A Model for Multiexponential Tryptophan Fluorescence Intensity Decay in Proteins

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ABSTRACT Tryptophan fluorescence intensity decay in proteins is modeled by multiexponential functions characterized by lifetimes and preexponential factors. Commonly, multiple conformations of the protein are invoked to explain the recovery of two or more lifetimes from the experimental data. However, in many proteins the structure seems to preclude the possibility of multiple conformers sufficiently different from one another to justify such an inference. We present here another plausible multiexponential model based on the assumption that an energetically excited donor surrounded by N acceptor molecules decays by specific radiative and radiationless relaxation processes, and by transferring its energy to acceptors present in or close to the protein matrix. If interactions between the acceptors themselves and back energy transfer are neglected, we show that the intensity decay function contain 2^N exponential components characterized by the unperturbed donor lifetime, by energy transfer rates and a probability of occurrence for the corresponding process. We applied this model to the fluorescence decay of holo- and apoazurin, ribonuclease T1, and the reduced single tryptophan mutant (W28F) of thioredoxin. Use of a multiexponential model for the analysis of the fluorescence intensity decay can therefore be justified, without invoking multiple protein conformations.

INTRODUCTION

In their review of tryptophan (Trp) fluorescence in proteins, Beechem and Brand (1985) have described how infrequently tryptophan fluorescence intensity decays monoexponentially even in proteins bearing a single tryptophan residue. Similarly, Trp fluorescence intensity decay in peptides having a single Trp, and even for free tryptophan itself in aequous solutions at neutral pH, is heterogeneous (Szabo and Rayner, 1980; Chen et al., 1991) and the popular interpretation is that each exponential recovered by data analysis reflects a specific rotamer, (Szabo and Rayner, 1980; Chen et al., 1991). An analogous interpretation for multiexponential Trp fluorescence decay in proteins is used to infer multiple conformations of the protein. By invoking such a model, one clearly assumes explicitly that there are interactions between the indole moiety and the protein matrix and/or solvent which are unique to each conformational state, and which therefore cause a specific lifetime to be evinced. In arriving at such a conclusion, one also assumes implicitly the actual *validity* of using a multiexponential model for analysis of the fluorescence intensity decay, irrespective of the precise analytical tool used to extract each pair of preexponential factors and fluorescence lifetimes.

The assumption of a multiexponential model for analysis and interpretation of fluorescence intensity decay data has been challenged, however, during the last decade. An alternative approach, employing the concept of a distribution of lifetimes, has been suggested (Alcala et al. 1987; James et al., 1985a; Siemiarczuk et al., 1990). For example, Alcala et al. (1987) have proposed that the dynamics of the Trp side chain allows the fluorophore to continuously sample multiple environments and hence be subjected to multiple, different interactions to yield a distribution of physical substates interconverting on the time scale of the fluorescence intensity decay. This approach is particularly appealing because it conveniently links the dynamics of the fluorophore–hence, indirectly, the dynamics of the protein matrix–with the fluorescence lifetimes. The width of the lifetime distribution is considered to be linked to the number and character of conformational substates and the rate with which these substates interconvert. Broad distributions would thus be indicative of substantial conformational freedom. An increase in temperature was shown by Alcala et al. (1987) to narrow the distribution of lifetimes, in keeping with what one would predict based on the physical model proposed.

The more traditional (multiexponential) and distribution of lifetime models now both enjoy substantial support, although the concept of distributed fluorescence lifetimes has spawned not inconsiderable skepticism. The problem is that there is no clear way of *proving* one or another model to be "correct." The assumption that a single fluorescence lifetime implies a unique conformation of the protein, or configuration of the Trp residue in that protein, seems reasonable, a priori. However, the appearance of multiple lifetimes and the inference therefrom of either unique conformations of the Trp side chain stable on the time scale of the fluorescence decay *or* of a distribution of conformational states carries with it distinct implications, for example, regarding the mechanism(s) of intramolecular quenching. The burden of proof must be placed on the investigator to *justify* the likelihood of multiple conformers in any particular protein *and* to rationalize the particular lifetime assigned on the

Received for publication 18 March 1993 and in final form 7 September 1993.
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0006-3495/93/12/2313/11 $2.00

basis of identifiable molecular interactions occurring in each conformation.

On the other hand, an assumption, again a priori, that the fluorescence lifetimes are distributed simply because the intensity decay data can be fit well with a distribution, is also difficult to justify especially if the concept is applied unquestioningly to the Trp fluorescence in any and all proteins. Inevitably, there will be examples in which the amplitude of fluorophore motion (libration) on the picosecond to nanosecond time scale is so restricted, or the fluorophore librational rate so high, that the motion of the fluorophore would be expected to "average" the environment sufficiently rapidly that a single exponential decay would be expected. In such situations, the need to invoke, or justification for using a distribution of substates to describe the data could be vitiated. (To posit that a single exponential represents simply a distribution of zero width is not really in the spirit of the concept of a distribution of lifetimes at least as described above.)

The fluorescence of the single Trp residue in RNAse T1 and in azurin provides good examples of the problem inherent in interpretations of fluorescence lifetimes in terms of either of these two physical models. The fluorescence decay of Trp 59 in RNAse T1 has been thoroughly investigated in several laboratories (James et al., 1985b; Chen et al., 1987; Gryczynski et al., 1988), and there is broad acceptance that at pH 5.5 the fluorescence decay is monoexponential, but at pH $\geq$7.0 two exponential terms are recovered. Likewise, Szabo and coworkers (Szabo et al. 1983; Hutnik and Szabo, 1989) and Fleming and coworkers (Hansen et al., 1990) have made especially careful measurements of the fluorescence of Trp in azurin and the general conclusion is that the fluorescence decay of holoazurin (of *Ps. aeruginosa*) is multiexponential (two or three lifetimes) while that of apoazurin is monoexponential. For both RNAse T1 and azurin, the heterogeneous intensity decay has been most frequently ascribed the existence of two or more conformers of each protein. Alcala et al. (1987) proposed instead a narrow distribution of states for both proteins.

The problem with the above interpretations for these two proteins is demonstrated in Fig. 1, which shows molecular graphic depictions of the packing of the Trp residues within the protein matrix of RNAse T1 and azurin, respectively. As shown by Fig. 1, and further evaluated for RNAse T1 with molecular dynamic simulations (Axelsen and Prendergast, 1989; MacKerrell et al., 1987), Trp 59 in RNAse T1 is sequestered in a tightly packed, largely nonpolar pocket in the protein matrix such that only high frequency of very low amplitude librations is plausible; additionally, there is markedly limited access for water, a potential quencher, to the Trp side chain (Axelsen and Prendergast, 1989). Furthermore, umbrella sampling calculations done in our group (C. Haydock and F. G. Prendergast, manuscript in preparation) show that the energetic barrier to rotational motion of the Trp in RNAse T1 side chain is so high that the probability seems small that there are many conformations of the indole side chain sampled more quickly than or attained during the fluorescence lifetime. Finally, although we do not yet have simulations done at specific pH values, judging from the equivalence of the steady-state Trp fluorescence anisotropy at pH 5.5 and 7.5 (T. Felmlee and F. G. Prendergast, unpublished data), we consider that any substantial change in Trp dynamics in RNAse T1 caused by changes in this pH range is improbable. In other words, there seems to be little basis for assuming multiple Trp conformations in RNAse T1. Rather, it would appear that the Trp side chain librational motion should be so rapid that a single fluorescence lifetime should be evinced irrespective of pH providing that the tertiary structure of the protein is not disrupted at pH >7.0. Admittedly we still need to show from simulations that within the entire set of χ^1, χ^2 dihedral angles defining orientations of the Trp side chain, only one configuration is plausible.

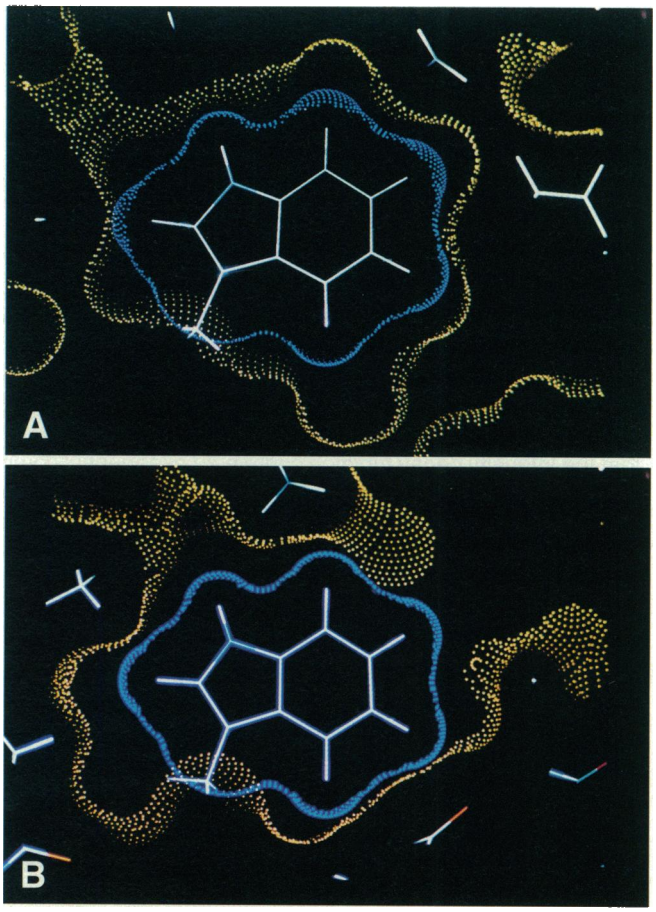

FIGURE 1 Computer graphic depictions of molecular packing around the tryptophan residues of azurin (*a*) and ribonuclease T1 (*b*). Atomic coordinates for these displays were obtained from the Protein Data Bank.

A similar situation holds for azurin. Here too the Trp indole moiety is ensconced in a densely packed hydrophobic pocket. However, in azurin there is in addition a single copper atom approximately 7 Å from the indole moiety (Adman, 1991). This copper atom is clearly responsible for the low quantum yield of holoazurin possibly because of electron transfer from the excited Trp (James et al., 1985b) or because

of fluorescence resonance energy transfer into a charge transfer band (Sweeney et al., 1991). Judging from the graphic depictions, even in the absence of molecular dynamics simulation data, multiple conformations of the Trp side chain do not seem tenable requiring as they would extensive disruption of a tightly packed region of the protein needed to allow the indole ring to flip. Hansen et al. (1990) reached a similar conclusion from their calculations regarding the limited mobility of the Trp side chain in azurin.

At this point we should note that the very arguments we mount against the possibility of multiple, unique conformations in azurin or, for that matter, *any* protein, *hold equally well for multiple conformational substates*, at least as the latter would need to be defined to justify the assertion of a continuous distribution of fluorescence lifetimes (cf. Alcala et al., 1987).

These and other examples of heterogeneous Trp fluorescence intensity decays have stimulated us to consider physical models other than the one just mentioned. We were particularly interested in approaches in which conformational changes or conformation substates were not an obligatory part of the model.

It will be apparent at the outset that irrespective of the precise physical model favored, the key issue(s) is(are) the intramolecular mechanism(s) influencing the excited state to ground state transition. Accordingly, the fundamental tenet in the model we present below is that deexcitation (quenching) of the excited fluorophore (in this instance tryptophan) can occur through a variety of mechanisms (channels), all fundamentally involving, obviously, some form of transfer of energy. For the conformationally determined models of quenching, such transfer of energy is assumed to be effected primarily through collisions with the quenching species in a manner analogous to that implied by the Stern-Volmer formalism. In the model we present, the deexcitation is (preferably) not at all dependent on collisions between the fluorophore and quenchers. We use the term "energy transfer" in a totally generic sense, realizing that all quenching of the excited state perforce requires a *transfer of energy*. Fluorescence resonance energy transfer is, in our parlance, simply one form of energy transfer, but electron transfer is justifiably another.

Although the precise form of that energy transfer is ultimately important, for our immediate purposes this issue is not of overriding importance. The primary consideration is that any such process of deexcitation occurs, by definition, with a specific rate and probability. We treat the fluorophore as the energy donor and assume the existence of one or more "acceptors" in the environment without specifying their molecular character, the precise molecular interactions or their absolute locations relative to the fluorophore. The fundamental objective was met, namely to show that the model provides a plausible interpretation for the data without the need to invoke conformational differences as the basis for multiexponential fluorescence intensity decay. Although the core of the model has been considered for some time (Prendergast et al., 1991), its development at this time was strongly influenced by the work of Blumen and Manz (1979) and Klafter and Shlesinger (1986).

THEORETICAL MODEL

The energetically excited donor surrounded by acceptor molecules will decay either by transferring its energy to an acceptor (or set of acceptors) or by donor-specific radiative or radiationless relaxation processes. These decay channels are independent so that the overall decay function $I(t)$ factorizes as (Blumen and Manz, 1979)

$$I(t) = I(0)e^{-t/\tau}\phi(t), \quad \phi(0) = 1. \tag{1}$$

where $e^{-t/\tau}$ is the donor-specific decay function and $\phi(t)$ represents a function describing the decay of the donor excitation due to energy transfer to the acceptors. $I(0)$ is the intensity at zero time. This formula is valid under the assumption that only one donor is present and thus donor-donor interactions are excluded. We will take into account only two body interactions between the donor and each acceptor, neglecting any interactions between acceptors. Also, any possibility of back energy transfer will be disregarded. We assume that N acceptors can be randomly distributed at N sites in configurations $K_{n,m} = \{i_1, i_2, \ldots, i_n\}$ where $n = 1, \ldots, N$ is the number of acceptors that occupy the sites $i_1, \ldots, i_n$ (Fig. 2). The index $m = 1, \ldots, C_n^N$ (with C_n^N being the binomial coefficient) enumerates different ways of distributing the n acceptors at N sites. $K_{0,1} = \{\emptyset\}$ (empty set) represents the configuration with no acceptors surrounding the donor. In mathematical terms $K_{n,m}$ corresponds to combination without repetition of order n from N elements. There are C_n^N such combinations and the total number of configurations is therefore $\sum_{n=0}^{N} C_n^N = 2^N$.

For mostly formal reasons it is useful to enumerate all configurations $K_{n,m}$ by a number

$$\kappa_{nm} = \sum_{k=0}^{n-1} C_k^N + m - 1, \quad n = 1, \ldots, N, \quad \kappa_{01} = 0, \tag{2}$$

which runs from 0 to $2^N - 1$. The incoherent direct decay of the donor excitation by energy transfer to acceptors in configuration $K_{n,m}$ can be then expressed by (Blumen and Manz, 1979)

$$\phi(\kappa_{nm}; t) = \prod_{i \in K_{n,m}} e^{-w_i t}, \tag{3}$$

where w_i is the decay rate for deexcitation due to energy transfer to the acceptor at site i. The product is taken for all i that are elements of $K_{n,m}$ and reflects the joint probability of decay for uncorrelated events. For example, if $N = 3$, then $\phi(1, t) = e^{-w_1 t}$, $\phi(6, t) = e^{-w_3 t}e^{-w_1 t}$, $\phi(7, t) = e^{-w_1 t}e^{-w_2 t}e^{-w_3 t}$. The decay rate w_i could be considered as dependent on distance $\mathbf{R}_i$ from the donor to the acceptor at site i: $w_i = w(\mathbf{R}_i)$. Specific examples of this dependence are often encountered for multipolar interactions, $w(R_i) = (1/\tau)(d/R_i)^s$, and for exchange interactions, $w(R_i) = w(R_i) = (1/\tau)\exp[\gamma(d - R_i)]$ (see e.g., Blumen and Manz,

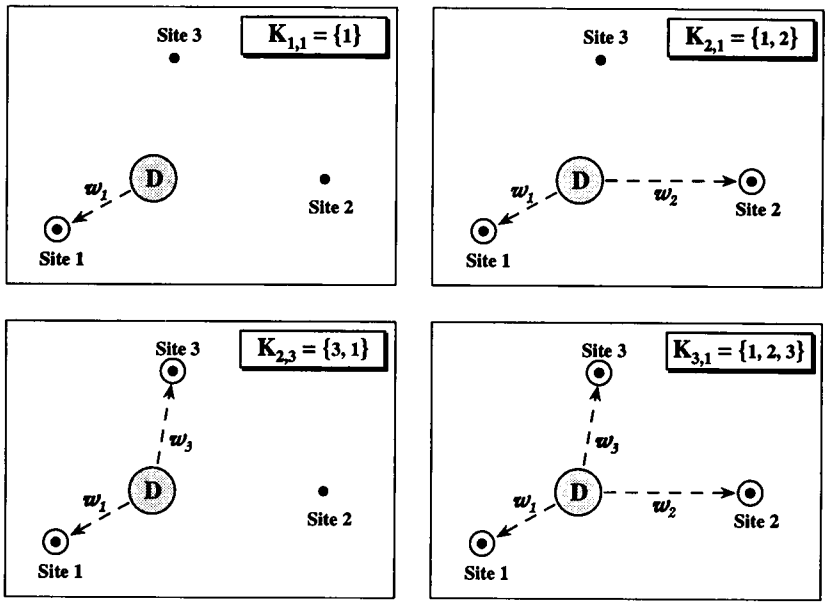

FIGURE 2 Schematic representation of some possible acceptor configurations $K_{n,m}$ for three sites. The acceptors could be of the same type or different types. Each energy transfer process (indicated by *arrow*) is strictly related to a given site i and corresponds to the same energy transfer rate w_i in any of the configurations.

1979). In further considerations we will keep w_i completely general.

The actually measured decay function in fluorescence experiments is the ensemble average over all possible acceptor configurations such that:

$$\phi(t) = \sum_{n=0}^{N} \sum_{m=1}^{C_N^n} p(\kappa_{nm})\phi(\kappa_{nm}; t) = \sum_{k=0}^{2^N-1} p_k \phi(k; t), \quad \sum_{k=0}^{2^N-1} p_k = 1. \tag{4}$$

Here $p_k = p(\kappa_{nm})$ is the probability of observing the acceptor configuration $K_{n,m}$. Equations 1, 3, and 4 yield to the decay function of the form:

$$\begin{aligned} I(t) = I(0)e^{-t/\tau}[\, & p_0 + p_1 e^{-w_1 t} + \ldots + p_N e^{-w_N t} \\ & + p_{N+1} e^{-(w_1+w_2)t} + \ldots + p_{2N} e^{-(w_1+w_N)t} \\ & + p_{2N+1} e^{-(w_2+w_3)t} + \ldots + p_{N+C_2^n} e^{-(w_{N-1}+w_N)t} \\ & + p_{N+C_2^N+1} e^{-(w_1+w_2+w_3)t} + p_{N+C_2^N+2} e^{-(w_1+w_2+w_4)t} \\ & + \ldots + \ldots + p_{2^N-1} e^{-(w_1+\ldots+w_N)t}] \equiv I_N(t) \end{aligned} \tag{5}$$

This decay function belongs to the family of general multiexponential functions

$$E(t) = \sum_j a_j e^{-t/\tau_j} \tag{6}$$

usually employed to describe the fluorescence intensity decay in terms of effective lifetimes τ_j and the corresponding amplitudes a_j. The decay model given by Eq. 5 exhibits more specific form than the general model given by Eq. 6. In the general model there are as many effective lifetimes, considered as independent parameters, as there are components, while in the model given by Eq. 5 with 2^N components only $N + 1$ rates $1/\tau, w_1, w_2, \ldots, w_N$ are independent parameters. Formally our model allows 1, 2, 4, . . . , 2^N components, but effectively any number of components may emerge when some probabilities tend to be zero. Thus, in principle, if the fluorescence intensity decay data were analyzed in terms of the general multiexponential model, the transformation of parameters makes possible interpretation of the decay in terms of an unperturbed lifetime τ, "energy transfer" rates w_1, $w_2, \ldots$, and probabilities $p_0, p_1, p_2, \ldots$ associated with occupancy of possible acceptor sites. This is illustrated in the Illustrative Examples section.

ILLUSTRATIVE EXAMPLES

We can now offer an alternative and plausible interpretation of the fluorescence decay in azurin (Hansen et al., 1990). The fluorescence intensity decay in apoazurin purified from *P. aeruginosa* is monoexponential with the lifetime τ_{apo} = 4.94 ns, which we interpreted as the *unperturbed* tryptophan fluorescence lifetime. In the native holoazurin, two fluorescence intensity decay components were observed and our decay model reveals the form:

$$I_1(t) = I_1(0)e^{-t/\tau}(p_0 + p_1 e^{-w_1 t}), \tag{7}$$

$$\begin{aligned} \tau = (4.2 \pm 0.1)\text{ns}, \quad w_1 = (9.38 \pm 0.36)\text{ns}^{-1}, \\ p_0 = 0.03 \pm 0.01, \quad p_1 = 0.97 \pm 0.01. \end{aligned} \tag{8}$$

(The errors in parameters were estimated from the errors of original parameters quoted in Hansen et al. (1990) by the use of error propagation rule.) Clearly, there is a high probability of deexcitation by energy transfer to the Cu ion–the acceptor in holoazurin. The small change in the apparent unperturbed tryptophan lifetime, between native azurin and apoazurin

(4.23ns versus 4.94ns) can be interpreted as being due to small change in nonradiative relaxation consequent upon removal of the copper atom. Another possible interpretation is given below in conjunction with measurement of Hutnik and Szabo (1989).

Hansen et al. (1990) consider the electron energy transfer to the Cu ion as dominant quenching mechanism in fluorescence intensity decay of holoazurin and calculate the rate of transfer as $1/\tau_1 - 1/\tau_{apo}$, where τ_1 is the lifetime of the fast component of the fluorescence decay of holoazurin. The rate they obtained was 9.41 ns^{-1}, which is very close to the value we obtained. The difference comes from the fact that τ is slightly different from τ_{apo}.

In another measurement (Hutnik and Szabo, 1989) of the fluorescence intensity decay of native azurin in apparently identical experimental conditions (except for the temperature which was 20°C compared with 22°C in Hansen et al. (1990), and 25 thousand counts at the peak versus 10 thousand in Hansen et al. (1990)), three exponentials were recovered. These can be interpreted within our model assuming two acceptors:

$$I_2(t) = I_2(0)e^{-t/\tau}[p_0 + p_1e^{-w_1t} + p_2e^{-w_2t} + p_3e^{-(w_1+w_2)t}] \tag{9}$$

$$\tau = 4.95\text{ns}, \quad w_1 = 9.80\text{ns}^{-1}, \quad w_2 = 2.66\text{ns}^{-1}, \quad p_0 = 0.02, \quad p_1 = 0.93, \quad p_2 = 0.05, \quad p_3 = 0 \tag{10}$$

Such interpretation is rather consistent with the above interpretation of data obtained by Hansen et al. (1990). The unperturbed tryptophan lifetime $\tau = 4.94$ ns for apozaurin as measured and analyzed by Hansen et al. (1990) agrees perfectly with that of holoazurin as measured and analyzed by Hutnik and Szabo (1989) and for both sets of data there is a high probability of deexcitation by energy transfer to the Cu ion with approximately the same rate. Basically the only difference is that for the data of Hutnik and Szabo interaction with a *second* acceptor needs to be postulated, although that interaction would have to proceed with a very small probability. Whether this phenomenon is a genuine result of the slight difference in temperature between those two measurements we cannot say, and is an issue to be decided by carefully designed measurements and data analysis. It is also conceivable that the small change in the apparent unperturbed tryptophan lifetime between holoazurin and apoazurin, which we observed in the data of Hansen et al. (1990), effectively accounts for the second acceptor observed in data of Hutnik and Szabo (1989). This shows that a thorough and accurate analysis of fluorescence decay data is critical.

Another example to be considered is the apparently biexponential decay of ribonuclease T1 Trp fluorescence (Bajzer and Prendergast, 1992). We can assume that Eq. 7 describes the fluorescence intensity decay. Accordingly, the excited single tryptophan in RNAse may transfer energy to an acceptor with rate w_1 and with a relatively low but still significant probability p_1, whence:

for pH = 7.5

$$\tau = (3.69 \pm 0.02)\text{ns}, \quad w_1 = (0.33 \pm 0.04)\text{ns}^{-1}, \quad p_0 = 0.85 \pm 0.02, \quad p_1 = 0.15 \pm 0.02,$$

and for pH = 5.5

$$\tau = (3.99 \pm 0.01)\text{ns}, \quad w_1 = (0.7 \pm 0.2)\text{ns}^{-1}, \quad p_0 = 0.961 \pm 0.004, \quad p_1 = 0.039 \pm 0.004.$$

(Here we actually refitted the decay curves analyzed in (Bajzer and Prendergast, 1992) by the one-acceptor model (Eq. 7) and obtained errors in parameters using a variant of Monte Carlo method (Press et al., 1986) with 100 simulations). From this result we would infer that while the unperturbed tryptophan lifetime does not change considerably with pH, the probability of energy transfer and its rate show significant changes.

In a third example we consider the fluorescence of the tryptophan residue in a reduced W28F mutant of *E. coli* thioredoxin (i.e., thioredoxin bearing a single Trp moiety at position 31). Time-correlated photon-counting measurements were performed at pH 8.0 under conditions of 10 ps channel widths and 20,000 counts at the peak. The intensity profile comprising 1900 channels was analyzed by use of the generalized Padé-Laplace method (GPL) (Bajzer et al., 1990), the maximum likelihood (ML) (Bajzer et al., 1991; Bajzer and Prendergast 1992) and the maximum entropy methods (MEM) (Livesey and Brochon, 1987; Vincent et al., 1988) assuming multiexponential function of the general form (Eq. 6). Four components were recovered by the GPL method (Table 1). The resulting lifetimes and preexponential factors are in good agreement with the result of the ML method (Table 1), when the estimates of errors in parameters are considered. Similarly, the results of MEM show clearly four overlapping peaks (Fig. 3). The corresponding values of lifetimes and preexponential factors are in agreement with two other methods (see Fig. 3 and Table 1) when the width of these peaks (characterized by the standard deviations for obtained parameters) is taken into account. Note that the lifetime distribution of Fig. 3 may give rise to speculations of a possible small fifth peak corresponding to a lifetime of about 0.8 ns.

The ML fit is excellent (Fig. 4) and adequately describes the decay data, as can be judged by statistical criteria (Table 1). Thus, the reduced Poisson deviance is $D/\nu = 1.036$, with the standard normal variate, Z, being well within acceptable limits of [−3,3]. The probability, Q, that the obtained deviance will exceed the ideal value of D, is larger than 0.1 (Bajzer and Prendergast, 1992; Press et al., 1986), which qualifies the four-component decay model as acceptable. Furthermore, the residuals appear random (Fig. 4) with a favorable run test statistic z (Bajzer and Prendergast, 1992). Separability and detectability indexes (Bajzer et al., 1991;

TABLE 1 Analysis of W28F mutant of thioredoxin

	τ_1	α_1*	τ_2	α_2	τ_3	α_3	τ_4	α_4
GPL‡	4.3 ± 0.8	0.05 ± 0.02	1.5 ± 0.7	0.06 ± 0.04	0.33 ± 0.07	0.32 ± 0.06	0.14 ± 0.02	0.56 ± 0.07
ML§	5.2 ± 0.1	0.033 ± 0.001	2.18 ± 0.07	0.071 ± 0.003	0.37 ± 0.01	0.30 ± 0.01	0.136 ± 0.004	0.60 ± 0.01
MEM‖	4.5 ± 0.9	0.04	2.3 ± 0.6	0.06	0.4 ± 0.2	0.34	0.16 ± 0.03	0.56
DA¶	τ	p_0	w_1	p_1	w_2	p_2	w_3	p_3
	5.2 ± 0.1	0.032 ± 0.002	0.259 ± 0.007	0.071 ± 0.001	2.38 ± 0.07	0.26 ± 0.01	5.4 ± 0.2	0.008 ± 0.033
		p_4		p_5		p_6		p_7
		0.001 ± 0.002		0.0007 ± 0.0042		0.29 ± 0.04		0.34 ± 0.03

* $\alpha_i = a_i/\Sigma_{j=1}^{4} a_j$ (see Eq. 6).

‡ The errors in parameters (second line) were estimated as described in (Bajzer et al., 1990).

§ $D = 1932$, $D/\nu = 1.036$, $Z = 1.12$, $Q = 0.135$, $z = 1.0$. The value of D is the obtained minimum of the Poisson deviance $D = 2\sum_{i=1}^{n}[c_i \ln(c_i/f_i) - c_i + f_i]$. Here c_i and f_i are the measured and calculated numbers of counts at channel i, respectively (see Bajzer et al., 1991; Bajzer and Prendergast, 1992), and ν is the number of degrees of freedom. The corresponding standard normal variate is $Z = (D - \nu)/(2\nu)^{1/2}$. Q is the probability that the obtained deviance will exceed its ideal value, $Q = \Gamma(\nu/2, D/2)/\Gamma(\nu/2)$, $\Gamma(a, x) = \int_x^\infty e^{-t}t^{a-1}\,dt$. $z = (r - \mu_r)/\sigma_r$ is the runs test statistic for residuals. Here r is a number of runs and $\mu_r = 1 + 2n_pn_n/n_s$, $n_s = n_p + n_n$ where n_p and n_n are the numbers of positive and negative residuals, respectively. $\sigma_r = [2n_pn_n(2n_pn_n - n_s)/(n_s^3 - n_s^2)]^{1/2}$. The residuals are defined (cf. Bajzer et al., 1991) $r_i = \sqrt{2}(c_i - f_i)[c_i \ln(c_i/f_i) - c_i + f_i]^{1/2}/|c_i - f_i|$. The errors in parameters (second line) were estimated as described previously (Bajzer and Prendergast, 1992).

‖ $\chi^2/\nu = 1.032$. Here $\chi^2 = \Sigma_{i=1}^{n}(c_i - f_i)^2/c_i$ where c_i is the measured number of counts and f_i the number of counts calculated as in (Livesey and Brochon, 1987). The values of τ_i, $i = 1, 2, 3, 4$, were obtained by standard methods as centroids of isolated peaks considered as unimodal probability distributions (Vincent et al., 1988) and the presented errors (second line) as SDs corresponding to these distributions. α_i, $i = 1, 2, 3, 4$, were obtained as the areas of isolated peaks subsequently normalized to yield the sum of 1.

¶ $D = 1928$, $D/\nu = 1.036$, $Z = 1.08$, $Q = 0.134$, $z = 1.4$. Definitions as in remark §. The errors in parameters are determined as SDs obtained by Monte Carlo method (Press et al., 1986) with 100 simulations.

Bajzer and Prendergast, 1992) are all above 17, indicating that these components are resolvable. The ML fit (Bajzer and Prendergast, 1992) also included the determination of zero time shift ($\delta = -0.005$ ns) and light scatter parameter ($\xi = 0.09$).

Subsequently we fitted (by the use of the ML technique) the same data with a model that assumes three acceptors:

$$I_3(t) = I_3(0)e^{-t/\tau}[p_0 + p_1e^{-w_1t} + p_2e^{-w_2t} + p_3e^{-w_3t} + p_4e^{-(w_1+w_2)t} + p_5e^{-(w_2+w_3)t} + p_6e^{-(w_3+w_1)t} + p_7e^{-(w_1+w_2+w_3)t}] \quad (11)$$

Examination of the resulting parameter values (Table 1) shows that it is very unlikely that an energy transfer process characterized by the rate w_3 can occur alone ($p_3 \approx 0$ within estimated uncertainty). The same is concluded for possible simultaneous processes characterized by the rates $w_1 + w_2$ and $w_2 + w_3$ ($p_4 \approx 0$, $p_5 \approx 0$ within uncertainties). Rather more probable are the processes characterized by the rate w_2 and the simultaneous processes characterized by the rates $w_3 + w_1$ and $w_1 + w_2 + w_3$. Interestingly the value of the unperturbed tryptophan lifetime in thioredoxin is quite similar to the value of unperturbed tryptophan lifetime of azurin.

The quality of the fit with the model given by Eq. 11 is the same as for the standard four-component model (Table 1). The likelihood ratio test for possible significant difference of fits for the three-acceptor and four-component model shows the equivalency of these models with respect to data. The difference between respective deviances is only 4, and it should be at least 13.3 to be significant with $Pr(X > 13.3) = 0.01$.

DISCUSSION

Origin of the model

The approach to modeling of decay law for fluorescence intensity we have presented here is basically similar to that of Blumen and Manz (1979). However, their aim was different. They wanted to derive a closed form expression when a large number of acceptors are distributed on an infinite regular lattice. As a final result, in continuum approximation they obtained a typically nonexponential decay law model that generalizes Förster type decay for fluorescence resonance energy transfer. They assumed a specific expression for the probability $p(\kappa_{nm})$, i.e., $p(\kappa_{nm}) = p^n(1-p)^{N-n}$ where p was the probability of a site being occupied by an acceptor. Such an expression is a result of their assumption that the occupancy of lattice sites by acceptors is random and uncorrelated.

We, however, retained the probability $p(\kappa_{nm})$ completely general and obtained the decay law model in the form of multiexponential functions. The number of components depends on the number of acceptors to which the energy can be transferred. For a small number of acceptors we deal with a multiexponential function specific in the form of its exponents. For a large number, N, of acceptors the multiexponential function of our model contains even more (2^N) components and could actually be considered as a representation of a nonexponential decay model.

Blumen and Manz first considered the case in which all acceptors were of the same type, and subsequently generalized their formulae to a more complex situation of acceptors belonging to different species. We have followed the reasoning of Blumen and Manz for a single type of acceptor.

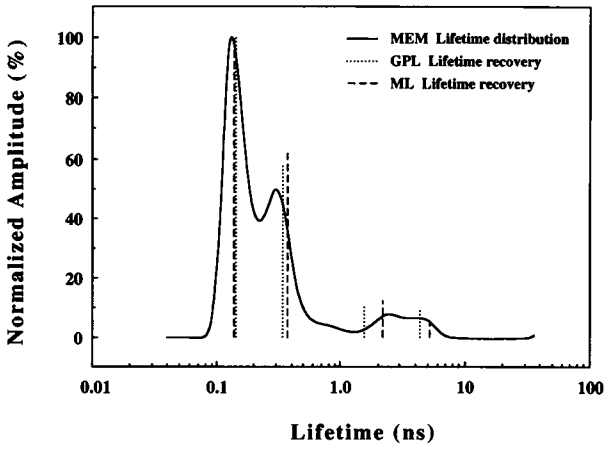

FIGURE 3 The distribution of lifetimes as recovered by the maximum entropy method and the discrete lifetimes (with amplitudes) as recovered by the maximum likelihood and generalized Padé-Laplace methods. For the lifetime distribution 290 logarithmically equidistantly spaced lifetimes were used in the range of 0.04–35 ns. The ordinates are expressed in percentage of the maximal value of the distribution function. Similarly, for discrete lifetimes the amplitudes are expressed in percentage of the maximal amplitude for a given method.

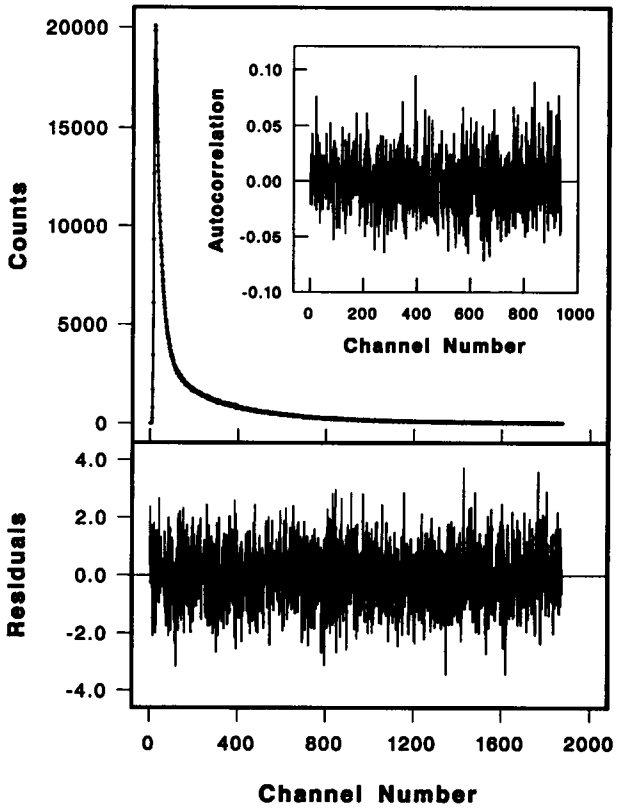

FIGURE 4 Fluorescence intensity decay of reduced W28F mutant of *E. coli* thioredoxin fitted to standard 4-exponential decay function by the ML method. Visual inspection of residuals and the corresponding autocorrelation function (calculated as in (O'Connor and Phillips, 1984) shows the randomness expected for a good fit. The statistical criteria of the goodness of the fit and the parameter values are given in Table 1.

However, by careful inspection of possible configurations $K_{n,m}$, we found that our formula remains valid even if N acceptors are of several types, providing that each type can occupy its own set of sites. In other words we do not take into account configurations in which two or more acceptors of different types may occupy exactly the same site. This restriction is really not of any importance because we have not defined the sites by a specific regular lattice, and two sites may be formally considered different even if they are infinitesimally close.

Our next comment is related to the recent paper of Sienicki et al. (1991) which leaves the impression that the multiexponential decay law is very approximate as it "...neglects the fact that the measured fluorescence decay profile is an average signal from all molecules excited by light." In other words, they suggested that the averaging procedure over all configurations leads inevitably to a nonexponential decay model. This appears true in the continuum approximation (except for the position-independent relaxation rate, as Sienicki et al. noted). However, for the model considered here, as long as the transition to the continuum is not performed, the multiexponential character of the decay law is in fact a *consequence* of configurational averaging. The same is true for the more specific decay model given by Eq. (2.7) of Blumen and Manz (1979) from which the continuum approximation employed by Sienicki et al. was actually derived. In that respect the nonexponential formulae of "Theoretical Considerations" of (Sienicki et al., 1991) could be considered equivalent to multiexponential functions with a large number of components.

Before going on to a discussion of the particular examples of Trp fluorescence we have presented, we wish to emphasize that our model formally represents a reparametrization of the traditional multiexponential model. Virtually any traditional multiexponential decay function can be reparametrized in terms of an unperturbed donor lifetime, "energy transfer" rates, and corresponding probabilities (of which some may vanish). As such, the decay function is interpretable in terms of a simple picture of a donor transferring its energy to acceptor(s) by certain mechanisms whose precise nature is *not crucial for the model.* To answer whether, or to what extent, this picture describes fluorescence decay in any protein, a detailed study would be required for each. Such a study should involve detailed examination of wavelength dependence, of the fluorescence decays, evaluation of pH and temperature effects, and the effects of other physical perturbations. Only then might some definitive answers be obtained and the appropriateness of this model be fully appreciated. However, within the scope of the present paper, we will briefly indicate possible interpretations in terms of the model proposed and point out the difficulties and dilemmas.

Specific Examples of Multiexponential Fluorescence Intensity Decays

In the case of the specific proteins considered in this paper, we can now offer interpretations of the multiexponential intensity decay that do not invoke conformational changes in

the protein. But now, the actual mechanisms of quenching become important in our deliberations. There are some unambiguous examples of the type of donor/acceptor model we propose, most notably Trp fluorescence in myoglobin and hemoglobin. In both of these proteins, the energy transfer process is clearly by Förster fluorescence resonance energy transfer (see e.g., Hochstrasser and Negus, 1984). In other proteins, the processes are less clear. For azurin, the role of copper seems incontrovertible, and the proposal that it acts as an electron acceptor has been well argued by Petrich et al. (1987) and later by Hansen et al. (1990). However, this view has been challenged by Sweeney et al. (1991). Using UV-resonance Raman techniques, these authors found no evidence for a radical Trp cation and concluded that quenching occurred by resonance energy transfer from Trp to Cu-protein charge-transfer band. Unfortunately Sweeney et al. did not provide any other direct evidence for such energy transfer to justify their inference. From our standpoint, irrespective of who is "right," the role of a Trp excited state-copper atom interaction is unchallengable, and in the model presented here, the copper atom effects clearly dominate the quenching processes in the holoprotein.

Without getting into the fray, we would also note simply that the precise mechanism of energy transfer is *not* critical to the model we describe here, but it is interesting that our model recovers the same rate (within the experimental error) for the quenching process as that calculated by Hansen et al. (1990) (9.38 and 9.41 ns^{-1}, respectively). As noted above the heterogeneous decay of fluorescence in holoazurin, which we propose, occurs because electron transfer is not perfect, i.e., does not occur with a probability of 1. As our model shows, one can then recover two exponential components assuming one acceptor, three assuming two acceptors all with statistically good results. The consonance of the results recovered by this new model for the rates and lifetimes from the data of both Hutnik and Szabo (1989) and Hansen et al. (1990) is reassuring in spite of the unresolved issue of the existence of the third component. Multiple protein conformations, which as we have noted are difficult to justify given the structure of azurin, need not be invoked. An important additional observation is that of all the interpretations we might use, only the model we propose here explains the recovery of the fluorescence lifetime of the *apoazurin* from the fluorescence lifetime intensity decay data from *holoazurin*.

At this junction two additional caveats are in order. The excellence of current experimental and analytic techniques is such that the "conformational" purity of protein samples used for fluorescence measurements becomes a real issue. Thus, one has to wonder of what real significance is a component that contributes only a small fraction (say $\leq 5\%$) of the fluorescence evinced. Furthermore, in the case of a protein such as azurin, how might slight displacements in the position of the copper atom influence the efficiency of electron transfer? Since we do not know the precise distance dependence of the effect of the Cu ion on Trp fluorescence, it is easy to postulate that a small change in the position of that bound ion relative to the indole side chain may be the basis for apparently different recovered lifetimes. But, as noted above, it is difficult to rationalize the recovery of the long lifetime component seen in holoazurin. (For example, how much would the copper atom have to be moved away in a (putative) second conformer to allow the appearance of the long lifetime component.) In principle, the structural fluctuations in the protein occurring on a picosecond-to-nanosecond timescale may be deemed to represent conformational substates. However, without a structurally credible description of how much of a conformational change is needed to effect a change in lifetime, the claim that heterogeneous lifetimes reflect either protein matrix dynamics *or* multiple protein conformations is found wanting. To our knowledge while there have been many allusions to, or assertions for, multiple conformers in proteins, little evidence has been adduced. As pointed out by a reviewer, our model does assume that the lifetimes are invariant with excitation and emission wavelength. Hence, Trp residues that exhibit distinguishable decay-associated spectra are not good candidates for the model. The second caveat, however, is relevant to the latter issue.

The popular view is that RNAse T1 exhibits a single lifetime at pH 5.5. Data from our laboratory have suggested a very small contribution from a quenched second component, and we *can* rationalize that result within the framework of our model. On the other hand, this latter result could also be due to no more than the presence of a very small fraction of protein which, for example, might be denatured. (Prendergast et al., 1991). A similar argument could be used for the protein at pH 7.5, even though under such conditions the apparent second component is prominent. The difficulty for our model is that in RNAse T1 there is no obvious candidate in the protein structure to assign as an "acceptor" except perhaps the carbonyl group of a single closely approximated proline residue (Axelsen and Prendergast, 1989). By the same token, there is also no obvious way to explain the appearance of a second lifetime component in terms of either two conformers or a distribution of substates.

Before considering the next protein, we must reiterate the importance of the choice of methods used for the analysis of the fluorescence intensity decay data primarily with regard to the ability to separate the individual components (in terms of preexponential factors and lifetimes) in a presumed multiexponential decay process. With time-correlated single photon counting data, for a number of sampled data, a given channel width and level of noise, and a particular algorithm for parameter recovery, there is a critical lifetime ratio and a critical ratio of preexponential factors beyond which the desired parameters *cannot* be resolved (Bajzer et al., 1990 Bajzer et al., 1991 Bajzer and Prendergast, 1992). The implications of this inescapable reality for interpretation of either single or multiple lifetimes are clearly exemplified by the case of azurin and ribonuclease T1. Therefore, to be able to extract reliable information from the experimental data, it is essential to use different methods of analysis, and several statistical criteria (including those we advocated in Bajzer and Prendergast (1992)). Such a thorough approach was exemplified in analyzing the fluorescence intensity decay curve

of W28F mutant of thioredoxin. It is also very useful to perform simulations which may suggest (Bajzer et al., 1991 Bajzer and Prendergast, 1992) that a given measured fluorescence intensity decay should be remeasured under different conditions with regard to the number of data points, and the level of noise and the extent of the tail portion of the decay curve included in the analysis. With these issues in mind, the results from the reduced single Trp mutant (W28F) of thioredoxin (TRX) are particularly interesting. Wild type thioredoxin (WT-TRX) has two tryptophan residues W31 and W28. Traditional analysis of the fluorescence intensity decay of WT-TRX yields four apparent lifetimes. Analysis of the fluorescence intensity decay of the single Trp mutant W31 (in the W28 mutant of TRX) also yields four apparent lifetimes in the oxidized and reduced forms of the protein. Assuming the existence of three acceptors, the data can be fit excellently with our model. Interestingly, a detailed 13C-NMR study of this protein labeled with Trp enriched at $C\delta_1$ of the indole ring with 13C (see e.g., Weaver et al. 1988) shows no evidence of multiple conformers (K. Nollet, F. G. Prendergast and M. D. Kemple., manuscript in preparation). Admittedly the NMR result could reflect simply the different time scales inherent in the NMR and fluorescence experiments but 13C-NMR relaxation data also showed a high order parameter for W31 in thioredoxin (Kemple et al., 1993), suggesting the restricted mobility of W31.

The disulfide bond in TRX has been proposed as the quenching agent responsible for the low quantum yield and short lifetimes found for the Trp fluorescence of oxidized wild type TRX (Holmgren, 1971; Mérola et al., 1989; Elofsson et al., 1991) and the evidence in favor of that inference is good. Molecular graphic depictions and molecular dynamic simulations (Silva, N. Haydock, C. and Prendergast F.G., unpublished observations) show that the direct collisional interactions between the indole rings of either W28 or W31 with the disulfide bond are not likely, implying that quenching mechanisms operating through space must exist, for example, by electron transfer. The fluorescence of W31 in the single Trp mutant would similarly be influenced by the disulfide bond in the oxidized protein, or by the individual sulfhydryl groups (therefore two potential acceptors) in the reduced protein. We do not have structural evidence for the putative third acceptor.

Throughout this discussion we have implicitly sided with the view that electron transfer may be *a* key, or possibly *the* key mechanism for quenching of the Trp fluorescence. However, it is also apparent that the model holds *conceptually* even if several different quenching mechanisms are operative providing only that the rates of the processes compete with the rate of the fluorescence emission. In fact, it is likely that in some proteins *multiple* mechanisms operate particularly if the Trp side chain is exposed to solvent. We would propose that the heterogeneity of decay arises from the *multiplicity of competing interactions* that result in the appearance of multiexponential fluorescence intensity decays or as nonexponential decays. The latter, in turn, might well be represented as a *distribution of lifetimes* (cf. Alcala et al. 1987). The conceptual significance and attractiveness of our model lies in validation of the multiexponential description of Trp fluorescence intensity decay *and* in the absence of a need to invoke conformational changes to explain the heterogeneity in Trp fluorescence lifetimes. The only critical considerations are the number of "acceptors" and the probabilities of their interaction with the excited state of the fluorophore.

CONCLUDING REMARKS

The single unambiguous conclusion that we can draw is that a variety of models can be applied equally effectively to the analysis of fluorescence intensity decay data. In other words, analysis per se cannot be used to prove a physical model. Currently, assignment of a physical model to a particular Trp fluorescence decay is influenced strongly by investigator bias. This situation is understandable because in general the structural elements in a protein matrix responsible for quenching are seldom identifiable making it difficult to *prove* any physical model. Our uncertainty is exacerbated by the fact that we do not know the precise mechanisms of intraprotein quenching.

As noted earlier there is usually an implicit assumption in the arguments for multiple conformational substates as the basis for multiple distinct or for distributed lifetimes, that quenching of Trp fluorescence arises from highly localized collisional interactions of the indole moiety with quenching groups. The already stated difficulty of identification of the specific quenching groups notwithstanding, this assumption is still problematic. Given apparent structural constraints imposed by protein packing around the Trp side chain in many proteins, the distance of separation between fluorophore and quencher is often so small that the rates of collision between the indole moiety and a putative quenching agent in the microscopic environments of the Trp side chain in such proteins would be exceedingly high, often $\geq 10^{10} s^{-1}$. Under such conditions one would find it difficult to explain why any fluorescence occurs at all, and if fluorescence is present why it is not monoexponential. The model we present here avoids this problem but also suffers from the difficulty of identifying the putative "acceptor" sites. However, it is not casuistic to point out that *all* models of intramolecular quenching of Trp fluorescence in proteins are beset by the difficulty inherent in the identification of the mechanisms responsible.

Regarding our suggestion that some proteins are conformationally so constrained, as to make multiple conformers unlikely, one of the reviewers of the paper pointed out that "protein models for side chains represent a single consensus structure that is derived from consensus-gathering methods (x-ray and NOE) that allow minority conformers to be "swept under the rug" unless they are both heavily populated and very long-lived." We agree, and we also agree on the limitation inherent in molecular dynamic simulations or umbrella-sampling techniques to detect conformers existing "in microsecond to millisecond equilibria." However, we hold that these simulations can provide reasonable insight into the energetic barriers to Trp side chain motion and hence

some indication regarding the likely fractional populations for different states, and barrier crossing rates between or among states (cf. Haydock, 1993). In the absence of data from other experimental techniques, and devoid of insights gleaned from simulations, how much confidence can we have in inferred conformational states?

As to mechanism of quenching of Trp fluorescence in proteins, *we* are frankly biased in our current thinking in favor of electron transfer as the principal (but by no means sole) mechanism of quenching of Trp fluorescence in peptides and proteins, at least insofar as the effects of the protein matrix per se are concerned (cf. Eftink et al., 1989). Additionally, when water is present, other factors, particularly exciplex formation, may contribute substantially, but in many, possibly most instances, water is *not* apparently a major reason for shortened Trp lifetimes. Indeed, several proteins with "long" Trp lifetimes have Trp residues, which from crystallographic data are substantially exposed to water (e.g., W15 of Liver alcohol dehydrogenase (Beechem and Brand, 1985)).

As a final remark we wish to emphasize that the model proposed here is not a panacea. No model is. The strong likelihood is that any one of the three models might be applied for the Trp fluorescence of a particular protein. Our intention is primarily to widen our perspective regarding the heterogeneity in proteins in terms of a simple, theoretically well-defined picture and plausible physical model. By no means does our approach a priori exclude more traditional models that invoke a "conformational" basis for heterogenous intensity decay. We are particularly concerned that wherever possible the interpretation of fluorescence lifetime data be corroborated by information from other experimental techniques; NMR might be the most useful (cf. Ross, 1992). Lastly, we agree with one of the reviewers that, as predicted by Lumry and Hershberger (1978) the effects of temperature may provide some of the most useful insights regarding the effects of the protein matrix on Trp fluorescence intensity decay.

The computer program DONAC for the analysis of fluorescence intensity decay curves in terms of the proposed model is available upon request.

We thank Dr Paul Axelsen for computer graphics depictions and Dr. Ken Nollet for providing single photon counting raw data for fluorescence intensity decay of W28F mutant of thioredoxin. We also thank Mrs. Jill Kappers for assistance in preparing the manuscript and Mr. Ken Peters for preparing the figures.

This work was supported by GM34847 of the PHS. Instrumentation for molecular graphics was provided by funds from the Ahmanson foundation.

REFERENCES

Adman, E. T. 1991. Copper protein structures. *Adv. Protein Chem.* 42: 145–197.

Alcala, J. R., E. Gratton, and F. G. Prendergast. 1987. Interpretation of fluorescence decays in proteins using continuous lifetime distributions. *Biophys. J.* 51:925–936.

Axelsen, P. H., and F. G. Prendergast. 1989. Molecular dynamics of tryptophan in ribonuclease-T1. II. Correlations with fluorescence. *Biophys. J.* 56:43–66.

Bajzer, Ž., J. C. Sharp, S. S. Sedarous, and F. G. Prendergast. 1990. Padè-Laplace method for the analysis of time resolved fluorescence decay curves. *Eur. Biophys. J.* 18:110–115.

Bajzer, Ž., T. M. Therneau, J. C. Sharp, and F. G. Prendergast. 1991. Maximum likelihood method for the analysis of time resolved fluorescence decay curves. *Eur. Biophys. J.* 20:247–262.

Bajzer, Ž., and F. G. Prendergast. 1992. Maximum likelihood analysis of fluorescence data. *Methods Enzymol.* 210:200–237.

Beechem, J. M., and L. Brand. 1985. Time-resolved fluorescence of proteins. *Annu. Rev. Biochem.* 54:43–71.

Blumen, A., and J. Manz, J. 1979. On the concentration and time dependence of the energy transfer to randomly distributed acceptors. *J. Chem. Phys.* 71:4694–4702.

Chen, L. X.-Q., J. W. Longworth, and G. R. Fleming. 1987. Picosecond time resolved fluorescence of ribonuclease T1. pH and substrate analogue binding study. *Biophys. J.* 51:865–873.

Chen, R. F., J. R. Knutson, H. Ziffer, and D. Porter. 1991. Fluorescence of tryptophan dipeptides: Correlations with the rotamer model. *Biochemistry.* 30:5184–5195.

Eftink, M. R., Y.-W. Jia, D. E. Graves, W. Wiczk, I. Gryczynski, J. R. Lakowicz, 1989. Intramolecular fluorescence quenching in covalent acrylamide-indole adductus. *Photochem. Photobiol.* 49:725–729.

Elofsson, A., R. Riegler, L. Nilsson, J. Roslund, G. Krause, A. Holmgren. 1991. Motion of aromatic side chains, picosecond fluorescence, and internal energy transfer in *Eschericia coli* thioredoxin studies by site-directed mutagenesis, time-resolved fluorescence spectroscopy and molecular dynamics simulations. *Biochemistry.* 30:9648–9656.

Gryczynski, I., M. R. Eftink, and J. R. Lakowicz. 1988. Conformation heterogeneity in proteins as an origin of heterogeneous fluorescence decays, illustrated by native and denatured ribonuclease T1. *Biochim. Biophys. Acta.* 954:244–252.

Hansen, J. E., J. W. Longworth, and G. R. Fleming. 1990. Photophysics of metalloazurins. *Biochemistry.* 29:7329–7338.

Haydock, C. 1993. Protein side chain rotational isomerization: A minimum perturbation mapping study. *J. Chem. Phys.* 98:8199–8214.

Hochstrasser, R. M., and D. K. Negus. 1984. Picosecond fluorescence decay of tryptophans in myoglobin. *Proc. Natl. Acad. Sci. (USA)* 81: 4399–4403.

Holmgren, A. 1971. Tryptophan Fluorescence study of conformational transitions of the oxidized and reduced form of thioredoxin. *J. Biol. Chem.* 247:1992–1998.

Hutnik, C. M., and A. G. Szabo. 1989. Confirmation that multiexponential fluorescence decay behavior of holoazurin originates from conformational heterogeneity. *Biochemistry.* 28:3923–3934.

James, D. R., D. R. Demmer, R. P. Steer, and R. E. Verral. 1985. Fluorescence lifetime quenching and anisotropy studies of ribonuclease T1. *Biochemistry.* 24:5517–5526.

James, D. R., Y. Liu, P. DeMayo, and W. R. Ware. 1985. Distributions of fluorescence lifetimes: Consequences for the photophysics of molecules adsorbed on surfaces. *Chem. Phys. Lett.* 120:460–465.

Kemple, M. D., K. E. Nollet, P. Yuan, and F. G. Prendergast. 1993. "Active Site" dynamics: A comparison of the wild type and single tryptophan variants of *E. coli* thioredoxin. *In* Techniques in Protein Chemistry IV. In press.

Klafter, J., and M. F. Shlesinger. 1986. On the relationship among three theories of relaxation in disordered systems. *Proc. Natl. Acad. Sci. USA.* 83:848–851.

Livesey, A. K., and J. C. Brochon. 1987. Analysing the distribution of decay constants in pulse-fluorometry using the maximum entropy method. *Biophys. J.* 52:693–706.

Lumry, R., and M. Hershberger. 1978. Status for indole photochemistry with special reference to biological applications. *Photochem. Photobiol.* 27: 819–840.

MacKerrell, A. D., Jr., R. Rigler, L. Nilsson, U. Hahn, and W. Saenger. 1987. A time-resolved fluorescence, energetic, and molecular dynamics study of ribonuclease-T1. *Biophys. Chem.* 26:247–261.

Mérola, F., R. Riegler, A. Holmgren, and J. C. Brochon. 1989. Picosecond tryptophan fluorescence of thioredoxin: Evidence for discrete species in slow exchange. *Biochemistry.* 28:3383–3398.

O'Connor, D. V., and D. Phillips. 1984. Time-Correlated Single Photon Counting. Academic Press, London. 184–186.

Petrich, J. W., J. W. Longworth, and G. R. Fleming. 1987. Internal motion and electron transfer in proteins: A picosecond fluorescence study of three homologous azurins. *Biochemistry*. 26:2711–2722.

Prendergast, F. G., Ž. Bajzer, P. H. Axelsen, and C. Haydock. 1991. Analysis and interpretation of tryptophan fluorescence intensity decays in proteins. *In* Proteins, Structure, Dynamics and Design. V. Renugopalakrishnan, P. R. Carey, I. C. P. Smith, S. G. Huang, and S. C. Storer, editors. Escom, Leiden. 208–219.

Press, W. H., B. P. Flannery, S. A. Teukolsky, and W. T. Vetterling. 1986. Numerical Recipes. Cambridge University Press, Cambridge.

Ross, J. B. A., H. R. Wyssbrod, R. A. Porter, G. P. Schwartz, C. A. Michaels, W. R. Laws. 1992. Correlation of tryptophan fluorescence intensity decay parameters with 1H NMR-determined rotamer conformations: [tryptophan2[-oxytocin. *Biochemistry*. 31:1585–1594.

Siemiarczuk, A., B. C. Wagner, and W. R. Ware. 1990. Comparison of the maximum entropy and exponential series methods for the recovery of distributions of lifetimes from fluorescence lifetime data. *Phys. Chem.* 94:1661–1666.

Sienicki, K., S. Blonski, and G. Durocher. 1991. Relaxation distributions of fluorescence decay. *J. Phys. Chem.* 95:1576–1579.

Sweeney, J. A., P. A. Harmon, S. A. Asher, C. M Hutnik, and A. G. Szabo. 1991. UV resonance Raman examination of the azurin tryptophan environment and energy relaxation pathways. *J. Am. Chem. Soc.* 113: 7531–7537.

Szabo, A. G., and D. M. Rayner. 1980. Fluorescence decay of tryptophan conformers in aqueous solutions. *J. Am. Chem. Soc.* 102:554–563.

Szabo, A. G., T. M. Stepanik, D. M. Wayner, and N. M. Young. 1983. Conformational heterogeneity of the copper binding site in azurin. A time-resolved fluorescence study. *Biophys. J.* 41:233–244.

Vincent, M., J. C. Brochon, F. Merola, W. Jordi, and J. Gallay. 1988. Nanosecond dynamics of horse heart apocytochrome *c* in aequeous solution as studied by time-resolved fluorescence of single tryptophan residue (Trp-59). *Biochemistry*. 27:8752–8761.

Weaver, A. J., M. D. Kemple, and F. G. Prendergast. 1988. Tryptophan sidechain dynamics in hydrophobic oligopeptides determined by use of ^{13}C nuclear magnetic resonance spectroscopy. *Biophys. J.* 54:1–15.