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Comparative Phosphorescence and Optically Detected Magnetic Resonance Studies of Pig and Yeast Glyceraldehyde-3-phosphate Dehydrogenase†

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ABSTRACT: A comparative optically detected magnetic resonance (ODMR) investigation has been made of the tryptophan (Trp) residues of glyceraldehyde-3-phosphate dehydrogenase (GAPD) from pig and yeast. We find that pig GAPD emits phosphorescence from only two of the three distinct Trp sites, while yeast GAPD exhibits resolved 0,0-bands from all three Trps. Heavy atom effects observed in the $CH_3Hg(II)$–sulfhydryl complex of pig GAPD resemble closely those reported earlier for the analogous rabbit GAPD–$CH_3Hg(II)$ complex. Trp-310, with a 0,0-band at 416 nm, undergoes a selective heavy atom perturbation as a result of $CH_3Hg(II)$ binding to the nearby Cys-281. The 416-nm peak in yeast GAPD is assigned to Trp-310 on the basis of ODMR, but no heavy atom effect of $CH_3Hg(II)$–sulfhydryl complexing is observed because of the absence of Cys-281 in yeast, thus supporting this assignment. The 406-nm 0,0-bands of pig and rabbit GAPD and the 409-nm band of yeast GAPD are assigned to Trp-193, located in a subunit contact region. This residue is solvent exposed in the yeast enzyme but appears to be buried in a polar environment in the mammalian GAPD. These differences may be related to variations in subunit cooperativity between species. Trp-84 appears to be quenched in pig and rabbit GAPD, most likely by His-108. In yeast GAPD, on the other hand, Trp-84 is not quenched, probably because His-108 is further removed. The Trp-84 0,0-band of the yeast enzyme peaks at 420 nm, making it the most red-shifted Trp origin reported thus far. The influence of local perturbations on the triplet-state properties of specific Trp sites in these enzymes is discussed.

Glyceraldehyde-3-phosphate dehydrogenase (GAPD,[1] EC 1.2.1.12) is a glycolytic enzyme, catalyzing the reaction

$$\text{glyceraldehyde 3-phosphate} + NAD^+ + P_i = \text{1,3-diphosphoglycerate} + NADH + H^+$$

It has a molecular weight of 144 000 and consists of four 36 000-dalton monomers with identical sequences. The sequences of the lobster, pig, and yeast enzymes are known (Olsen et al., 1975), as well as those of human GAPD (Nowak et al., 1981) and GAPD from chicken heart (Domdey et al., 1983) (93%) and from two thermophilic bacteria (Walker et al., 1980; Hocking & Harris, 1980). The sequence is not known for rabbit GAPD (which along with the yeast enzyme is commercially available). X-ray studies have been done on lobster GAPD (Moras et al., 1975) and of the *Bacillus stearothermophilus* enzyme (Biesecker et al., 1977). Although the monomer sequences are the same for each species, small differences may occur in their conformations. The lobster GAPD appears to be an asymmetric dimer of dimers, with identical so-called "red" and "yellow" subunits as well as identical "blue" and "green" subunits (Moras et al., 1975). The sequences of GAPD's tend to be highly conserved (Domdey

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[1] Abbreviations: ODMR, optical detection of magnetic resonance; GAPD, glyceraldehyde-3-phosphate dehydrogenase; zfs, zero-field splittings; AM-PMDR, amplitude-modulated phosphorescence/microwave double resonance; NAD, nicotinamide adenine dinucleotide.

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Table I: ODMR Frequencies and zfs Parameters of Rabbit, Pig, and Yeast GAPD

organism	origin (nm)	$\nu_1(\Delta\nu)^b$	$\nu_2(\Delta\nu)^b$	D (cm^{-1})	E (cm^{-1})
rabbit[a]	405.5	1.91	2.09	0.0985	0.0348
	415.7	1.67	2.37	0.0952	0.0395
pig	406.3	1.87 (160)	2.12 (227)	0.0977	0.0353
	416.0	1.67 (210)	2.43 (200)	0.0962	0.0405
yeast	409.0	1.680 (117)	2.453 (218)	0.0969	0.0409
	415.9	1.648 (56)	2.390 (120)	0.0948	0.0398
	420.0	1.564 (44)	2.590 (94)	0.0953	0.0432

[a] Data from Hershberger & Maki (1980); the line widths were not given. [b] Peak ODMR frequencies in GHz; the values given are the average of frequencies observed sweeping in both directions. The numbers in parentheses are line widths (fwhm) in MHz.

et al., 1983). Notable is the fact that the three Trps per monomer are conserved in all eukaryotic GAPD's with known sequences.

Rabbit muscle GAPD exhibits two resolved Trp phosphorescence 0,0-bands at 406 and 415.7 nm (Hershberger & Maki, 1980). When methylmercury iodide, which binds only to sulfhydryls (Rabenstein, 1978), is added, it undergoes a specific heavy atom perturbation affecting only the Trp origin at 415.7 nm, which appears to be shifted and broadened. A Trp $D + E$ signal and a short decay component are observed when monitoring the phosphorescence of the CH_3Hg–GAPD complex. In previous work (Davis & Maki, 1982), we used AM-PMDR to extract the underlying broad phosphorescence spectrum of the perturbed Trp in the $CH_3Hg(II)$ complex of rabbit muscle GAPD from the total emission. We also reported that yeast GAPD, which has Ser in place of Cys at position 281, does not experience a heavy atom perturbation. The external heavy atom effect is a short-range interaction, requiring the chromophore and perturber to be in van der Waals contact. Although rabbit muscle GAPD has not been sequenced, it has often been assumed to be homologous to the pig enzyme. On the basis of the X-ray crystal structure of lobster muscle GAPD (Moras et al., 1975) and the sequence of pig muscle GAPD (Olsen et al., 1975), the perturbation was assigned to an interaction between Trp-310 and methylmercury bound to Cys-281. In the crystalline lobster enzyme the distance between the Ser-281 oxygen and Trp-310 is about 6 Å. Since pig GAPD is known to have three Trp residues in its sequence, while only two resolved phosphorescence spectra and corresponding ODMR signals were found in the rabbit enzyme (Hershberger & Maki, 1980), two possibilities were considered likely: (1) phosphorescence from one of the Trp sites is quenched or (2) the rabbit enzyme may be missing one of the Trp of the pig sequence.

In this paper, we report the wavelength dependence of the ODMR signals of Trp for pig as well as for yeast GAPD. It is known (von Schütz, et al., 1974) that the presence of phosphorescence from different Trp sites of a protein can be uncovered by monitoring the ODMR with narrow monochromator slits throughout the 0,0-band region. These measurements are made in order to shed light on the "missing" Trp phosphorescence of the rabbit enzyme. In order to confirm further our assignment of the $CH_3Hg(II)$-perturbed site to Trp-310, ODMR and AM-PMDR measurements have been carried out on the pig GAPD–$CH_3Hg(II)$ complex.

Materials and Methods

GAPD from pig muscle was isolated from a freshly slaughtered animal by using a modification of the method of Harrigan & Trentham (1973). When purified, it ran as a single band on an SDS–polyacrylamide gel (Tanner & Boxer, 1972). It was stored as a suspension in an 85% saturated $(NH_4)_2SO_4$ solution. When a sample was prepared for spectroscopy, the suspension was centrifuged for about 20 min at 12000g and the pellet was dissolved in a 20 mM potassium phosphate buffer, pH 7.2, containing 1–5 mM Na_2EDTA and 2 mM mercaptoacetic acid or 2-mercaptoethanol or 10 mM dithiothreitol. The solution generally was applied to a Pharmacia PD-10 desalting column and eluted with the same buffer, although it was found that both the phosphorescence and the ODMR spectra were unchanged when this step was omitted. These operations were carried out between 0 and 5 °C. The protein concentration in the aqueous sample was approximately 20 mg/mL, although it varied from sample to sample. The protein concentration was determined from its absorbance at 280 nm (Fox & Dandliker, 1956). The aqueous sample was diluted 1:1 (vol/vol) with ethylene glycol (Mallinckrodt analytical reagent grade). The aqueous sample was diluted 1:1 with 10 mM CH_3HgI[2] in ethylene glycol to make the $CH_3Hg(II)$–GAPD complex.

GAPD from rabbit muscle was obtained from Sigma as a crystalline suspension in 2.6 M $(NH_4)_2SO_4$. The preparation of samples for spectroscopy was the same as for the pig GAPD.

GAPD from yeast was obtained as a lyophilized powder (Sigma). It was dissolved in pH 7.0, potassium phosphate buffer, 0.1 M, and mixed 1:1 with ethylene glycol (Matheson Coleman and Bell). The concentration was between 10 and 25 mg/mL in the final solution.

Phosphorescence and ODMR measurements were made using the apparatus described by Hershberger & Maki (1980). The apparatus for the AM-PMDR experiment is described by Davis & Maki (1982). The excitation band-pass was 16 nm for all spectra, except for the yeast GAPD phosphorescence spectra, where it was 10 nm. The other conditions are given in the figure captions.

Results

The phosphorescence spectra of rabbit, pig, and yeast GAPDs are compared in Figure 1. The phosphorescence spectrum of pig GAPD is very similar to that of the rabbit enzyme. The positions of the peaks do not differ by more than a few angstroms. The yeast GAPD spectrum is clearly different. Table I lists the ODMR signals for pig, rabbit, and yeast GAPDs, measured at the peaks of some prominent phosphorescence bands. The ODMR signals of pig and rabbit GAPDs are very similar, consistent with similar Trp environments. The yeast signals are quite different, except for those measured at the 415.9-nm origin, which produces ODMR signals similar to the 416-nm peaks of the other two species. The yeast signals at 416 nm are considerably narrower than those in the mammalian enzymes, however.

The occurrence of discontinuities in the zfs with changing observed wavelength may be indicative of emission from

[2] Organomercurials are extremely toxic. Care was taken to avoid inhalation or contact with the skin. The $CH_3Hg(II)$ compounds, waste solutions, and solid waste were kept isolated in a separate, dedicated hood in which all sample preparation was carried out.

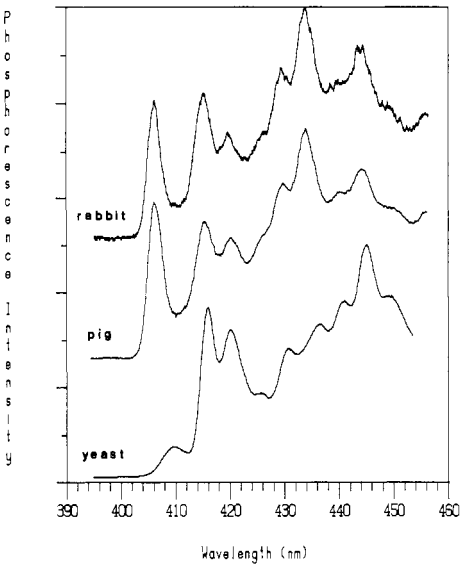

FIGURE 1: Phosphorescence spectra of rabbit muscle GAPD, pig muscle GAPD, and yeast GAPD. Excitation wavelength is 300 nm. The temperature is 4.2 K for yeast and rabbit and 1.1 K for pig. The excitation wavelength is 300 nm.

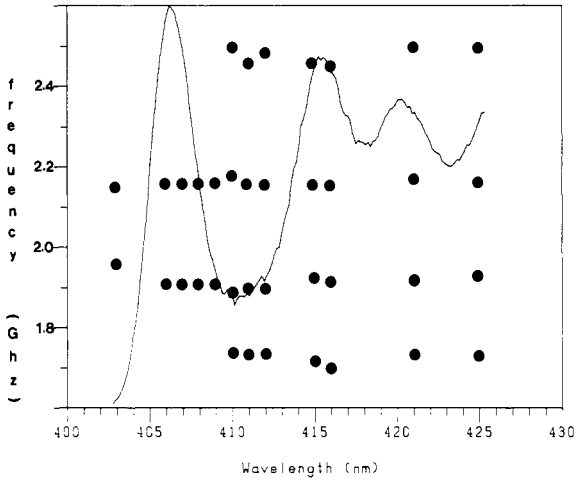

FIGURE 2: Frequencies of $2E$ (above 2 GHz) and $D - E$ (below 2 GHz) transitions of pig GAPD measured at varying wavelengths by using 1.3-nm slits. The phosphorescence spectrum is shown for reference.

distinct Trp sites in the enzyme. Frequently, also, resolved doublet ODMR signals are observed in overlap regions between vibronic bands, which may indicate emission from distinct Trp sites (von Schütz et al., 1974). Because of the vibronic structure emitted by Trp, however, and the variation of the zfs with wavelength throughout an inhomogeneously broadened emission band (Lemaistre & Zewail, 1979; van Egmond et al., 1975), these effects also can be obtained from a *single Trp site* as the observed wavelength *passes from one vibronic band to another* (Kwiram et al., 1978). In this case, the zfs will repeat the wavelength dependence in each resolved vibronic band since the same set of Trps is being observed repeatedly. Emission from distinct sites may be demonstrated if the zfs does not repeat itself from band to band and also if it can be shown that the bands have different excitation spectra. In order to determine which phosphorescence peaks correspond to distinct 0,0-bands, the ODMR signals of both pig and yeast GAPDs were measured at various wavelengths throughout the 0,0-band region using ca. 1.3-nm slits. In Figure 2, the D–E and 2E transition frequencies of pig GAPD are plotted vs. wavelength. Figure 3 shows the variation in

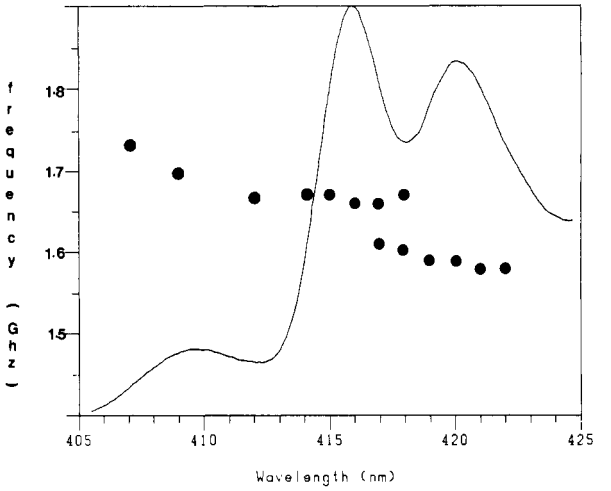

FIGURE 3: Frequency of the $D - E$ transition of yeast GAPD measured at various wavelengths by using 1.3-nm slits. The phosphorescence spectrum is shown for reference.

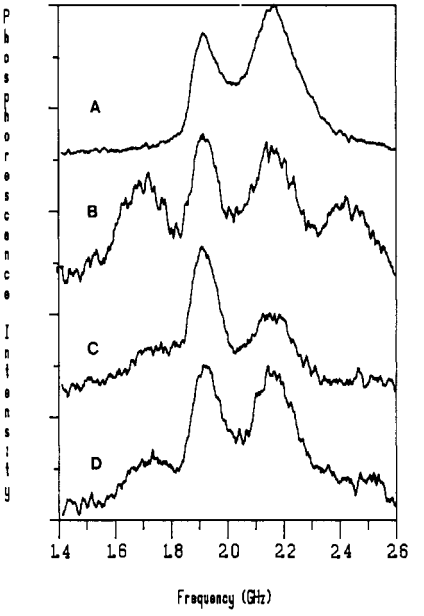

FIGURE 4: Slow passage ODMR spectra of pig GAPD. The microwaves are swept from 1.4 to 2.5 GHz in 30 s. The temperature is 1.1 K and the signals result from 60 accumulations. The phosphorescence is monitored at (A) 406, (B) 416, (C) 421, and (D) 425 nm with 1.3-nm slits.

the $D - E$ signal frequency with observing wavelength for yeast GAPD. Monitoring the 406-nm band of pig GAPD we see a single set of signals (Figure 4, spectrum A) whose frequencies are nearly wavelength independent (Figure 2). To the red of about 410 nm, a second set of signals are observable. The second set of signals are most intense at the 416-nm peak wavelength of the second band (Figure 4, spectrum B), indicating that this is the 0,0-band of a second site. Additional evidence that these bands are due to distinct sites are their different excitation spectra, and the distinct (and nonrepeating with wavelength) zfs. No new ODMR signals are observed at 421 or 425 nm (Figure 4, spectra C and D). For this reason, we assign the peak at 421 nm (Figure 2) as a vibronic band of the 406-nm origin. Analogous vibronic bands are observed in the well-resolved phosphorescence spectrum of the single Trp of ribonuclease T_1 (Hershberger et al., 1980). We thus find that pig GAPD gives evidence of phosphorescence from only two of its three Trps. Any ODMR signals from the third Trp are beyond our limit of detectability.

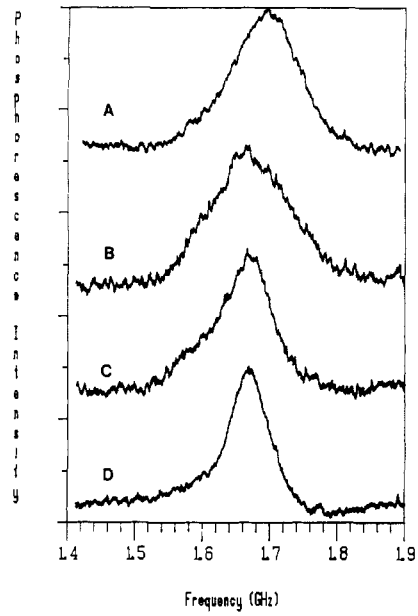

FIGURE 5: Slow passage ODMR spectra of yeast GAPD at 1.1 K using 1.3-nm slits. The microwaves were swept from 1.5 to 1.9 GHz in 32 s. The phosphorescence was monitored at (A) 409.3 nm (120 scans), (B) 412 nm (136 scans), (C) 414 nm (63 scans), and (D) 415 nm (38 scans).

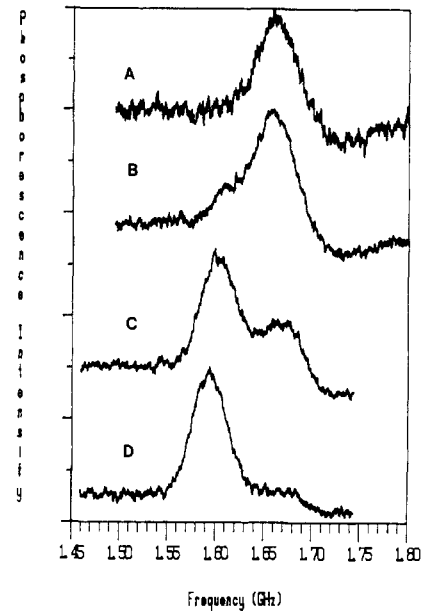

FIGURE 6: Slow passage ODMR spectra of yeast GAPD at 1.1 K using 1.3-nm band-pass slits. (A) The phosphorescence was monitored at 416 nm. Microwaves were swept from 1.5 to 1.8 GHz in 60 s (13 scans). (B) The phosphorescence was monitored at 417 nm. Microwaves were swept from 1.5 to 1.8 GHz in 60 s (98 scans). (C) The phosphorescence was monitored at 418 nm. Microwaves were swept from 1.4 to 1.7 GHz in 60 s (87 scans). (D) The phosphorescence was monitored at 419 nm. Microwaves were swept from 1.4 to 1.7 GHz in 60 s (26 scans).

The signals associated with the 409- and 416-nm peaks of yeast GAPD are close enough in frequency that they overlap strongly. An inflection, rather than a sharp break, is observed in Figure 3 as the wavelength changes from one emission band to the other. Strong evidence that these signals originate from separate Trp sites is given by the dramatic change in line width in this frequency range as shown in Figure 5. As the 416-nm emission begins to dominate, the D–E signal becomes very much narrower. It is widest at about 412 nm, where the two phosphorescence bands overlap. Additional evidence that the 409- and 416-nm bands are 0,0-bands originating from distinct Trp sites is given by their distinguishable excitation spectra (vide infra). Since they are both quite narrow, the signals observed from the 416-nm band and the 420-nm band are clearly resolved in the region where the two phosphorescence peaks overlap (Figure 6). Figure 3 shows that the zfs observed when monitoring the 420-nm band is distinct from that observed when monitoring either the 409- or 416-nm band, indicating that it is a 0,0-band of a distinct Trp site. The fact that the 409-, 416-, and 420-nm peaks of yeast GAPD are distinct 0,0-bands of three Trp sites is confirmed by the phosphorescence excitation spectra shown in Figure 7. The relative intensity of the 409-nm peak increases as the excitation is shifted to the blue, while that of the 420-nm peak increases as the excitation is shifted to the red. The 420-nm band of yeast GAPD is the most red-shifted Trp 0,0-band thus far verified in a protein.

Rabbit GAPD, monitored at 420.5 nm, shows strong ODMR signals at 1.88 and 2.09 GHz, a weaker signal at 1.67 GHz, and a very weak signal at around 2.4 GHz. These are consistent again with the assignment of the 420.5-nm peak as a vibronic band of the 406-nm origin. These results are very much like those we obtained with the pig enzyme (Figure 4, spectrum C). Because rabbit and pig GAPDs contain bound NAD when isolated, while yeast GAPD does not, we were concerned that NAD might be quenching the phosphorescence of one of the Trps of the mammalian GAPDs. To test this, we removed NAD from rabbit GAPD. After passage through charcoal, the ratio of the absorbance at 280 nm to the absorbance at 260 nm had changed from 1.2 to 1.7, indicating that on the average less than one-third of an NAD was bound to each GAPD (Fox & Dandliker, 1956). The phosphorescence spectrum of the apo-GAPD was not noticeably different from that of the rabbit GAPD, which still contained bound NAD (Figure 1).

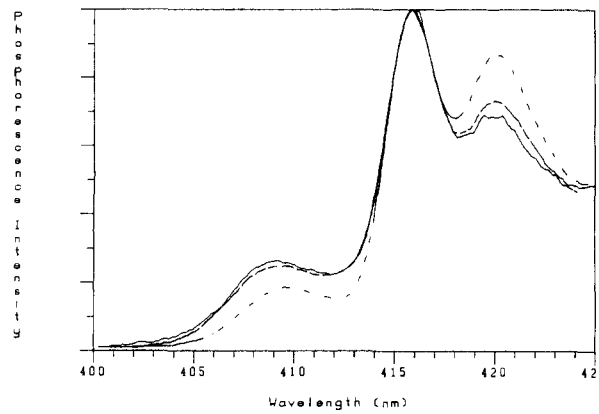

FIGURE 7: Phosphorescence spectra of yeast GAPD excited at 280 (—), 290 (– – –), and 302.5 nm (- - -).

Aside from the extremely red shifted 0,0-band in the yeast GAPD, the other distinctive features of these GAPDs are some unusually small frequencies for the $2E$ transition. Figure 8 is a plot of the parameter E vs. the peak wavelength of the 0,0-band for a large number of proteins and peptides. The unfilled circles are values for 16 different peptides and proteins that were compiled by Hershberger et al. (1980). These are examples of typical E values. Note that among the data presented in this paper, only the 409-nm peak of yeast GAPD has a fairly typical value for E. The other values of E are considerably lower than would be expected from their 0,0-band maxima.

When CH_3HgI is added, the pig GAPD phosphorescence spectrum (Figure 9, spectrum A) shows a perturbation like

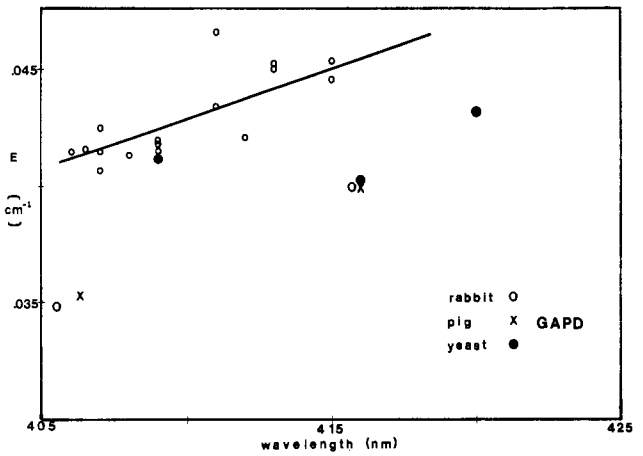

FIGURE 8: Value of parameter E vs. the phosphorescence 0,0-band wavelength for Trp in several peptides and proteins. The small circles are values from Hershberger et al. (1980), and the line is the least-squares linear regression for those points. The other points are for rabbit (O), pig (X), and yeast (●) GAPD.

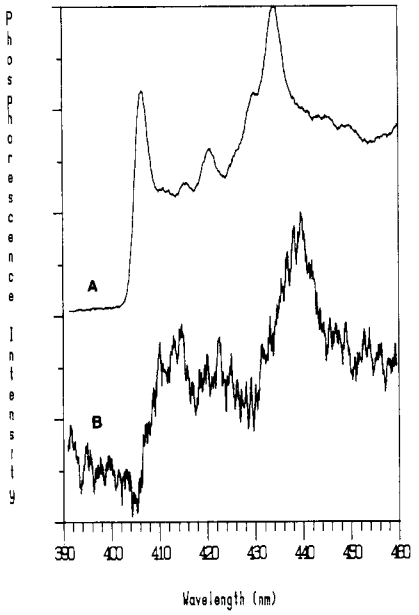

FIGURE 9: (A) Phosphorescence spectrum of the pig GAPD–$CH_3Hg(II)$ complex. (B) AM-PMDR spectrum of the pig GAPD–$CH_3Hg(II)$ complex at 1.1 K. FM range is 4.0-4.5 GHz. FM frequency is 2 kHz. AM frequency is 17 Hz, and the spectrum is scanned at 20 nm/min. The signal results from 46 accumulations. The emission slits are 3.2 nm and the excitation wavelength is 300 nm for (A) and (B).

that exhibited by rabbit GAPD (Hershberger & Maki, 1980). Yeast GAPD shows very little change in its phosphorescence spectrum when treated with $CH_3Hg(II)I$ in a similar manner. The $CH_3Hg(II)$ complex of pig GAPD shows an induced $D + E$ ODMR transition at 4.2 GHz analogous to that of the rabbit enzyme (Hershberger & Maki, 1980). A decay component of approximately 15 ms is observed when the phosphorescence decay is monitored at 416 nm but not at 406 nm. When monitored at 416 nm under slow sweep conditions, the $2E$ and $D–E$ signals associated with the 416-nm peak in the native enzyme are no longer observed. These results are all indicative of selective heavy atom perturbation of the Trp having the 416-nm 0,0-band and are the same as were reported previously for rabbit GAPD (Hershberger & Maki, 1980). When the 4.2-GHz transition is driven in an AM-PMDR experiment, a spectrum similar to that seen with rabbit GAPD (Davis & Maki, 1982) is produced. The AM-PMDR spectrum of $CH_3Hg(II)$–pig GAPD is compared with its phosphorescence spectrum in Figure 9. The 0,0-band and major vibronic band seeem to be somewhat better defined in the pig as compared with the rabbit GAPD AM-PMDR spectrum. This may indicate a slightly different interaction geometry. These results, and the absence of a heavy atom effect in yeast GAPD, provide evidence to support our identification of the 416-nm Trp as Trp-310 and the binding site of the Hg perturber as Cys-281.

Table II: Neighbors of Trp-310[a]

lobster	pig	yeast	comments
Val-237	Val	Val	nonpolar, conserved
Val-239	Val	Val	nonpolar, conserved
Ser-281	Cys	Ser	polar
Phe-283	Phe	Phe	nonpolar, aromatic, conserved
Asp-293	Asp	Asp	polar, H bond to Trp, conserved
Val-308	Ile	Val	nonpolar

[a] Residues that have some atom within 4 Å of Trp-310 in the lobster GAPD crystal structure. The assumption is then made that residues of the same number in the three species are in the same positions relative to tryptophan. All residues are in the same subunit.

Table III: Neighbors of Trp-193[a]

lobster	pig	yeast	comments
Arg-194[b]	Arg	Arg	basic, conserved
Ser-189[b]	Ser	Ser	polar, conserved
Gly-195[b]	Asp	Gly	acidic in pig, uncharged in yeast
Glu-38[c]	His	Asp	acidic in lobster and yeast, basic in pig
Tyr-39[c]	Tyr	Tyr	aromatic, conserved
Tyr-42[c]	Tyr	Tyr	Trp may be "sandwiched" between two Tyr
Val-278[d]	Val	Val	nonpolar, conserved
Asp-277[d]	Gln	Ala	in lobster, Trp-193 is H bonded to Asp-277
Asp-276[d]	Asp	Asp	polar, conserved

[a] Residues that have an atom within 4 Å of Trp-193 in the red subunit of the lobster GAPD crystal structure. [b] Residues in the red subunit. [c] Residues in the green subunit. [d] Residues in the yellow subunit.

Table IV: Neighbors of Trp-84[a]

lobster	pig	yeast	comments
Asn-6	Asn	Asn	polar, conserved
Ala-29	Ala	Ala	nonpolar, conserved
Asn-31	Asn	Asx	polar, may be conserved
Ile-82	Ile	Leu	nonpolar
Ala-89	Thr	Ser	
Ile-92	Val	Ala	nonpolar
His-108	His	His	basic, conserved
Gly-4	Gly	Ala	nonpolar
Phe-74	Phe	Tyr	aromatic

[a] Residues that have an atom within 4 Å of Trp-84 of the lobster GAPD crystal structure. All residues are in the same subunit.

Discussion

Assignment of the Resolved Tryptophan Emissions. Using methylmercury(II) as a probe, we have been able to identify Trp-310 of the pig GAPD structure with the 416-nm Trp 0,0-band in its phosphorescence spectrum. Assignment of the other Trps will necessarily be more speculative, but previous work on protein triplet states (Purkey & Galley, 1970; Hershberger et al., 1980) provides some basis for further assignments. The probable neighbors of Trp-84, -193, and -310 are listed in Tables II–IV. These are based on the crystal structure of lobster GAPD and the amino acid sequences of pig and yeast GAPD. Tryptophans-84 and -310 are buried in the interior of the subunits, while Trp-193 is in the subunit contact region. The 409-nm Trp of yeast GAPD has a phosphorescence 0,0-band wavelength, zfs, and ODMR as well as phosphorescence line widths that are typical of solvent-exposed Trp. These features suggest strongly that the 409-nm

origin of yeast GAPD should be assigned to Trp-193 rather than to Trp-84, which is buried in the protein interior. Trp-310 (also buried) has been assigned to the 416-nm 0,0-band, already, by analogy with the pig and rabbit GAPD data. Trp-84 would be responsible, as a result, for the very red-shifted 0,0-band in yeast GAPD. The narrow ODMR and phosphorescence line widths associated with the 420-nm 0,0-band also indicate a buried Trp.

The 406-nm origin of pig GAPD is quite narrow and considerably more blue-shifted than one would expect from solvent exposure. These features are indicative of a buried Trp that experiences polar interactions that destabilize the triplet state relative to the ground state. This type of Trp is exemplified by the single Trp site of ribonuclease T_1 (Hershberger et al., 1980). Ribonuclease T_1 exhibits considerably narrower ODMR line widths (50 MHz for the $D - E$ transition and 125 MHz for the $2E$ transition) than does the pig GAPD 406-nm origin, however. Trp-193 has many more polar neighbors than does Trp-84, and on this basis we assign the 406-nm origin of pig GAPD to Trp-193. The remaining Trp-84 is the site whose phosphorescence and ODMR we do not observe in the pig and rabbit GAPD, on the basis of this assignment.

We now will discuss the relationship of the observed parameters with the neighboring group interactions that each Trp may undergo on the basis of these assignments.

Neighboring Group Interactions. A general correlation between zfs and the 0,0-band wavelength has been observed for Trps in proteins, with D becoming smaller and E becoming larger with increasing wavelength (Hershberger et al., 1980). Shifts in phosphorescence wavelength and zfs may have a common Stark effect origin (Clark & Tinti, 1980). Two models for this (Lemaistre & Zewail, 1979; van Egmond et al., 1975) predict a linear correlation between the zfs and the phosphorescence wavelength. Specific interactions with neighboring residues may perturb the electron distribution, directly affecting the spin–spin dipolar interaction that is primarily responsible for the zfs of Trp. E is likely to be largely determined by the spin density on the C=C group of the five-membered indole ring (Zuclich, 1970; Harrigan & Hirota, 1975). The lowest E value previously observed for Trp (0.0387 cm^{-1}) was for the C subunit of protein kinase (Ross et al., 1980). In that case, it was suggested that the small value of E might be due to an interaction of Trp with phosphate bound to the protein. Although GAPD binds phosphate, it appears that the anion binding sites are not particularly close to any of the Trp.

The 409-nm Trp of pig (and rabbit) GAPD, which we have assigned to Trp-193, has a considerably smaller E value than even the C-subunit Trp. This is in contrast to Trp-193 of yeast GAPD, with its very typical zfs. It is of interest to examine the neighboring residues of pig GAPD for possible interactions that might strongly affect E and to compare these with interactions in yeast GAPD. Unfortunately, the orientation of the residues in the neighborhood of Trp-193 in pig and yeast GAPD and their distances from the indole ring are rather uncertain, since there are some significant changes in this region in going from the X-ray crystallographically investigated lobster to the pig or the yeast enzyme. Olsen et al. (1975) suggest that "changes in the character of residue located in the S-shaped loop", in which Trp 193 is located, "... might be the cause of differences in subunit cooperativity between the different species". Our ODMR results suggest that Trp-193 in yeast GAPD is solvent exposed, rather than buried in the subunit contact region, while the converse appears to be the case in the pig and rabbit enzymes. One thing that is apparent from Table III is that particularly in pig GAPD, the environment is highly polar and contains several charged groups that may affect E. Of particular interest are Arg-194 and Asp-195, which may form a salt link. The Trp-193 environment of yeast GAPD is rather polar also, but residue 195 is Gly, so the salt link cannot occur. The permanent dipole created by the nearby salt link in the pig enzyme may be a factor in the very small value of E exhibited by the 406-nm Trp.

Other possible perturbers of Trp-193 that should be mentioned are His-38, found only in pig GAPD, and Asp-276, Tyr-39, and Tyr 42, found in lobster, pig, and yeast GAPD. His-38 will probably only be protonated, and thus charged, if it interacts with Asp-276. Whether it does or not is uncertain. An interaction with the nearby tyrosines might also affect Trp-193. Tyr-39 and Tyr-42 lie in planes roughly 3–4 Å above and below the Trp. Their exact positions are not clear in the X-ray crystal structure. In addition to the different interactions with charged neighbors, the Trp-193 of pig and yeast GAPD differ in the possibility of hydrogen bonding. Trp-193 in lobster is hydrogen bonded to Asp-277. In pig, Asp-277 is replaced by Gln, also a polar residue. In yeast GAPD, residue 277 is nonpolar Ala.

Serine-281 in the blue subunit hydrogen bonds with Asn-202 in the red subunit. It has been proposed that reaction of mercurials with Cys-281 in the rabbit and pig enzymes may cause disruption of subunit contacts (Olsen et al., 1975). However, Trp-193 also participates in hydrogen bonding across the red–blue boundary. If Trp-193 has been correctly assigned as the 406-nm tryptophan, then dissociation into dimers must not be occurring in our $CH_3Hg(II)$–GAPD samples of rabbit and pig GAPD, since the 406-nm ODMR signals are completely unaffected upon the addition of mercurials.

The 416-nm Trp, which we have assigned to Trp-310 on the basis of the methylmercury perturbation, also has a small E value for its 0,0-band wavelength in all three species. In each enzyme, hydrogen bonding occurs between Trp-310 and neighboring Asp-293. Both residues are conserved in all the GAPDs that have been sequenced (lobster, yeast, pig, *B. stearothermophilus*, *Thermus aquaticus*, chicken, and human).

In general, however, the fact that a Trp participates in hydrogen bonding is not sufficient to give it a very small E value. The nature of the hydrogen acceptor is significant. Hydrogen bonding with water, which presumably occurs in solvent-exposed Trp, results in a rather large value of E. On the other hand, hydrogen bonding with charged groups, such as aspartate, may effect a large reduction of E. It is possible that electric fields originating at charged atoms located in the indole plane may induce mixing of T_1 with T_n ($n > 1$) states with $^3(\pi, \pi^*)$ configurations. Such Stark mixing would perturb primarily the value of E.

The zfs of the 416 nm Trps in pig, rabbit, and yeast GAPDs are quite similar, and as noted before, somewhat anomalous. Since the environment around Trp-310 is highly conserved among species, it is reasonable to believe that the 416-nm origin arises from Trp-310 in all three species. While the ODMR line widths of yeast GAPD are extremely narrow, as one would expect from a buried Trp, those of pig GAPD are significantly wider. Although the sequences of the monomers are identical, lobster GAPD is shown by its crystal structure to be asymmetrical; that is, it exists as an asymmetric pair of symmetric dimers. It is not known whether this asymmetry exists in the apoenzyme or whether it is induced by NAD binding (Henis & Levitski, 1980). (*B. stearothermophilus*

GAPD is symmetrical both as the holoenzyme and as the apoenzyme (Biesecker et al., 1977).) As a consequence of the asymmetry that may occur in some species, the environment of Trp may differ slightly between subunits. If this occurs in pig GAPD, but not in yeast, the resulting site heterogeneity provides one possible explanation for the larger line width in the pig enzyme. A similar explanation has been advanced for the line widths observed in alcohol dehydrogenase (Rousslang et al., 1979).

The 416-nm origins attributed to Trp-310 are quite red-shifted. Interaction with Cys-281 was suggested originally as a possible cause for the red-shifted 416-nm 0,0-band in rabbit GAPD (Hershberger & Maki, 1980). Yeast GAPD, however, also has an origin at 416 nm. Since this enzyme has Ser rather than Cys at position 281, interaction with sulfur cannot play a significant role in determining the 0,0-band wavelength of this Trp. Tryptophan-310 is in a rather nonpolar environment. It is also parallel to and about 4.2 Å away from Phe-283. This stacking interaction could contribute to its red shift. Another possible contribution to the red shift could arise from hydrogen bonding between Trp-310 and Asp-293 as discussed earlier.

Since we have shown that only two Trps emit detectable phosphorescence in pig GAPD and we have associated Trp-310 with the 416-nm origin using the heavy atom perturbation, either Trp-84 or Trp-193 must be largely quenched. As we will discuss below, Trp-84 has a more probable quenching mechanism than does Trp-193. Quenching of phosphorescence could occur by several mechanisms. These might include energy transfer to another chromophore or interaction with other residues or cofactors that might cause an increase in radiationless decay processes or the loss of an electron from the excited state. No likely acceptor for quenching by energy transfer is apparent. Quenching of Trp fluorescence on coenzyme binding has been observed (Velick, 1958). NAD is not a quencher of Trp-84 in the rabbit (or pig) enzyme, since we observed no effect of the removal of NAD on the GAPD phosphorescence. Spectral overlap between Trp and NAD is so small that energy transfer would be expected to be negligible at the distances between these chromophores in GAPD (Abdallah et al., 1978; Montenay-Garestier, 1975).

There is little information in the literature concerning phosphorescence quenching of Trp by interaction with neighboring amino acids. Several fluorescence quenching interactions have been reported for Trp, however. These have been reviewed by Longworth (1971). Molecules that are good electron scavengers are reported to be good quenchers of indole fluorescence (Steiner & Kirby, 1969). Among these are the His cation and Cys, which are considerably better quenchers than other amino acids. Since the only Trp that is close to a cysteine is Trp-310, and this is known not to be quenched, a more likely candidate for a quencher is protonated His. Besides forming a charge transfer complex, His probably quenches fluorescence and phosphorescence by electron transfer from the excited Trp (Steiner & Kirby, 1969). Both Trp-193 and Trp-84 have His in their general vicinity (see Tables III and IV). Although we cannot determine the precise relationship between Trp-193 and His-38 in the pig enzyme from the information we have, the direct replacement of Glu-38 in the lobster structure with His would place the latter more than 10 Å from the indole ring. His-38 may or may not be protonated. With a substantial, but not impossible, relocation from the Glu-38 position of the lobster structure, His-38 could hydrogen bond with Asp-276. This would raise its pK_a above 6, the value in free histidine, and thus it could be protonated at ca. pH 7. Tryptophan-84, on the other hand, appears to interact closely with His-108. In the lobster GAPD crystal structure, the histidine lies about 3 Å above the N and C-3 of the indole ring. Pig GAPD is very similar to the lobster enzyme in this region, with the only change being the replacement of Ile-92 with Val. His-108 is almost certainly protonated. The crystal structure of lobster GAPD shows that His-108 is hydrogen bonded to Glu-94. Such an interaction would greatly raise the pK_a. A similar His has been reported to be still entirely protonated at pH 9 (Scholberg et al., 1980). Shinitzky & Fridkin (1969) suggest a structure for the Trp–His charge transfer complex where the C(2)–C(3) bond of indole, believed to be the electron-donating region (Shifrin, 1968), lies directly under the positively charged center of the imidazolium ring. This is only slightly different from the configuration of Trp-84 and His-108 seen in the lobster GAPD structure. In subtilisin Novo (Shopova & Genov, 1983), His-238 appears to completely quench the fluorescence of Trp-241. According to the X-ray structure, the two residues are parallel and the average distance between them is about 3.5 Å. Although His-108 also occurs in yeast GAPD, none of its Trps is completely quenched. Instead, Trp-84 has an extremely red-shifted phosphorescence origin. There are two differences between pig and yeast GAPD that may be sufficient to move His-108 far enough from Trp-84 so that quenching is incomplete. These changes are the replacement of Glu-94 in the pig enzyme with Asp, which is one carbon shorter, and the replacement of Val-92 in the pig enzyme with Ala. His-108 might be expected to move farther away from Trp in order to remain hydrogen bonded to the smaller Asp-94. The His and Asp would be expected to stay in close contact for energetic reasons, since they are the only charged groups in an otherwise hydrophobic region.

Histidine's positive charge in the vicinity of Trp-84 in the yeast GAPD may still have an effect on the Trp triplet state. The phosphorescent state is believed to be ^3L_a (Konev, 1967), which is largely a localized excitation involving the ethylenic 2–3 bond (Lami, 1977). The excited state contains a considerable amount of diffuse, Rydberg character. The stabilization of the diffuse electron density by the positively charged His-108, along with the polarizable character of other close neighbors of Trp-84, could be responsible for the extreme red shift we see in the 420-nm origin of yeast GAPD.

Summary and Conclusions

We have identified Trp-310 with a particular resolved phosphorescence 0,0-band of GAPD (416 nm) using methylmercury(II) as a probe. Line width and 0,0-band wavelength considerations allow us to make reasonable assignments for the other Trps. Our arguments lead to the conclusion that Trp-84 in pig and rabbit GAPD is quenched, apparently by an interaction with His-108. Trp-84 in yeast GAPD is not quenched, although this enzyme also contains His-108, but its phosphorescence is observed to be extremely red-shifted. Perturbation by a more distant His-108 may be partially responsible for the phosphorescence red shift and anomalous zfs. Trp-193 in pig and rabbit GAPD (406 nm) shows an unusually small E value. Local electric fields may be responsible, particularly one produced by a possible salt link between nearby Arg-194 and Asp-195. This salt link cannot occur in yeast GAPD in which residue 195 is Gly. The most blue-shifted 0,0-band (409 nm) of yeast GAPD is assigned to Trp-193. The ODMR and phosphorescence characteristics of this Trp indicate that it is solvent exposed. This is in contrast to the corresponding site in the pig and rabbit enzymes (406 nm), where the data are consistent with Trp that is buried in the subunit contact region in which it undergoes polar interactions.

Registry No. GAPD, 9001-50-7; Trp, 73-22-3.

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