Time-Resolved Room Temperature Protein Phosphorescence: Nonexponential Decay from Single Emitting Tryptophans

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ABSTRACT The single room temperature phosphorescent (RTP) residue of horse liver alcohol dehydrogenase (LADH), Trp-314, and of alkaline phosphatase (AP), Trp-109, show nonexponential phosphorescence decays when the data are collected to a high degree of precision. Using the maximum entropy method (MEM) for the analysis of these decays, it is shown that AP phosphorescence decay is dominated by a single Gaussian distribution, whereas for LADH the data reveal two amplitude packets. The lifetime-normalized width of the MEM distribution for both proteins is larger than that obtained for model monoexponential chromophores (e.g., terbium in water and pyrene in cyclohexane). Experiments show that the nonexponential decay is *fundamental*; i.e., an intrinsic property of the pure protein. Because phosphorescence reports on the state of the emitting chromophore, such nonexponential behavior could be caused by the presence of excited state reactions. However, it is also well known that the phosphorescence lifetime of a tryptophan residue is strongly dependent on the local flexibility around the indole moiety. Hence, the nonexponential phosphorescence decay may also be caused by the presence of at least two states of different *local* rigidity (in the vicinity of the phosphorescing tryptophan) corresponding to different ground state conformers. The observation that in the chemically homogeneous LADH sample the phosphorescence decay kinetics depends on the *excitation* wavelength further supports this latter interpretation. This dependence is caused by the wavelength-selective excitation of Trp-314 in a subensemble of LADH molecules with differing hydrophobic and rigid environments. With this interpretation, the data show that interconversion of these states occurs on a time scale long compared with the phosphorescence decay (0.1–1.0 s). Further experiments reveal that with increasing temperature the distributed phosphorescence decay rates for both AP and LADH broaden, thus indicating that either 1) the number of conformational states populated at higher temperature increases or 2) the temperature differentially affects individual conformer states. The nature of the observed heterogeneous triplet state kinetics and their relationship to aspects of protein dynamics are discussed.

INTRODUCTION

The description of proteins as static, topologically well ordered systems (as often schematically represented based on crystallographic experiments) has evolved over time as new techniques have yielded valuable insight into their dynamic nature. This study of dynamics is at the heart of investigations into the protein structure-function problem because functional proteins must show structural flexibility and undergo conformational change for such processes as ligand binding and release, catalysis, and transport. Techniques such as molecular dynamics modeling, fluorescence, and the temperature dependence of x-ray diffraction Debye-Waller factors have extended our knowledge of biomolecular dynamics on the picosecond to nanosecond time scales, whereas methods such as NMR and hydrogen isotope exchange have been used to probe motions on the tens of nanoseconds to hour time range.

Room temperature phosphorescence (RTP) of intrinsic tryptophan residues is useful in the study of protein dynamics over the submillisecond to second time scale of its emission. Unlike tryptophan fluorescence decay, which is sensitive to a variety of intrinsic quenchers and other effects such as solvent relaxation, tryptophan phosphorescence lifetime (in the absence of disulfide groups, the only known intrinsic quenchers (Li and Galley, 1989)) is determined primarily by local rigidity (Strambini and Gonnelli, 1985). Thus, for free indole in aqueous solution the phosphorescence lifetime, τ, is 11.6 μs (Bent and Hayon, 1975; Ghiron et al., 1988), whereas if constrained in a rigid polymer such as polyvinyl alcohol τ approaches the 6 s observed in frozen glasses (Kuntz, 1968). The long-lived tryptophan RTP in purified proteins is necessarily associated with those tryptophans located in exceptionally inflexible regions, and this lack of local flexibility is consistent with phosphorescence anisotropy measurements that indicate that phosphorescent tryptophans are immobilized by the protein matrix on the time scale of the decay for a variety of tryptophan-containing biomolecules (Papp and Vanderkooi, 1989). Tryptophan RTP thus provides an ideal probe, of great sensitivity, for structural studies of the core of globular proteins. Such regions are of critical importance to overall protein stability and as such are associated with the nascent protein folding core (Kim et al., 1993).

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Abbreviations used: AP, *Escherichia coli* alkaline phosphatase; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; IEF, isoelectric focusing gel electrophoresis; LADH, horse liver alcohol dehydrogenase; MEM, maximum entropy method; NADH, nicotinamide adenine dinucleotide; RTP, room temperature phosphorescence; Tris, tris(hydroxymethyl)aminomethane.

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The first report of intrinsic protein RTP and its assignment to tryptophan was in the 1974 work of Saviotti and Galley on horse liver alcohol dehydrogenase (LADH) and *Escherichia coli* alkaline phosphatase (AP). Monoexponential decays of 130 and 800 ms were found for LADH and AP, respectively. In the intervening 20 years, LADH and AP have been used extensively in room temperature phosphorescence studies (Kai and Imakubo, 1979; Domanus et al., 1980; Calhoun et al., 1983; Strambini, 1983; Strambini and Gonnelli, 1985; Gonnelli and Strambini, 1986; Strambini and Gonnelli, 1986; Strambini, 1987; Strambini et al., 1987; Strambini and Gabellieri, 1987; Calhoun et al., 1988; Gabellieri et al., 1988; Strambini and Gonnelli, 1988; Strambini and Gabellieri, 1990; Strambini and Gonnelli, 1990; Strambini et al., 1990; Vanderkooi et al., 1990; Wright et al., 1992). Although the lifetimes reported in these investigations range from 120 to 680 ms for LADH and from 800 to 2000 ms for AP (reflecting the inherent difficulties in thoroughly removing the ubiquitous triplet state quencher oxygen from the samples), in all instances they were reported to be monoexponential. More recent work from our laboratory has demonstrated, however, that by improving the signal-to-noise in the decay curve it becomes evident that the triplet state decay of LADH is not a single exponential (τ_{ave} = 634–650 ms) (Schauerte et al., 1992a, b).

It has been generally believed that tryptophan RTP decays exponentially; this is a reasonable assumption because its decay in solid media at liquid nitrogen temperature is invariably reported to be a single exponential (Longworth, 1971). Nonexponential RTP has been observed previously in complex systems. The ligand-induced biphasic kinetics observed in glutamate dehydrogenase and LADH (Strambini et al., 1987; Strambini et al., 1990) have thus been interpreted as arising from macroscopic changes in enzyme conformation. Multi-tryptophan-containing proteins such as glucose-6-phosphate dehydrogenase show complex kinetics attributed to contributions from more than one tryptophan (Schauerte et al., 1992b). Solidified species such as crystallin aggregates (Berger and Vanderkooi, 1989) and lyophilized powders (Strambini and Gabellieri, 1984) also exhibit nonexponentiality because of protein-protein interactions.

In the current work, we show that the RTP decays of both AP and LADH in fluid solutions are intrinsically nonexponential despite the fact that this emission originates from a single tryptophan residue in each of the two proteins. We extend our previous work on LADH by making use of a distributional model in the analysis of the decay data and by studying the excitation wavelength and temperature dependence of the phosphorescence decay of the chemically well characterized samples. It is concluded that the phosphorescence data may reveal an unexpected long-lived heterogeneity of emitting protein species.

MATERIALS AND METHODS

$Tb(Cl)_3$ was from Aldrich (Milwaukee, WI). *E. coli* alkaline phosphatase type III-S (Sigma Chemical Co., St. Louis, MO) and horse liver alcohol dehydrogenase (Boehringer-Mannheim, Indianapolis, IN) were exchanged from the solutions in which they were received into 10 mM Tris pH 8.0 and 10 mM HEPES pH 7.4 buffers, respectively, using ultrafiltration devices (Amicon, Beverly, MA). Before spectroscopic measurements, both samples were filtered through a 0.2 μm filter to remove solid contaminants. From the absence of nicotinamide adenine dinucleotide (NADH) absorbance at 340 nm (extinction coefficient = 6220 M^{-1} cm^{-1} (Coleman and Weiner, 1973)) and from the signal-to-noise ratio of this measurement, we conservatively estimate an upper limit of 2% for the amount of NADH present in the LADH sample. LADH from Boehringer-Mannheim is known to be of the isomeric form designated EE (Johansson et al., 1991; Ehrig et al., 1992). Because of the nonexponential decays obtained for LADH, the question of sample homogeneity must be addressed. Our material showed a single band with sodium dodecyl sulfate-polyacrylamide gel electrophoresis and a specific alcohol activity commensurate to that reported by the suppliers. The lack of steroid activity is consistent with the absence of the LADH isomers designated ES and SS, which are active towards both alcohols and steroids. CM-Sephadex chromatography of the Boehringer-Mannheim enzyme showed less than 5% of a species that eluted at lower ionic strength than the majority of LADH and may have been caused by a column-induced precipitate. Isoelectric focusing (IEF) of the Boehringer-Mannheim sample on agarose gels with 8–9.5 pH ampholytes gave a single band at a pI of 8.4 with Coomassie brilliant blue staining (determined relative to pI standards). From the IEF results, we estimate that if other charged species are present they must contribute less than 5% to the total protein. Based upon these experiments, the apo-LADH sample used in our studies appears chemically homogeneous. Our AP sample, which as recieved is a mixture of three isozymes, was used without further purification.

The observation of room temperature phosphorescence of proteins in solution depends critically on the removal of the effective triplet state quencher oxygen from the sample solution. Deoxygenation was achieved by subjecting two- to four hundred microliter protein samples, sealed in 1 cm × 1 cm cuvettes, to several hours of a continuous stream of purified argon (Englander et al., 1987) in the top part of the cuvette. This method is time consuming and depends on the rate of partitioning of gases at the liquid-gas interface. We have recently modified our deoxygenation procedures and now use argon (<1 ppm $O_2(g)$), which is further purified (<50 ppb $O_2(g)$) by use of an inline cartridge (Labclear, Oakland, CA). Samples in sealed cuvettes are slowly and repeatedly cycled between a water-aspirated vacuum and one atmosphere argon with due care to minimize the formation of denaturing bubbles. This method takes 10–20 min to achieve oxygen levels comparable with those obtained after 2–3 h using the aforementioned technique. There are no discernible differences in spectroscopic results obtained using samples prepared with the two different methods.

Phosphorescence measurements were made using a time-resolved, laser-based instrument similar to that described previously (Mersol et al., 1991). The frequency-doubled output of a Nd:YAG laser-pumped dye laser was used as an excitation source. Uncorrected emission spectra were obtained by exciting the sample with a laser pulse (5 ns, generally at 10 Hz) and electronically gating the emission to exclude short times (generally no less than 1 ms of the initial signal is excluded) in which prompt fluorescence and scattering are present. A one-third meter monochromator (2.4 nm resolution) dispersed the light, which was detected using photon counting techniques. Phosphorescence decays were collected at a single wavelength using a multi-channel scaler. Count rates were kept well below the experimentally determined instrumental bandwidth to avoid distortion of the decay curves. Individual decay curves were accumulated long enough to obtain good signal-to-noise. Typically 10^5-10^6 counts in the peak channel were obtained, and the initial intensity of the decay was followed over 3 to 4 orders of magnitude corresponding to between 6.9 and 9.2 lifetimes for a monoexponential decay. Before analysis, background counts were subtracted from the data.

Data were analyzed in two ways. The discrete model assumes that the phosphorescence decay, $P(t)$, is composed of a finite sum of components, $P(t) = \sum_i^N \alpha_i \exp(-t/\tau_i)$, where N is the number of components and the associated amplitudes are normalized, $\sum \alpha_i = 1$. An analysis is determined to be adequate if the reduced chi-squared, χ^2, is close to one and a visual

inspection of residual and autocorrelation plots show a random dispersion with respect to zero. In situations where no a priori physical information is known concerning the complexity of a luminescence decay, however, it can be shown that the maximum entropy method (MEM) analysis is an unbiased predictor (Livesey and Brochon, 1987). The MEM algorithm maximizes the function $Q = \lambda S - C$ where S is a Shannon-Jaynes entropy-like function:

$$S = -\sum_i^m \alpha_i(\tau)\log\left(\frac{\alpha_i}{\alpha_{tot}}\right) \qquad \alpha_{tot} = \sum_i^m \alpha_i.$$

λ is a Lagrangian scaler, and C is a chi-squared constraint

$$C = \frac{1}{N}\sum_i^N \frac{(I_i^{calc} - I_i^{obs})^2}{(\sigma_i)^2}.$$

N is the total number of data channels and σ_i is the variance in the intensity of the ith channel, I_i. With experimental data subject to Poisson statistics, σ_i is taken as the square root of the number of counts in the ith channel. For all of the MEM results presented here, the number of equally spaced lifetimes in log τ space, m, was set to 100 corresponding to the number of exponents in the series $\sum \alpha_j \exp(-t/\tau_j)$. The algorithm initially sets all of the amplitudes equal then maximizes Q until the reduced chi-squared is ~1.0, at which time only the entropy, S, is adjusted.

The recovered spectrum of amplitudes from the MEM program is in log τ space. Transformations to linear τ space and to decay rate space that correctly preserve distributional information were performed as previously outlined (Siemiarczuk et al., 1990). The fractional contribution of a given peak to the total distribution spectrum is defined to be the ratio of the integrated area of the peak, with limits of integration being local minima that bound the peak, to the total integrated area of the spectrum. Both discrete and MEM software routines were supplied by PTI, Inc. (South Brunswick, NJ).

RESULTS

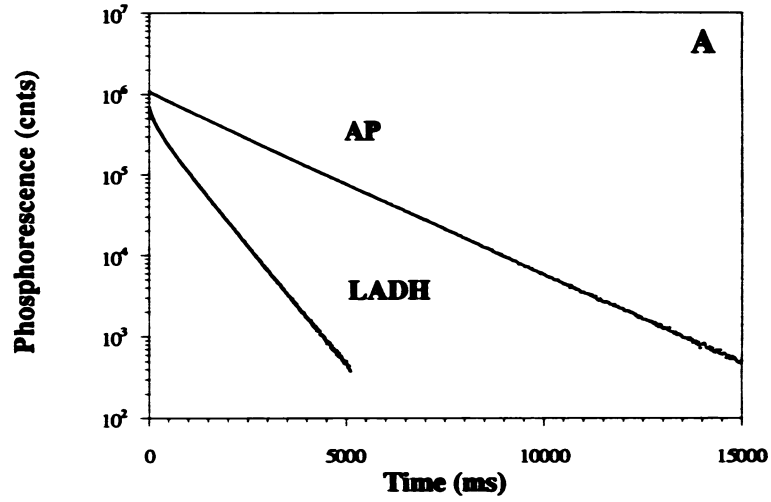
The RTP spectra of AP and LADH are typical of tryptophan in proteins and show, in each case, a single resolved 0,0-band that arises in the 410 to 412 nm region (data not shown). Although AP contains 3 tryptophans per monomer, whereas LADH contains 2, only Trp-109 in AP and Trp-314 in LADH are phosphorescent in fluid solution (Domanus et al., 1980; Strambini and Gabellieri, 1990; Mersol et al., 1991). The assignment of LADH phosphorescence to Trp-314 is particularly well established. At liquid nitrogen temperatures, LADH shows well resolved dual phosphorescence with 0,0-band wavelengths of 405 and 412 nm (Purkey and Galley, 1970; Strambini and Gabellieri, 1990). Furthermore, the most red-shifted phosphorescence can be selectively excited using wavelengths that lie on the red side of the proteins' absorption (Purkey and Galley, 1970), consistent with a tryptophan in a hydrophobic environment. A short lifetime component associated with the 405 nm band in LADH is observed to disappear distinctly as the sample is warmed through the glass transition temperature of 220 K (Strambini and Gabellieri, 1990). These observations unambiguously assign the 405 nm-based phosphorescence spectrum to the solvent-exposed LADH Trp-15. In our experiments, no spectral shifts could be observed upon changing the excitation wavelength for either AP or LADH. The resolution of previous time-resolved spectral measurements did not allow for detection of structural rearrangements occurring on the time scale of the excited state lifetime (over the time interval 0.1–5 s) for AP or LADH (Calhoun et al., 1983). The data strongly support the notion that the RTP from each of these two multi-tryptophan-containing proteins arises solely from a single residue.

The phosphorescence decay curves for AP and LADH at 20°C are shown in Fig. 1 *A*. The decays are not monoexponential as clearly demonstrated by the residual plots (Fig. 1 *B*) obtained using the discrete model and a number of components. Indeed, for the quality of the data presented here, three and four discrete components were required to model accurately the phosphorescence decays of AP and LADH, respectively. An important observation is that fewer components are required to achieve an adequate fit when using the discrete model if the phosphorescence decays are collected to fewer counts per channel.

We previously reported that LADH in 100 mM phosphate buffer (pH 7–8) exhibits an attenuated lifetime with respect to that found in HEPES buffer (Schauerte et al., 1992a), raising the possibility of protein-solvent interactions. It is important to note that although the average phosphorescence lifetime is diminished in phosphate buffer, possibly because of a specific phosphate-LADH interaction or an unidentified contaminant quencher such as a trace metal, the decay is *distinctly nonexponential.* Measurements of LADH phosphorescence in 50 mM glycine/sodium hydroxide buffer at pH 10.0 and in 50 mM 2-(*N*-morpholino)ethanesulfonic acid at pH 6.0 show changes in average phosphorescence lifetime (data not shown), but like LADH in phosphate buffer the decay is *unmistakably nonexponential.* In addition, we note that the LADH phosphorescence decay is nonexponential for concentrations between 0.2 to 1.8 mg/ml and, therefore, cannot be the result of protein-protein interactions.

To gain further insight into the observed complex triplet state decays, the data were analyzed assuming lifetime distributions. The MEM analysis of the data presented in Fig. 1 is shown in Fig. 2 (*open circles*), where the results of the discrete analysis with heights proportional to amplitudes are included as lines for comparison. The results for LADH are dominated by a long-lifetime distribution (fractional contribution of 67%) centered at 712 ms with a full width at half-maximum, $\Delta\tau$, of 210 ms. The remaining 23% of the spectrum at shorter lifetimes is relatively complex, showing at least two local maxima with the most prominent local maximum found at 140 ms. The four component discrete analysis of the data shows some correspondence to the MEM result, especially with respect to the 712 ms peak, which might be correlated with the 769 ms (43%) component. Similarly, the 169 ms (20%) and 41 ms (11%) discrete components appear to coincide with the 140 ms peak area of the MEM image. However, the four-component model shows a significant contribution, 26%, of a 519 ms component, falling between the two peaks in the MEM analysis: a decay component that is not suggested by the MEM analysis.

In contrast to the distribution analysis of LADH, the MEM image of AP shows primarily a single Gaussian (97% fractional contribution) with a mean of 1920 ms and a $\Delta\tau$ of 290 ms. Additionally, a small contribution (less than 3% of the



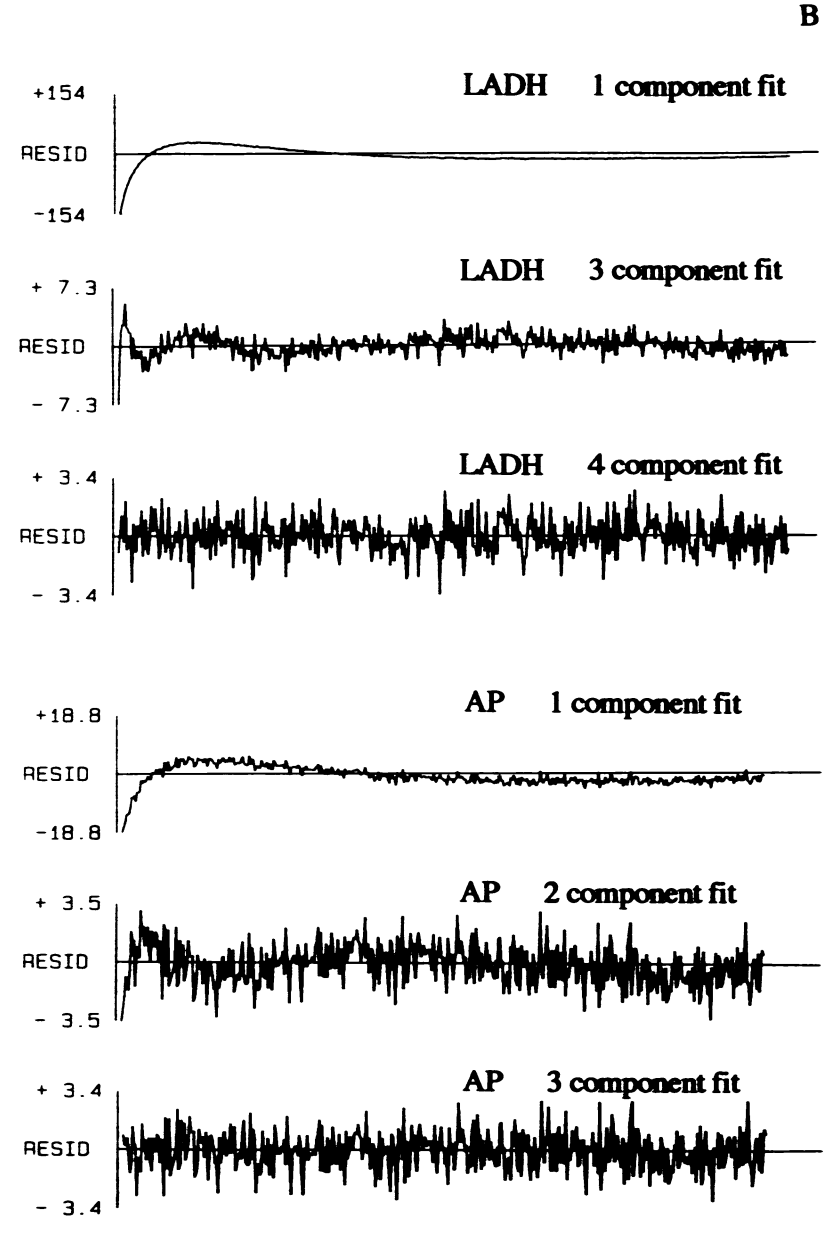


FIGURE 1 (*A*) Pulse-induced (280 nm) phosphorescence decay of solutions of LADH (0.7 mg/ml, 10 mM HEPES pH 7.4) and AP (0.8 mg/ml, 10 mM Tris pH 8.0) at room temperature (20°C). (*B*) From top to bottom, the weighted residuals for 1, 3, and 4 discrete components for LADH, and for 1, 2, and 3 discrete components for AP, are shown. Parameters for LADH: one component ($\chi^2 = 423.52$): $\alpha_1 = 1.000$, $\tau_1 = 655$ ms; three components ($\chi^2 = 1.65$): $\alpha_1 = 0.200$, $\tau_1 = 86$ ms, $\alpha_2 = 0.236$, $\tau_2 = 349$ ms, $\alpha_3 = 0.564$, $\tau_3 = 740$ ms; four components ($\chi^2 = 1.01$): $\alpha_1 = 0.112$, $\tau_1 = 41$ ms, $\alpha_2 = 0.198$, $\tau_2 = 169$ ms, $\alpha_3 = 0.260$, $\tau_3 = 519$ ms, $\alpha_4 = 0.430$, $\tau_4 = 769$ ms ($\tau_{ave} = 655$ ms). For AP: one component ($\chi^2 = 10.63$): $\tau_1 = 1908$ ms; two components ($\chi^2 = 1.48$): $\alpha_1 = 0.049$, $\tau_1 = 841$ ms, $\alpha_2 = 0.951$, $\tau_2 = 1932$ ms; three components ($\chi^2 = 1.28$): $\alpha_1 = 0.018$, $\tau_1 = 228$ ms, $\alpha_2 = 0.072$, $\tau_2 = 1247$ ms, $\alpha_3 = 0.910$, $\tau_3 = 1945$ ms ($\tau_{ave} = 1910$ ms).

$$\tau_{ave} = \frac{\sum(\alpha_i \tau_i^2)}{\sum(\alpha_i \tau_i)}.$$

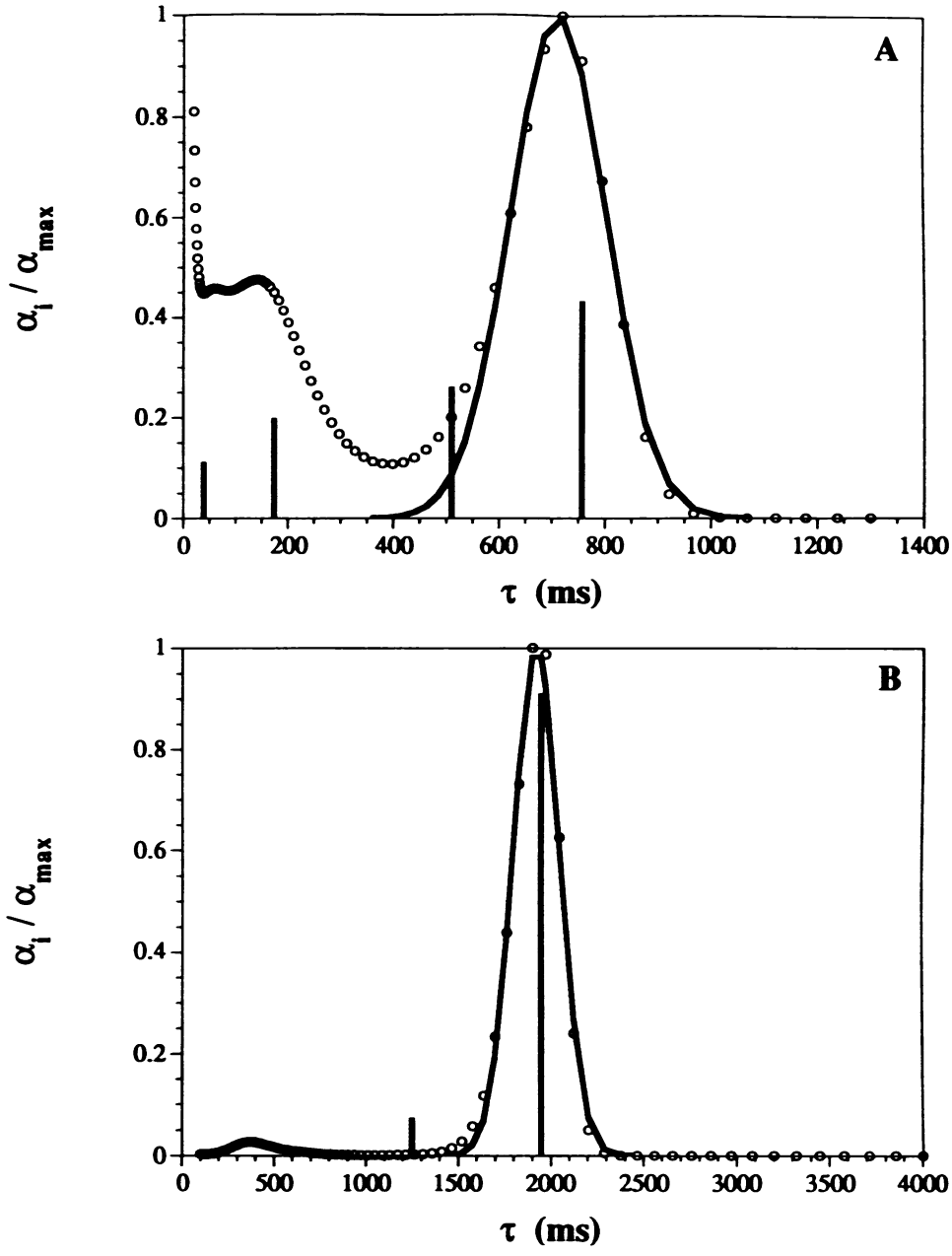


FIGURE 2 MEM distribution analysis of the data shown in Fig. 1. The amplitudes (○) are normalized to peak amplitudes. (*A*) LADH. A line shows a Gaussian approximation to the major component with a peak at 712 ms and $\Delta\tau$ of 210 ms. (*B*) AP. The shown fit to the MEM amplitudes is a Gaussian with a peak at 1920 ms and $\Delta\tau$ of 290 ms. Also shown in *A* and *B* by vertical lines are the results of the discrete analysis (4- and 3-component fits for LADH and AP, respectively), the residuals of which are displayed in Fig. 1 *B*.

total distribution) is found centered at 380 ms. The discrete analysis agrees well with the distributional analysis for the long-lived component of the decay; however, it is less than satisfactory for the shorter-lived, minor constituents of the overall decay. It must be noted that, unlike our LADH sample, the AP material is composed of several isozymes, and we cannot exclude the possibility of their contribution to the phosphorescence lifetime distribution width.

When the distribution analysis results for LADH and AP are replotted after their scaling by the lifetime associated with the center of the Gaussian envelope (shown in Fig. 3 with amplitudes normalized to their maximum values), the full width at half-maximum amplitude of the major lifetime distribution of Trp-314 in LADH ($\Delta\tau/\tau_{mean}$ = 0.30) is a factor of two greater than for Trp-109 in AP ($\Delta\tau/\tau_{mean}$ = 0.15). We also note that even the latter tryptophan has a distribution width in the unitless representation that is much greater than for model monoexponential chromophores. More specifically, our analysis of Tb(III)*aq* shows τ_{mean} = 440 μs, $\Delta\tau/\tau_{mean}$ = 0.02, whereas pyrene in cyclohexane gives τ_{mean} = 383 ns, $\Delta\tau/\tau_{mean}$ = 0.02 (Siemiarczuk et al., 1990). Both are much closer to pure monoexponential.

The results of experiments probing the temperature dependence of AP and LADH phosphorescence are shown in Fig. 4. An increase in temperature from 4 to 41°C results in an order of magnitude decrease in the mean lifetime of LADH, from 1290 to 125 ms, and an increase in the fractional contribution of the long-lifetime distribution from 73 to 85% as shown in Fig. 4 *A*. (Note that the 67% fractional contribution of the long-lived distribution, shown above in Fig. 3

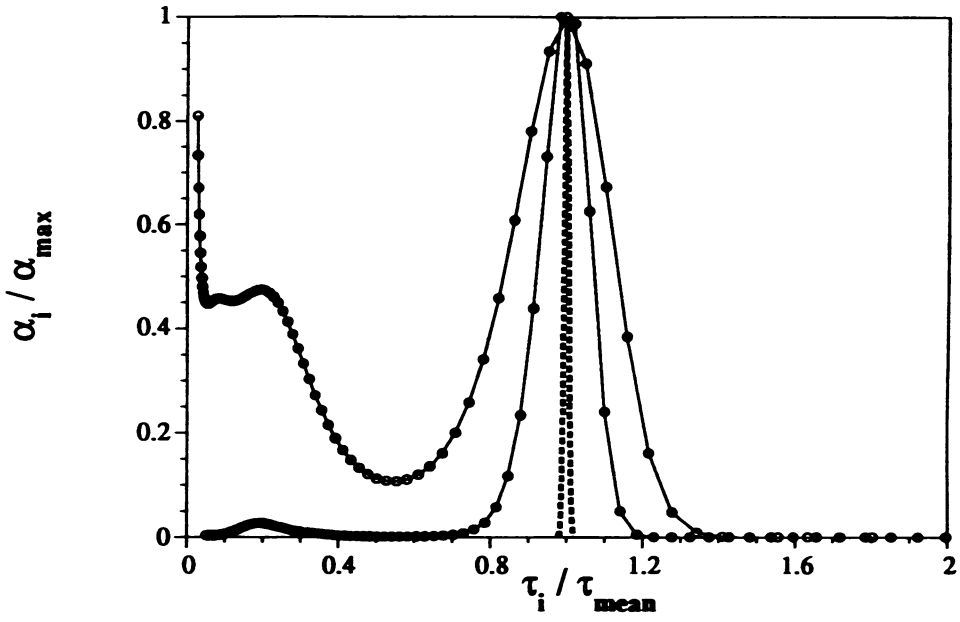


FIGURE 3 The results of the distributional analysis for LADH (○), AP (●) and Tb(III)*aq* (—). The amplitudes of each decay are arbitrarily normalized by their respective peak amplitudes.

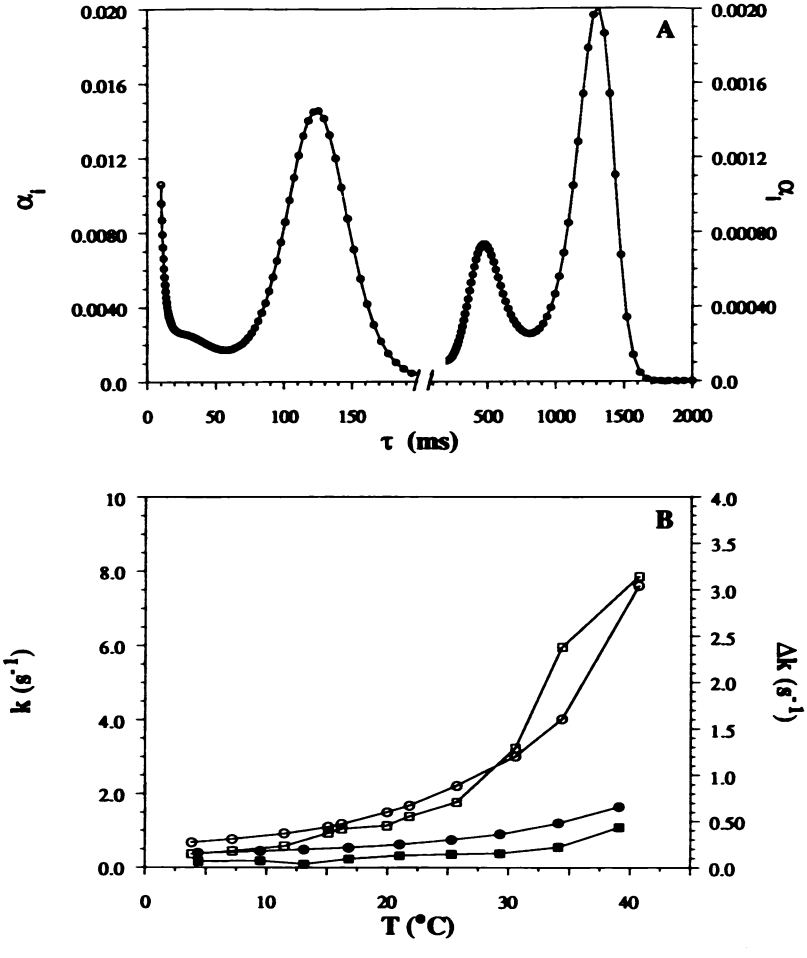


FIGURE 4 LADH (1.3 mg/ml) and AP (1 mg/ml) phosphorescence temperature dependence. (*A*) Results of a distribution analysis for LADH at 4°C (●) and at 41°C (○). Note the discontinuity and difference in scaling of the abscissa between the two temperatures. The amplitudes are adjusted so that the area under each curve equals one. (*B*) The temperature dependence of the mean decay rates, k_{mean} s^{-1} (*circles*), and the distributional rate width, Δk s^{-1} (*squares*), derived from the MEM analysis for AP (*filled symbols*) and LADH (*open symbols*). The parameters k_{mean} and Δk were graphically determined from plots of the inverse lifetimes.

for LADH at 20°C, is not bracketed by the fractional contributions reported in Fig. 4 *A* for higher and lower temperatures as one might expect. Different LADH samples were used for the 20°C and temperature-dependent measurements, and it is a general observation that there is variability in the contribution of lifetime components for different LADH preparations.) The temperature-dependent measurements on a single sample of LADH were made by starting

at room temperature, taking several measurements at lower temperature, then several high temperature measurements, and finally measurements were made again at room temperature. No significant changes in the room temperature data were observed between the first and last measurements made on a single sample.

The temperature dependence of the mean of the distribution of decay rates, $k_{mean} = (\tau_{mean})^{-1}$, and rate distribution widths at half-maximum amplitude, Δk, for AP and LADH are displayed in Fig. 4 *B*. There is a larger temperature dependence of k_{mean} and Δk for LADH (*open symbols*) than there is for AP (*filled symbols*). The Arrhenius activation energies determined for LADH, 11 kcal mol^{-1}, and for AP, 7 kcal mol^{-1}, are in line with those reported previously (Wright et al., 1992). The correspondence between the change in the mean rate of decay of the phosphorescent tryptophan (*circles*) and of its distributional width (*squares*) is readily apparent.

Further information concerning the Trp-314 environment in LADH may be gained from the dependence of the phosphorescence on excitation wavelength. Such an experiment is shown in Fig. 5, where the phosphorescence is excited at 280 and 295 nm. As the excitation wavelength is red-shifted, the average lifetime increases from 552 to 676 ms. Indeed, the two logarithmic decays are not parallel at long decay times, clearly demonstrating that longer lifetime components are selected with the 295 relative to 280 nm excitation. In addition, the 280 nm selected phosphorescence exhibits increased curvature, indicative of short lifetime components not observed in the 295 nm excitation experiment. Thus, the excitation dependence of lifetimes appears to reflect not only a partitioning of populations among lifetime components but a qualitative change in the selected lifetime species. The complication of photochemistry, an alternative interpretation of the data, is discussed below. Within the precision of our measurements, no LADH phosphorescence spectral shifts were observed as a function of excitation wavelength, which is consistent with previous observations (Calhoun et al., 1988). Furthermore, no changes in decay were observed as a function of emission wavelength. In contrast to LADH, similar photoselective experiments performed on AP show the decay kinetics to be independent of the excitation energy.

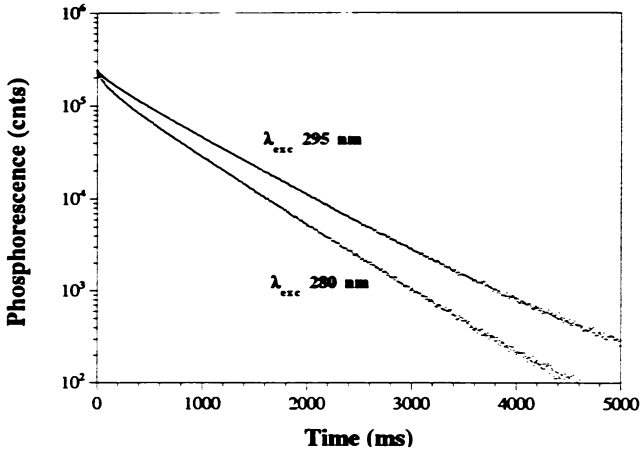


FIGURE 5 Excitation wavelength dependence of the phosphorescence decay of LADH (1.7 mg/ml) at 20°C. The average lifetime associated with the decays are 552 and 676 ms for the excitation wavelengths 280 nm (*bottom*) and 295 nm (*top*), respectively.

DISCUSSION

Nonexponential phosphorescence decay behavior

To demonstrate that the complex phosphorescence lifetime distribution observed in LADH was intrinsic, the enzyme was characterized to ensure that the contribution from impurities or aggregated proteins was minimal. Chromatography, electrophoresis, and isoelectric focusing analysis as well as activity assays all suggested that the LADH sample was homogeneous before spectroscopic measurement. Because the question of sample homogeneity is paramount to the interpretation of the data, we elaborate on several potential areas of concern in the following discussion.

Apo-LADH and NADH-bound LADH exhibit different phosphorescence lifetimes (Strambini and Gonnelli, 1990); hence, if NADH were present it may be a source of potential lifetime heterogeneity. The absence of detectable NADH absorbance at 340 nm argues against this. Furthermore, when LADH is saturated with NADH the phosphorescence intensity decreases by 85%, largely because of quenching of the singlet state of Trp-314 (Strambini and Gonnelli, 1990). A 2% contribution of NADH-bound LADH (the upper limit of NADH in our sample), which is 85% quenched, is inconsistent with the significant complexity in LADH lifetime that we observe.

Other potential sources of chemical inhomogeneity are changes in the state of protonation of amino acid residues, or covalent modifications of residues that vary from one LADH molecule to another. The nonexponential phosphorescence decay observed for LADH is clearly independent of different protonation states of the enzyme because the complex decay behavior is observed at values from 6 to 10 pH. The possibility of covalent modifications to some of the LADH molecules is more difficult to rule out. If such modifications lead to a change in charge, then their existence would be detected upon isoelectric focusing. Because the LADH IEF results show that there is an upper limit of 5% of total protein with a pI different then 8.4, any covalent modifications present must not result in a charge change.

Chemical inhomogeneity could also arise from substitution of one of the two Zn(II) per monomer with another metal. This is unlikely because the association constant for Zn(II) is large with respect to other metals commonly found to contaminate buffers. Furthermore, addition of millimolar amounts of Zn(II) to the LADH, which would displace trace metal contaminants, had no effect on the phosphorescence lifetime (data not shown).

Freely diffusing external agents known to cause triplet state quenching, the most likely species present being residual oxygen, will not give rise to a heterogeneous decay

unless the access to the tryptophan varies from molecule to molecule. Therefore, external quenchers appear not to be the cause of nonexponential phosphorescence decays. The only known intrinsic triplet state quencher in proteins, disulfides, is absent in LADH.

The explicit correlation of phosphorescence lifetime with the microviscosity at the site of the emitting tryptophan suggests that the origin of the nonexponential phosphorescence decay may be caused by ground state heterogeneity corresponding to different conformational states of local rigidity around Trp-109 in AP and Trp-314 in LADH. Such a model was also proposed earlier to interpret recent time-resolved phosphorescence experiments in the multi-tryptophan-containing yeast enzyme phosphoglycerate kinase. In this case, RTP was shown to arise from a single buried tryptophan (Cioni et al., 1993), and the biphasic decay observed using a discrete analysis was interpreted as evidence for slowly interconverting conformers corresponding to a large structural change in the protein. This model was also used to interpret the nonexponential decay found at room temperature for Trp-177 of tryptophan synthase (Strambini et al., 1992; Strambini et al., 1992). Such behavior is indeed surprising because it is usually assumed that at room temperature the activation energy for interconversion between protein conformer states is small compared with kT. Although not discussed in these references, an alternative explanation, assuming issues of sample purity and absence of triplet state quenchers are excluded from consideration as done above for LADH, is that the nonexponential decay is caused by the presence of a reversible excited state reaction that is yet to be identified. Because phosphorescence reports on molecular structure and dynamics associated with the (excited) triplet state, it is difficult to rule out absolutely such an alternative model. Indeed, such effects were clearly important in the study of triplet state dynamics on the shorter time scale character of indole and related derivatives (Bent and Hayon, 1975) that are free in solution. However, the great sensitivity of phosphorescence lifetime to changes in local viscosity (Strambini and Gonnelli, 1985) appears to support a model based on ground state heterogeneity. Indeed, the data shown below on the excitation wavelength dependence for LADH phosphorescence are consistent with the model that the ground state of chemically homogeneous LADH is heterogeneous in rigidity in the environment of Trp-314.

Nature of the heterogeneous decay behavior

The demonstration of nonexponentiality in the RTP decays of AP and LADH is the result of the higher level of resolution of the phosphorescence decay kinetics achievable through an increase in the dynamic range and in the total number of photons counted. Decay data with lower signal-to-noise ratio may be well fit with a single exponential as judged by a χ^2 close to one and a random distribution of residuals with respect to zero. With further data collection, a single exponential decay analysis fails and the use of additional discrete components in the fitting analysis is required to achieve an acceptable result. The number of components required continuously increases if the data do not agree with the discrete model (Siemiarczuk et al., 1990). This is the case for the AP decays presented here where data collected to low counts per channel can be modeled adequately as a single exponential, but as the number of photon counts and dynamic range increases, the number of exponentials required to obtain the same χ^2 increases. The observations for LADH are analogous to those of AP; however, because LADH is less "single exponential" than AP, the single exponential model fails even at a low level of data accumulation. To analyze these complex nonexponential decays, a distribution analysis based on the maximum entropy method was used rather than the more model-dependent discrete component method (Brochon et al., 1990).

It is important to note that the dependence of the recovered χ^2 on the number of counts in a time-correlated single-photon counting experiment is the subject of a recent paper (Lami and Piemont, 1992), where it was determined that the counts per channel were not Poisson-distributed because of the contribution of instrumental factors that were correlated with total number of counts collected. Our apparatus was tested for such errors by using the model exponentially decaying Tb(III)*aq*, and it was found that the recovered distribution width was independent of the total number of counts in an experiment.

By way of comparison to model emitters, we note that MEM analysis of the monoexponential chromophores Tb(III)*aq* and pyrene in cyclohexane resulted in images with relative widths of 2%. The corresponding width for AP, 15%, is significantly larger. We conclude, therefore, that the underlying decay associated with the apparent Gaussian at 1920 ms in the MEM spectrum is more complex than a single discrete exponential. We note, however, that the MEM analysis, when applied to simulated data of similar quality as used here, is unable to resolve two discrete lifetimes separated by a factor of less than 1.4 (Siemiarczuk et al., 1990). Hence, based on the MEM analysis, our data do not allow us to state unequivocally whether the apparent AP distribution at 1920 ms is a true distribution (i.e., a continuum of lifetimes) or the unresolved analysis of two (or more) discrete components with similar lifetimes. Following the same line of reasoning, the relative width of the LADH MEM image centered at 712 ms is also greater than that of the model chromophores and, therefore, is more complex than a single exponential.

Simulations were performed to test whether the long-lived apparent AP distribution could be successfully resolved into a sum of more than two discrete lifetimes if the quality of data were improved. Seven discrete components were used to construct a lifetime distribution corresponding to the recovered MEM results for AP (τ_{mean} = 1920 ms, $\Delta\tau$ = 290 ms). A dwell time per channel of 30 ms extending over 512 channels with added statistical noise was used to duplicate the experimental conditions as the total number of counts in the simulation were varied. We found that a two component discrete analysis of a simulated decay curve containing 10 times

the total number of counts of the AP experimental data fits to a χ^2 of ~1. Thus, the discrete analysis is unable to differentiate between a two component decay and more complex decay behavior for the quality of our data.

Temperature dependence of distribution widths

It is significant that the width of the decay rate distribution, Δk, increases with temperature for both AP and LADH. This observation can best be interpreted by considering a simple model where the "distribution" is composed of two similar states distinguishable only by different tryptophan decay rates, k_A and k_B. Physically, if we assume that the nonexponential decay is caused by ground state heterogeneity and not by excited state reactions, then these states may arise from slight variations in protein conformation leading to differences in accessibility to solution quenchers, or to changes in the local rigidity of the tryptophan environment. We assume that states A and B are in equilibrium and that the rate of interconversion, k_{AB}, is small with respect to the rates of decay, i.e., $k_{AB} < k_A, k_B$. In this model, the decay rate width, Δk_{AB}, is the difference in rates between the two states, $\Delta k_{AB} = |k_A - k_B|$. If state-specific quenching terms, Q_A and Q_B, containing all of the temperature-dependent processes, are introduced as the result of a change in temperature, the new decay rate width is $\Delta k^*_{AB} = \Delta k_{AB} + Q_A - Q_B$. Our observation that Δk increases with increasing temperature shows that $Q_A - Q_B \neq 0$ and, hence, the two states are not identical with respect to quenching processes. The data suggest, therefore, that either 1) different distributional states have quenching processes that show unique temperature susceptibility or 2) additional conformational states with different phosphorescence lifetimes are being sampled with increasing temperature.

Excitation wavelength dependence

Upon increasing the excitation wavelength from 280 to 290 nm, we observed a change in the RTP decay of LADH Trp-314. There is an overall increase in lifetime (Fig. 5), reflecting the involvement of new longer-lived states and the loss of shorter-lived states. Within experimental accuracy, no changes were observed for AP when performing a similar experiment. *The observation of an excitation-dependent triplet state lifetime suggests the presence of ground state heterogeneity in the environment of* Trp-314. It is well established that chromophores in hydrophobic environments have red-shifted absorption (Calhoun et al. (1983) and references therein) and that an increase in the local viscosity leads to longer phosphorescence lifetime (Strambini and Gonnelli, 1985). Our excitation-dependent decays are consistent with the selective excitation of a subensemble of Trp-314 in ground state configurations, which are observed to be in a more rigid and more hydrophobic environment as the excitation wavelength is increased. This is consistent not only with the loss of the short-lived states of the ensemble but also with the increase in lifetime of the long-lived states. It is important to note that if the molecules in the ensemble are quickly interconverting on the time scale of the experiment, then no excitation dependence would be observed.

Only two excitation wavelengths, at 280 and 295 nm, are shown in Fig. 5 to emphasis the difference in observed lifetimes. The observation is a general one, however; ie., for wavelengths between 280 and 295 nm, one finds increasing average lifetime because of the appearance of longer-lived components and loss of short-lived components with increasing excitation wavelength. In addition, for a single sample, the observed decay at a given wavelength is independent of the sample's history and the sample may be cycled many times between two different excitation wavelengths with no change in average lifetime at a given wavelength. It has been suggested by a reviewer that the decreased lifetime at 280 nm is caused by photochemical depletion of the emitting state. According to this argument, excitation at 295 nm results in less photochemistry. If photochemistry occurs to some extent over the triplet state lifetime of LADH, then the process must be reversible as the lack of hysteresis in the excitation wavelength experiments clearly demonstrates. It is noteworthy that AP phosphorescence shows no excitation wavelength dependence, although its excited state lifetime is 3 times that of LADH. Therefore, if tryptophan RTP photochemistry is responsible for nonexponential decay behavior, it is not a function of triplet state occupancy time but rather influenced by the local protein matrix.

Possible models

We report here the existence of considerable complexity in tryptophan RTP decay in LADH and, to a lesser extent, AP: proteins that were heretofore reported to decay as single exponentials. LADH was chemically characterized as homogeneous using traditional techniques, and (neglecting the possibility of covalent modification) on this basis it is concluded that the complex decay behavior does not likely arise from sample impurity. The temperature dependence of the decay distribution widths, and even more significantly the phosphorescence lifetime excitation dependence, suggest that we are observing states of different microviscosity. This in turn implies that the interconvertion time between the states is no shorter than the time scale of our phosphorescence lifetimes, about 1 s.

Assuming the above interpretation to be correct, it is tempting to speculate on a molecular model. More specifically, from crystallographic studies it is known that LADH undergoes a major structural change upon coenzyme binding with the catalytic domains in each subunit displaced towards the coenzyme-binding domain. Previous phosphorescence measurements of LADH in solution and in the crystalline state have demonstrated a decrease in lifetime upon coenzyme binding (Gabellieri et al., 1988; Strambini and Gonnelli, 1990), and it was argued that the open conformation was the more stable one in solution. We are of the opinion that the observed bimodality is unrelated to the open and closed conformational states. This follows from a con-

sideration of rhodanase, a monomeric enzyme that shows structural similarity to LADH in that two rigid domains are interconnected by a flexible hinge and in which the equilibration between open and closed forms was suggested to occur on the microsecond time scale (Koloczek and Vanderkooi, 1987). This is six orders of magnitude faster than in LADH if a similar mechanism is to be invoked for both enzymes.

A possible physical model of the system that focuses on the presence of ground state conformational heterogeneity in LADH (and most likely AP) would be that of a shallow potential energy hypersurface with multiple energy valleys and with high energy barriers separating conformational substates. Such a glass-like model has been developed by Frauenfelder and co-workers (Frauenfelder et al., 1988, 1991) to explain the nonexponential ligand rebinding kinetics in myoglobin below 200 K. The myoglobin substates are populated by rapid equilibrium fluctuations at room temperature; however, as the temperature is lowered the transitions between conformers become slower and the protein ensemble condenses into spectroscopically distinct states which, according to the model, may themselves be structured.

The long time scale of the dynamics implied by our decay experiments is on the order of those observed in hydrogen exchange and NMR experiments for rigid core regions of proteins. The slowest exchanging protons in a number of proteins are invariably found in "knot" regions composed of β-sheet and α-helical units with a high proportion of aliphatic and aromatic neighbors (Gregory and Lumry, 1985). This is the type of environment that encases AP Trp-109 and LADH Trp-314. In the absence of unfolding, the exchange mechanism for knot protons is by rare conformational events involving cooperative local distortions in the extended β-structures. The same class of collective displacements of many atoms may account for the complexity in the dynamics we find. Thus, the distributed decays we observe for Trp-314 and Trp-109 may be ascribed (in part) to an ensemble of conformational states at the sites of the tryptophans which, while in equilibrium, undergo slow interconversion because of the small probability of structural change in their knot-like environments. We observe, however, that although the above picture merits careful consideration, the details of our experiments do not permit us to determine the scale length of the heterogeneity; i.e., we cannot discriminate between large scale protein conformational changes and small scale changes in the immediate vicinity of the tryptophan.

In summary, the use of sensitive RTP techniques to monitor the dynamics of the rigid protein cores of AP and LADH reveal a previously unreported and unexpected complexity in the phosphorescence decay. Given the strong dependence of phosphorescence lifetime on local rigidity and the dependence (in the case of LADH) of phosphorescence decay dynamics on excitation wavelength, it is likely that a portion of the heterogeneity arises because of the presence of multiple ground state configurations. Because the intrinsic room temperature protein phosphorescence is only observed for tryptophans in viscous environments, our results suggest that distributed tryptophan phosphorescence decays in proteins are the rule and not the exception.

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