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Tryptophan phosphorescence as a monitor of the solution structure of phosphoglycerate kinase from yeast

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Abstract

The enzyme phosphoglycerate kinase from yeast possesses two tryptophan residues whose phosphorescence spectrum in low-temperature glasses is resolved into two distinct components with 0–0 vibronic bands centered at 408 and 412.5 nm. The thermal profile of the phosphorescence intensity and lifetime shows that the red (longer wavelength) component is quenched in fluid solutions so that the long-lived phosphorescence observed at ambient temperature in buffer is due entirely to the blue (shorter wavelength) component. The remarkable heterogeneity in flexibility of the two chromophores' sites inferred from the thermal behaviour, when analyzed in terms of the crystallographic structure, allows to make a straightforward assignment of the long-lived emission to internal Trp-333. Because in buffer the phosphorescence is due to only one Trp residue the biphasic nature of the decay reveals the presence of stable, slowly interconverting, conformers with profound differences in the internal fluidity of the C-domain. Further, according to the triplet lifetime, complex formation with substrates affect the protein structure in a very selective way. Thus, while 3-phosphoglycerate has practically no influence on the average lifetime, Mg ATP and Mg ADP increases τ by a factor of 1.9 and 5.3, respectively. The change in lifetime implies a remarkable stiffening of the C-domain which is partly relaxed in ternary complexes with 3-phosphoglycerate. These findings are discussed in terms of ligand-induced "closed" conformations of the protein.

Keywords: Tryptophan; Phosphorescence; Phosphoglycerate kinase; Binding

1. Introduction

To understand the mechanism of action of enzymes knowledge of the solution structure and the details of ligand induced conformational changes is of paramount importance. The potential of Trp room temperature phosphorescence, RTP, for the study of structural and dynamical features of proteins has been recognized only in recent years. Although this spectroscopic approach is still in its infancy, when compared to the numerous biochemical applications of Trp fluorescence, there are features peculiar of the delayed emission that make it attractive for its resolution and exquisite sensitivity. First, the well-resolved vibrational structure of the phosphorescence spectrum often allows the assignement of distinct spectral components to individual tryptophyl side-chains [1]. Second, and most

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important, triplet lifetime and phosphorescence yield display a strong dependence on the effective viscosity of the embedding protein matrix [2]. This correlation between lifetime and microviscosity provides a direct measure of the flexibility of the protein structure at the chromophore site. Moreover, residues which are at the surface or in unstructured region of the macromolecule will undergo dramatic thermal quenching when glassy solutions are warmed above the glass transition temperature. As a result, RTP, is often due solely to one or few residues buried in rigid cores of the macromolecule [3–5]. In numerous proteins, the triplet lifetime at room temperature was found to represent a sensitive monitor of the conformational state of the macromolecule and be capable of revealing even subtle structural changes associated with binding of substrates [6] and allosteric effectors [7], change in physical state (solid, crystalline, micellar, etc.) [8–10] of the protein or the presence of cosolvents and stabilizing/destabilizing agents [11,12].

Phosphoglycerate kinase (PGK) (EC 2.7.2.3) is an enzyme required for ATP generation in glycolysis. The reaction catalyzed by PGK is that of the transfer of a phosphoryl group from the acyl phosphate of 1,3-diphosphoglycerate (1,3-DPG) to ADP thus forming ATP and 3-phosphoglycerate (3-PG).

The enzyme from a variety of sources is a monomer (Mw 44 500) folded into two well separated domains of about equal size that correspond to the N and C terminal parts of the chain. X-ray crystallographic studies [13,14] show that the catalytic nucleotide binding site is in C domain, while phosphoglycerate substrates bind to the N domain, about 12 Å from the nucleotide. The bilobal feature of the enzyme, and the large distance between the substrates binding sites have lead to the hypothesis of an hinge bending movement of the two domains in order to complete a catalytically competent (closed) form of the enzyme [13,14]. Evidence supporting substrate-induced conformational changes has come from a number of studies employing techniques such as proton NMR [15], low angle X-ray scattering [16], ultracentrifugation [17], photochemically induced nuclear polarization NMR [18] and Trp fluorescence [19]. Inspite of the effort, the nature of structural changes is not clear and, what is more, there is no agreement on the precise substrate requirements for producing what is supposed to be the closed form [13].

The yeast enzyme contains two Trp residues and both of them are in the C domain. Trp-308 is a surface residue in an unstructured region distant from the active site while Trp-333 is deeply buried in an extended 6-strands β-sheet which is topologically similar to the dinucleotide-binding fold in dehydrogenases [14], an assembly that invariably gives rise to highly inflexible cores. Indications from both fluorescence quenching by acrylamide [20] and proton NMR [21] are that, in contrast to Trp-308, the site of Trp-333 is indeed rigid and solvent inaccesible. On these grounds we may anticipate RTP from PGK, the emission originating exclusively from the internal chromophore.

In this work we have determined the Trp phosphorescence emission of PGK both in low temperature glasses and at room temperature in buffer. The results show that the phosphorescence is long-lived even in buffer and that at ambient temperature the delayed emission originates only from the internal residue (Trp-333). The sensitivity of the triplet probe to the dynamical structure of the C-domain reveals both the existence of at least two stable conformers of the enzyme in solution and marked conformational changes upon complex formation with the substrates.

2. Materials and methods

All chemicals were of the highest purity grade available from commercial sources and water, doubly distilled over quartz, was employed throughout. The magnesium salt of ATP, the potassium salt of ADP and the disodium salt of D-(−)-3-phosphoglyceric acid (3-PG) were purchased from Sigma Chemical Co. Yeast phosphoglycerate kinase was supplied by Boehringer (Mannheim) as a crystalline suspension in ammonium sulfate. The protein showed a single band on SDS/PAGE and was used without further

purification. Prior to use the enzyme was thoroughly dialyzed at 4°C against 20 mM triethanolamine acetate buffer pH 7.5 and centrifuged to remove any small residues. The protein concentration was measured spectrophotometrically by using $E_{280} = 0.49$ mg^{-1} ml cm^{-1} [22]. All protein solutions were used within two days of preparation.

To obtain reproducible phosphorescence data in fluid solution, it is of paramount importance to remove thoroughly all dissolved oxygen. The procedure followed to obtain satisfactory deoxygenation was described in a previous report [6]. All phosphorescence measurements were carried out at a protein concentration of 1 mg/ml.

A conventional home-made instrument was employed for all phosphorescence measurements [23]. The exciting light was provided by a high pressure 100 W Hg-lamp (HBO/W2, Osram). The excitation was selected by a 250 nm grating monochromator (Jobin-Yvon, H25) and the emission was detected with an EHI 9635QB photomultiplier. Luminescence decays in fluid solution were obtained following pulsed excitation by a frequency doubled flash pumped dye laser (UV500 M-Candela) with a pulse duration of 1 μs and an energy per pulse typically of 1–10 mJ. The decay of tryptophan luminescence was monitored at 430 nm by an electronic double shutter arrangement permitting the emission to be detected 1 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. Subsequent analysis of decay curves in terms of a sum of exponential components was carried out by a non-linear least-squares fitting algorithm implemented by the program Global Analysis (Global Unlimited, LFD University of Illinois, Urbana).

3. Results

3.1 Low temperature Trp phosphorescence

The well resolved vibrational structure of the phosphorescence spectrum of Trp allows, in favorable cases, to distinguish the emission spectrum between residues which lie in environments of different polarity [1,3,4]. Yeast PGK possesses two Trp residues, and their phosphorescence spectrum in the glass formed with propylene glycol/buffer at 160 K is shown in Fig. 1. Upon excitation at 280 nm the phosphorescence emission which, as commonly found in proteins, is dominated by Trp shows an heterogeneous vibronic structure with a 0–0 vibronic band made up of two distinct contributions centered at 408 nm (shoulder) and at 412 nm. Red edge excitation (305 nm) has the effect of sharpening the spectrum with the loss of the 408 nm shoulder. This finding demonstrates that there is heterogeneity also in the absorption spectrum and that the red emitting component can therefore be selectively excited.

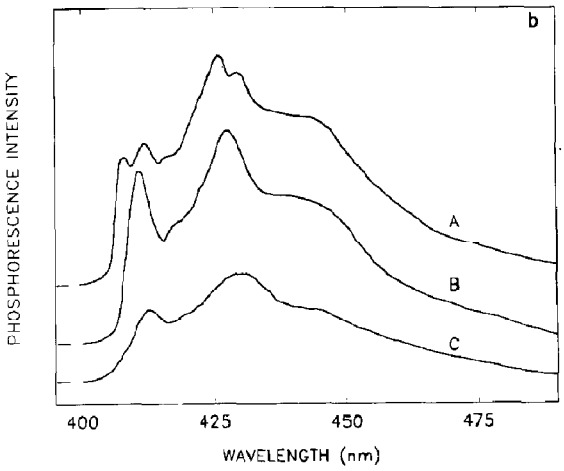

Fig. 1. Phosphorescence spectrum of PGK at different temperatures and excitation wavelength. (a) PGK at 160 K; (b) PGK ($\lambda_{ex} = 295$ nm) at 220 K (A) and 274 K (C). (B) is the same as (A) upon addition of both 0.5 mM ADP and Mg^{2+}. All samples are in 50 : 50 (v/v) propylene glycol/triethanolamine acetate buffer, 0.02 mM pH 7.5. The enzyme concentration is 1 mg/ml.

The spectral energy is related to the solvation energy of the chromophore which in turn depends on the chemical nature of the environment. As regards to general solvent effects, in going from aqueous to non-polar media the 0–0 band of Trp shifts from 408 nm to 411–412 nm, respectively. In practice a asymmetric distribution of charges in the protein environment may cause

internal residues to phosphoresce anywhere between 404 and 421 nm [2]. Thus, even if the 408 nm band could be assigned to Trp exposed to the aqueous phase it will be apparent from the thermal quenching profile that the blue component (408 nm) is due to buried Trp-333 and the red one (412 nm) to Trp-308 which is on the surface.

In the presence of saturating amounts (using dissociation constants in buffer) [22] of 3-PG (13 mM) or Mg ADP (0.5 mM) the shoulder at 408 nm disappears almost completely, its maximum having presumably shifted towards the more intense 412 nm band. Anticipating the assignment of the 408 nm emission to Trp-333 this spectral shift is direct proof that a change in the environment of the internal residue occurs upon complex formation. Since the indole ring is at least 11–12 Å removed from both nucleotide and 3-PG binding sites [14] we conclude that the ligand-induced shift reflects a change in conformation of the C domain rather than a perturbation due to the juxtaposition of positive/negative charges carried by the ligands.

In glasses the Trp phosphorescence of PGK decays essentially in a monoexponential fashion. After subtracting 2–3% of the total intensity due to a rapid component, typical of solvent impurities emission, the decay yielded a triplet lifetime of 6.4 ± 0.1 s, irrespective of the substrate present.

3.2 Trp phosphorescence in fluid solution

Raising the temperature above the glass transition of the solvent mixture invariably causes strong quenching of Trp phosphorescence in proteins, a phenomenon due to the shortening of the triplet state lifetime by viscosity-dependent radiationless process [2]. In PGK, above 190 K, both intensity and lifetime decrease rapidly upon warming. The loss in intensity is accompanied by spectral changes which emphasize preferential quenching of the 412 nm band (Fig. 1). Above 230 K the 0–0 vibrational band of the spectrum is comprised entirely of the blue component (408–410 nm).

To quantify the drop in intensity in terms of the fraction of residues that is thermally quenched

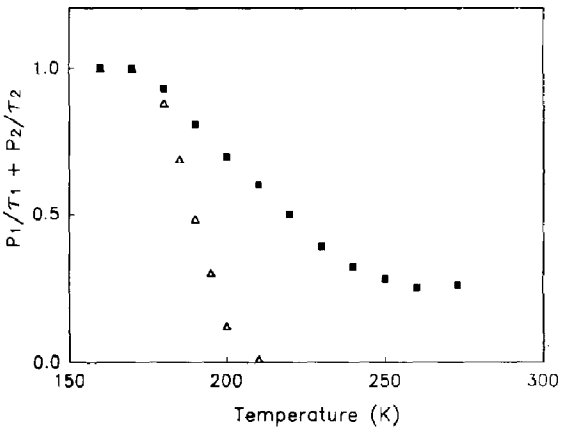

Fig. 2. Phosphorescence thermal profile of PGK in 50:50 propylene glycol/triethanolamine acetate buffer 0.02 M, pH 7.5 (■). The excitation wavelength was 295 nm, λ_{em} 440 nm, the protein concentration was typically 1 mg/ml. (△) Refer to the decrease in phosphorescence intensity of a 2×10^{-5} M solution of tryptophan under the same conditions.

(i.e. the fraction whose τ is less than 1 ms, the resolution limit of the apparatus) we have plotted (Fig. 2) the thermal profile of the lifetime normalized phosphorescence intensity, $\Sigma P_i/\tau_i$, P_i being the fractional intensity decaying with lifetime τ_i. Above the glass transition temperature, the decay was fitted to a biexponential law throughout even if in the range 210–240 K the statistics, χ^2 and plot of residuals, indicated the presence of additional components. From inspection of the thermal profile one observes that about 75% of the emission is thermally quenched below 240 K and that above this temperature the normalized intensity is practically constant up to ambient temperature. Spectral decomposition shows that at glassy temperatures the blue band contributes about $27 \pm 3\%$ to the total emission intensity. This and spectral data collected along the thermal profile confirm that the decrease in normalized intensity is fully accounted for by quenching of the red component.

If spectral heterogeneity stresses different interaction energies between indole rings and their embedding matrix, the thermal profile emphasizes heterogeneity in dynamical structure of these sites. From the correlation between local viscosity and triplet lifetime [2] we deduce that the thermally quenched red component must be associ-

ated to a chromophore in a rather flexible region of the macromolecule (typical of residues near or at the surface) as opposed to the blue component whose long lifetime attests to a tight and rigid site (typical of residues in buried β-sheet).

The presence of substrates, 3-PG and Mg·ADP, does not affect appreciably the thermal behaviour of Trp emission. Although the Mg·ADP complex displays consistently longer triplet lifetimes relative to uncomplexed enzyme both transition temperature and the plateau value of $\Sigma P_i/\tau_i$ are quite similar. This implies that whatever the conformational change undergone by the protein upon 3-PG or Mg·ADP binding, the flexibility in the structure of the thermally quenched residue is largely unaffected.

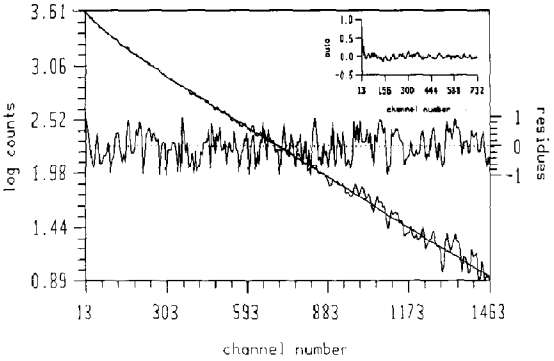

Fig. 3. Phosphorescence decay of PGK (1 mg/ml), ADP (1 mM), Mg (1 mM), 3-PG (13 mM) in triethanolamine acetate buffer 0.02 M pH 7.5 at 0°C ($\lambda_{ex} = 295$ nm, $\lambda_{em} = 440$ nm). The fitting to a biexponential law yields a value of χ^2 of 0.9. The time interval for each channel is 1.3 ms.

3.3 Trp phosphorescence in buffer

Although the triplet lifetime is somewhat larger in the presence of the organic cosolvent, the spectrum and the lifetime normalized intensity are practically the same in buffer as in PG/buffer. At ambient temperature the phosphorescence spectrum displays broad, little resolved, vibrational bands whose maxima are 4–5 nm red shifted with respect to the cold temperature spectrum. While red shifts do indicate a certain degree of relaxation of the protein matrix on the phosphorescence time scale (10–100 ms) the lack of resolution could in part be due to structural heterogeneity in the macromolecule.

As with PG/buffer, the phosphorescence decay in buffer is clearly biphasic. Fitting statistics (Fig. 3) indicate that a two component model is adequate to represent the data. Since only one Trp residue is presumably phosphorescent at ambient temperature (see discussion in Section 4) the observation that the phosphorescence decays in an highly non-exponential fashion implies that chromophores in different molecules are not equivalent in the dynamic properties of their environment, when averaged over a time window of the order of τ.

The formation of binary and ternary complexes with 3-PG, ADP, ATP, Mg·ADP and Mg·ATP can have a profound influence on the decay kinetics of the protein phosphorescence. Ligand-induced changes of the triplet state decay rate at 1°C are displayed in Fig. 4 and the parameters obtained from a biexponential analysis of

Table 1

Lifetimes and preexponential terms obtained from a biexponential fitting of phosphorescence decays in the presence of various substrate combinations. The average lifetime, $\tau_{av} = \alpha_1\tau_1 + \alpha_2\tau_2$, is also included. Experimental conditions are the same as in Fig. 4. The concentration of ATP is 1 mM

Substrate	τ_1 [a] (ms)	τ_2 (ms)	α_1 [a]	τ_{av} (ms)	χ^2
PGK	196	25	0.20	59	0.9
PGK·3-PG	74	38	0.50	56	1.5
PGK·Mg·ADP	392	120	0.71	313	1.1
PGK·Mg·ADP	311	56	0.24	117	1.6
PGK·Mg·ADP·3-PG	243	60	0.70	188	1.2
PGK·Mg·ATP·3-PG	112	39	0.32	62	0.8

[a] The typical error in lifetime and amplitude data is about 3–5% and 8–9%, respectively.

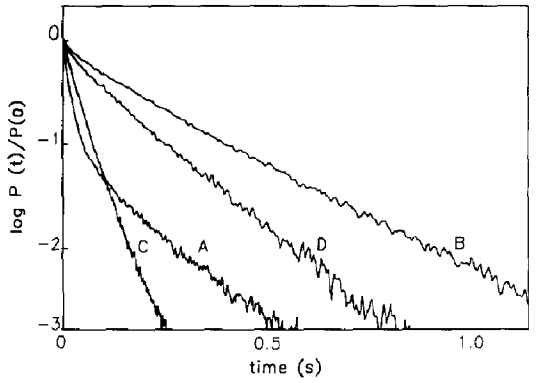

Fig. 4. Decay of phosphorescence intensity with time (λ_{ex} = 295 nm, λ_{em} = 440 nm) at 1°C in 0.02 *M* triethanolamine acetate buffer pH 7.5 for PGK (1 mg/ml) (A) and its complexes with Mg (1 m*M*), ADP (1 m*M*) (B), 3PG (13 m*M*) (C) and ADP (1 m*M*) Mg (1 m*M*) 3PG (13 m*M*) (D).

the data are collected in Table 1. Except for the complex PGK · 3-PG, for which the decay is almost exponential and the average lifetime, $\tau_{av} = \alpha_1\tau_1 + \alpha_2\tau_2$, is practically identical to that of PGK, the binding of nucleotides brings about a substantial lengthening of τ_{av}. It is in the presence of Mg^{2+}, hovewer, that we observe a dramatic increase in the triplet lifetime, τ_{av} being twofold and sixfold larger with Mg · ATP and Mg · ADP, respectively. As opposed to the free enzyme. The addition of 3-PG to the Mg · ADP complex reduces τ_{av} by almost a factor of two.

The addition of 3-PG to the Mg · ATP complex gives rise to products of the reaction so that at equilibrium PGK will be present mostly as PGK · Mg · ADP · 1,3-PG and PGK · Mg · ATP · 3-PG undergoing rapid interchange. For this mixture of molecular species in dynamic equilibrium the decay is still distinctly biphasic and τ_{av} is not too dissimilar from that of the abortive ternary complex with Mg · ADP.

Parallel to drastic tightening of the protein structure in Mg-nucleotide complexes (as inferred from the larger values of τ_1 and τ_2) there is a shift in the weight of the preexponential terms from the short-lived to the long-lived component. Should α_i represent emission from the same species of PGK in free enzyme and in its complexes the large change in the ratio α_1/α_2 would indicate a strong influence of nucleotide ligands on the equilibrium relating the two conformations of the protein.

4. Discussion

Yeast PGK possesses two Trp residues and from the crystallographic structure [12] it is known that they are both in the C-domain. While Trp-308 lies in an unstructured region on the surface of the protein, away from the active site, Trp-333 is buried in strand L of the 6-strands β-sheet which in turn is enveloped by long α-helical rods. The separation of the latter residue from the nucleotide binding site in the C-domain is greater than 12 Å [14]. Their phosphorescence emission at low temperature is clearly heterogeneous, the two spectrally resolved contributions emphasizing a different polarity of the chromophores' environment. The heterogeneity is even more drastic in terms of the dynamical structure of these sites. Indeed, the thermal profile of the phosphorescence intensity and lifetime shows that the red spectral component is totally quenched even at relative low temperatures, whereas the blue component remains long-lived up to and above room temperature. Since, barring specific quenching reactions, the triplet lifetime and quantum yield depend almost exclusively on the local viscosity at the indole ring [2], (decreasing upon lowering the viscosity) the thermal behaviour indicates that the red/blue emission must be associated with residues in flexible/rigid environments respectively. Based on the crystallographic structure of PGK the assignment of these spectral components to individual residues appears straightforward. Namely, the red component can only be due to Trp-308 which being at the surface in an unstructured region of the macromolecule is bound to enjoy great mobility. Trp-333, on the other hand, lies in an α/β structural motive analogous to that of the coenzyme-binding domain in dehydrogenases. There is ample evidence, NMR, H-exchange and tryptophan phosphorescence, that such an assembly of secondary structure in proteins gives rise to exceptionally rigid cores, environments that are an essential requisite for long (100 ms) phosphorescence life-

times at ambient temperature. Evidence of a compact structure about Trp-333 comes also from proton NMR relaxation rates [21], fluorescence anisotropy [19] and fluorescence quenching [20] studies. What is more, these investigations confirm also that Trp-308 is in a solvent accessible, highly mobile region. Thus, following this assignment, we must conclude that only Trp-333 is detectably phosphorescent in buffer at ambient temperature a conclusion also in keeping with the fraction of the cold temperature phosphorescence intensity remaining under these conditions.

For a monomeric protein with a single emitting center, a biphasic phosphorescence decay provides direct, unequivocal, evidence of heterogeneity in molecular structure. The conformers of PGK, as identified by their distinct triplet lifetimes, interconvert slowly in the phosphorescence time scale (10–100 ms) and their relative weight is influenced by temperature as well as binding of substrates. Judging from the difference in triplet lifetime the C-domain of these conformers could have substantially different structures. Utilizing the relationship between τ and microviscosity [2] we obtain effective viscosities at the site of Trp-333 that differ by roughly a factor of 30 [$\eta_1/\eta_2 \approx (\tau_1/\tau_2)^{1.7}$].

Since crystallographic studies have revealed an "open" cleft bilobal structure for PGK there has been a continuing search for evidence of catalytically competent, "closed", conformers of the protein. Recent NMR data has been interpreted in terms of closed and open conformers in rapid equilibrium, closed forms being stabilized by nucleotide substrates [15]. Clearly the two solution structures identified by the triplet probe are so stable and long-lived states of the protein that cannot be associated with the open/closed conformers inferred by NMR. Further, since the interconversion is slow even in catalytically competent ternary complexes, whose turnover rates are in the ms time scale [20], we deduce that these structures cannot represent functionally open/closed forms of the enzyme. It should be pointed out that although Trp phosphorescence provides the first direct evidence for the existence of stable conformers of PGK in solution, there are anomalous kinetic [24] and thermodynamic data [25] that perhaps could be rationalized by such structural heterogeneity. Many enzyme kinetic studies have reported biphasic double-reciprocal plots which have proved difficult to understand. While Scopes [24] has suggested that the reason for the nonlinearity could be due to direct substrate activation, the presence of conformers with different catalytic properties might account as well for it. Likewise, there is an asymmetry in the differential scanning calorimetry curve, for the enzyme and its complexes, that does not indicate a single transition in the thermal unfolding reaction [25]. While the data could not be fitted by models involving either two sequential or two independent two-state steps the presence of conformers with different thermal stability might account for it.

More recent calorimetric data [26,27] suggest that the thermal unfolding profile can be accounted by irreversible effects or by separate unfolding of N- and C-domains.

Binding of substrates to either the C- or the N-domain alters the internal conformation of the C-domain in that both the spectrum and the triplet lifetime of Trp-333 are affected. Among the substrates, 3-PG, which binds to the N-domain, has practically no influence on τ_{av} but renders the two decay components more similar in magnitude. Because, in the PGK · 3-PG complex the two conformers are hardly distinguishable by the triplet probe we must conclude that either they have equivalent structures or they interconvert to some extent during the excited state lifetime.

The nucleotide-binding site is in the C-domain and the distance to the indole ring of Trp-333 is at least 12 Å. Given the large separation, any change reported by the triplet probe upon nucleotide binding is bound to reflect structural changes that are transmitted over large distances. According to τ_{av} the nucleotides induce a stiffening of the protein structure the effect being drammatically enhanced by the presence of Mg^{2+} ions. A more rigid, presumably more thermally stable structure, is fully in agreement with the conclusions drawn from depolarized Rayleigh scattering studies [28] and the higher temperature of thermal denaturation [25], but opposite to the

conclusion derived from fluorescence anisotropy data on the horse enzyme [19]. The latter study reports a decrease in the degree of polarization in binary and ternary complexes with Mg-nucleotides which is attributed to a greater mobility of Trp-335 (equivalent to Trp-333 in yeast). The horse enzyme, however, has 4 Trp residues and in the absence of spectral resolution a detailed interpretation of anisotropy changes is clearly unwarranted.

Although a tightening of the polypeptide in the C-domain would be expected from the closure of the cleft (compare triplet lifetimes on alcohol dehydrogenase complexes [6]), the information available is insufficient to specify the nature of the structural change brought about by Mg-nucleotides. Proton NMR studies infer the existence of "closed" conformations in that bound nucleotides alter the chemical shift of surface residues in the opposite N-domain. Thus, it is possible that the lengthening of τ for each stable conformer brought about by Mg-nucleotides be due to a shift of the equilibrium towards "closed", structurally more rigid states. Like proton NMR data, τ points out substantial differences between Mg–ADP and Mg–ATP complexes. But, whereas the former imply a greater shift of the equilibrium towards "closed" forms upon increasing the number of phosphates, the phosphorescence lifetime shows that the average structure is more flexible with Mg–ATP than with Mg–ADP, a conclusion consistent also with the lower temperature of thermal denaturation [25] of the triphosphate complex.

Ternary complexes, abortive or catalytically competent, display intermediate flexibilities. The effect of 3-PG appears to be one of loosening the stiff structure achieved in the binary complex with nucleotides. In contrasts to low-angle X-ray scattering studies where a decrease of the observed radius of gyration in the ternary complex was taken to represent a 20° rotation of the lobes towards each other there is no evidence, according to the triplet probe, of such a presumably structure tightening conformational change that would be triggered by binding of both substrates.

The present investigation on the Trp phosphorescence properties of PGK emphasizes that Trp-333 is selectively luminescent at ambient temperature in buffer. The sensitivity of phosphorescence to the chemical nature and dynamical structure of the protein environment makes Trp-333 an intrinsic, natural, probe of the conformational state of the C-domain. Besides exploring ligand induced effects it may be suitable to report on the connectedness between domains and on perturbations that may arise from amino acid substitutions or the addition of cosolvents that lead to cold denaturation [29].

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