.
28 D.V. Bent and E. Hayon, J. Am. Chem. Soc. 97 (1975) 2612.
29 R.F. Steiner and E.D. Kirby, J. Phys. Chem. 73 (1969) 4130.
30 G.B. Strambini and M. Gonnelli, J. Phys. Chem. 92 (1988) 2850.
31 M. Gonnelli and G.B. Strambini, J. Phys. Chem. 92 (1988) 2854.
32 P. Cioni and G.B. Strambini, J. Mol. Biol. (1989) in the press.
33 P. Cioni, G.B. Strambini and A. Puntoni, Biochemistry (1989) in the press.