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Biophysical Chemistry 52 (1994) 25–34

Biophysical Chemistry

Heterogeneity of protein conformation in solution from the lifetime of tryptophan phosphorescence

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Received 28 October 1993; accepted in final form 27 February 1994

Abstract

The decay of Trp phosphorescence of proteins in fluid solutions was shown to provide a sensitive tool for probing the conformational homogeneity of these macromolecules in the millisecond to second time scale. Upon examination of 15 single Trp emitting proteins multiexponential decays were observed in 12 cases, a demonstration that the presence of slowly interconverting conformers in solution is more the norm rather than an exception. The amplitude of preexponential terms, from which the conformer equilibrium is derived, was found to be a sensitive function of solvent composition (buffer, pH, ionic strength and glycerol cosolvent), temperature, and complex formation with substrates and cofactors. In many cases, raising the temperature, a point is reached at which the decay becomes practically monoexponential, meaning that conformer interconversion rates have become commensurate with the triplet lifetime. Estimation of activation free energy barriers to interconversion shows that the large values of ΔG^* are rather similar among polypeptides and that the protein substates involved are sufficiently long-lived to display individual binding/catalytic properties.

Key words: Tryptophan phosphorescence; Protein conformation; Substates

1. Introduction

Current views of protein structure have been shaped largely by results from X-ray crystallography. Such structural determinations, although extremely useful, provide only a static model of the protein and the structure often is determined only for one set of conditions. Only recently attempts have been made to analyze X-ray diffraction in terms of more than one conformation in the crystal [1].

Theoretical considerations made on the basis of the unique physical characteristics of a biomolecule strongly indicate the existence of many different substates in proteins. Experiment and computer simulations show that a protein does not exist in only one particular conformation but can assume a large number of slightly different structures [2–4]. Based on the relaxation characteristics over a wide temperature range, Frauenfelder and co-workers have argued that a single monomeric protein can possess many nearly isoenergetic conformations [2]. The individual substates are believed to be separated by barriers of different heights. The dynamics of the internal

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SSDI 0301-4622(94)00039-M

motions in proteins spanning several decades in time is thought to be a direct consequence of the complexity of the underlying energy landscape. Depending on temperature and on the state of the surrounding, substates separated by activation barriers $\Delta G^* < k_B T$ are in rapid equilibrium while those for which $\Delta G^* > k_B T$ are essentially static.

In liquid solutions at ambient temperature, relaxation times for protein substates that have been mainly investigated are in the ns time-scale or shorter. The presence of substates interconverting in this time-scale has been detected for several proteins and it seems to be quite general [2]. In principle the existence of more grossly different substates separated by barriers of several kcal/mol is also plausible. Experimental evidence for their existence in solution is beginning to accumulate from a number of biophysical techniques among which flash photolysis [2], luminescence [5,6] and NMR [7–9] methods are the principal ones. In particular, within the μs–ms nuclear relaxation times, NMR data often resolve more than one environment for certain side-chain, although, given the large protein concentration involved in these studies (mM), the formation of protein aggregates may sometime be responsible for the heterogeneity.

In the absence of quenching reactions the phosphorescence lifetime of Trp is largely dominated by the effective viscosity of the medium [10]. Consequently the intrinsic lifetime of Trp residues in proteins reflects by and large the local flexibility of the protein matrix. Since the segmental mobility of the polypeptide is closely related to the conformation adopted by the macromolecule Trp phosphorescence has proven to be a sensitive and direct monitor of conformational changes of these biopolymers in solution. High sensitivity coupled to the long lifetime (ms–s) render this approach suitable to reveal distinct conformers of the macromolecule that persist for times longer than τ. Recently, multicomponent phosphorescence decays have been observed even in proteins with a single Trp residue in the polypeptide [6,11,12].

Integrating published data with new experiments, in this work we consider the decay characteristics of a number of single Trp emitting proteins under a variety of experimental conditions, and seek to establish: (1) whether the phosphorescence decay can provide an appropriate monitor of conformational heterogeneity of the macromolecule and (2) whether the existence of long-lived conformers is a general property of proteins in solution. The outcome of this analysis confirms that almost in every case grossly different substates coexist in solution and that their equilibrium is promptly affected by solvent conditions (buffer, pH, ionic strength and cosolvent) and ligand binding.

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.

Holo-$\alpha_2\beta_2$ complex of tryptophan synthase from *Salmonella typhimurium* was a generous gift of Dr. Mozzarelli, (University of Parma, Italy). Apoenzyme and reduced holo-$\alpha_2\beta_2$ were prepared and checked according to Strambini et al. [13]. *E. coli* alkaline phosphatase (AP) and glyceraldehyde-3-phosphate dehydrogenase (GPDH) from *B. Stearothermophilus* were purchased from Sigma Chemical Co. (St. Louis, MO). Horse liver alcohol dehydrogenase (LADH), yeast phosphoglycerate kinase (PGK), bovine glutamate dehydrogenase (GDH), GPDH from yeast and from rabbit muscle were obtained from Boehringer (Mannheim, Germany). GPDH from *E. coli* was a gift of Dr. G. Branlant (University of Nancy, France). Ribonuclease T_1 (RNase T_1) was purchased from Calbiochem Co. (San Diego, Ca). Apoazurin from *Pseudomonas aeruginosa* was a gift of Dr. Rosato (University of Rome, Tor Vergata, Italy) and F_1-ATPase was a gift of Dr. Solaini (SSSUP Sant'Anna, Pisa, Italy). Isobutirimmide (IBA) was from Aldrich (Germany), NADH Boehringer (Mannheim), whereas DL-α-glycerol 3-phosphate (GP), guanosine 2′-monophosphate (disodium salt) (2′-GMP), guanosine 3′-monophosphate (trisodium salt) (3′-GMP), the magnesium salt of ATP, the potassium salt of

ADP and the disodium salt of D-(-)3-phosphoglyceric acid (3-PG) were obtained from Sigma Chemical Co. (St. Louis, MO).

Prior to luminescence measurements all the proteins were extensively dialyzed in their respective buffers as reported in Table 1. Each GPDH, was treated with charcoal (Norit A, from Serva), as described by Henis and Levitzki [14] to eliminate strongly bound NAD^+. Proteins concentration in the phosphorescence measurements were typically 3–5 μM. To avoid quenching effects and to obtain reproducible phosphorescence measurements, oxygen was removed from the samples as described in a previous report [15].

Phosphorescence spectra and decays measurements were obtained with a conventional homemade instrument [16]. The excitation provided by a Cermax xenon lamp (LX 150 UV: ILC Technology) was selected by a 250 nm grating monochromator (Jobin-Yvon, H25), and the emission was detected with an EMI 9635 QB photomultiplier. Luminescence decays in fluid solution were obtained following pulsed excitation (λ_{ex} 292 nm) 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 Trp phosphorescence was monitored at 440 nm by an electronic shutter arrangement permitting the emission to be detected 1 ms after the excitation pulse. The decaying signal was digitized (12 bits resolution) and averaged by a Computerscope EGAA system (RC Electronics Inc., Santa Barbara, Ca). The decay curves were analyzed in terms of a sum of exponential components by a non linear least-squares fitting algorithm.

3. Results and discussion

3.1. Trp phosphorescence lifetime as a monitor of protein conformation

The fluorescence lifetime of indole is sensitive to the chemical nature of the environment. Distinct lifetimes are observed for each conformational rotamer of tryptophan (Trp) and the decay of Trp fluorescence in proteins is, with few exceptions, generally nonexponential [17,18]. By contrast, the phosphorescence decay of free Trp and single Trp proteins in glasses is exponential and the lifetime is between 5 and 7 s apparently insensitive to the nature of the solvent or protein microenvironment [19]. Shorter lifetimes in pro-

Table 1
Lifetime and preexponential terms obtained from a biexponential fitting of Trp phosphorescence decay of proteins in solution. The standard error in τ and α is typically below 8%

Protein	T (°C)	τ_1 (ms)	τ_2 (ms)	α_1	χ^2	Solvent	Ref.
Apoazurin	20	600	–	1	0.9	50 mM tris-Cl pH 7.5	[34]
Alkaline phos.	20	2060	–	1	1.0	10 mM tris-Cl pH 8	[35]
GPDH · *E. coli*	20	62	–	1	1.1	20 mM K phosphate pH 7.2	
GDH	−10	3300	1500	0.5	1.3	100 mM K phosphate pH 7.5 [a]	
LADH	20	697	249	0.83	0.9	50 mM tris-Cl pH 7.5	
GPDH · rabbit	20	918	140	0.77	1.9	20 mM K phosphate pH 7.2	[36]
GPDH · yeast	20	150	90	0.72	1.3		[36]
GPDH · *B. Stearot.*	20	91	23	0.88	1.0		[37]
Trp Synth. apo	0	55	17	0.55	3.0	50 mM bicine pH 7.8	[6]
Trp Synth. holo	0	48	28	0.54	2.8		[6]
Trp Synth. red	0	79	29	0.44	2.8		[6]
RNase T_1	0	107	25	0.17	1.0	50 mM cacodylate pH 5.5	[11]
F_1 ATPase	20	8.2	2.8	0.29	1.2	100 mM tris-SO_4 pH 8	
myoglobin tuna	−40	1210	640	0.45	1.8	100 mM K phosphate pH 7.5 [a]	[38]
PGK	0	25	196	0.8	2.9	20 mM triethanolamine acetate pH 7.5	[12]

[a] Buffer/propylene glycol (50/50, V/V).

teins are found when the chromophore is within interaction distance of disulphides or of prosthetic groups that exchange energy/electrons with it. In fluid solutions τ decreases dramatically with the reduction of solvent viscosity reaching 15–20 μs for free Trp derivatives in buffer at ambient temperature [20]. Under the same conditions buried Trp residues in proteins display lifetimes ranging from 2 to 5×10^{-4} s [21,22]. Although diffusion mediated quenching reactions with a number of solutes can affect the decay rate the relationship established between τ and microviscosity suggests that τ reflects predominantly the flexibility of the surrounding protein matrix [10]. In any case, a monoexponential decay from a single Trp protein in fluid solution indicates homogeneity in the dynamical structure surrounding the triplet probe a condition met whenever the τ-averaged structure of the macromolecule is unique.

Because of the long lifetimes involved, phosphorescence measurements in fluid solutions are particularly exacting on the control of quenching impurities. Ideal conditions are not easily attained and a number of artifacts can in principle obscure the determination of intrinsic lifetimes and also give rise to nonexponential decays. Potential artifacts may originate from both thermal and quenching impurities gradients or from slow rearrangements of protein structure subsequent to excitation of the chromophore. Impurity gradients across the sample excited by the actinic light beam can be generated by nonuniform light intensity. A nonuniformely intense actinic beam causes residual O_2 in solution to be preferentially destroyed [16] in the more intense region where at the same time the concentration of photogenerated quenching impurities, photoelectron, neutral and ionic radicals, will be highest.

Rigorous tests on the influence of these factors on the decay kinetics of Trp phosphorescence were carried out with apoazurin, a single Trp protein with a relatively long triplet lifetime. The results showed that upon thorough removal of O_2 and at excitation intensities less than 5 mJ/cm^2 ($\lambda_{ex} = 292$ nm), the phosphorescence of apoazurin decays monoexponentially both in buffer and in glycerol/buffer mixtures. An example of the decay in buffer monitored over a time range 10^{-3} to 3.6 s is reported in Fig. 1. From the goodness of the fit we may conclude that under carefully controlled conditions no artifacts are introduced that can give rise to nonexponential decays. Conversely, the detection of nonexponential phosphorescence decays in this time window are most likely a manifestation of conformational heterogeneity of the macromolecule. This may originate from either different intramolecular quenching configurations, accessibility to external quenchers or to differences in the local flexibility of the protein matrix.

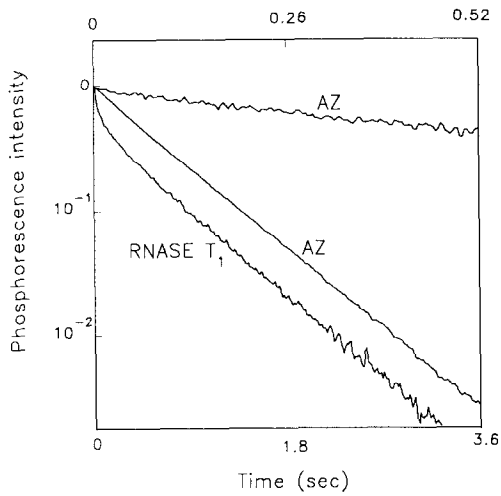

Fig. 1. Comparison between the decay of tryptophan phosphorescence intensity ($\lambda_{ex} = 292$ nm; $\lambda_{em} = 440$ nm) of RNaseT_1 in 70% glycerol at 0°C (upper timescale) and apoazurin in buffer at 20°C (lower timescale). To show that there is no short-lived component in the decay of apoazurin, its phosphorescence intensity is displayed also in the smaller time window.

3.2. Nonexponential decays in single Trp emitting proteins

Among the proteins examined apoazurin, RNase T_1, F_1-ATPase and Trp synthase have a sole Trp residue in the polypeptide. Although the other proteins have 2 or 3 of them, previous experiments have shown that at room temperature only one Trp, generally the most buried, is detectably phosphorescent. Consequently, in all

cases we are dealing with single Trp emission. Hence, should these polypeptides adopt a predominant conformation in solution one, based on the results with apoazurin, would anticipate monoexponential decays throughout.

Table 1 summarizes the phosphorescence lifetime in buffer of several proteins near room temperature. Among them only apoazurin, AP and GPDH from *E. coli* yielded a monoexponential phosphorescence decay. This implies that over the period of the triplet lifetime, the probe detects a single average structure for the polypeptide. Should multiple conformations average out in this time window, upon lowering the temperature interconversion would slow down so that structural heterogeneity might become apparent. The decay of apoazurin in propylene glycol/buffer (50/50, V/V) was found to remain strictly monoexponential down to the glass transition temperature (≈ 200 K) where τ has increased and reached the low temperature limit of 6 s. With AP and GPDH low temperature decays have multiple components. Such components cannot be interpreted unambiguously in terms of structural heterogeneity of the polypeptide because at lower temperature/higher solvent viscosity other Trp residues with a different lifetime may contribute to the overall emission.

In contradistinction to apoazurin we note that most single Trp emitting proteins of Table 1 have multiexponential decays. Accordingly, the triplet probe provides direct evidence of conformational heterogeneity of these macromolecules; conformers that are sufficiently stable to preserve their identity for times of the order of the phosphorescence lifetime (> ms) or longer. For convenience, all decays were fitted with a sum of two exponential functions even if three components or alternatively a broad distribution of lifetimes would fit better the data. For GPDH (rabbit), Trp synthase and PGK χ^2 is > 1 and it is evident that two components do not give an adequate description of the decay. This suggests that three or more long-lived substates of the macromolecule are coexisting. Of course, with multimeric proteins any heterogeneity in the decay could also be due to quaternary structure asymmetry. In such cases, however, the amplitude of the preexponential terms would be simply related to the number of subunits in the polymer. For instance, a value of $\alpha_1 \approx \alpha_2$ with hexameric GDH is compatible with an hexamer made up asymmetric trimers. On the other hand $\alpha_1 = 0.2$ in dimeric LADH cannot be accounted by asymmetry.

Preexponential terms (α) are related to the population of each substate. However, it must be pointed out that α_i is identical to the fraction, f_i, of macromolecules in the ith conformation only if the phosphorescence quantum yield is constant in each conformer and the rate of interconversion between substates is smaller than $1/\tau$. The static, or slow interconverting, regime can be verified by comparing the values of α derived upon continuous and pulsed excitation. The relationship between preexponential terms is given by [23]:

$$\alpha'_i = \alpha_i \tau_i / \Sigma \alpha_i \tau_i ,$$

where α'_i and α_i refers to the preexponential amplitudes upon continuous and pulsed excitation respectively.

At 0°C the emission from PGK, LADH and GPDH (yeast) was measured with both excitation modes. The good agreement between measured and predicted values of α demonstrates that for these proteins there is virtually no interconversion during the long, > 200 ms, lifetimes.

In conclusion the triplet probe emphasizes the prevalence of multiple conformers of protein molecules in solution. The technique differentiates substates mainly in terms of dynamical features of the structure. Although it provides no structural details, from the 2 to 8 folds difference in τ between lifetime components of the same macromolecule (Table 1), which corresponds to a factor of roughly 4 to 60 in effective internal viscosity [10], we deduce that the polypeptide must assume substantially different structures. Also, from the magnitudes of α (0.1 to 0.5) we note that even the least represented substates make up a non minor fraction of the protein population. In energetic terms ($\Delta G^\circ = -RT \ln \alpha_1/\alpha_2$) the conformers differ in thermodynamic stability by not more than 1 kcal/mol. Further, expressing conformer interconversion rate constants in terms of transition state theory ($k = (k_B T/h) \exp(-\Delta G^*/RT)$) we find that rates

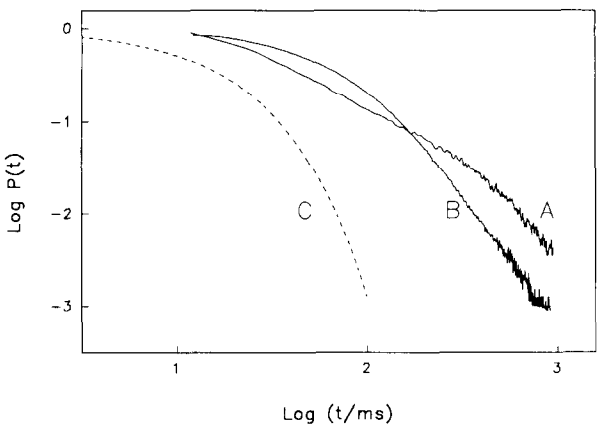

Fig. 2. Decay of Trp phosphorescence intensity with time following pulsed excitation: (A) PGK in triethanolamine buffer at 5°C, (B) RNase T1 in 70% glycerol at 0°C and (C) simulated monoexponential decay. The behaviour of RNase T1 is representative of all other proteins in Table 1.

of interconversion less than 10 s^{-1} entail activation free energy barriers higher than 15 kcal/mol.

We have chosen to treat nonexponential decays as a sum of discrete lifetime components and consequently discuss protein heterogeneity in terms of few stable conformations of the macromolecule. The alternative is a distribution of lifetimes which then would imply a large number of conformational substates. A reviewer has pointed out that by plotting the logarithm of the phosphorescence intensity, $P(t)$, versus log t one, by analogy to CO rebinding kinetics in myoglobin [24], might distinguish between taxonomic (few) and statistical (many) substates since in the former case $\log[P(t)]$ will decrease in a multiple step fashion while in the latter the decrease will be uniform. Representative $\log[P(t)]$ versus log t plots are shown in Fig. 2. Except for PGK, for which a slight shoulder is evident in the log–log plot all other proteins have a smooth decrease in intensity. This distinction, we believe, is no proof that only PGK displays taxonomic substates. Simulations of double exponential decays demonstrate that structure in a log–log plot is appreciable only if two components differ in τ by at least a factor of 10. Inspection of Table 1 shows that except for PGK the lifetime components of all other proteins differ by at most a factor of 5 and hence a smooth log–log plot is anticipated irrespective of whether the substates are few or many. All we know about the substates implied by the heterogeneous emission is that they interconvert extremely slowly. At room temperature they require fractions of a second to equilibrate, a long time in comparison to microseconds typical for the taxonomic substates of tier 0 in myoglobin [25]. Such slow protein motions in the energy landscape imply large free energy of activation barriers, a parameter that places the substates at the top of the hierarchy where the number is small.

3.3. Influence of environmental factors on the conformer distribution

The stability of a given protein substate is likely to depend on experimental conditions such as solvent composition, pH, temperature, and complex formation with other macromolecules or small ligands [26]. Consequently, the relative amplitude of preexponential terms of the phosphorescence decay should be modulated by these factors.

With protein phosphorescence, changes of τ and α due to varying solvent composition, namely type of buffer, pH, ionic strength and addition of organic cosolvents, are very common. For instance, with RNase T_1 the pH dependence of the preexponential terms (at $T = 2$°C) indicates that below pH 7 the conformer equilibrium is largely independent of pH, but between pH 7 and 8 the short lived component becomes dominant and the decay at the highest pH is practically homogeneous. At alkaline pH the protein is only marginally stable, and if conformer interchange were facilitated by the lower stability, the loss of heterogeneity might be due to rapid isomerization.

Such an effect may also be induced by large concentrations (> 0.5 M) of NaCl or KCl which alter τ and render the phosphorescence decay well approximated to a single exponential law. On the other hand, upon increasing the solvent viscosity with the addition of glycerol, the decay slows down and, as opposed to buffer (20°C)

where it is monoexponential, becomes distinctly heterogeneous [11].

The type of buffer can also bring about subtle changes in conformation which are probably caused by distinct affinities for buffering salts. For LADH, on going from TES to phosphate buffer (pH 7) the preexponential terms of LADH change from 0.2 and 0.8 to 0.4 and 0.6 and the lifetimes from 249 and 697 ms to 237 and 516. At high ionic strength, 0.4 M NaCl, the triplet lifetime is slightly smaller ($\tau \approx 300$ ms) but, as for RNase T_1, the decay is practically monoexponential and independent of the type of buffer.

Recently, a variation of preexponential terms was found also with a number of proteins when subjected to pressures of 1–3 kbar [27].

Binding of ligands may have profound effects on the phosphorescence decay kinetics. A sample of the variation of τ and α upon complex formation with substrates, cofactors and allosteric effectors is given in Table 2. In many cases complex formation induces isomerization of the protein structure that results in considerable changes, more often an increase, in triplet lifetime. The decay is often heterogeneous even after complex formation. Since measurements were carried out at over 95% saturation of binding sites the heterogeneity is not due to partial saturation but rather it implies that interconversion between conformers is slow relative to $1/\tau$ even for the bound form of the macromolecule. Phosphorescence lifetimes with Mg–nucleotide complexes of PGK, indicate a drastic tightening of the protein structure accompanied by a shift in the weight of preexponential terms from the short-lived to the long-lived component [12]. Substantial changes in the amplitude of preexponential terms are also induced by coenzyme binding to LADH and binding of allosteric effectors such as glycerol phosphate to Trp synthase [16], ADP and GTP to GDH [24]. The latter enzyme provides a singular case of conformer modulation. Activators, such as ADP, induce a tight configuration of the coenzyme binding domain whereas inhibitors, such as GTP, promote a more flexible one. The free enzyme, which only at lower temperature manifests two distinct conformations, has a phosphorescence lifetime intermediate of that observed with positive and negative effectors its actual value being identical to that given by the two conformations in rapid equilibrium. A model of the hexameric macromolecule based on asymmetric, rapidly interchanging, trimers accounts for both spectroscopic and catalytic properties of the enzyme [28]. Although most complexes have multicomponent decays, binding of 2′-GMP and 3′-GMP to RNase T_1 apparently removes any structural heterogenity. Again, this result could be due either to the stabilization of a predominat form or to the lowering of activation barriers for interconversion among coexisting forms.

Temperature effects on the phosphorescence lifetime are generally large, τ increasing as the temperature is lowered and the greater solvent viscosity/smaller thermal energy reduce segmental motions of the polypeptide. Also at lower

Table 2
Effect of ligand binding on the Trp phosphorescence decays of some proteins of Table 1. All decays were fitted with a biexponential law. Standard errors in τ and α are less than 8%

Protein	Ligand	T (K)	τ_1 (ms)	τ_2 (ms)	α_1	χ^2	Ref.
RNase T_1	2′GMP	273	60	–	1	1.0	[11]
RNase T_1	3′GMP	273	80	–	1	1.1	[11]
PGK	Mg · ADP	273	392	120	0.71	2.0	[12]
PGK	Mg · ATP	273	311	56	0.24	2.2	[12]
PGK	Mg · ADP · 3PG	273	243	60	0.7	2.3	[12]
PGK	Mg · ATP · 3PG	273	112	39	0.32	2.0	[12]
Trp synth. holo	GP	273	66	33	0.7	3.0	[6]
Trp synth. apo	GP	273	53	10	0.14	2.8	[6]
Trp synth. red	GP	273	178	27	0.25	3.0	[6]
LADH	IBA-NADH	293	847	244	0.61	1.1	–

temperature interconversion slows down and more and more substates freeze out so that distinct conformers should be detected with greater resolution. This appears to be the case since at subzero temperatures all the protein examined, but apoazurin, display nonexponential decays which, judging from the magnitude of χ^2, require, relative to ambient temperature, a greater number of exponential components in order to be adequately fitted. Examples of this are provided by RNase T_1, Trp synthase, and F_1 ATPase single Trp proteins which at -30°C have at least three distinct phosphorescence lifetimes.

With multitryptophans proteins interpretation of low temperature decays is complicated by the possibility that under these conditions of large solvent viscosity even Trp residues in mobile sites of the macromolecule, that are quenched at room temperature, may become detectably phosphorescent. In practice, the distinction between protein substates and multi-Trp emission can be made only with precise determination of emission yields in that new contributions to the overall intensity are accompanied by an increase in lifetime-normalized phosphorescence intensity (P/τ). With GDH the constancy of P/τ between 0 and -30°C assigns the emerging heterogeneity in the decay at subzero temperatures to the freezing out of distinct conformers [28].

At sufficiently high temperatures interconversion will eventually become too rapid for conformer resolution to be possible in the phosphorescence time scale and the emission will be characterized by a single average lifetime τ_m. This was confirmed with a number of the proteins of Table 1 and the temperature, T_m, at which heterogeneity was no longer observed is reported in Table 3. Exceptions are Trp synthase and F_1 ATPase for which the phosphorescence intensity is drastically reduced above 40°C, meaning that a large portion of chromophores is too short-lived for detection.

Near T_m, interconversion rates have a magnitude comparable to $1/\tau_m$. This convergence allows for a rough determination of average interconversion rates at T_m and from them, applying transition state theory, the corresponding activation free energies, ΔG^*. In Table 3 we note that the values of ΔG^* derived from this analysis ($1/\tau_m = k = k_B T/h \exp(-\Delta G^*/RT)$) are all greater than 14.4 kcal/mol and surprisingly similar among monomeric and multimeric proteins. There is about 2 kcal/mol lower activation barrier in monomeric proteins. A plausible rationalization for it might be found in the absence of possible quaternary structure restrictions to conformer transition.

If the existence of grossly distinct long-lived conformations of proteins in solution is prevailing among the systems so far investigated, confirming evidence from other spectroscopic techniques is either scanty or of ambiguous interpretation. Namely, for PGK, the presence in solution of several states interconverting slowly on the nanosecond time scale has been inferred from site specific labelling and time-resolved fluorescence energy transfer [5]. Evidence for two conformers of the β subunit of tryptophan synthase in solution has recently been provided by spectrophotometric and electrophoretic methods [29].

Table 3
Phosphorescence lifetimes, τ_m, at the lowest temperature, T_m, at which the decay becomes monoexponential. Catalytic rates, k_{cat}, are compared to the conformer interconversion rates, k_{in}, predicted by ΔG^* at the same temperature (T)

Protein	T_m (K)	τ_m (ms)	ΔG^* (kcal/mol)	k_{cat} (s^{-1})	Ref.	T (K)	k_{in} (s^{-1})
GDH	273	2300	16.3	3.5–194	[39]	298	6.2
RNase T_1	285	38	14.7	30–350	[40]	298	94.0
LADH	323	27	16.6	23	[41]	296	3.1
PGK	290	20	14.7	570–1650	[42]	298	94.0
GPDH yeast	313	56	16.5	360	[43]	293	2.7
Trp synthase	303	8 [a]	> 14.8	15	[44]	310	< 218
F_1 ATPase	303	4 [a]	> 14.4	556	[45]	303	< 237.8

[a] For these proteins the decay is not monoexponential and τ_m represents the average lifetime $= \alpha_1\tau_1 + \alpha_2\tau_2$.

Different long-lived conformers in solution were shown to exist for both *Staphylococcal* nuclease [8] and dihydrofolate reductase [7] by NMR techniques. For the latter protein, an activation free energy barrier of 17.8 kcal/mol was determined from the time of interconversion. However, NMR findings are open to criticism because in the mM range of protein concentration at which experiments are typically carried out, protein–protein interactions could give rise to heterogeneous long-lived aggregates.

Indirect evidence of stable and functionally distinct conformers for enzymes in solution is often provided by complex ligand binding and catalytic rate behaviours. While for multimeric proteins non-linear kinetics are invariably taken as an indication of cooperativity among subunits, with monomeric proteins, PGK being a notable example [30], anomalous kinetic plots imply either a multiplicity of long-lived conformations or inhomogeneous protein samples. In Table 3 the turnover numbers of some enzymes are compared to the conformer interconversion rates as predicted for the same temperature by ΔG^*. What is striking is that in all cases separate conformations of the macromolecule persist for times longer than the catalytic cycle. If, as plausible, distinct catalytic rates were associated to each structural form complex kinetic behaviours rather than an anomaly would be expected to represent the norm. Certainly the prevalence of multiple gross conformations in solution together with the prompt modulation of substates equilibria by effector molecules and environmental conditions unveils a subtle link between structure and function that, although still little understood, will have to be taken into account when formulating models that describe biological function. For example, the origin of cooperativity in oligomeric enzymes of identical subunits is still an important problem. The concerted model of Monod et al. [31] proposes that two or more protein structures coexist in equilibrium, whereas the sequential model of Koshland et al. [32], requires that different conformers of the proteins exist only after substrate binding. The present investigation points out that multiple stable conformations often coexist in solution and that invariably ligand binding alters both their structure and relative stability, and it therefore emphasizes that a more integrated model is probably required for describing cooperativity.

Recent experimental and theoretical work on the conformational energy landscape and motions in proteins has established that conformational substates can be roughly classified into a hierarchy with several tiers [2]. At the highest levels macrostates are few, interconvert slowly and, in the case of myoglobin, were shown to possess distinct functional properties [33]. Within this framework it is clear that the phosphorescence lifetime can distinguish among macrostates of the protein near the top of the hierarchy. The finding that such heterogeneity in structure may be a 'universal' feature of proteins in solution has important implications for the interpretation of folding, kinetic and regulation processes.

Acknowledgement

The authors are grateful to Ms. Claudia Neri for typing the manuscript and to Mr. Alessandro Puntoni for his technical assistance.

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