Long-Lived Tryptophan Fluorescence in Phosphoglycerate Mutase†

Joseph A. Schauerte*,‡ and Ari Gafni§
Institute of Gerontology and Department of Biological Chemistry, University of Michigan, Ann Arbor, Michigan 48109
Received July 14, 1988; Revised Manuscript Received October 28, 1988

ABSTRACT: Phosphoglycerate mutase (PGM; EC 2.7.5.3) isolated from rat and rabbit muscle has been shown to possess an unusually long-lived fluorescence component when excited by ultraviolet light below 310 nm. On the basis of spectral and physical measurements, this 16.4 (±0.2) ns fluorescence lifetime at room temperature is assigned to a tryptophan residue in an unusual environment. The emission profile of this long-lived tryptophan is red shifted from the other tryptophans of PGM by approximately 25 nm. PGM has been crystallized and sequenced from yeast where it has been shown to be a tetramer with 29K subunits. However, we have not been able to detect the existence of an unusually long-lived fluorescence component in the yeast isomer. The long fluorescence lifetime is lost upon denaturation of rabbit PGM and is partially restored upon introduction of the protein to a nondenaturing environment, suggesting the long lifetime is not the result of a covalent modification. The PGM molecule was studied by a number of techniques including time-resolved tryptophan fluorescence, quenching studies of tryptophan fluorescence, and enzyme activity studies. The long-lived fluorescence has been shown to be statically quenched by Br^-, I^-, and Cu^{2+} in the submillimolar region while the acrylamide quenching shows the tryptophan is marginally accessible to solvent. Characterization of the long-lived fluorescence and its possible sources are discussed.

Phosphoglycerate mutase (PGM; EC 2.7.5.3)[1] catalyzes the conversion of 3-phosphoglycerate to 2-phosphoglycerate in glycolysis. The reaction involves phosphorylation at one of two active-site histidine residues by 2,3-diphosphoglycerate and subsequently a transfer of the phosphate group between carbons of the substrate according to the postulated "Ping-Pong" mechanism (Grisolia & Cleland, 1968). PGM isolated from mammalian sources is thought to have a structural similarity to yeast PGM except that the active species is a dimer of molecular weight 60000 compared to a tetrameric structure for yeast PGM and monomeric wheat germ PGM (Grisolia, 1962). PGM has been crystallized from yeast (Winn et al., 1981) and was shown to be comprised of α-helices and β-pleated sheets in a configuration that characterizes the familiar α/β structural class of proteins.

The structure of PGM and the location of its active site have been determined from crystal structure studies and from the identification of the active-site histidine that is phosphorylated (Han & Rose, 1979). Several studies have investigated the involvement of ions and cofactors on the enzyme activity of PGM. A study by Rose and Kaklij (1984) included an evaluation of the univalent anions Cl^- and I^-, showing these anions to increase the enzyme activity up to an ionic concentration of several millimolar while at higher concentrations, these anions inhibited PGM activity. Two univalent anions were needed for complete activation while a single divalent anion (*p*-hydroxybenzoate) was shown to activate the enzyme. These anions have been shown to affect PGM activity by lowering the K_m for the substrate 3-phosphoglycerate. The effect of these anions on the K_m is the result of enhanced access of the substrate to the active site due to PGM conformational changes brought about by the binding of the small anions. The anion binding site was not established; however, it should be noted that an arginine residue has been shown to be essential for enzyme activity (Borders & Wilson, 1976), and covalent modification of this arginine is prevented by the 2,3-diphosphoglycerate or 3-phosphoglycerate.

In addition, Cu^{2+} has been shown to affect the activity of muscle PGM (Cowgill & Pizer, 1956). Cu^{2+} is known to interact with sulfhydryl groups, which have been shown to be essential for activity of the muscle isomer (Ray & Peck, 1972). Bartrons and Carreras (1982) discussed the difference between isoenzymes of PGM from mammalian muscle tissues in terms of their differences in heat stability and sensitivity to sulfhydryl reagents. The brain (type B) and muscle (type M) differ in terms of an essential -SH group necessary for activity in muscle PGM. The muscle isoenzyme is fully inactivated by sulfhydryl oxidizing reagents such as tetrathionate (Bartrons & Carreras, 1982), while the brain isoenzyme is resistant.

In order to assess the binding of cofactors and anions to PGM, tryptophan fluorescence characteristics were studied. In this paper, we describe the effects of I^-, Br^-, and Cu^{2+} binding on an unusually long-lived fluorescence found in rat and rabbit PGM, but not found in yeast PGM. The long-lived fluorescence is attributed to a tryptophan residue, and its fluorescence properties depend upon experimental conditions that are known to modulate PGM enzyme activity.

Materials and Methods

Rabbit phosphoglycerate mutase was purchased from Sigma Chemical Co. (St. Louis, MO). The enzyme activity of rabbit PGM was 550 units/mg of protein, compared to the value of 605 units/mg given by Sigma. Phosphoglycerate mutase from rat was isolated by the procedure of Scopes (1977) with the modification that PGM was selectively eluted from a final CM-Sephadex column with 1 mM NaI. The purities of Sigma rabbit PGM and rat PGM were greater than 95% on the basis of polyacrylamide gel electrophoresis (results not shown).

† This work was supported by the Presidential Initiative Fund award to the University of Michigan from the W. K. Kellogg Foundation and by a grant from the U.S. Office of Naval Research. J.A.S. was supported by a training grant from the National Institute on Aging (Contract T32AG00114).
‡ Institute of Gerontology.
§ Department of Biological Chemistry.

[1] Abbreviations: BES, 2-[bis(2-hydroxyethyl)amino]ethanesulfonic acid; PGM, phosphoglycerate mutase; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; nm, nanometer.

0006-2960/89/0428-3948$01.50/0

Yeast PGM was isolated according to the procedure of De La Morena et al. (1968). Other materials were reagent grade.

Steady-state fluorescence measurements were performed on a Spex Fluorolog II fluorometer with a 0.25-m single-grating excitation monochromator and a 0.25-m double-grating emission monochromator. The fluorescence excitation scans were done in the ratio mode. The band-pass for excitation was 0.9 nm and 1.2 nm for the emission. Optical densities of the protein samples used were approximately 0.2–0.4 at the excitation wavelength.

Time-resolved fluorescence was measured by the correlated single photon counting method (Ware, 1971). The fluorescence decays were measured by a Photon Technology International (PTI) picosecond fluorescence system (London, Ontario). The fluorescence excitation light source was a frequency-doubled (KDP crystal "R6G", Inrad) laser pulse from a Coherent Model 701-3 dye laser using rhodamine-6G dye. The dye laser was driven by a Quantronix Model 416 Nd:YAG laser with a pulse stabilizer and an acousto-optical pulse modulator yielding a pulse rate of 76 MHz. The 1.06-μm radiation from the Nd:YAG was frequency doubled to 532 with a time-average energy output of 0.8–1.0 W of 532-nm radiation with a pulse width of approximately 70 ps. The dye laser was equipped with a Coherent 7220 cavity dumper to reduce the pulse rate to 1 MHz with a pulse width of approximately 10 ps. The fluorescence was measured through a 0.25-m PTI monochromator, with an acquisition rate less than 2% of the fluorescence excitation frequency. The PTI picosecond fluorescence system utilized a Tennelec TC 536 counter and timer, a Tennelec TC 455 Quad constant fraction discriminator for the fluorescence emission "start" pulse, and an Ortec Model 457 biased time to pulse height converter. The "stop" pulse was from the output of the dye laser and was detected by a Hamamatsu 1P21 photomultiplier tube with discrimination from an Ortec Model 436 discriminator. The fluorescence "start" pulse was detected by a photon counting Hamamatsu 1P28 photomultiplier tube in a Pacific Instruments Model 33 cooled PMT housing unit, operating at −20 °C. Data were stored on a Tracor Northern Model TN-7200 multichannel analyzer operating in the pulse height analysis mode. Measurements typically contained a total of 1 million counts. The decay kinetics were deconvoluted and analyzed by a Marquardt nonlinear least-squares algorithm (Marquardt, 1963). Due to a wavelength dependence of the instrument response function, this response, as determined by scattering from a sample of Ludox spheres at the excitation frequency, would alter the results of the deconvolution at the longer fluorescence wavelengths. We used a mimic technique (James et al., 1983) to reconstruct an excitation time profile from the fluorescence decay of a sample with known decay kinetics. *p*-Terphenyl in cyclohexane was used here, having a short lifetime and excitation and emission profiles similar to the amino acid tryptophan. A minor contaminant in *p*-terphenyl amounting to less than 0.1% of the total counts was not considered in the mimic as it had no measurable effect on the calculation of the lifetimes.

The decay-associated spectra were derived from time-resolved emission spectra of PGM. These spectra were deconvoluted, and the relative intensities of the long-fluorescence decay and the remaining components were scaled at each wavelength in relation to the steady-state intensity at the wavelength according to Donzel et al. (1974)

$$I_i(\lambda) = I(\lambda)A_i(\lambda)\tau_i / \sum_i A_i(\lambda)\tau_i$$

where $I(\lambda)$ is the steady-state fluorescence, $A(\lambda)$ and τ are the preexponential factor and lifetime, respectively, and the subscripts represent the individual decay components. The short decay components were not individually resolved.

The enzyme assay procedure used was provided by Sigma Chemical Co. (St. Louis, MO). This assay couples the activity of PGM to pyruvate kinase, enolase, and lactate dehydrogenase and measures the oxidation of NADH spectrophotometrically at 340 nm. The assay is done in the presence of 1.3 mM 2,3-diphosphoglycerate, which is a cofactor for the reaction catalyzed by PGM. The specific activity of PGM is defined as the number of micromoles of 2-phosphoglycerate converted to 3-phosphoglycerate per minute per milligram of protein. Enzyme concentrations were determined by Bio-Rad protein assay using bovine serum albumin as standard.

The assessment of the quantum yield of tryptophan fluorescence in PGM was based upon a value of 0.14 for the quantum yield of tryptophan in water (Eisinger, 1969). Values for the quantum yield of PGM were determined with excitation at 280 or 290 nm and using the integrated fluorescence emission spectra from 300 to 500 nm. The excitation at 280 nm might also include contributions from tyrosine residues while the excitation at 290 nm would preferentially excite tryptophans.

Protein Denaturation/Renaturation. PGM in 10 mM BES, 5 mM mercaptoethanol, and 0.5 mM EGTA at pH 7.2 was denatured by addition of guanidine hydrochloride to a final concentration of 4 M. Renaturation was done by a 40-fold dilution of the denatured PGM into denaturant-free buffer. Final protein concentrations for renaturation were kept below 5 μg/mL to minimize precipitation of the enzyme.

Results

Fluorescence lifetime studies of rat and rabbit PGM reveal an unusually long-lived fluorescence component not normally measured in protein molecules. This component can be seen in the top panel of Figure 1, where PGM is excited at 290 nm and the fluorescence is measured at 350 nm. In an attempt to determine whether the long-lived fluorescence was the result of a covalent modification of an amino acid residue or the result of an unusual microenvironment, the PGM molecule was denatured in 4 M guanidine hydrochloride at pH 7.2. The bottom panel of Figure 1 (and Figure 2 for steady-state fluorescence) shows that the long-lived fluorescence is destroyed in the denatured molecule. When PGM was allowed to refold following a 40-fold dilution into a nondenaturing buffer, the molecule partially regained the long-lived fluorescence decay component of 16.4 (±0.2) ns; however, the appearance of turbidity indicated that some of the PGM was irreversibly denatured. The yield of refolded active PGM becomes very low above PGM concentrations of approximately 4 μg/mL [see Hermann et al. (1983)].

The fluorescence spectra associated with the long and short decay components are compared to the steady-state fluorescence of PGM in Figure 2. It can be seen that the short-lifetime fluorescence (all fluorescence components excluding long-lived fluorescence) has a maximum at approximately 345 nm, while the long-lived fluorescence component peaks at a longer wavelength of 368 nm. There also is some difference between the maximum excitation wavelengths for the long-lived and short-lived fluorescence components. The difference between the excitation profiles for fluorescence observed at several emission wavelengths is shown by excitation difference spectra in Figure 3A. It can be seen that the red-shifted fluorescence emission is preferentially excited by the red-shifted absorption shoulder of PGM. 4 M guanidine hydrochloride, which destroys the long-lived fluorescence, removed virtually

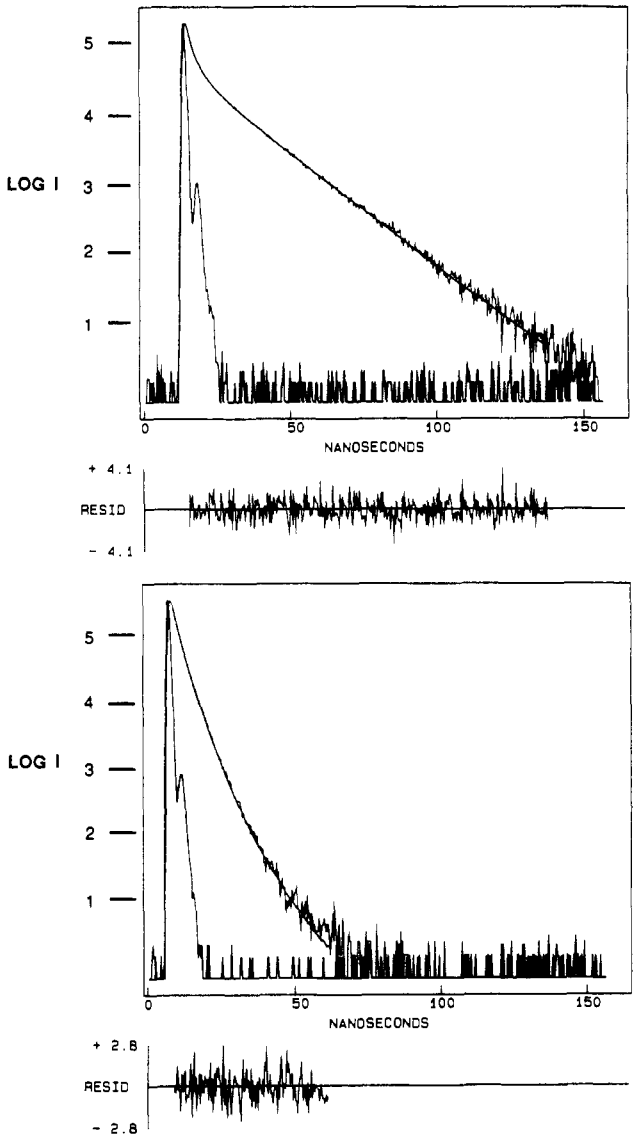

FIGURE 1: Fluorescence decay kinetics of rabbit PGM at 20 °C. Excitation wavelength = 290 nm; emission wavelength = 350 nm. (Top panel) PGM under native conditions (in 10 mM BES, 5 mM mercaptoethanol, and 0.5 mM EGTA, pH 7.2). A three-exponential decay fits the data; $A_1 = 0.60$, $T_1 = 1.37$ ns, $A_2 = 0.24$, $T_2 = 4.41$ ns, $A_3 = 0.16$, $T_3 = 16.5$ ns. $\chi^2 = 1.10$ for this fit. (Bottom panel) PGM under denaturing conditions; the above buffer with 4 M guanidine hydrochloride, pH 7.2. This decay is fit with a three-exponential decay with $A_1 = 0.52$, $T_1 = 1.6$ ns, $A_2 = 0.46$, $T_2 = 3.8$ ns, $A_3 = 0.02$, and $T_3 = 8.1$ ns. $\chi^2 = 1.03$ for this fit.

all of the excitation wavelength dependence of the remaining fluorescence. 4 M guanidine hydrochloride also induces alterations in the absorption spectrum of PGM. Figure 3B indicates that native PGM has a pronounced absorption shoulder at 290 nm that is removed in denaturing conditions. This may be an indication of an unusual ground state for one of the tryptophan residues.

Figure 4 presents the quenching of the long-lived fluorescence component in PGM by acrylamide (A) and by I^- (B). The Stern–Volmer plot in Figure 4A clearly shows that quenching by acrylamide is dynamic and the rate constant K_q calculated from the data was 1.2×10^8 M^{-1} s^{-1}, suggesting the fluorophore is moderately accessible to solvent. This is substantiated by our finding that the PGM fluorescence lifetime in D_2O was increased by less than 4% compared to that in H_2O, as opposed to the fluorescence decay time of free tryptophan in water which is increased by a factor of 2 (Kirby & Steiner, 1970). It was determined that I^- was very effective

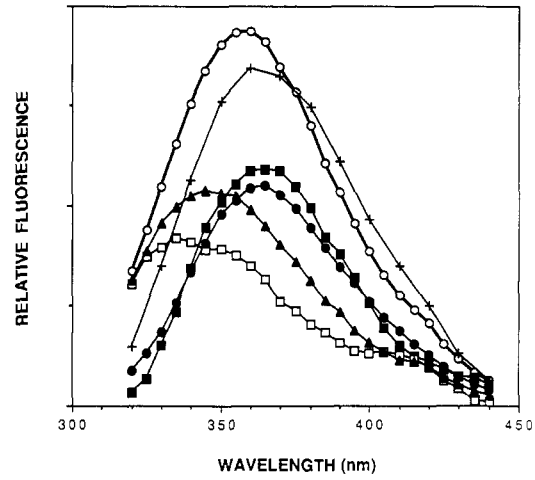

FIGURE 2: Relative fluorescence emission of phosphoglycerate mutase excited at 290 nm. (○) Steady-state fluorescence of PGM. (■) Decay-associated spectrum of the long-lived PGM fluorescence (lifetime = 16.4 ns at 20 °C). (□) Decay-associated spectrum of the remaining "short" fluorescence lifetime components (<6 ns). The individual "short" components were not resolved in this figure. (▲) Total PGM steady-state fluorescence in presence of 1.0 mM NaI. (●) Total PGM fluorescence minus four-fifths of the fluorescence in the presence of 1 mM I^- to estimate the fluorescence spectrum lost with I^- binding to PGM. (+) PGM fluorescence in the presence of 4 M guanidine hydrochloride. This sample lost all of the long fluorescence decay component.

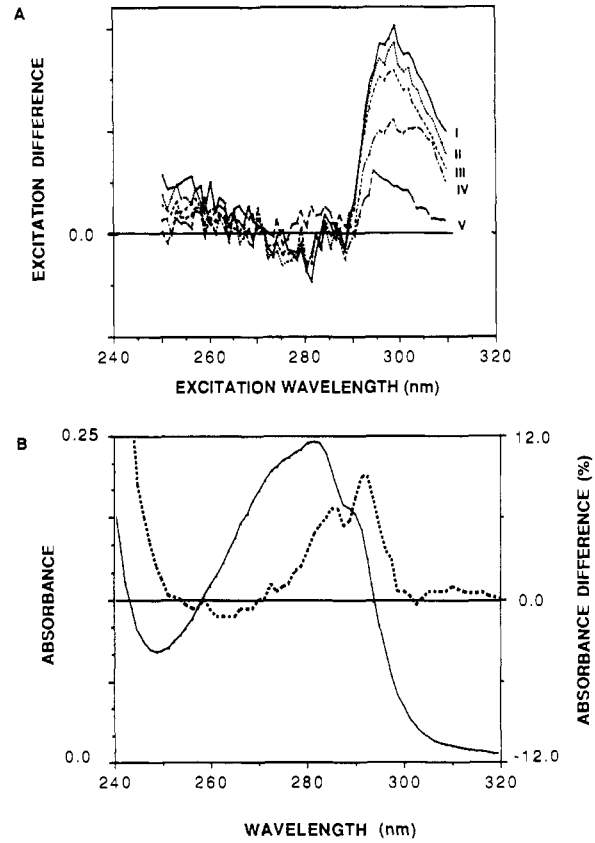

FIGURE 3: (A) Difference between the fluorescence excitation spectra of PGM for emission at 320 nm and at (I) 400, (II) 380, (III) 360, and (IV) 340 nm. In addition, the difference between PGM fluorescence excitation viewed at emission wavelengths of 400 and 320 in the presence of 4 M guanidine hydrochloride is shown (V) to indicate the importance of the native conformation for the fluorescence excitation curves. (B) Ultraviolet absorption spectrum of rabbit PGM under native conditions (solid line). Difference spectra of PGM upon denaturing PGM in 4 M guanidine hydrochloride pH 7.2 (dotted line).

at quenching the long-lived fluorescence of PGM. Analysis of iodide quenching of this fluorescence (Figure 4B) indicates

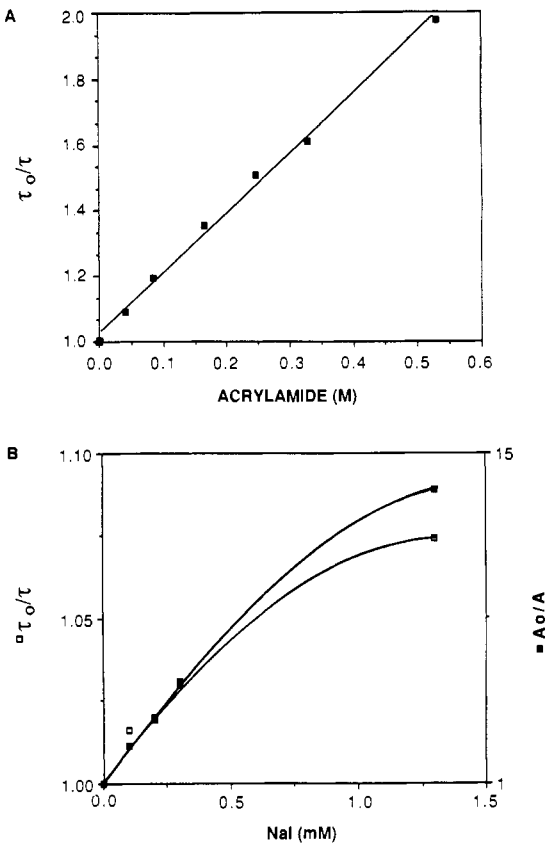

FIGURE 4: Quenching plots of the long-lived fluorescence component of PGM. (A) Effect of acrylamide on PGM fluorescence. The τ_0 was 16.4 ns, and the calculated $K_q = 1.2 \times 10^8$ M^{-1} s^{-1}. (B) Effect of I^- on PGM fluorescence. (□) Stern–Volmer plot presenting the dependence of the lifetime on I^- concentration. (■) Dependence of the preexponential factor on I^- concentration. Calculation of the quenching constant based upon the lifetime gives $K_q = 6.0 \times 10^9$ M^{-1} s^{-1}, while the binding constant calculated from the changing preexponential factor yielded $K_d = 60$ μM.

the existence of two quenching mechanisms. Figure 2 shows that a concentration of 1.0 mM I^- dramatically quenched the steady-state fluorescence of PGM and subsequent fluorescence lifetime analysis showed that the long-lived fluorescence was reduced approximately by a factor of 10 and produced a new short-lived fluorescence decay with a lifetime of 3.5 ns. this quenching, however, is predominantly static. To illustrate, both the preexponential factor and the lifetime of the long-lived component are plotted versus iodide concentration in Figure 4B. The dissociation constant calculated from the preexponential factor is $K_d = 60$ μM while the quenching rate constant calculated from the fluorescence lifetime is $K_q = 6.0 \times 10^9$ M^{-1} s^{-1}. Br^- also competitively binds to this site and induces a change in the fluorescence lifetime from 16.4 (±0.2) ns to 11.0 (±0.2) ns. In addition, it was determined that Cl^- competes with I^- binding and will reverse the effects of I^- quenching of the long-lived fluorescence. The binding constants of Br^-, I^-, and Cl^- are within 20% of each other, with Cl^- binding having a negligible affect on the long-lived fluorescence lifetime.

We attempted to evaluate the quantum yield of the long-lived fluorescence by exciting the molecule at 280 or 290 nm. Assuming a quantum yield for tryptophan in water of 0.14 (Eisinger, 1969), the quantum yield of native PGM fluorescence was determined to be 0.11. With the addition of 1.0 mM I^-, the quantum yield is reduced by 38% to 0.07. Assuming there are five tryptophans in mammalian PGM with only the long-lived tryptophan being affected by this concentration of I^-, the quantum yield of this long-lived tryptophan in the absence of I^- is 0.31 (±5%). The addition of 1 mM I^- produces the new total steady-state fluorescence spectra shown in Figure 2. We attempted to correlate the affects of I^- on the steady-state fluorescence profiles with the results from the decay-associated spectrum performed with the fluorescence lifetime instrumentation. The difference between the steady-state fluorescence of native PGM and the fraction of fluorescence spectrum lost in the presence of 1 mM I^- can be estimated from the steady-state fluorescence spectra of Figure 2. Assuming that rabbit PGM has five tryptophans that are all equally excited at 280 nm (and that I^- binding does not produce a conformational change that affects the remaining tryptophan residues), then the long-lived fluorescence profile that is quenched by I^- is found by subtracting four-fifths of the fluorescence profile in the presence of 1 mM I^- (since four-fifths of the tryptophans are unaffected by low concentrations of I^-) from the total steady-state fluorescence. This result is also shown in Figure 2. It can be seen that this calculated fluorescence profile is comparable to the decay-associated spectrum of the long-lived fluorescence determined by fluorescence lifetime analysis.

Table I: Affect of Cu^{2+} on the Long-Lived Fluorescence Decay and Enzyme Activity of PGM

$[Cu^{2+}]$ (mM)	% enzyme act.	τ/τ_0 long decay
0.0	100	1.0
0.5	79	
1.0	68	0.67
2.0	41	0.51

The effect of Cu^{2+} on the lifetime of the long-lived fluorescence corresponds to its effect on enzyme activity of PGM. Both values are reduced by a factor of 2 at approximately 2 mM Cu^{2+}, as shown in Table I. Unlike I^- binding to PGM, however, the quenching by Cu^{2+} is not accompanied by a shift in the fluorescence emission. Addition of Cu^{2+} at 2.0 mM resulted in a turbid sample, making further titration difficult.

The quenching mechanisms of the long-lived fluorophore were analyzed by determining the temperature dependence of the fluorescence lifetime. The quenching of fluorescence may be distinguished into temperature-dependent and -independent mechanisms, the former being characterized by an activation energy for the quenching process (Kirby & Steiner, 1970). Figure 5 shows the determination of the activation energy for the temperature-dependent fluorescence quenching of the long-lived tryptophan by a plot of $\ln [(Q^{-1} - 1) - A_0]$ vs $1/T$ where $Q = t/t_0$. t_0 was determined to be 53 ns based upon a quantum yield of 0.31 and a lifetime of 16.4 ns at room temperature. A_0 represents the proportion of the temperature-independent quenching mechanism. The best fit for the A_0 parameter was 0.7, and this value was substituted for A_0 in Figure 5. The activation energy determined from the slope of this plot is 4.2 kcal/mol. This compares to a value of 6.6 kcal/mol for acetyltryptophanamide in water or 3.1 kcal/mol for acetyltryptophan methyl ester in dioxane (Kirby & Steiner, 1970).

Discussion

Source of the Long-Lived Fluorescence in PGM. Rat and rabbit muscle PGMs were found to display an unusually long fluorescence decay. Our data imply that the fluorophore responsible is most likely a noncovalently modified tryptophan residue. This tryptophan appears to be in an environment that substantially affects its ground state as shown by the absorption spectra of rabbit and rat PGM which are significantly different

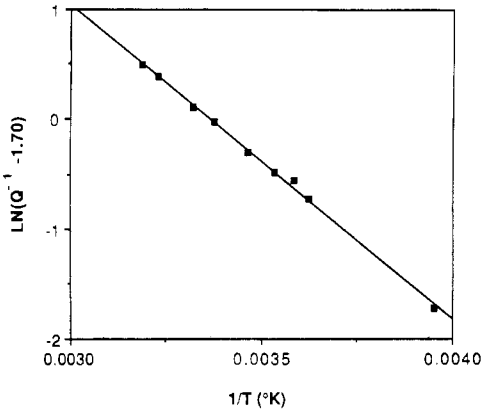

FIGURE 5: Determination of the activation energy for the temperature-dependent quenching of the long-lived fluorescence of PGM. The activation energy is derived from the slope of ln $[(Q^{-1} - 1) - A_0]$ versus $1/T$ based upon the relation $Q^{-1} - 1 = A_0 + A_1 \exp(-E_a/RT)$, where $Q = \tau/\tau_0$, E_a is the activation energy, A_0 is the temperature-independent quenching process, and A_1 is the preexponential factor for the temperature-dependent quenching process (Kirby & Steiner, 1970). τ_0 for the long-lived fluorescence was determined to be 53 ns (see text for details). The best-fit value of A_0 was determined to be 0.70. The activation energy for the temperature-dependent of the long-lived fluorescence calculated from the slope of this curve is 4.2 kcal/mol.

than that of the yeast isomer, which does not show the long-lived tryptophan fluorescence component. There are five tryptophans in the yeast PGM monomer, two tryptophans in the intersubunit interface, and two tryptophans near the active site. It is unclear whether these residues are in different environments in rat and rabbit PGM than they are in yeast PGM or whether a different tryptophan may be responsible.

The long-lived tryptophan fluorescence is quenched by both the anions Br^- and I^- and the cation Cu^{2+}; however, it is unlikely that the quenching mechanism is similar for the three ions. Cu^{2+} is an effective electron scavenger and may facilitate an electron transfer from the excited tryptophan residue. In the case of the static quenching by Br^- and I^-, it is possible that these anions are attracted to a positively charged site and may interact with a tryptophan at that site. It is interesting to note that Br^- and I^- do not quench the long-lived tryptophan completely but rather shorten the fluorescence lifetime. This partial quenching indicates that the iodide binding site is at some distance from the long-lived tryptophan residue. Br^- and I^- in direct contact with tryptophan would be expected to completely quench the long-lived fluorescence. In addition, bromide and iodide binding to PGM affect the long-lived fluorescence without substantially altering the absorption spectrum in the 290-nm region, indicating no dramatic alterations in the ground state of the chromophore. The pronounced absorption band at 290 nm has not been directly correlated with the long lifetime but remains unique to the PGMs so far studied that have the long lifetime. Alternatively, binding of charged species to PGM may alter the conformation of the enzyme such that the environment of a tryptophan is altered without directly interacting with the residue. However, since Cl^- effectively competes with Br^- and I^- for binding to PGM without causing the quenching of the long-lived tryptophan, it seems more likely that the effect of Br^- and I^- is through proximal interactions.

Significance to Enzyme Activity. Amato et al. (1984) analyzed the structure of PGM from crystallographic studies and observed that the structure can be described as three lobes that can move with respect to each other. The active site is located at an interface of two of the lobes, and access to this site is possible from two directions and can be altered by movement of the lobe interface. The active site was shown to possess a number of arginine and lysine residues in close proximity to the histidine residue that is phosphorylated during catalysis. It has also been shown that certain lysine and arginine residues are required for activity and that two arginines are in close proximity to the active site as evidenced by their protection against covalent modification by butanedione upon addition of the PGM substrate and cofactor 2,3-diphosphoglycerate (Borders & Wilson, 1976). In addition, it has been shown that the active region of PGM has an electrostatic field gradient that may serve to direct the negatively charged substrates toward the active site (Warwicker, 1986). It is tempting to speculate that the lysine and arginine residues may play a role in the binding of the negatively charged ions that modulate the activity and accessibility of the substrate 3-phosphoglycerate to the active site. It seems plausible from the fluorescence lifetime studies presented above that the unusual tryptophan may either be in close proximity to the PGM active site or be close to an interface of one of the three lobes of PGM such that conformational changes in the structure are readily transferred to this tryptophan residue.

Rose and Kaklij (1984) showed that millimolar concentrations of univalent anions such as Cl^- and I^- activate PGM. This activation occurs with the binding of two anions by lowering the K_m for the substrate 3-phosphoglycerate. A conformational change is postulated to occur with the binding of the anions, increasing the accessibility of 3-phosphoglycerate for the active site. It should be noted that the effect of I^- on the enzyme activity occurred at higher concentrations of I^- than those necessary for effective quenching of the long-lived tryptophan fluorescence. Therefore, the binding of the first of two I^- to PGM may be the event responsible for quenching the long-lived fluorescence.

While the effect of Cu^{2+} on quenching the long fluorescence lifetime may be associated with its electron scavenging ability, Cu^{2+} is also known to form complexes with sulfhydryl groups on proteins. Since Cu^{2+} is similarly efficient at destroying enzyme activity and quenching the long-lived fluorescence, it remains to be determined whether these effects are related by structural changes in the protein or result from proximal interactions of Cu^{2+} with the active site and the long-lived tryptophan. It has been shown that there is an essential sulfhydryl group for enzyme activity and also that there is a sulfhydryl group in close proximity to the active site (Hartman & Norton, 1976).

Environment of the Long-Lived Tryptophan. One prominent spectroscopic characteristic of indoles and of the long-lived tryptophan of PGM is the large fluorescence Stokes shift. A number of theories have been presented including an excited-state-induced dipolar relaxation of the solvent (Jacyno & Kawski, 1974) resulting in stabilization of the excited state and red shifting the fluorescence. Also, formation of excited-state complexes, exciplexes, with stabilization through dipole–dipole (Hershberger et al., 1981) and charge transfer states (Walker et al., 1967) has been suggested to account for the solvent-dependent Stokes shift and the increase in the quenching efficiency in polar solvents. In addition, there is evidence that indole and tryptophan are involved in excited-state processes including photooxidation–reduction following excitation to the lowest singlet states (Masuhara et al., 1978), or photoinduced e^- ejection from the excited state (Grossweiner & Joschek, 1965). Lami (1977) suggested that solvated mixed-valence–Rydberg states could account for the Stokes shift. Static charge perturbation of indole absorbance and fluorescence has also been suggested (Illich et al., 1988). An

explanation of PGM fluorescence must account for the long fluorescence lifetime, the unusually large Stokes shift, the relatively high quantum yield, and the small activation energy for the temperature-dependent quenching processes.

We have demonstrated that the long-lived tryptophan fluorescence has red-shifted excitation and fluorescence spectra relative to the other tryptophans of PGM. Solvent effects can affect the absorption and fluorescence characteristics of the indole chromophore by perturbing the ground and/or excited states (Ananthanarayanan & Bigelow, 1969; Bailey et al., 1968). In hydrophobic environments, the L_b state is the lowest excited state followed by the L_a state which has a larger absorption oscillator strength (Strickland & Billups, 1973; Andrews & Forster, 1974). It has been noted that an absorption red shift of the L_b transition occurs with N–H hydrogen-bond formation (Catalan et al., 1986), consistent with the PGM absorption spectra. The small activation energy for the temperature-dependent quenching may be the result of L_b being the major radiative state (Glasser & Lami, 1981). Fluorescence from the L_b state, having a lower oscillator strength, would be associated with increased fluorescence lifetime, but with a smaller Stokes shift, contrary to the PGM long-lived fluorophore. Conversely, in a hydrophilic environment, the dipole moments of polar solvents interact with the excited indole chromophore and alter the energies of the excited states. Since the L_a transition is associated with a larger excited-state electric dipole moment (Sun & Song, 1977), it is more effectively stabilized by the solvent dipole moment than the L_b transition which can result in the L_a state becoming the lowest energy excited state. The molecules excited to the L_b state would relax into the L_a state such that fluorescence would occur from only one excited state, L_a, exhibiting a large solvent-induced stabilization and Stokes shift. If the L_a/L_b inversion that occurs in polar solvents establishes the L_a as the lowest energy state, then the lifetime would be associated with this larger fluorescence oscillator and hence become shorter. The L_b state is not susceptible to solvent effects and therefore will not account for the large Stokes shift (Lami, 1977), while the unperturbed L_a state will not have the long radiative rate observed. Therefore, solvent effects are not capable of explaining both the long lifetime and large Stokes shift seen in PGM tryptophan fluorescence, and an excited-state mechanism should be considered.

The formation of solvated electrons following indole excitation has been reported (Grossweiner & Joschek, 1965), consistent with increases in its electronic dipole moment in the excited state. Indole excitation is also known to increase the acidity of the N–H group such that dissociation is more favorable in the excited state (Hopkins & Lumry, 1972). As our data suggest that D_2O and acrylamide are not fully accessible to the long-lived tryptophan, it is unlikely that a solvent molecule would be the recipient of the N–H hydrogen, or of a free electron. Another group within PGM may hydrogen bond to the excited-state N–H hydrogen. Whether a neighboring group of the tryptophan may be involved in hydrogen bonding with the N–H hydrogen or a positively charged group may form an ionic association with a photoejected e^- remains unresolved. In light of the proximity of an anionic binding site to the long-lived tryptophan, a cationic acceptor may be plausible. Aubailly et al. (1972) have noted that heavy atoms can be chemical traps for photoejected e^-, this may be consistent with the influence of I^- and Br^- in reducing the fluorescence lifetime of the long-lived tryptophan. The long-lived fluorescence may be associated with the rate of recombination of the photoejected electron with the tryptophan residue. Again, the work of Kirby and Steiner (1970) does not support photoejection since the large charge separation involved would be associated with a large activation energy. Moreover, photodissociation and photoejection are efficient quenching mechanisms of the excited state and would be expected to result in a larger reduction in the quantum efficiency of the long-lived tryptophan.

There is evidence that solvent dipoles are reorientated upon excitation of indole (Milton et al., 1976). However, Walker et al. (1967) have argued that solvent–solute interactions alone cannot energetically account for the large Stokes loss in indole. There is evidence that certain molecules, such as methanol and 1-butanol, can interact with the electronically excited indole molecule. Exciplex formation between indole and other molecules can yield a stable excited-state association and result in significant fluorescence Stokes shifts. These excited-state complexes are stabilized by dipole–dipole interactions (Hershberger et al., 1981) or charge transfer (CT) interactions (Walker et al., 1967). However, the restricted solvent exposure of the long-lived tryptophan suggests that an exciplex formation would be within the PGM molecule and not involve a solvent molecule. Meech et al. (1983) have described the susceptibility of the L_a state to CT due to its higher excited-state dipole moment. This state, $CT/^1L_a$, would have an ability to interact with neighboring static charge groups. Stabilization of the excited state would reduce its energy (red shifting fluorescence), and if the molecule is not physically modified (through photoejection of e^- or hydrogen bonding of N–H), it would not be as susceptible to the more efficient quenching mechanisms. It is known that there are very efficient quenching mechanisms for the Frank–Condon excited L_a state (Lami & Glasser, 1986), and therefore this state may mix with the closely lying L_b state to produce a state with lower fluorescence oscillator strength and hence longer lifetime.

Static charge perturbations may also affect the nature of the transition dipole moments of indole and tryptophan. Andrews and Forster (1972) showed that static charges can perturb the absorption spectra of indoles, with the two lowest electronic transitions having different susceptibilities to the static charge. Ilich et al. (1988) have performed PPP-SCF/INDO molecular orbital calculations of indole and have assessed the role of static charge perturbations on the absorption characteristics of indole and correlated these effects with a tryptophan residue in ribonuclease. Both shifts in absorption and changes in the oscillator strength can result from the static charge perturbations. The effect was more pronounced for static charges that were out of the plane of the indole group. Berlman (1970) pointed out that an aromatic molecule in a charged environment may be differentially affected between the ground and excited states. He has shown that a number of molecules can undergo a change in the nuclear configuration upon excitation to alter the planar geometry of either the ground or the excited state. In the terminology of Berlman, the long-lived tryptophan of PGM might best be explained by a fluorophore of type IV wherein the molecule might be expected to lose some planar structure upon excitation. In the ground state, the binding energy of the mobile π electrons can confer stability to a planer structure whereas in the excited state, an increase in the bond distances (decrease in bond order) can allow for decreased resistance to perturbations that may distort the plane of the aromatic residue. It has been suggested that the excited state of indole carboxylic derivatives may become more extended (Mani & Lombardi, 1969) and less planar (Aaron et al., 1985). A change in the planar structure would result in a large Stokes

shift and a reduced oscillator strength (hence increasing the fluorescence decay time) for the S_1–S_0 transition. A charged group out of the plane of the molecule may accomplish both effects, maximally perturb the electronic transition moments for the lowest excited singlet states in addition to providing a static charge perturbation that could physically distort the geometry of the CT state of indole following excitation. Static charge distributions can have an effect in stabilizing an excited state with charge transfer character. If significant CT or even photoejection occurs upon excitation of the tryptophan residue, than an out-of-plane static charge interaction can distort the plane of the indole chromophore and result in both a significant Stokes shift and loss of vibrational structure that is seen with the PGM fluorescence spectra. The long fluorescence lifetime may correspond to the rate the molecule reestablishes its planar structure to fluorescence, or the fluorescence may occur from the nonplaner state. It is not possible for us to distinguish among all of the aforementioned possibilities; however, the fact that the quantum efficiency is maintained relatively high would suggest that the indole ring is probably not involved in any direct physical complex with another PGM or solvent molecule. The tryptophan residue involved in the interaction would probably be in the L_a state, as it would be more effectively stabilized by static charge interaction through the $CT/^1L_a$ state. Also, interactions between histidine and tryptophan may alter the excited-state properties of tryptophan (Shinitzky & Goldman, 1967). Aromatic–aromatic interactions as well as amine–aromatic interactions have been studied by Burley and Petsko (1985, 1986) and may provide possible groups to interact with a tryptophan residue. However, the spectroscopic nature of these interactions had not been elucidated.

Summary

The characteristics of the long-lived fluorescence of PGM are most likely described by a static charge interaction between a tryptophan residue and a second residue that perturbs both the ground and excited states of the chromophore. This interaction may lead to a CT interaction between the perturbing molecule (histidine, arginine, lysine, or cysteine) and the tryptophan molecule through the increased dipole moment of the excited state. This state could stabilize the excited state of the tryptophan residue to lead to a red shift and a smaller fluorescence transition dipole moment, increasing the lifetime of the excited state.

Registry No. PGM, 9032-62-6; L-tryptophan, 73-22-3.

References

Aaron, J. J., Tine, A., Wojiechowska, M. E., & Parkanyi, C. (1985) *J. Lumin. 33*, 33.

Amato, S. V., Rose, Z. B., & Liebman, M. N. (1984) *Biochem. Biophys. Res. Commun. 121*, 826.

Ananthanarayanan, V. S., & Bigelow, C. C. (1969) *Biochemistry 8*, 3717.

Andrews, L. J., & Forster, F. A. (1972) *Biochemistry 11*, 1875.

Aubailly, M., Bazin, M., & Santus, R. (1972) *Chem. Phys. Lett. 14*, 422.

Bailey, J. E., Beaven, G. H., Chignall, D. A., & Gratzer, W. B. (1968) *Eur. J. Biochem. 7*, 5.

Bartrons, R. C., & Carreras, J. (1982) *Biochim. Biophys. Acta 708*, 167.

Berlman, I. S. (1970) *J. Phys. Chem. 74*, 3085.

Borders, C. L., & Wilson, B. A. (1976) *Biochem. Biophys. Res. Commun. 73*, 978.

Burley, S. K., & Petsko, G. A. (1985) *Science 229*, 23.

Burley, S. K., & Petsko, G. A. (1986) *FEBS Lett. 203*, 139.

Catalan, J., Perez, P., & Acuna, A. U. (1986) *J. Mol. Struct. 142*, 179.

Cowgill, R. W., & Pizer, L. I. (1956) *J. Biol. Chem. 223*, 885.

De La Morena, E., Santos, I., & Grisola, S. (1968) *Biochim. Biophys. Acta 151*, 526.

Donzal, B., Gauduchon, P., & Wahl, P. (1974) *J. Am. Chem. Soc. 96*, 801.

Eisinger, J. (1969) *Photochem. Photobiol. 9*, 247.

Glasser, N., & Lami, H. (1981) *J. Chem. Phys. 74*, 6526.

Glasser, N., & Lami, H. (1986) *J. Mol. Struct. 142*, 193.

Grisolia, S. (1962) *Methods Enzymol. 5*, 236.

Grisolia, S., & Cleland, W. W. (1968) *Biochemistry 7*, 1115.

Grossweiner, L. I., & Joschek, H. I. (1965) *Adv. Chem. Ser. No. 50*, 279.

Han, C. H., & Rose, Z. B. (1979) *J. Biol. Chem. 254*, 8836.

Hartman, F. C., & Norton, I. L. (1976) *J. Biol. Chem. 251*, 4565.

Hermann, R., Rudolph, R., Jaenicke, R., Price, N. C., & Scobbie, A. (1983) *J. Biol. Chem. 258*, 11014.

Hershberger, M. V., Lumry, R., & Verrall, R. (1981) *Photochem. Photobiol. 33*, 609.

Hopkins, T. R., & Lumry, R. (1972) *Photochem. Photobiol. 15*, 555.

Ilich, P., Axelson, P. H., & Prendergast, F. G. (1988) *Biophys. Chem. 29*, 341.

Jacyno, G., & Kawski, A. (1974) *Bull. Acad. Pol. Sci., Ser. Sci., Math., Astron. Phys. 22*, 339.

James, D. R., Demmer, D. R., Verrall, R. E., & Steer, R. P. (1983) *Rev. Sci. Instrum. 54*, 1121.

Kirby, E. P., & Steiner, R. F. (1970) *J. Phys. Chem. 74*, 4480.

Lami, H. (1977) *J. Chem. Phys. 67*, 3274.

Lami, H., & Glasser, N. (1986) *J. Chem. Phys. 84*, 597.

Mani, A., & Lombardi, J. R. (1969) *J. Mol. Spectrosc. 31*, 308.

Marquardt, D. W. (1963) *J. Soc. Ind. Appl. Math. 11*, 431.

Masuhara, H., Saito, T., Maeda, Y., & Mataga, N. (1978) *J. Mol. Struct. 47*, 243.

Meech, S. R., & Phillips, D. (1983) *Chem. Phys. 80*, 317.

Milton, J. G., Purkey, R. M., & Galley, W. C. (1978) *J. Chem. Phys. 68*, 5396.

Ray, W. J., Jr., & Peck, E. J., Jr. (1972) *Enzymes* (3rd Ed.) *6*, 459.

Rose, Z. B., & Kaklij, G. S. (1984) *Biochem. Biophys. Res. Commun. 121*, 834.

Scopes, R. K. (1977) *Biochem. J. 161*, 253, 263.

Shinitzky, M., & Goldman, R. (1967) *Eur. J. Biochem. 3*, 139.

Strickland, E. H., & Billups, C. (1973) *Biopolymers 12*, 1989.

Sun, M., & Song, P. S. (1977) *Photochem. Photobiol. 25*, 3.

Teale, F. W. J. (1960) *Biochem. J. 76*, 381.

Walker, M. S., Bednar, T. W., & Lumry, R. (1967) *J. Chem. Phys. 47*, 1020.

Ware, W. R. (1971) *Creation and Detection of the Excited State* (Lamola, A. A., Ed.) Vol. 1A, p 213, Marcel Dekker, New York.

Warwicker, J. (1986) *J. Theor. Biol. 121*, 199.

Warwicker, J., & Watson, H. C. (1982) *J. Mol. Biol. 157*, 671.

Winn, S. I., Watson, H. C., Harkins, R. N., & Fothergill, L. A. (1981) *Philos. Trans. R. Soc. London B293*, 121.