On the involvement of electron transfer reactions in the fluorescence decay kinetics heterogeneity of proteins

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Abstract

Time-resolved fluorescence study of single tryptophan-containing proteins, nuclease, ribonuclease T1, protein G, glucagon, and mastoparan, has been carried out. Three different methods were used for the analysis of fluorescence decays: the iterative reconvolution method, as reviewed and developed in our laboratory, the maximum entropy method, and the recent method that we called “energy transfer” method. All the proteins show heterogeneous fluorescence kinetics (multiexponential decay). The origin of this heterogeneity is interpreted in terms of current theories of electron transfer process, which treat the electron transfer process as a radiationless transition. The theoretical electron transfer rate was calculated assuming the peptide bond carbonyl as the acceptor site. The good agreement between experimental and theoretical electron-transfer rates leads us to suggest that the electron-transfer process is the principal quenching mechanism of Trp fluorescence in proteins, resulting in heterogeneous fluorescence kinetics. Furthermore, the origin of apparent homogeneous fluorescence kinetics (monoexponential decay) in some proteins also can be explained on the basis of electron-transfer mechanism.

Keywords: Tryptophan; photophysics; time-resolved fluorescence; electron transfer

Comprehension of the structure–function relationship in proteins is critical for a detailed understanding of the molecular mechanisms of their biological activity. For this purpose, tryptophan fluorescence has been used widely to obtain information about the structure and dynamics of proteins in solution. This largely arises from the high sensitivity of tryptophan to the physical and chemical nature of its local environment (Creed 1984; Eftink 1991). Unfortunately, its complex photophysics has made this task challenging (Beechem and Brand 1985). For example, the association of the fluorescence decay parameters to structural and dynamic features of proteins is still controversial.

Single tryptophan-containing proteins have been found in many cases to show multiexponential fluorescence decay (heterogeneous fluorescence kinetics) (Beechem and Brand 1991; Bombarda et al. 1999; Tanaka and Mataga 1992). The origin of the complex fluorescence kinetics of Trp in proteins was a subject of extensive debate during the last two decades. The most interesting interpretations are the rotamer model (Szabo and Rayner 1980; Petrich et al. 1983; Kim et al. 1993; Dahms and Szabo 1997) and the recently proposed energy transfer model (Bajzer and Prendergast 1993). Nevertheless, some points remain unclear. One major problem with the rotamer model is the absence of a well-specified mechanism for fluorescence quenching. On the other hand, the energy-transfer model confronts a crucial problem of acceptors. The latter model is based on the occurrence of transfer of the excited-state energy from the fluorophore to acceptors located elsewhere in the protein matrix. In the case of tryptophane, no energy acceptor could

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be identified. Nevertheless, Bajzer and Prendergast (1993) report that the term “energy transfer” is used in a totally generic sense, claiming that all quenching processes of the excited state require a transfer of energy.

Thus, a detailed and consensual interpretation of fluorescence kinetics in proteins appears an urgent task. In this work, we have carried out a time-resolved study of Trp fluorescence emission on five proteins taken as model systems, mimicking the most frequently encountered features of Trp environment. The five proteins are nuclease (Nucl), ribonuclease T1 (RNT1), protein G (ProtG), glucagon (Gluc), and mastoparan (Mastp). The fluorescence decays were analyzed by three different methods: the iterative reconvolution method (IRM) (Vix and Lami 1995), the maximum entropy method (MEM) (Livesey and Brochon 1987), and the energy transfer method (ETM) (Bajzer and Prendergast 1993). With this study, we try to contribute to the interpretation of the fluorescence kinetics heterogeneity in proteins in terms of electron-transfer process. Moreover, we attempt to show that electron-transfer process also could explain the existence of apparent homogeneous fluorescence kinetics in some proteins, such as ribonuclease T1.

Results

Protein structure

To investigate fluorescence kinetics of Trp residues, we have chosen five proteins where Trp displays very different

degrees of exposure to the solvent. In Nucl, the side chain of Trp-140 residue lies near the surface with a solvent-accessible surface area (SASA) of 73 Å². In RNT1, the Trp-59 is highly shielded from the aqueous phase with SASA of 1 Å², while Trp-48 in ProtG is in the interior pocket of the protein with SASA of 67 Å². As comparison, the SASA of the totally exposed Trp residues in Gluc and Mastp are 218 Å² and 241 Å², respectively. While all protein structures are known, the structure of Mastp, which is totally unstructured in aqueous solution at neutral pH (McDowell et al. 1985), was modeled as random structure.

Fluorescence kinetics

The analysis of the fluorescence decays of the five single-Trp proteins was carried out using three methods, IRM, MEM, and ETM. All the protein models indicate heterogeneous fluorescence kinetics (Table 1). With IRM analysis, all proteins display biexponential decays. Using ETM, the best fit is obtained with one-acceptor model (equation 2) in all cases. In contrast, some differences among the proteins are observed when using MEM analysis. Indeed, a bimodal distribution was obtained for RNT1, ProtG, and Mastp, while a trimodal distribution was found in the case of Nucl and Gluc. When comparing these results, we found an excellent agreement between the longest lifetime recovered by MEM and IRM and the specific lifetime τ recovered by ETM. Additionally, the preexponential terms α_i recovered by IRM are consistent with the probabilities p_i recovered by ETM.

Table 1. Fluorescence decay parameters of proteins models recovered by MEM, IRM, and ETM

Protein $\lambda_{em}(nm)$	MEM			IRM			ETM		
	τ_i (ns)	α (τ_i)	χ_r^2	τ_i (ns)	α_i	$\chi_r^2 CF$	w_1 (ns ⁻¹) τ (ns)	$p_1 P_0$	χ_r^2
Nucl 340	0.99 ± 0.09	0.16 ± 0.01	1.28	1.41 ± 0.19	0.29 ± 0.01	0.99–1.10 1.65	0.32 ± 0.01	0.36 ± 0.01	1.38
	2.94 ± 0.23	0.27 ± 0.01		5.39 ± 0.08	0.71 ± 0.01		5.73 ± 0.03	0.64 ± 0.01	
	5.78 ± 0.06	0.57 ± 0.02							
RNT1 335	0.83 ± 0.09	0.16 ± 0.03	1.19	0.86 ± 0.19	0.18 ± 0.01	0.98–1.01 1.50	0.53 ± 0.05	0.19 ± 0.01	1.36
	3.86 ± 0.13	0.84 ± 0.03		3.82 ± 0.04	0.82 ± 0.01		3.89 ± 0.02	0.81 ± 0.01	
ProtG 340	1.51 ± 0.16	0.10 ± 0.01	1.41	1.90 ± 0.67	0.12 ± 0.01	0.99–1.01 1.54	0.24 ± 0.05	0.12 ± 0.02	1.50
	6.11 ± 0.02	0.90 ± 0.01		6.19 ± 0.08	0.88 ± 0.01		6.24 ± 0.04	0.88 ± 0.02	
Gluc 350	0.90 ± 0.05	0.31 ± 0.02	1.25	1.13 ± 0.05	0.47 ± 0.01	0.99–1.05 1.82	0.44 ± 0.01	0.58 ± 0.01	1.44
	2.41 ± 0.15	0.41 ± 0.03		3.73 ± 0.04	0.53 ± 0.01		4.11 ± 0.02	0.42 ± 0.01	
	4.32 ± 0.14	0.28 ± 0.03							
Mastp 350	0.97 ± 0.06	0.25 ± 0.01	1.48	1.23 ± 0.05	0.33 ± 0.01	0.99–1.05 1.68	0.42 ± 0.02	0.37 ± 0.01	1.42
	3.35 ± 0.04	0.75 ± 0.01		3.56 ± 0.02	0.67 ± 0.01		3.64 ± 0.02	0.63 ± 0.01	

λ_{em} : the emission wavelengths. The errors with IRM are given as $\pm$ confidence intervals at the 5% level of significance, and with ETM are $\pm$ SD estimated by Monte Carlo simulations. For more details, see text in Materials and Methods.

ETM, energy transfer method; IRM, iterative reconvolution method; MEM, maximum entropy method.

Our results (Table 1) can be satisfactorily compared with those reported by other groups, taking into account the differences in experimental conditions and the data-acquisition techniques. For example, with IRM, Eftink et al. (1989) found $\tau_1 = 2.79$ ns, $\tau_2 = 6.09$ ns, and $\alpha_1 = 0.31$ for Nucl at pH 7.0, and Chen et al. (1987) reported $\tau_1 = 1.6$ ns, $\tau_2 = 3.95$ ns, and $\alpha_1 = 0.1$ for RNT1 at pH 7.0. In the case of ProtG, it was reported that $\tau_1 = 0.44$ ns, $\tau_2 = 5.9$ ns, and $\alpha_1 = 0.18$ (Tcherkasskaya et al. 2000), nevertheless only a qualitative comparison is possible, as pH was 5.7 and their protein was truncated for the first five residues. For Gluc, at pH 8.2, $\tau_1 = 1.10$ ns, $\tau_2 = 3.26$ ns, and $\alpha_1 = 0.42$ were found (Cockle and Szabo 1981). Additionally, for Mastp at pH 7.0, it has been reported the average lifetime = 3.3 ns (McDowell et al. 1985) that corresponds to 2.8 ns in our case. Furthermore, Swaminathan et al. (1994), using MEM analysis, reported $\tau_1 = 0.49$ ns, $\tau_2 = 3.11$ ns and $\alpha_1 = 0.16$ for RNT1 at pH 7.0, and $\tau_1 = 0.53$ ns, $\tau_2 = 1.99$ ns, $\tau_3 = 3.19$ ns, $\alpha_1 = 0.28$, and $\alpha_2 = 0.35$ for Gluc at pH 7.0. Finally, with ETM for RNT1 at pH 7.5, $w_1 = 0.33$ ns⁻¹, $\tau = 3.69$ ns and $p_1 = 0.15$ has been reported (Bajzer and Prendergast 1993).

To get further insight into the fluorescence deexcitation pathways, the fluorescence decays were studied over 10–45°C temperature range. The theoretical temperature dependence of the fluorescence lifetime splits the nonradiative rate (k_{nr}) into temperature-independent and temperature-dependent contributions that can be expressed as follows:

$$1/\tau_i = k_r + k_{nr} = k_r + k_{nr}^o + k_{nr}(T) = k_o + A_o \exp(-E_a/RT) \quad (1)$$

k_o is the total temperature-independent decay rate and presumably $k_o = k_r + k_{ic} + k_{isc}$, where k_r is the radiative rate, k_{ic} is the internal conversion rate, and k_{isc} is the intersystem crossing rate. A_o is the frequency factor and E_a is the activation energy, which characterizes the principal dynamic quenching process. We are principally interested in the short component of the fluorescence decay, as its appearance is clearly related to specific nonradiative deexcitation pathway(s). Nonlinear least-squares analysis of $1/\tau_1$ as a function of $1/T$ (Fig. 1) was performed using the program ORIGIN (Microcal Software, Inc.), and the recovered parameters are gathered in Table 2. In the case of RNT1, the short component shows a very low A_o and $E_a \leq 0$. This suggests that the nonradiative process involved in the deexcitation pathway leading to this short component is energy barrierless, or that this component is probably temperature-independent. In the other proteins, the short component shows A_o ranging from $1.35 \cdot 10^{12}$ to $5.58 \cdot 10^{13} \text{ s}^{-1}$ and E_a from 5.23 to 7.01 kcal/mol. These values are consistent with electron-transfer process as the main quenching mechanism in the short component for these proteins (Petric et al. 1983; Ricci and Nesta 1976; Chen et al. 1996).

Fig. 1. Temperature dependence of the short lifetime recovered by maximum entropy method (solid square) and iterative reconvolution method (open square). The curves through the points are the least-squares fits analyzed as $\text{Ln}(1/\tau_1)$ vs $1/T$.

Discussion

The heterogeneity of fluorescence kinetics in single Trp-containing proteins translates the existence of interactions between the excited indole and its environment. However, it is unclear how this heterogeneity should be interpreted in terms of a physical model. Usually, the heterogeneous fluorescence kinetics of proteins has been attributed to the existence of multiple ground state conformers of Trp residue (Szabo and Rayner 1980; Petrich et al. 1983). The existence of stable Trp rotamers has been observed by NMR (Dezube et al. 1981) and by molecular supersonic jet (Sipior et al.

Table 2. Arrhenius parameters of the nonradiative decay rate of the short component in protein models

	ko (s ⁻¹) ±Δko	Ao (s ⁻¹) ±ΔAo	Ea (kcal/mol) ±ΔEa	ko (s ⁻¹) ±Δko	Ao (s ⁻¹) ±ΔAo	Ea (kcal/mol) ±ΔEa
	MEM			IRM		
Nucl						
τ ₁	9.02E + 08 3.99E + 06	1.00E + 12 3.96E + 06	4.99 0.07	5.66E + 08 2.83E + 06	1.00E + 12 2.79E + 06	5.06 0.04
RNT1						
τ ₁	2.73E + 08 3.48E + 06	3.88E + 08 3.60E + 06	-0.54 0.02	2.27E + 08 6.57E + 06	2.11E + 08 7.68E + 06	-0.85 0.04
ProtG						
τ ₁	3.75E + 08 2.19E + 06	1.00E + 12 2.14E + 06	5.01 0.02	4.93E + 08 1.76E + 06	1.60E + 13 1.72E + 06	6.80 0.08
Gluc						
τ ₁	7.84E + 08 3.36E + 06	1.00E + 12 3.34E + 06	4.62 0.04	6.27E + 08 6.44E + 05	1.00E + 13 6.38E + 05	6.14 0.01
Mastp						
τ ₁	7.44E + 08 2.75E = 6	1.00E + 14 2.73E + 06	7.63 0.04	4.45E + 08 3.44E + 05	100E + 12 3.12E + 05	4.65 0.003

IRM, iterative reconvolution method; MEM, maximum entropy method.

1987; Sulkes and Arnold 1992). However, only in few cases has the correlation of the observed rotamers and fluorescence lifetimes been possible (Haydock et al. 1990; Ross et al. 1992; Willis and Szabo 1992).

In RNT1, Trp-59 is buried in the protein matrix in a tightly packed pocket, and the analysis of its 3D structure shows a B factor of indole atoms ranging from 5.5 to 8.3 Å², except for the carbon C_{ε3} and C_{η2} whose B factors are 10.3 and 11.0 Å², respectively. This means that a high frequency of a very-low-amplitude motion is plausible. Furthermore, our molecular mechanics study of Trp-59 reveals the existence of quasi-one rotamer, which corroborates the prediction from 3D structure (Ababou 1998). These structural features are against multiple Trp conformations, nevertheless RNT1 shows a heterogeneous fluorescence kinetics (see Table 1 and Chen et al. 1987). Consequently, it is difficult to interpret this heterogeneity in terms of rotamer model. Furthermore, it was reported that the variant lysozyme T4-W138 shows heterogeneous fluorescence kinetics (Harris and Hudson 1990), while molecular modeling studies show a rotamer conversion to the observed geometry in a few picoseconds (Harris and Hudson 1991). Again, the rotamer model cannot apply; indeed, this model requires that the individual rotamers interconvert slowly on the fluorescence time scale. On the other hand, for peptides, the rotamer model seems plausible because of low energy barriers that could exist between different rotameric states. However, without experimental proof on the existence of populated rotamers, the question on how to relate the extent of fluorescence kinetics heterogeneity to the existence of rotamers remains unanswered (Ababou 1998).

Bajzer and Prendergast (1993) proposed an alternative model to describe the fluorescence kinetics heterogeneity of Trp residue in proteins, without invoking multiple protein conformations. This model, originally proposed in solid state, was based on the occurrence of transfer of the excited-state energy from a donor to randomly distributed acceptors (Blumen and Manz 1979). In solution, this model was extrapolated to an intramolecular interaction between a fluorophore as donor and acceptors located elsewhere in the protein matrix. The idea is very attractive, but in the case of Trp residue as the donor, no indications for energy acceptors have been found. Undoubtedly, this is the weakness of the model. Nevertheless, the authors elegantly defended possible interpretations in terms of this model. The most plausible interpretation can be an electron-transfer process between Trp and acceptors that, in this case, exist and are relatively known. Obviously, energy and electron-transfer theories are different and complex but there are some analogies in their transfer rates (Hopfield 1974).

The possible fluorescence-quenching mechanisms of Trp in proteins are reported. Internal conversion is apparently small and intersystem crossing seems to be insensitive to protein functional groups (Chen et al. 1996). Photoionization, which might depend on the environment (Bent and Hayon 1975), reduces the fluorescence quantum yield, but has no effect on the fluorescence lifetime (Mialocq et al. 1982). Exciplex formation was suggested to occur in proteins with exposed Trp residues (Lumry and Herschberger 1978). We have not found in the literature convincing experimental evidence about exciplexes formation in proteins, although there are hypotheses (Sopokova et al. 1994). Water

quenching may marginally affect the Trp lifetime in the presence of an efficient quenching process (Chen et al. 1996). The excited-state proton transfer occurs from a strong proton donor to an adjacent aromatic carbon of the indole moiety (Yu et al. 1992; Chen et al. 1995), but it is difficult to prove its occurrence in proteins except for N-terminal tryptophans. Finally, the excited-state electron-transfer process has been proposed as the principal mechanism of quenching of indole fluorescence in peptides and proteins (Petrich et al. 1983; Eftink et al. 1989; Ricci and Nesta 1976; Chen et al. 1996; Chen and Barkley 1998). However, a detailed understanding of this fluorescence-quenching mechanism in proteins still is not fulfilled, for instance, no acceptor in electron-transfer reaction are clearly specified and no explicit use of electron-transfer theory was made to calculate the rate constant of this mechanism.

Accordingly, to contribute to the understanding of Trp photophysics in proteins, we assume that the electron transfer (ET) process plays an important role in the origin of fluorescence kinetics heterogeneity in proteins. Indeed, the energetics and dynamics of ET are shown to depend on the structure of the reactants (donor and acceptor), the distance separating the reactants, their relative orientations, the nature and polarity of the medium, and the coulombic effects (Marcus and Sutin 1985). We consider that an excited state of Trp residue might be subjected to all known deactivation channels: fluorescence, nonradiative processes in general, and among these, ET. To simplify our strategy in this work, we consider for all proteins a short component with lifetime τ_S and a long component with lifetime τ_L . Because there are ET reactions that compete efficiently with fluorescence, we suggest that in the short component the fluorescence is accompanied by an efficient ET process, which does not occur in the long component.

$$1/\tau_S = k_r + k_{nr}' + k_{ET} \quad (2)$$

$$1/\tau_L = k_r + k_{nr}' \quad (3)$$

where k_r is the radiative rate (fluorescence), k_{nr}' is the total rate for nonradiative processes, except ET, occurring in τ_S , and k_{ET} is the ET rate merely given by (Oevering et al. 1987; Eftink et al. 1989):

$$k_{ET} = 1/\tau_S - 1/\tau_L \quad (4)$$

For clarity, we have used the equations (2) and (3) to calculate k_{ET} with the hypothesis that ET is a dominant non-radiative process in the short lifetime. Our aim is to verify that the appearance of short components in fluorescence decay of proteins is essentially the result of ET process. However, because of other ET reactions that could occur in both lifetimes (for example an ET event to the solvent), we include them in k_{nr}' . In other words, k_{nr}' could be written as

a sum of nonradiative processes including other ET reactions, which are less competing with fluorescence, and may occur in both lifetimes.

Moreover, k_{ET} can be calculated by subtracting the non-radiative rate of an indole analog with nonquenching side chain, such as 3-methylindole (3MI), from the nonradiative rate k_{nr} of the short component (Petrich et al. 1983; Eftink et al. 1995)

$$k_{ET} = k_{nr} - k_{nr}^{3MI} \quad (5)$$

The equations (4) and (5) will be used for IRM and MEM, while in the case of ETM, we assume that k_{ET} corresponds directly to the recovered w_1 rate ($k_{ET} = w_1$).

To verify our assumption, comparison of the experimental rate k_{ET} with theoretical rate is required. The rate of nonadiabatic ET reactions is well established (Bixon and Jortner 1982; Marcus and Sutin 1985) and the theory predicts

$$k_{ET} = 2\pi/\hbar |H_{DA}|^2 / (4\pi \lambda k_B T)^{1/2} [\exp(-(\Delta G^\circ + \lambda)^2 / 4\pi \lambda k_B T)] \quad (6)$$

where H_{DA} is the electronic coupling matrix element, k_B is the Boltzmann constant, and T is the temperature. λ is the total reorganization energy and $\lambda = \lambda_v + \lambda_s$ where λ_v is the reorganization energy as a result of vibrations within the reactants and λ_s is the reorganization energy within the surrounding solvent molecules. ΔG° is the free energy change of the ET reaction and can be given by the Rhem-Weller equation (Rehm and Weller 1970)

$$\Delta G^\circ = E_{ox}(D/D^+) - E_{red}(A^-/A) - E_{oo}(D) + E^*(P) - e^2/\epsilon_s r \quad (7)$$

where $E_{ox}(D/D^+)$ and $E_{red}(A^-/A)$ are the oxidation potential of the donor and the reduction potential of the acceptor respectively. $E_{oo}(D)$ is the energy of the zero-zero transition to the lowest excited singlet state of the donor. $E^*(P)$ represents the energy of an excited state product and takes into account that the product of the ET reaction may be an excited product (Petrich et al. 1987; Suppan 1992). The last term in eq 7 is a correction for the coulomb energy changes associated with charge separation, but it was omitted in our calculation because its contribution is expected to be small (Miller et al. 1982). λ_v and λ_s in eq 6 can be estimated as follows (Marcus and Sutin 1985; Mataga and Miyasaka 1994):

$$\lambda_v = \sum_j [f_j^r f_j^p / (f_j^r + f_j^p)] \Delta q_j^2 \quad (8)$$

$$\lambda_s = e^2 (1/2r_D + 1/2r_A - 1/R) (1/\epsilon_{op} - 1/\epsilon) \quad (9)$$

where f_j^r and f_j^p are the j^{th} normal mode force constants in the reactants and in the products respectively. Δq_j is the change in the equilibrium value of the normal coordinate j^{th} , e is the charge transferred from one reactant to the other, and r_A and r_D are the radii of the spherical approximation of the two reactants. R is the center-to-center separation distance between reactants, and ϵ and ϵ_{op} are the static and optical dielectric constants of the medium, respectively.

Because H_{DA} is an unknown parameter, eq 6 is inapplicable to determine k_{ET} and thus it is necessary to find a strategy to allow the comparison between experimental and theoretical k_{ET} . Determination of H_{DA} is not the subject of this paper despite its crucial importance in the comprehension of an ET reaction (Stuchebrukhov 1996; Gehlen et al. 1996). However, in homogeneous barrier (Hopfield 1974; Redi and Hopfield 1980) and superexchange models (Miller and Beitz 1981), H_{DA} is well known to depend exponentially on donor-acceptor distance R ($H_{\text{DA}} \propto \exp(-\alpha R)$) (Langen et al. 1995). Hence, to avoid the appearance of H_{DA} , we calculate the ratio of $k_{\text{ET}}(T)$ at two different temperatures T_1 and T_2 :

$$k_{\text{ET}}(T_2)/k_{\text{ET}}(T_1) = (T_1/T_2)^{1/2} \exp[-(\Delta G^\circ + \lambda)^2/4\pi\lambda k_B (1/T_2 - 1/T_1)] \quad (10)$$

The temperatures were $T_1 = 10^\circ\text{C}$ and $T_2 = 40^\circ\text{C}$.

To specify the acceptor in the photoinduced ET reaction where Trp is the donor, it is worth noting that different potential quenchers of Trp fluorescence exist. Indeed, peptide-bond carbonyl groups, histidine and cysteine side chains, as well as disulfide bridges, have been reported as potential electron acceptors (Chen et al. 1996; Chen 1990; Williaert and Engelborghs 1991; Chen and Barkley 1998). However, to avoid or at least to minimize interferences between different acceptors, which would complicate our calculations and interpretations, we have chosen our protein models with histidines and cysteine groups being far from Trp (the shortest distance found was 10.2 Å with His40 in RNT1, and for all proteins, His and Cys or Cys-Cys were between 11.5 and 43.5 Å), or simply absent as in the case of ProtG. Hence, in this work we will consider only the peptide-bond carbonyl group as acceptor.

To estimate λ and ΔG° , we have proceeded as follows: R was evaluated from the 3D structure of the proteins (Table 3), r_D for the indole, and r_A for the backbone carbonyl group were evaluated to be 4 Å and 1.1 Å, respectively. ϵ is 78 for Gluc and Mastp, because the Trp residue is totally exposed. The estimation of the dielectric constant in the remaining proteins was based on recent studies, which infer that the value of ϵ in the core of a protein is higher than usually considered (Schaefer et al. 1997; Garcia-Moreno et al. 1997). The protein interior should be rich in strong dipoles,

Table 3. Measured and calculated parameters for theoretical ET rate

Protein	Residue	R (Å) ^a	E_∞ (D) (eV) ^b	λ (eV) ^c	ΔG° (eV) ^d
Nucl	Ile-139	4.832	4.081	0.574	-0.271
RNT1	Pro-60	4.522	4.167	0.543	-0.357
ProtG	Thr-58	4.989	4.072	0.580	-0.262
Gluc	Trp-25	4.275	4.063	0.561	-0.253
Mastp	Val-1	5.329	4.061	0.607	-0.251

^a Distances are measured from geometric center to center of the indole moiety and C=O bond.

^b Zero-to-zero excited state energy.

^c Total reorganizational energy.

^d Free energy change of ET reaction (see text in Discussion).

as N-methylformamide, which is taken as the solvent analog of the peptide groups and has a very high dielectric constant, $\epsilon = 186$ (Richardi et al. 1997). The calculated dielectric constant can be very low if only electronic polarization is taken into account, while quite different are the values when the nuclear motions are accounted for. Indeed, the dielectric dispersion curves in proteins, derived from molecular dynamics simulations, demonstrate a behavior that is very analogous to highly viscous polar liquid resulting in very small dielectric response on a very fast time scale and strong response with effective dielectric constant as large as 30–40 at longer times (Smith et al. 1993). We have verified the effect of ϵ values on the calculation of the theoretical ratio with equation 10. Figure 2A shows that the theoretical ratio varies between $\epsilon = 4$ and $\epsilon = 8$, while for $\epsilon > 8$, no significant changes are observed. Consequently, we assume $\epsilon = 20$ for Nucl and ProtG and $\epsilon = 10$ for RNT1, according to the different degree of solvent exposure of the Trp residue in these proteins. Additionally, we assumed $\epsilon(T_1) = \epsilon(T_2)$ because the temperature range is narrow. The optical dielectric constant ϵ_{op} of the medium is fixed to unity. λ_v is attributed principally to the vibrational energy of the backbone carbonyl groups (the acceptor) and estimated to be 0.22 eV.

The values of $E_{\text{oo}}(\text{D})$ have been determined from the absorption and fluorescence emission spectra of proteins (Lakowicz 1983) and gathered in Table 3. $E^*(\text{P})$ is obtained from the absorption spectra of Trp radical cation and was 2.21 eV (Bent and Hayon 1975), while $E_{\text{ox}}(\text{D}/\text{D}^+)$ is 1.1 V (DeFelippis et al. 1989). Because of the lack of reported redox potentials for amide compounds to model peptide linkage, the value of $E_{\text{red}}(\text{A}^-/\text{A})$ was approximated to be -0.5 V according to previous studies (Bent and Hayon 1975; Rao and Hayon 1974a,b; Amouyal et al. 1982). These works reported that the backbone carbonyl group is an acceptor of electrons with low redox potential, and most of them exhibit negative redox potentials. The estimations of the total reorganization energy, the free energy change of ET reaction,

Fig. 2. Variation of theoretical ratio of k_{ET} (A) as function of dielectric constant of proteins models, N, Nucl; R, RNT1; P, ProtG; G, Gluc; and M, Mastp; where in the case of Gluc and Mastp, the ϵ was fixed to $\epsilon = 78$. (B) as function of distance R between donor and acceptor. R_structure represents the shortest distance calculated from the protein structure.

the energy of the lowest singlet excited state of donor, and the separating distance between reactants are given in Table 3.

Because the backbone carbonyl groups are distributed all around Trp at different distances in the protein matrix, we have tested the distance donor-acceptor (R) effect on the theoretical ratio. Figure 2B shows the plot of the theoretical ratio for each protein at the shortest distance calculated from the structure (R_structure) and using R equal to an intermediate distance of 8 Å and to a long range distance of 20 Å. The result shows that the theoretical ratio does not change dramatically with the distance, especially in the 8–20 Å range. ET process depends strongly on the distance between donor and acceptors and this dependence is contained in H_{DA} (Closs and Miller 1988; Langen et al. 1995; Miller 1987). Consequently, in our case, the ratio of two k_{ET} at two different temperatures is expected not to change dramatically, as H_{AD} is absent. Furthermore, in our case, (i) the R value will affect only slightly the λ_s and no other parameter in the theoretical ratio, and (ii) we assume a unique value of

$E_{red}(A^-/A)$ for all the peptide-bond carbonyls, and hence ΔG° was constant at any R value. However, as we will discuss below, the acceptor affinity for electrons may be affected by its local environment and especially by residue side-chain effect, as a result of the polarization and charge flow between residues. This result suggests that the ratio masks the real R effect as it is expected for k_{ET} , which contain H_{DA} . Hence, we will use in our comparison only those peptide-bond carbonyls at $R = R_{structure}$.

The experimental ratio was obtained using the ratio of the rates calculated by equation 4 or 5 using the lifetimes recovered by IRM and MEM analysis. While in the case of ETM, the ratio is calculated directly using the recovered w_1 rates as function of temperature.

Figure 3 shows the comparison between the experimental and the theoretical ratios of $k_{ET}(T)$ at two different temperatures ($T_1 = 10^\circ\text{C}$ and $T_2 = 40^\circ\text{C}$). In Figure 3A, the

Fig. 3. Plot of the experimental (with maximum entropy method (MEM), iterative reconvolution method [IRM], and energy transfer method [ETM]) and theoretical ratio (electron transfer [ET] theory) of k_{ET} at $T_1 = 10^\circ\text{C}$ and $T_2 = 40^\circ\text{C}$ as function of the protein size. N, Nucl; R, RNT1; P, ProtG; G, Gluc; and M, Mastp. Using for MEM and IRM (A) equation 4, and (B) equation 5. ETM and ET theory ratio are the same in both panels (A) and (B). The error calculations of the experimental ratio were done according to the incertitude propagation law (Taylor 1982).

comparison reveals a good agreement in three cases (Nucl-RNT1-Mastp) with MEM, in four cases (Nucl-RNT1-Gluc-Mast) with IRM, and in three cases (RNT1-Gluc-Mast) with ETM. Comparison of the shape of the curves given by connecting points shows how good the global agreement is. While in Figure 3A a global good agreement is present for all proteins, in Figure 3B a significant agreement between experiment and theory is still observed, except for Gluc and Mastp with MEM and IRM analysis. This can be attributed to the weakness of Arrhenius' model in case of flexible structures, such as Gluc and Mastp. Interestingly, it is worth noting that in the case of ETM, the substantial agreement between theoretical and experimental ratio indicates that w_1 can be associated to ET rate. This is in line with the possible ET interpretation of w_i 's rates (Bajzer and Prendergast 1993). The global agreement between the theoretical and experimental ratios in Figure 3A and at least for Nucl, RNT1, and ProtG in Figure 3B show that the experimental data are consistent with our prediction. This result emphasizes the importance of the ET process as the principal fluorescence-quenching mechanism leading to fluorescence kinetics heterogeneity in proteins.

In our approach, we consider the backbone carbonyl group as the acceptor in the ET reaction, which corroborates earlier inferences and thus our assumption. However, some questions need to be addressed. (i) Why is the most probable acceptor the peptide bond carbonyl group? (ii) What could be the role of the peptide bond carbonyl in the nature of the ET reaction (i.e., one-step or multi-step)? (iii) If the peptide bond carbonyl is the acceptor, why do proteins show different fluorescence kinetics heterogeneity? (iv) Why do some proteins show homogenous fluorescence kinetics?

Why peptide bond carbonyl is the most probable acceptor?

Quantum mechanics calculations suggest the appearance of a net-positive charge on the peptide bond carbonyl carbon, and hence the availability of this carbon to accept an electron (Milner-White 1997). The carbonyl group often was indicated as a fluorescence quencher in proteins, and recently it was reported that the peptide bond quenches indole fluorescence (Chen et al. 1996). Because of involvement of ET reactions in many biochemical processes, a number of studies were performed to get further insight into these reactions in proteins, which often involve distant donors and acceptors (Gray and Malmström 1989; Farver et al. 1996a,b). Because of the importance of the structure of the intervening protein medium in electron tunneling, Onuchic and Beratan (1990) have developed a tunneling-pathway model (Beratan et al. 1987). The pathway model was used successfully to study ET in proteins where the backbone path often is involved directly in electron transport (and/or hole transport) (Onuchic and Beratan 1990; Beratan et al.

1992). This indicates that the peptide bond carbonyl is at least involved as an acceptor-mediator to ET between redox sites in proteins.

In a recent ET study of azurin, the H_{DA} calculations show the pathway through peptide backbone to be 10-fold more probable than a through-space jump pathway (Farver et al. 1996a). This result shows the direct involvement of the peptide bond carbonyl in electron transport between redox sites in a protein. Beratan and coworkers (1991) reported that the secondary structure and its tertiary arrangement define the distance dependence of electronic coupling in a protein. Furthermore, it has been shown that long-distance ET in β -sheet protein is expected to be more efficient than in α -helical protein (Langen et al. 1995). Peptide bond carbonyl is directly involved in formation of secondary structures, hence if differences exist between these secondary structures in the context of ET reaction, the peptide bond carbonyl should play an important role.

Thus, the peptide bond carbonyl is a good candidate to be an acceptor/mediator in ET reactions in proteins in a structure-dependent way (Langen et al. 1995; Onuchic and Beratan 1990; Evenson and Karplus 1993; Siddarth and Marcus 1993), as it is directly involved along the peptide backbone and in secondary structure formation.

What could be the role of peptide bond carbonyl in the nature of the electron transfer reaction?

As the donor is the indole moiety and the acceptor is the peptide bond carbonyl, one has to wonder if the peptide bond carbonyl of the Trp residue has a particular role, implying a one-step reaction. In a model system with two acceptors, the characteristic time of two-step ET was reported to be far shorter than one-step ET, even in a molecule with a shorter bridge (Mataga et al. 1984). These data agree with theoretical estimates according to which multistep process of ET along the gradient of the redox potential of the acceptors ensures a higher efficiency of charge separation than a one-step process (Nishitani et al. 1983). Evidence for multistep ET in biological systems is beginning to emerge (Iverson et al. 1998; Jortner et al. 1998). Hence, the peptide bond carbonyl in proteins should play a crucial role in photoinduced ET from Trp, by favoring multistep ET reaction. Recent studies show that the amino acid side-chain may play a role in affecting the electrophilicity of the peptide bond carbonyl (Gehelen et al. 1996; Stuchebrukhov 1996). Furthermore, partial charges calculation of the hydroxamate inhibitors IND1 and IND2 (peptide-like molecules) shows clearly that Trp side-chain affect the electrophilicity of the peptide bond carbonyl by decreasing the partial positive charge on the next carbonyl carbon (Toba et al. 1999). Consequently, a one-step ET reaction between the indole moiety and the peptide bond carbonyl of Trp still is possible, depending on the effect of the other side chains on the sur-

rounding peptide bond carbonyls. However, it may have low occurrence in proteins; otherwise the fluorescence kinetics of Trp in proteins should show no real difference. Thus, multistep ET in proteins is expected to be the most favored (Mataga et al. 1984; Nishitani et al. 1983).

Why do proteins show different fluorescence kinetics heterogeneity?

ET rate is controlled by the distance between donor and acceptor, the nature of intervening medium between donor and acceptor, the reaction free energy change, and the reorganization energy that accompany ET reaction. As confirmed by the studies quoted here, it is clear that proteins will show different ET rates even if they display the same donor-acceptor couple (Trp-peptide bond carbonyl). Consequently, because ET rates are different in proteins, the fluorescence kinetics heterogeneity will be different.

Why do some proteins show homogenous fluorescence kinetics?

In this work, we show that heterogeneous fluorescence kinetics in proteins principally is because of the ET-quenching mechanism. Although the observation of homogenous fluorescence kinetics (monoexponential decay) in proteins is rare, it still is a reality. The well-known examples are ribonuclease T1 at pH 5.5 (James et al. 1985) and apoazurin (Huntik and Szabo 1989). Because of the ET-quenching mechanism, heterogeneous fluorescence kinetics in proteins appears if ET process takes place in the time scale of fluorescence. Indeed, for example, if $k_{ET} \geq 10^{12} \text{ s}^{-1}$, no heterogeneity can be observed with the present instrumental resolution. Accordingly, in a previous study aimed to estimate k_{ET} in a model system where the donor and acceptor are separated by an increasing number of nonconjugated bridges, the authors concluded that, at short separating distances, $k_{ET} \geq 10^{11} \text{ s}^{-1}$, because no short-lived fluorescence component occurred (Oevering et al. 1987). Thus, if the fluorescence decay of RNT1 is monoexponential at pH 5.5 (James et al. 1985) and biexponential at pH 7.4 (Table 1), it seems plausible that the change in ET rate with pH could be concerned. In studies of long-distance ET in peptide models and proteins, it has been shown that at $\text{pH} < 6$, the ET rate increases (Weinstein et al. 1991; Bobrowski et al. 1997). Additionally, in different wild-type and single-mutated azurins, a significant increase in ET rate was observed upon decreasing the pH from 8 to 4 (Farver et al. 1996b). To explain this behavior, the authors suggested a pH-induced change either in the ET distance or in the electronic coupling (H_{DA}). We have made no specific investigation of the pH effect on photoinduced ET rates. Nevertheless, in the case of RNT1, on the basis of the results of Farver and coworkers (1996b), we speculate that ET rate increases at

pH 5.5 and thus homogenous fluorescence kinetics is obtained.

Conclusion

The data and the preliminary calculations presented above provide evidence for the involvement of ET process as the principal quenching mechanism in proteins and peptides, leading to heterogeneous fluorescence kinetics in single Trp-containing proteins. In our calculations, we assume the peptide bond carbonyl as acceptor and we have tried to explain its importance and implication in ET reactions in proteins. We have detailed the difference in ET rates that can arise between proteins even if the acceptor is the peptide bond carbonyl, and we have speculated about the reasons of the apparent homogenous fluorescence kinetics in certain proteins. Our work emphasizes the utility of time-resolved fluorescence as a tool to extract structural dynamic information in proteins, as the ET process is conformation dependent (distance between reactants, relative orientation of reactants, nature and polarity of the medium between reactants). Nevertheless, further investigations are required to understand the implication of the peptide bond carbonyl as the acceptor in photoinduced ET in proteins. The knowledge of the electronic structure of proteins although difficult, is fundamental for understanding the ET reactions and their pathway. Our next step in this direction will be the use of a recent semiempirical divide-and-conquer quantum mechanical methodology for proteins (Dixon and Mertz 1997; Gogonea and Mertz 1999) to gain further information about the efficiency of the peptide bond carbonyl as ET acceptor, the side-chain effect on the peptide bond carbonyl, the secondary structure effect in ET reaction, and hence the importance of ET mechanism in fluorescence kinetic heterogeneity in proteins. Finally, we should not forget that electron recombination reaction (ET back to Trp) is an important event that occurs between a donor and an acceptor of electrons. Although an explicit treatment of recombination reactions is not easy and such reactions usually are slower than *forward* ET reactions by orders of magnitude (Mataga et al. 1988a,b; Paddon-Row et al. 1988), it will be useful taking them into account, at least for short donor-acceptor distances, to extend our understanding of the details in ET mechanism in fluorescence kinetics of proteins.

Materials and methods

Chemicals and sample preparation

Nuclease (*Staphylococcus aureus*), ribonuclease T1 (*Aspergillus oryzae*), protein G (*Streptococcus sp.*), glucagon, and mastoparan (*polistes jadwagea*), were purchased from SIGMA-France and used without further purification. The proteins were dissolved in freshly prepared buffer containing 50 mM HEPES, 100 mM KCl,

pH 7.4 at 21°C. Protein concentration in the range 4–10 μM was used routinely. The samples were continuously stirred with a Teflon[®] magnetic bar throughout experiments.

Fluorescence lifetime measurements

The fluorescence lifetime measurements were performed with a laser apparatus that was described previously (Bombarda et al. 1999). The excitation wavelength was 295 nm. The emission wavelength was selected with a monochromator of 4 nm slit width and it was kept at 340 nm for nuclease and protein G, 335 nm for ribonuclease T1, and 350 nm for glucagon and mastoparan. Before each experiment, a corrective factor was carefully determined by taking N monoexponential decays of the fluorescent standard indazole ($\lambda_{\text{ex}} = 295 \text{ nm}$, $\lambda_{\text{em}} = 320 \text{ nm}$, and $\tau = 3.2 \text{ ns}$). The value of the corrective factor is obtained when all the N monoexponential decays are accepted (Vix and Lami 1995).

Fluorescence decay data analysis

The fluorescence decay data were analyzed with IRM as a sum of exponential, $I(t) = I_0 \sum_i \alpha_i \exp(-t/\tau_i)$, where $I(t)$ and I_0 are the intensities at times t and $t=0$, respectively, α_i is the normalized preexponential term, and τ_i is the lifetime. The adequacy of the fit was judged by the reduced χ^2 (χ_r^2), as a general rule; only deconvolutions yielding $\chi_r^2 = 1 \pm 3(2/\nu)^{1/2}$ were retained, ν being the number of degrees of freedom. In practice, the good analysis of the N decays allowed the acceptance of all the N decays with χ_r^2 near 1, otherwise a component was added in the analysis (Vix and Lami 1995). In IRM, the probability law governing the distribution of the counts per channel (CPC) is a compound Poisson law (Vix and Lami 1995). The weighting factors in χ_r^2 are no longer the inverse of the CPC, as is the case for the Poisson law, but they must be estimated from a sample of N decays. In the case of multiexponential decay law, a set of $N \approx 20$ decays should be used for analysis. In this work, $N = 15$ was used, because no change in results was observed.

Analysis of the fluorescence decays also was carried out by MEM using Pulse5 software (Livesey and Brochon 1987). A lifetime domain spanning 200 values, ranging from 1 ps to 10 ns equally spaced in a logarithmic scale, was routinely used in the analyses.

Analysis using ETM assumes that the decay function $I(t)$ is factorized as follows (Blumen and Manz 1979)

$$I(t) = I_0 \exp(-t/\tau) \phi(t) \quad (11)$$

where $\exp(-t/\tau)$ is the donor-specific decay function, $\phi(t)$ represents a function describing the decay of the donor excitation due to energy transfer to the acceptors, and I_0 is the intensity at $t=0$ (Bajzer and Prendergast 1993). In the analysis procedure $\phi(t)$ is expressed as follows:

$$\text{For 1 acceptor} \quad \phi(t) = p_0 + p_1 \exp(-w_1 \cdot t) \quad (12)$$

$$\text{For 2 acceptors} \quad \phi(t) = p_0 + p_1 \exp(-w_1 \cdot t) + p_2 \exp(-w_2 \cdot t) + p_3 \exp(-(w_1 + w_2) \cdot t) \quad (13)$$

where p_i are the probabilities associated with occupancy of possible acceptor sites and w_i are the rate constants of "energy transfer."

Structure analysis

The protein structures are imported from Brookhaven Data Bank, except mastoparan, whose structure was modeled (see Results). The files are 1SNC (nuclease), 9RNT (ribonuclease T1), 1IGD (protein G), and 1GCN (glucagon). The structural analysis and the modeling of the Mastp structure were performed by SYBYL program (Tripos Inc.). The calculation of the accessibility of Trp residues was carried out by TURBO-FRODO program (Bio Graphics) using a probe radius of 1.4 Å.

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