PROTEIN FLUORESCENCE AND CONFORMATIONAL SUBSTATES:
THE DYNAMICS OF HUMAN SUPEROXIDE DISMUTASE

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I. INTRODUCTION

The time-resolved fluorescence intensity and anisotropy decay of single tryptophan (Trp) containing proteins offers the unique opportunity of studying protein dynamics in a sensitive and non-perturbing manner.\textsuperscript{1,2} Other spectroscopic fields have underlined the importance of understanding protein dynamics through experiments which cover a wide range of temperature $T$ and viscosity $\eta$.\textsuperscript{3-7} In fact, some of these studies have successfully fit their data to a $T$ or $\eta$ dependent model of the protein system.\textsuperscript{4,5,8}

However, no satisfactory fit of a $T$ dependent physical model has been achieved on the time-resolved fluorescence intensity decay of any single Trp protein. There are several reasons for this situation. The first reason concerns the interpretation of the heterogeneity in the time-resolved fluorescence decay of single Trp proteins. Traditionally, the fluorescence decay is fit to a discrete set of 2-4 lifetimes.\textsuperscript{9} It is assumed that each lifetime corresponds to a particular protein conformation.\textsuperscript{10} However, previous studies have demonstrated problems with this approach.\textsuperscript{11,12} The most prominent problem is the growing body of evidence for the existence of many ($\gg 4$) protein conformations, termed as conformational substates, from a variety of experimental and theoretical sources.\textsuperscript{4,6,13,14} These results suggest that a distribution of lifetimes may be appropriate toward modeling the heterogeneity in the fluorescence decay of single Trp proteins. Indeed, some single Trp proteins have their fluorescence intensity decay best described by a distribution of lifetimes containing less parameters than a equivalently good discrete fit.\textsuperscript{2,15} Of course, with less fitting parameters comes increased statistical confidence in those parameters. More importantly, the distributional approach satisfies many predictions concerning the behavior of, say, the width of the lifetime distribution as a function of $T$ or denaturant concentration.\textsuperscript{2,16}
A second reason for the failure of fitting a physical model to the $T$ dependent time-resolved fluorescence decay of single Trp proteins is the growing departure between all the previously proposed models and the experimental data as the system temperature is decreased. Tanaka and Mataga introduced one of the first attempts toward addressing the increased heterogeneity of the fluorescence intensity decay of single Trp proteins as temperature is decreased.\textsuperscript{17} However, their approach assumed that the Trp residue resided in a single potential well and poor data fits resulted at low temperatures indicating that their model cannot describe the temperature dependence of the lifetime and anisotropy. The introduction of conformational heterogeneity in physical models through 1) a single potential well with a distribution of substates\textsuperscript{2} and 2) a double-potential well with a distribution of interconversion rates\textsuperscript{18} was a step in the right direction. The fits of these distribution models over $T$ were relatively successful. However, they suffered the same problem as the Tanaka and Mataga model because of the assumption that the Trp-protein system resided in one or two potential wells. In particular, progressively bad fits to the model resulted as the system temperature decreased. These results implied greater conformational heterogeneity in the Trp-protein system than was previously assumed.

Model formulation is very dependent on the experimental data available. In particular, a model is very robust if it can satisfy a wide range of experimental conditions. However, protein dynamics studies that have tried to fit a model for the $T$ dependent, time-resolved fluorescence decay of single Trp proteins have only considered a relatively small range of the interval $\Delta = ((T/\eta)_i - (T/\eta)_f)/(T/\eta)_i$ where $(T/\eta)_i$ and $(T/\eta)_f$ are extreme values of $T/\eta$ with $(T/\eta)_i < (T/\eta)_f$. In particular, such studies\textsuperscript{17,2,18} were done in aqueous solution and covered a temperature range from 5 to 50°C which corresponds to a $\Delta$ range of 2.2. This situation is in contrast to other spectroscopic studies of protein dynamics which
typically cover a $\Delta$ range greater than 200 (e.g., as attained in going from -20°C to 50°C in an 80% glycerol solution (w/w)).19 Understandably, thermal denaturation and solution freezing are a concern for aqueous solution experiments. However, with respect to protein dynamics, residue diffusional motion is strongly dependent on $T/\eta$ and one should probe a wide timescale of motions by increasing the range of $\Delta$ so as to formulate a satisfactory model. No doubt, there have been biochemical fluorescence studies which satisfy $\Delta \gg 2.2$. However such studies have, at best, proposed qualitative models which primarily addresses the dipolar relaxation mechanism their experiment was designed to observe in the first place.20-22

The point of dipolar relaxation brings us to an experimental aspect of investigating protein fluorescence at low $T/\eta$. Clearly, there are many contributions to the dynamics of excited state Trp residues. They include dipolar relaxation, protein matrix interactions and solvent viscosity, to name a few. Decoupling some of these effects can be done if the right experiment is chosen. In particular, if a focus of protein matrix and solvent viscosity effects on Trp dynamics is desired, then the entire emission of the Trp residue should be observed. Only in this manner can we exclude dipolar relaxation effects on the fluorescence intensity decay. One must remember that evidence for dipolar relaxation comes from observing only part of the emission in a narrow bandpass region. No doubt dipolar relaxation will affect the Trp residue just as rotational motions will. However, just as choosing the magic angle excitation polarization allows us to eliminate rotational effects on the fluorescence intensity23 decay so will viewing the entire emission allow us to eliminate dipolar relaxation effects on the same intensity decay. An analytical demonstration of this point will be emphasized in the discussion.

Thus, given the unsuccessful record of those trying to fit a model to the time-resolved
fluorescence decay of single Trp proteins as a function of $T$ it seems more than reasonable, at this stage, to introduce a model whose resulting qualitative predictions rationalizes non-dipolar relaxation effects on the fluorescence intensity decay over a wide range of $\Delta$. Development of such a model will at least provide general guidelines toward understanding the most significant features necessary in the future formulation of a model which gives reliable quantitative predictions. For this work we have developed a model whose thermal behavior is in qualitative agreement with time-resolved entire emission of a single Trp protein over a wide range of $\Delta$. The model, called the bifurcating ultrametric system (BUS), is qualitative based on the complexity of protein systems but it accounts for the low temperature deficiencies of previous models. Furthermore, BUS only requires several general conditions which gives qualitative agreement with the observed data. These conditions are: 1) a hierarchy of free energy barriers separating each conformational state, 2) each conformational state is degenerate in energy but not in entropy and 3) each conformational state is characterized by a temperature independent fluorescence decay rate after the Trp residue is excited.

We have investigated a single Trp protein, human superoxide dismutase (HSOD), to test the BUS model. HSOD is a copper-zinc protein composed of two identical subunits. The single Trp residue is located on the protein surface away from the dimer interface. For our study, a wide range of $\Delta$ is obtained by placing HSOD in 80% glycerol (GLOH) because: 1) GLOH is a viscous antifreeze, 2) GLOH is well characterized through such parameters as viscosity and 3) GLOH is known to stabilize protein structure. N-acetyl-tryptophanamide (NATA) in 80% GLOH served as our control since it closely resembles a Trp residue containing peptide bonds.
II. EXPERIMENTAL

II.A. Samples

Time-resolved fluorescence decay experiments were performed on NATA (from Chemical Dynamics Corp., South Plainfield, New Jersey) in 80% GlOHa (v/v) using a 0.1M sodium phosphate buffer at pH 6.2. GlOHa was redistilled from A.C.S. grade glycerine (from Fisher Scientific, Pittsburg, Pennsylvania). The redistilled GlOHa had relatively small fluorescence over the emission range covered (less than 0.1% of the NATA emission).

Time-resolved fluorescence decay experiments were also performed on holo- and apo-HSOD in 80% GlOHa using a 0.01 M potassium phosphate buffer at pH 7.6. Holo-HSOD was prepared in the laboratory of Prof. Alessandro Finazzi-Agro and Dr. Nicola Rosato (Universita di Roma "Tor Vergata", Rome, Italy) using published procedures. Apo-HSOD was generously provided by Victor Buckwold of ABI Biotechnology Inc., Winnipeg, Canada. All time-resolved fluorescence decay experiments were performed from -30°C to 50°C in steps of 5°C for NATA and Apo-HSOD and in steps of 10°C for Holo-HSOD. In addition, steady state and time-resolved fluorescence anisotropy of Apo-SOD in 80% GlOHa was measured from -30°C to 50°C in increments of 5°C. The temperature was continuously monitored during experimentation by attaching a thermocouple to the sample cuvette. The temperature readings of the thermocouple were monitored by an Omega DigiCator (from Omega Engineering, Stamford, Connecticut) with an accuracy of ±0.1°C.

II.B. Time-resolved measurements and analysis

All time-resolved fluorescence intensity and anisotropy decay measurements were performed at the Laboratory for Fluorescence Dynamics of the University of Illinois at Urbana-Champaign, Urbana, Illinois. Frequency domain techniques were used to measure the fluorescence decay of all samples in the range of 10 to 300 MHz. The 295 nm excita-
tion was polarized at the "magic angle" for intensity decay measurements. The entire wavelength range of emission was observed using an optical filter combination of UV34 and U340 (from Oriel Corp., Stratford, Connecticut). Thus, for this study, the effects of dipolar relaxation on the observed fluorescence decay essentially cancels out (a more analytical treatment of this result is discussed below). All samples had an optical density of 0.1 at 295 nm. Data analysis was performed by minimizing the reduced \( \chi^2 \) with a routine based on the Marquardt algorithm using the Globals Unlimited software.

III. RESULTS

III.A. Time-resolved fluorescence decay

The time-resolved fluorescence decay of aqueous HSOD is best described by a distribution of lifetimes. Similar results have been obtained for HSOD in 80% GIOH whose fluorescence decay is best described by a gaussian distribution of lifetimes (all distributions are in fractional intensities). In particular, for holo-HSOD, \( \chi^2_d/\chi^2_g = 1.6 \) at -30°C and \( \chi^2_d/\chi^2_g = 1.1 \) at 50°C where \( \chi^2_d \) is the reduced chi-square from a double exponential fit and \( \chi^2_g \) is the reduced chi-square from a gaussian distribution fit. (This effect will be later discussed in the context of the BUS model.) Though at high temperatures \( \chi^2_d \) approaches \( \chi^2_g \) it should be noted that a double exponential fit contains more parameters than a gaussian fit (4 versus 3). Thus, compared to the discrete fit, the gaussian distribution fit is statistically superior.

A similar result applies for apo-HSOD in 80% GIOH.

Another important feature is the thermal behavior of the gaussian center (mean) and width (standard deviation), for both HSOD samples, as \( T \) increases (Figure 1). In particular, above 0°C both parameters decrease with increasing temperature. The decrease in the width of the lifetime distribution agrees with the hypothesis of conformational sub-states. In other words, as temperature increases the Trp residue interconverts between more
conformational substrates and effectively sees an “averaged” environment during its excited lifetime. Below 0°C the center and width behavior changes markedly with a reduced growth of the center and a slow decrease of the width in the case of holo-HSOD. For apo-HSOD, both parameters are relatively constant below -5°C. In fact, below this temperature the width of apo-HSOD is essentially constant within error (±0.1 nsec). Furthermore, Figure 1 shows that the lifetime parameters of apo-HSOD in 80% G1OH are generally larger than that of holo-HSOD in 80% G1OH which is consistent with previous observations in aqueous solution.16

The fluorescence decay of NATA in 80% G1OH can be fit to a double exponential decay or alternatively to a gaussian distribution of lifetimes plus a discrete component with equivalent χ² values (the discrete component contributes about 5% of the total fractional intensity). The time-resolved fluorescence decay of aqueous NATA is typically fit to a single exponential decay while the fluorescence decay of aqueous Trp is best fit to a double exponential decay.32,33 However, molecular dynamics studies of Trp and evidence for an excited-state reaction in the fluorescence decay of aqueous Trp suggest that Trp may be interconverting between conformations about its χ₁ or χ₂ dihedral angles within the fluorescence time scale.34,35 For NATA, a viscous environment like 80% G1OH may slow down the interconversion rate between conformers giving rise to heterogeneous fluorescence decay. Consequently, we will only consider the gaussian lifetime distribution fit of NATA in 80% G1OH (Figure 1). The additional discrete component used with the gaussian distribution will not be considered due to its relatively low fractional intensity.

III.B. Time-resolved anisotropy decay and steady state anisotropy

The time-resolved anisotropy decay of apo-HSOD in 80% G1OH, r(t), can be adequately described by two rotational correlation times. This finding agrees with previous results for
the time-resolved anisotropy of aqueous HSOD.\textsuperscript{16} The \( r_o \) value used for our analysis is the literature value of 0.278 for 295 nm excitation.\textsuperscript{36} The use of two rotational correlation times (of which one is relatively short) can be interpreted as a Trp residue confined to free diffusion within a cone semi-angle \( \theta \) while the whole protein molecule undergoes isotropic rotational diffusion.\textsuperscript{37} This free diffusional approach is approximate considering that the Trp residue interconverts between conformational substates over a complex energy landscape. However, we believe the average behavior of the system can be captured by using the free diffusional model of a probe attached to a macromolecule.\textsuperscript{37}

Figure 2 shows the rotational diffusion coefficient, \( D_{pro} \), of apo-HSOD in 80\% GLOH obtained from \( r(t) \) as a function of temperature.\textsuperscript{37} If we estimate the molecular volume \( V \) through the Stokes-Einstein relation\textsuperscript{38} we obtain values on the order of \( 10^3 \) cm\(^3\)/mole. Such values of \( V \) are at least an order of magnitude lower than the dry volume of HSOD (42,403 cm\(^3\)/mole).\textsuperscript{39} As a result, the whole protein is essentially immobilized and \( D_{pro} \) is probably reflective of segmental motion.

Figures 3 shows the temperature dependence of the Trp wobbling diffusion coefficient, \( D_{Trp} \), and the cone semi-angle \( \theta \) obtained from \( r(t) \).\textsuperscript{37} Figures 2 and 3 show that all three parameters generally increase with increasing temperature, which is an effect that has been previously observed in other protein systems.\textsuperscript{1,40,41}

Previous work done by Weber and co-workers\textsuperscript{12-44} showed an interesting correlation between the low temperature steady state anisotropy of proteins in 76\% GLOH (v/v) and Trp in 76\% GLOH. They considered the quantity \( Y \) defined as

\[
Y = \ln((r_o/r) - 1) - \ln(RT\tau/V) \tag{1}
\]

where \( r \) and \( \tau \) are the steady state anisotropy and fluorescence lifetime, respectively, of the sample at a given temperature \( T \) (\( R \) is the gas constant). Figure 4 shows the resulting plot

9
of Y versus T for apo-SOD in 80% GLOH where we have replaced τ of equation (1) with the center of the lifetime distribution. Two temperature regions are clearly displayed in Figure 4. One is a low temperature region between -31°C and -5°C. A linear regression of the data in this region yields a slope of 0.067 which is in close agreement with that obtained by Weber and co-workers for Trp in 76% GLOH (they obtained a slope of 0.07 between -30°C and 15°C). It is reasonable to assume that this slope would be the same for NATA in 80% GLOH since the low temperature slope of Y versus T is about 0.07 for several small molecules in 76% GLOH.42

The high temperature region of Figure 4 (between -5°C and 50°C) yields a lower slope (0.029) which is in qualitative agreement with the results of Weber and co-workers for other proteins in 76% GLOH.44 Thus, a transition occurs around -5°C such that below this temperature the mobility of the Trp residue in apo-HSOD is primarily due to the solvent. Interestingly, the temperature region below -5°C also corresponds to where the center and width of the lifetime distribution of apo-HSOD reaches a maximum and stays relatively constant. Supporting evidence for this transition is also seen in Figure 3 where θ and D_{Trp} below -5°C, drop sharply in value and then vary slowly.

IV. DISCUSSION

IV.A. The possibility of observing dipolar relaxation

Dipolar relaxation is used to explain the emission wavelength dependence of the apparent fluorescence lifetime. In particular, some fluorophores in viscous environments show an increase in their fluorescence lifetime with increasing emission wavelength.21 In order to demonstrate that our measurement of the fluorescence intensity decay minimizes dipolar relaxation effects we consider a more general form of the well known dipolar relaxation model introduced by Lakowicz and co-workers.21 This extended model is schematically portrayed
in Figure 5 which represents each excited state $i$ as a different dipole relaxed state that has a fluorescence decay rate $k_i$. Emission wavelength and dipolar relaxation increases with $i$ (from left to right) and due to dipolar relaxation the forward rate from state $i$, $k_i^f$, is much greater than the backward rate from state $i$, $k_i^b$. In the model introduced by Lakowicz and co-workers only two states were considered and the backward rate was set to zero.

What we wish to show is a disappearance of dipolar relaxation effects on the fluorescence decay when the entire emission is observed. Consider $N$ possible emission states in Figure 5 ($i = 1, \ldots, N$). Then the time-evolution of the population of state $i$, $n_i(t)$, is given by the differential equation

$$\frac{dn_i}{dt} = -kn_i - (k_i^f + k_i^b)n_i + k_{i-1}^f n_{i-1} + k_{i+1}^b n_{i+1}$$

(2)

where it is understood that

$$k_0^f = k_1^b = k_N^f = k_{N+1}^b = 0$$

(3)

If we sum equation (2) over all $N$ states we get

$$\frac{dI}{dt} = -kI - \sum_{j=1}^{N} (k_j^f + k_m^b)n_j + \sum_{l=1}^{N} k_{l-1}^f n_{l-1} + \sum_{p=1}^{N} k_{p+1}^b n_{p+1}$$

(4)

where $I = \sum_i n_i$ is proportional to the fluorescence intensity decay observed over the entire emission range. As a result, we need to solve for $I(t)$ in equation (4). Taking advantage of the relations in equation (3) and rearranging equation (4) gives

$$\frac{dI}{dt} = -kI - \sum_{j=1}^{N} k_j^f n_j + \sum_{l=2}^{N+1} k_{l-1}^f n_{l-1} - \sum_{m=1}^{N} k_m^b n_m + \sum_{p=0}^{N-1} k_{p+1}^b n_{p+1}$$

(5)

Letting $j = l - 1$ for the first summation and $m = p + 1$ for the last summation causes all terms on the right hand side of equation (5) to cancel except the term $-kI$. As a result, $I(t)$ is given by

$$I(t) = I_0 \exp(-kt)$$

(6)
which is completely devoid of forward and backward dipolar relaxation rates. Thus, observing the entire emission cancels out any dipolar relaxation effects on the fluorescence intensity decay. Of course, the underlying assumption is that the detection system is wavelength independent. We verify that in our system, for the emission range investigated, the detection system is reasonably flat.

IV.B. Contributions of conformational heterogeneity to the fluorescence intensity decay

While there seems to be close similarities in the thermal behavior of the center and width of holo- and apo-HSOD there is a difference between the HSOD samples and NATA. The first difference is the large heterogeneity in the fluorescence decay of HSOD as compared to NATA. This difference can be characterized through the width/center ratio of NATA and HSOD. In particular, the width/center ratio of holo- and apo- HSOD hovers around 0.5 for all temperatures while for NATA it plateaus to a maximum of \( \approx 0.25 \) below \(-20^\circ\text{C}\) and then decreases with increasing temperature to values below 0.1 above \(15^\circ\text{C}\). A gaussian width/center ratio that is less than 0.1 implies that the fluorescence decay is indistinguishable from a single exponential decay.\(^{12}\) Thus, holo- and apo-HSOD can be distinguished from a single exponential decay while NATA is distinguishable from a single exponential decay only for temperatures below \(15^\circ\text{C}\).

Another difference between NATA and HSOD is the thermal behavior of the width in Figure 1 which shows that an increase of about \(35^\circ\text{C}\) is needed for NATA to reach homogeneity from maximum heterogeneity at \(-17^\circ\text{C}\). In contrast, apo-HSOD has not reached homogeneity even after an increase of \(55^\circ\text{C}\) from maximum heterogeneity at \(-5^\circ\text{C}\). The comparatively slow growth of the width for HSOD suggests that its energy landscape is more complex than that of NATA.
The final difference between apo-HSOD and NATA is the low temperature state of both systems (Figure 1). Since both samples are in 80% GIOH the constant behavior of the width of the lifetime distribution of apo-HSOD below -5°C is due to the protein. In other words, the protein seems to be frozen during the excited state lifetime below -5°C. Physically, the Trp residue of a particular protein molecule is locked in a particular valley of the potential energy curve. The large inhomogeneity implies that many different valleys are isoenergetic. Such a confined Trp residue would likely have its rotational mobility determined by nearby residues and the surrounding solvent. In fact, the Y plots of Figure 4 suggest that the solvent is the main determinant for the mobility of Trp below -5°C.

The approach toward homogeneity with decreasing temperature for NATA in 80% GIOH (below -15°C) is probably the result of a minimum energy conformation whose mean fluorescence lifetime (at low temperatures) seems to be below 6.5 nsec (Figure 1). Holo-HSOD seems to approach homogeneity with decreasing temperature below 1°C. However, the marginal decrease of its width as compared to NATA suggests that holo-HSOD may not have a single minimum energy state.

NATA is probably able to reach a minimum energy conformation because of its relatively small number of degrees of freedom. In contrast, apo-HSOD may have too many constraints to achieve a single minimum energy state. These constraints may be due to steric hindrances and long-range interactions affecting backbone and residue conformations. In other words, a system as complex as a protein may be frustrated in its efforts toward finding a minimum energy conformation.

The spin glass analogies (frustration and complex energy landscapes) suggested by our results prompted us to adopt a spin glass model whose framework has been studied by Ogielski and Stein. They presented a model which describes random walks in a bifurcating
ultrametric space (BUS). Our choice of an ultrametric space may be questioned since it has been shown that a self-similar hierarchical system with no ultrametric topology displays the same dynamical behavior as the BUS model. Nevertheless, as a starting point, we use the BUS model to provide a preliminary interpretation for the thermal behavior of the time-resolved fluorescence of HSOD in 80% ClOH.

IV.C. A bifurcating ultrametric system for single Trp proteins

IV.C.1. Model description

In the BUS model we classify different protein motions through a hierarchy which is dependent on the energy needed to produce a particular motion. It should be reminded that the energy landscape of a protein is generally a function of many conformational variables. In our model, we classify the three tiers of motion according to a notation given by Frauenfelder. That is, at the tier 2 level there is motion that may only involve the adjustment of the Trp residue and nearest neighbor residues. Tier 1 level motion may require conformational changes in the Trp residue and a larger number of local residues than in tier 2. The final level (tier 0) may require motion of the Trp residue and large adjustment of the polypeptide backbone. We assume that each of these motions are binary jumps and, as a result, the Trp-protein system can occupy eight different conformational states.

Any state can interconvert to another through one jump. The energy needed for these motions increases as tier number decreases. Figure 6 schematically displays the energy needed to produce the various tier motions. $\Delta_i$ is the energy needed for tier $i$ motion which is assumed to be constant over conformational space for a given tier. A main assumption of the model is that the substates are degenerate in energy, but not necessarily in free energy.
We assume that the rate of interconversion from state $j$ to any state over the $i$'th barrier, $k_{ji}$, is thermally activated and given by the Arrhenius expression

$$k_{ji} = \frac{k_0 g_o}{2f-(i+1)g_j} \exp\left(-\frac{\Delta_i}{RT}\right)$$  

(7)

where $k_0$ is the frequency factor, $g_o$ is the degeneracy of the transition state, $f$ is the total number of tier levels ($f=3$ in our case), $g_j$ is the degeneracy of the $j$'th state, $\Delta_i$ is the energy needed for tier $i$ motion, $R$ is the gas constant and $T$ is the temperature. As assumed by equation (7) the degeneracy of all transition states are equal. The entropy of the $j$'th state is given by $R\ln(g_j)$. We avoid any occurrence of negative free energy barriers by imposing $g_o \leq g_j$. We also assume that all degeneracies are temperature independent.

Upon excitation by ultraviolet light we assume that the energy landscape of Figure 6 remains the same except for fluorescence decay from each state. Since Trp is an environmentally sensitive fluorophore we assume that its fluorescence decay is state dependent. The de-excitation of Trp involves many processes which include radiative decay, charge transfer, proton transfer and photo-ionization.\textsuperscript{49-53} Most of these processes are temperature dependent. However, for the sake of simplicity, we assume that all fluorescence decay rates are temperature independent. We also assume that the fluorescence from each state is observed with equal efficiency. Figure 6 shows the fluorescence lifetime decay values, $\tau_j$, assigned to each $j$'th state. These assignments are done only for the purpose of illustrating the model and they are not related to any specific experimental assignment.

Whether the degeneracy $g_j$ should be state dependent can be tested by fitting the low temperature data of HSOD in 80% GLOH to a distribution that is uniform in population. The resulting fractional intensity distribution would be trapezoidal in shape. This test is appropriate because at low temperatures substrate interconversion is frozen and the amplitude of the fluorescence decay from the $j$'th state will be determined by its initial population.
In particular, the initial population of the $j$'th state is proportional to the degeneracy $g_j$. A fit of the apo-HSOD in 80% GlOH data at -27°C to a uniform population distribution yields a chi-square greater than 30.0 Therefore, variation in $g_j$ with conformational substate $j$ should be included.

The resulting time-resolved fluorescence decay of the BUS model is then obtained by solving the differential equation
\[
\frac{d\vec{p}}{dt} = \mathbf{B} \cdot \vec{p}
\]  
(8)

$\mathbf{B}$ is the $N_s \times N_s$ matrix ($N_s = 8$ in our case) describing the BUS fluorescence decay and interconversions of Figure 6. $\vec{p}(t)$ is the time evolution population vector whose $j$'th component is the population of the $j$'th state. With $p_j(0)$ being proportional to $g_j$, one obtains the time-resolved intensity $I(t)$ as
\[
I(t) = (1/N_s) \sum_{j=1}^{N_s} p_j(t)
\]  
(9)

The phase and modulus of the Fourier transform of $I(t)$, at frequency $\omega$, gives the phase $\phi(\omega)$ and modulation $M(\omega)$ of the BUS system which is used for comparison with experimental results.

Though $I(t)$ consists of a sum of $N_s$ exponential terms, it should be stressed that we have not abandoned the continuous distribution approach. The continuous distribution was used because it is a physically meaningful way to characterize a multitude of conformational substates.\textsuperscript{12} The number of conformational substates in proteins are less than infinite yet they can be quite large ($\gg 10^3$).\textsuperscript{13} Fitting the time-resolved fluorescence data of single Trp proteins to four or more lifetime terms will always present problems with multiple chi-square minima and large uncertainties in the obtained parameters. Thus, using a distribution characterized by two parameters represents a statistical advantage over the discrete method.
provided that one has a physical model to explain its behavior with environmental changes.

IV.C.2. BUS simulations and comparisons to HSOD in 80% GLOH

To illustrate the thermal behavior of BUS, we consider the following simulation of the system described in Figure 6. The tier energies are set as $\Delta_2 = 3.0$, $\Delta_1 = 3.9$ and $\Delta_0 = 4.7$ kcal/mole. The degeneracy ratio $g_j/g_o$ is proportional to a gaussian curve with a mean of 3 and a standard deviation of 3 ($g_3/g_o = 4.05$). Finally, the frequency factor is set to $k_o = 10^3$/nsec. Figures 7a-7d show the resulting fractional lifetime distribution (shown as vertical lines) at four different temperatures. In addition, for a given temperature, the phase and modulation data of the BUS system was generated for 20 frequencies between 20MHz and 360MHz and a gaussian distribution of lifetimes were fit to the data (noise typical of experimental conditions were also added to the data). The $\chi^2$ values for these fits ranged from 2.4 to 1.1. The resulting gaussian lifetime distributions are shown as dashed curves in Figures 7a-7d. In this particular simulation the fluorescence decay is essentially a single exponential decay for temperatures greater than 120°C. In this temperature range the Trp residue is interconverting so fast between the conformational substates that it only experiences an average environment during the fluorescence lifetime. For temperatures below -173°C the Trp residue is locked in its conformational substates and the resulting lifetime distribution ceases to change from its broad shape which is determined by substate degeneracy (Figure 7d). Thus, a clear feature of the simulations in Figures 7a-7d is the rise of new lifetime components as the system temperature decreases. An analysis using discrete lifetimes would need an increasing number of components as temperature decreases (assuming very good signal to noise ratio).

In order to see how the BUS system compares with the experimental observables of Figure 1 we have followed the previously described method for generating frequency domain
data over a wide temperature range at 2.5°C increments. The data was then fit to a gaussian distribution of lifetimes at a given temperature. The reduced $\chi^2$ values from the individual gaussian fits ranged from 2.4 to 1.1.

Figure 8 shows the resulting thermal behavior of center and width (in filled symbols for the BUS model described in Figure 6. Both curves are sigmoidal and decrease with increasing temperatures. Figure 6 also shows that below a critical temperature both parameters are frozen. The qualitative behavior of the simulation in Figure 8 closely portrays the experimental thermal behavior of the center and width of the lifetime distribution for apo-HSOD in 80% G1OH (Figure 1). This suggests that the general features of the BUS model (isoenergetic substrates, state dependent fluorescence decay rates and a hierarchy of energy barriers) provides a physical explanation for the thermal dependence of the fluorescence decay of apo-HSOD in 80% G1OH. In the case of holo-HSOD, a set of conformational substates may be slightly lower in energy than the rest giving rise to the slight decrease in its width as temperature is decreased below 0°C.

BUS also explains why the lifetime distribution width of apo-HSOD has a different thermal behavior as compared to the width of NATA in 80% G1OH. Consider a variant of the previous system which is exactly the same as Figure 6 except for the range of energy barriers covered: $\Delta_2 = 3.0$, $\Delta_1 = 3.02$ and $\Delta_0 = 3.04$ kcal/mole. The range of energy barriers is relatively small for this system and we call it an $\alpha$ system. The previous system having a larger range of energy barriers is denoted as a $\beta$ system. Since the $\alpha$ and $\beta$ system have the same value of $\Delta_2$ we would expect that their resulting center and width would cease to change below a common critical temperature. However, due to the range of energy barriers, the $\alpha$ system would progress toward homogeneity faster than the $\beta$ system with increasing temperature. A gaussian lifetime distribution fit to the phase and modulation
data (plus noise) generated by the $\alpha$ and $\beta$ systems show this expected difference in Figure 8. Displayed in Figure 8 are two regions, $\Delta T_{\alpha}$ and $\Delta T_{\beta}$, where the width attains half of its maximum value for the $\alpha$ and $\beta$ system, respectively. Clearly, $\Delta T_{\beta}$ is greater than $\Delta T_{\alpha}$, indicative of the extreme hierarchical nature in the energy landscape of the $\beta$ system. A relatively simple system as NATA in 80% GlOH may serve as an approximate $\alpha$ system. However, NATA is not the ideal candidate for an $\alpha$ system because: 1) it displays at least one minimum energy state 2) it is like a single amino acid "protein" displaying few degrees of freedom, 3) its value for $\Delta_2$ may not be the same as that for the Trp residue in HSOD and 4) the spread of fluorescence decay lifetimes $\tau_j$ from each substate may not be the same as that for HSOD. Nevertheless, we wish to argue, in an approximate sense, that HSOD is a $\beta$ system when compared to NATA in 80% GlOH.

When NATA and HSOD in 80% GlOH reach maximum heterogeneity this tells us that Trp in both systems experience full environmental diversity. We have argued that the width of apo-HSOD in 80% GlOH is essentially constant below -5°C, as is the case if we take into account an error of $\pm 0.1$ nsec for the width. Yet, the width of apo-HSOD actually reaches a maximum at about -17°C. Thus, maximum heterogeneity of NATA and HSOD in 80% GlOH is reached at roughly the same temperature region suggesting that the assumption of $\Delta_2$ being equal for the two systems is not exaggerated. A complication is presented by the expectation that HSOD probably has a greater spread of fluorescence decay rates from each conformational substate than NATA. However, assuming, to first order, that the absolute magnitude of the width of the lifetime distribution is proportional to the number of substates and the spread of fluorescence decay rates from each conformational substate, we need only consider how large a temperature increase is needed to decrease the width to, say, half of its maximum value (Figure 8). As a result, Figure 9 shows that one needs at least a
temperature increase of $\Delta T_\beta = 40^\circ\text{C}$ for apo-HSOD before the width reaches half maximum from $-5^\circ\text{C}$. This temperature increase is large compared to the $\Delta T_\alpha = 20^\circ\text{C}$ increase needed to decrease the maximum width of NATA at $-15^\circ\text{C}$ by half. This comparison suggests that HSOD is a $\beta$ system. Thus, from the general features of BUS one can gain an understanding of the thermal behavior of the width of the lifetime distribution of single Trp proteins.

The ratio of the center of the lifetime distribution of NATA to apo-HSOD varies between 0.65 and 0.75 over temperature. The relatively close thermal behavior of the center of NATA with the center of HSOD may be due to a specific effect of GLOH on the Trp residue. This conclusion is plausible considering that the Trp residue of HSOD is on the surface of the protein. In fact it may be appropriate to include the effect of viscosity on substrate interconversion through the use of the Kramer's relation, which would entail the division of equation (7) by a friction constant which can approximated by Stokes law. Future work on the BUS model is being done to take into account the possibility of solvent friction.
V. CONCLUSIONS

In summary, the thermal behavior of HSOD in 80% G10H is consistent with the hypothesis of conformational substates. In particular, below a critical temperature time-resolved fluorescence and anisotropy decay measurements support the existence of isoenergetic conformational substates in HSOD. A frozen state occurring below the critical temperature can also be seen from the Y plots of figure 4. Furthermore, due to the character of the resulting low temperature lifetime distribution, substate dependent entropy must be considered for apo-HSOD in 80% G10H.

The BUS model, which only requires several general conditions (isoenergetic substates, a hierarchy of energy barriers between substates and substate dependent fluorescence decay rates), provides a physical picture which is in qualitative agreement with the data. In particular, BUS predicts the freezing of the center and width of the lifetime distribution of a single Trp-protein system below a critical temperature. With respect to the plausible assumption that the energy landscape of NATA is more like an α system than HSOD, BUS correctly predicts the qualitative differences in the thermal behavior of the width of the lifetime distributions between the two samples. Simpler models would have to make more specific conditions than BUS on various parameters in order to be in qualitative agreement with the data. BUS thus provides a very general physical framework for interpreting the time-resolved fluorescence decay of proteins.

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Fig 6

Tier Level

0

1

2

Substate: 1 2 3 4

$\tau_j$ (nsec): 0.5 1.5 2.5 3.5

Energy

$\Delta_1$

$\Delta_2$

$\Delta_0$

5 6 7 8

4.5 5.5 6.5 7.5