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Correlating Protein Structure and Protein Fluorescence

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INTRODUCTION

The ultimate goal of a time-resolved fluorescence study of a protein is the quantitative description of the pathway of the excited state energy dissipation and transfer within the protein, and the explanation of each pathway in terms of the structure and the dynamics of the protein. This has been partially possible for a number of proteins, such as barnase [1–3] and DsbA [4–6] and many others. The same is true for the spectral properties. Although the rational way is to start with single tryptophan containing proteins, the study of multitryptophan proteins has contributed a lot to the current insight and is very often more interesting. A crucial contribution is coming from site directed mutagenesis and consists in the production and the characterisation of single-Trp containing mutants by replacing all but one of the tryptophan residues by phenylalanine (preferably) or by tyrosine depending on the exposure. Generally this can be done without harming the protein's structure and function too much. In the absence of any energy transfer among the tryptophan-residues and any conformational effect of the mutations, the fluorescence properties (e.g., the amplitude average lifetime and the quantum yield) of the multitryptophan protein can be calculated by averaging the data for the individual tryptophan-mutants. Additivity has been observed for a number of proteins, e.g., colicin [7], actin [8], and DNA-binding protein [9]. Lack of additivity is an indication for the possibility of energy transfer among the tryptophan residues, conformational effects, and/or the variation of the extinction coefficients of individual tryptophan residues.

Replacing one tryptophan and subtracting the quantum yield of the mutant (WxyF) from that of the WT allows the characterisation of the quantum yield of the replaced tryptophan in the presence of the others. Comparing with the individual tryptophan allows to identify the effect of the other tryptophan residues via conformation or via energy transfer.

ANALYSIS OF ENERGY TRANSFER AMONG TRYPTOPHAN RESIDUES VIA QUANTUM YIELDS

Energy transfer among tryptophan residues can be bidirectional, such as in Barnase [1–3], and in this case the kinetics are governed by the equations derived by Porter [10] and Woolley et al. [11]. These equations show that when mutual energy transfer is occurring between two tryptophan residues, the measured lifetimes can be calculated from the lifetimes observed for the individual tryptophan residues and from the rate constants for energy transfer. However, with multiple tryptophan residues and multiple lifetimes, the situation becomes soon too complex to be dealt with in this way. Therefore the analysis of quantum yields is much more simple. We made an extensive analysis of energy transfer among the four tryptophan residues in PAI (plasminogen activator inhibitor) on the basis of the quantum yield [12]. Important is also to take into account the proper extinction coefficients when combining the quantum yields.

ANALYSIS OF SINGLE TRYPTOPHAN RESIDUES

The remaining task then consists in the explaining of the observed lifetimes in terms of the structure and the dynamics of the immediate environment of the individual residues. In a previous review we have extensively discussed this matter [13] and here we elaborate on some new information. A thorough analysis of the properties of a single tryptophan in a protein starts with its spectral properties.

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Statistical Analysis of the Spectral Properties of Single Tryptophan Residues

Although one would expect that the dynamic polarizability of the environment of a fluorophore which is responsible for the Stokes shift [14] would be able to produce a continuous shift of λ_{max}, it has been suggested, already some time ago, that the fluorescence emission spectrum of a protein can be decomposed into a limited number of discrete classes of tryptophan residues [15,16]. The fitting of the emission spectrum to log-normal function has allowed to isolate different components [17]. These components can be limited to five types going from extremely isolated tryptophans (like the one in Azurin) to fully exposed ones. An extensive statistical proof of the discreteness of the tryptophan classes has been made [18]. A detailed characterization of the environment of the tryptophan in each class was made in terms of hydrogen bonding, solvent accessibility, packing density, relative polarity, temperature factor, and dynamic accessibility. The assignment of log-normal components to individual tryptophans belonging to multitryptophan proteins was done on the general interpretation of the Stokes shift implying that tryptophan residues that are more accessible emit at longer wavelengths. The whole analysis was applied to more than 300 log-normal spectra [19]. This work is an important achievement in the characterization of the environment of each tryptophan and the linkage to the spectral properties. So far, the same database has not been used to extract correlations between fluorescence lifetimes and the different classes of tryptophan residues.

The method has demonstrated its applicability by identifying the tryptophan residues that are responsible for the increase of the protein fluorescence of myosin S1 on ATP binding [20].

We observed that the decay-associated spectra of a single tryptophan residue can be described very nicely by the log-normal representation, and we now use it as a standard fitting procedure [21]. Our interpretation is that multiple species of a single tryptophan exist and eventually probe a different environment and therefore can be linked to a different log-normal component.

Statistical Analysis of the Lifetimes of Single Tryptophan Residues

The tryptophan emission decay of β-glycosidase from the extremophilic archaeon *Sulfolobus solfataricus* has been investigated by frequency domain fluorometry. This protein is tetrameric and has 17 tryptophan residues for each monomer [22,23]. The data were analyzed in terms of sums of discrete lifetimes as well as in terms of quasi-continuous lifetime distributions of different shape. At neutral pH the emission decay is characterized by two components: a long-lived component centered at 7.4 ns, and a short one at 2.7 ns, irrespective of the decay scheme used for the interpretation of the experimental results. The effects of an irreversible inhibitor (cyclophellitol) and that of the denaturant guanidinium hydrochloride (GdHCl) on the dynamics of the protein have been investigated by observing the changes induced in the two components of the tryptophan emission decay. The addition of cyclophellitol to native β-glycosidase reduces the contribution of the short-lived component but does not affect the long-lived one: Increasing concentrations of GdHCl differently affect the contributions of the two emission components. Higher concentrations were required to unfold the molecular regions containing the long-lived components. These results indicate that the long-lived contribution arises from tryptophan residues deeply buried in the interior of the protein matrix, whereas the short-lived one includes residues located in less rigid and more solvent accessible regions, some of which are located in functionally important parts of the protein. The knowledge of the crystallographic structure of β-glycosidase allowed the evaluation of some average parameters for the microenvironment of each tryptophan such as hydrophobicity, structural flexibility, and ability of side chains to act as fluorescence quenchers. These results permitted to divide the tryptophan fluorescence of β-glycosidase in the contribution of two emitting clusters: one consisting of eight closely positioned tryptophan residues (i.e. Trp 33, 36, 60, 84, 151 174, 425, and 433) responsible for the long-lived emission component and the other cluster, composed of nine tryptophan residues nearer to the subunit surface (i.e., Trp 12, 156, 192, 287, 288, 316, 361, 376, 455) associated to the short-lived emission component. Finally, the examination of the tryptophan emission decay of the mesophilic β-glycosidase from *Escherichia coli* and its temperature dependence indicated that the environments of the tryptophan residues of the mesophilic enzyme are homogeneous as a consequence of much more pronounced protein dynamics.

A molecular dynamics simulation approach has been utilized to understand the bimodal distribution of the lifetims of this protein [24]. From the examination of the trajectories of the side chains capable of causing intramolecular quenching for each tryptophan microenvironment and using a modified Stern-Volmer model for the process of fluorescence quenching, the authors calculated the fluorescence lifetime for each tryptophan residue at two different temperatures, i.e., 300 and 365 K. The highest temperature was chosen because in this condition the protein shows a maximum in its catalytic activity and

is stable for a very long time. The calculated lifetime distributions overlap those experimentally determined. The tryptophan lifetimes appear to be a complex function of several variables, such as microenvironment viscosity, solvent accessibility, the chemical structure of quencher side chains, and side-chain dynamics. The results of this study are certainly very interesting, but as in the case of the spectral properties, one spectrum and one lifetime for each tryptophan is more often the exception than the rule.

EXCITED STATE PROCESSES

The different excited state processes that can take place in a protein and in solution are summarized by Chen and Barkley [25]. They can be considered as parallel pathways. Therefore (in the absence of fluorescence energy transfer) the global rate constant (the inverse of the lifetime) can be described as a sum of the different rate constants, as has been worked out by Chen and Barkley [25]:

$$1/\tau = k_r + k_{isc} + k_{sol} + \Sigma k_{qi} \qquad (1)$$

where k_r is the radiative rate constant, k_{isc} is the rate constant for intersystem crossing, k_{sol} for solvent quenching, and k_{qi} for quenching by quenchers either present in solution or in the protein itself, using a variety of mechanisms i.e. proton and electron transfer. The different values for these rate constants are discussed in great detail [13,25].

The Radiative Rate Constant

The value of k_r is fundamentally defined by the absorption and the emission band of a fluorescent group as expressed in the relation proposed by Strickler and Berg [26]. Using the log-normal representation of a tryptophan emission spectrum and this fundamental relation we were able to construct a simple relation between the radiative rate constant (k_r) and λ_{max}:

$$k_r = -0.1138 + 54.0104/\lambda_{max}\ (ns^{-1}), \text{ with } \lambda_{max} \text{ in nm}$$

This relation is a simplification of a previously determined polynomial [21] that turned out to be very instable, i.e., very sensitive to the exact numerical values of the factors. The foundation for this relation is described by Sillen et al. [21] and has been further extensively elaborated by Tuszynski et al. [27]. (Unfortunately the minus sign of the constant term is missing in the equation of that paper.) This equation accounts for a variation of k_r between 0.054 (320 nm) and 0.036 (360 nm). However, sometimes lower values are obtained. This could be due to the fact that experimental values for k_r are calculated from $Q/\langle\tau\rangle$ where Q could be underestimated due to static quenching, or $\langle\tau\rangle$ could be overestimated due to the presence of extremely short lifetimes that are too fast to be detected [28,29].

A variability comes also from the shape of the absorption spectrum. Although a statistical study of the absorption coefficient in a set of 30 proteins [30] showed that the molar extinction coefficient for Trp at 280 nm is 5540 M^{-1} cm^{-1}, the extinction coefficient at 295 can differ considerably, indicating a change in the shape of the absorption spectrum (or a different ratio of the contribution from ^{1}La and ^{1}Lb transitions). We measured the extinction coefficients at 295 nm of three individual tryptophan residues in the protein barnase [2] and of four tryptophan residues in mutants of PAI [12] and these results clearly support the variability of ε at 295 nm.

Quenchers of Tryptophan Fluorescence in a Protein

By determining the k_q in solution many side chains of amino acids were identified as efficient quenchers [25]. The best known are disulfide bridges, thiols, and protonated histidine residues. Although Arg was not found to be a solution quencher of tryptophan [25], a warning was given in the same paper that proximity and/or local polarity may change the quenching ability. Several examples exist that support this statement. In CRABP1 [31] Arg is shown to be an efficient quencher. Replacing that residue causes a substantial increase of the fluorescence intensity of the protein. However, no lifetime study was made. Inspection of the structure with Rasmol shows that the guanidium moiety points into the aromatic ring. In the calcium-binding protein of *Nereis diversicolor* (NSCP) Trp57 is strongly quenched in the calcium-bound state [32]. Here again an Arg residue is very close. However, a full lifetime study reveals that Arg does not shorten any lifetime but changes the balance of the microstates. Inspection of the structure in Rasmol indicates that the guanidinium moiety in this case is not in contact with the Trp side chain but makes a salt bridge close to the peptide backbone of Trp57. Also interactions with aromatic residues can lead to considerable quenching [33].

Electron Transfer to the Peptide Backbone

Apart from the possibility of having a quencher in the immediate neighborhood of a tryptophan residue, the ultimate mechanism that could be responsible for the presence of different lifetimes is the mechanism of elec-

tron transfer to the peptide backbone [25], a mechanism that would be different for the different rotamers. The difficulty with this mechanism is that although it has been studied extensively in theory [34–37], it is very difficult to prove experimentally. The same is true for the existence of tryptophan rotamers. A very convincing study was done on conformationally restricted cyclic peptides containing one tryptophan residue for which the conformation was determined by 1D and 2D ^{1}H-NMR. One conformation for the peptide backbone was found, but three rotamers of tryptophan were determined and linked to the three observed lifetimes by the similarity of the amplitudes of the lifetimes and the populations of the rotamers. The existence of the different lifetimes for the different rotamers was explained on the basis of electron transfer to the peptide bond [38]. The mechanism of electron transfer from the indole to the peptide bond carbonyl is extensively elaborated by Ababou and Bombarda [39] and applied to 12 lifetimes obtained from 5 different proteins.

Strong convincing evidence for electron transfer from the excited state is the recent observation of the reduction of SS bridges that are found in the immediate environment of a Trp. The first example is present in the protein Cutinase from *Fusarium solani pisi* [40–42]. The fluorescence of the single Trp69 is highly quenched in the native protein. This quenching is due to the presence of a nearby disulfide bridge between Cys31 and Cys109 and disrupting the disulfide bridge with DDT leads to a strong increase of the protein fluorescence just as in Dsba [4–6]. Very surprisingly, however, the quantum yield increases about tenfold upon extensive UV irradiation of the protein. This effect of UV-irradiation is also due to the breaking of the disulfide bridge. The formation of a thiol can be demonstrated by thiol reagents like DTNB. The fact that the fluorescence increases proves that the tryptophan is not itself irreversibly oxidized in the process.

The fluorescence properties of Trp69 are also influenced by an aromatic-amide hydrogen bond between the indole side chain of Trp69 and the peptide amide from Ala32, but NMR studies show that this hydrogen bond remains intact in the irradiated protein and cannot explain the observed effects. Oxidation of two disulfide bridges was also observed in the protein goat-alfa-lactalbumine [43]. Here again the formation of one thiol per disulfide bridge is observed and in this case the formation of a thioether linkage between Cys73 and Trp60 can be demonstrated.

Electron transfer can explain multiple lifetimes for a single tryptophan residue if the different rotamers show different electron transfer rates and if interconversion of rotamers is slower than the nanosecond time scale.

TRYPTOPHAN MICROSTATES

In this review, I limit myself to the study of tryptophan residues for which the time resolved fluorescence can be described by discrete lifetimes or discrete lifetime distributions (i.e., fitting the fluorescence decay at different wavelengths gives the same lifetimes but different amplitudes). Tryptohan dynamics and excited state reactions can also lead to multiple lifetimes, as has been reviewed before [13]. The discrete lifetimes considered must be due to the presence of microstates in the ground state, i.e., rotamers of Trp or alternative conformations of the side chains of other residues in the immediate neighborhood.

The link between multiexponential decay and the presence of rotamers about the $C^\alpha-C^\beta$ bond was made for Tyr by Gauduchon and Wahl [44] and for tryptophan by Szabo and Rainer [45]. The importance of rotations around the $C^\beta-C^\gamma$ bond in the amino acid tryptophan have been suggested by Engh et al. [46] because according to their molecular dynamics calculations $C^\alpha-C^\beta$ rotamers interconvert rapidly, while rotations about the $C^\beta-C^\gamma$ are much more infrequent. In proteins one might expect that all these rotations are even more hindered. Nevertheless anisotropy studies in a variety of proteins yield rotational correlation times between 0.03 and 50 ns [47,48] indicating that no extrapolation from one protein to the other can be made. On top of that, X-ray structure analysis usually gives only one rotamer position for each tryptophan in a protein. The possibility to populate more than one rotamer which slowly interconvert is therefore not easy to prove. A correlation between the presence of different lifetimes and different rotamers by ^{1}H-NMR data was done in oxytocin for Tyr [49] and for Trp [50]. The results for Trp are surprising in the sense that the authors conclude that $C^\alpha-C^\beta$ rotations are more relevant than $C^\beta-C^\gamma$ rotations, in contrast to what is expected from the molecular dynamics calculations mentioned before [46]. Also for cyclic enkephalin analogues containing a single tryptophan, the lifetimes were correlated with theoretical conformational calculations [51].

The existence of different rotamers of tryptophan in different proteins present in the databases have been studied by several authors [52–54]. McGregor et al. [55] show a link with the secondary structure and the absence of g+ in the helical conformation due to steric clashes (see Fig. 1 in Ref. 13 for the definition of the rotamer

angles, and note that in the literature many different definitions are used. In this manuscript we always refer to Fig. 1). Schrauber et al. (1993) [56] show by a statistical analysis of the data of 70 polypeptide chains that many deviations of the six standard rotamers occur in native proteins.

The existence of multiple discrete rotamers for a *single* tryptophan in a protein is a more difficult matter to prove. Crystal structures never show the existence of Trp-rotamers. Yet evidence of conformational heterogeneity was obtained in the time-resolved fluorescence decay measurements of the individual protein crystals of erabutoxin b [57]. NMR data are generally not available or also do not give indications for rotamers. This is, for example, the case for the single Trp in the mutant F102W of Carp Parvalbumin where two lifetimes are observed but only one rotamer exist in solution as determined by NMR [58]. The absence of evidence for rotamers for a *single* tryptophan in these NMR studies clearly indicate that other explanations have to be found for the existence of the different lifetimes in these cases. Up to now the number of cases studied is, however, too small to exclude the existence of rotamers in other proteins. MD simulations are often used to "demonstrate" the existence of rotamers. This is, for example, the case for the HIV-protease. The fluorescence of the two-tryptophan protein HIV-protease was characterized by lifetime analysis, acrylamide quenching and molecular dynamics simulations [59]. The protein forms a dimer and two tryptophan-residues are present in each monomer (Trp6 and Trp42). Two lifetimes are observed (19.2% of 2.7 ns and 80.8% of 5.7 ns) and a different kq value for acrylamide quenching is associated with each of them (6.85×10^9 $M^{-1}s^{-1}$ is associated with the short lifetime and 1.88×10^9 $M^{-1}s^{-1}$ with the long lifetime. A 500-ps molecular dynamics simulation of the dimer shows a few transitions between two different rotameric states for Trp42 (in monomer B) with one of them exposed to the quencher Tyr59 and therefore responsible for the short lifetime, and the other rotamer not in contact with a quencher and responsible for the longer lifetime. Trp6 does not show any transitions between rotameric states and is supposed not to contribute to the fluorescence and have a subnanosecond lifetime but the exact origin of this quenching is not identified. The MD-simulations show a few transitions in the rotameric states (both χ_1 and χ_2) of Trp42 in 500 ps trajectory indicating that the lifetimes of the rotameric states are subnanosecond, and that the observed fluorescence lifetimes might be the solutions of two coupled differential equations between emission rate constants and interconversion rate constants similar to the equations used for mutual energy transfer [10,11]. In the presence of the inhibitor acetyl pepstatin, the fluorescence data can be fitted as well with a single lifetime as with a double, but a single k_q (1.78×10^9 $M^{-1}s^{-1}$) is now observed and no interconversions are present in the MD simulations, showing only one rotameric state.

DsbA is probably the only protein where the rate of interconversion between two rotamers of Trp has been inferred from a study of the reaction with NBS [4–6]. The process is rather slow in a mutant protein where more space was made available to the Trp.

If the existence of rotamers is not easy to prove, the correlation between Trp-rotamers and their lifetime is even more problematic. The most convincing indication for the existence of several microstates of a single Trp seems to be the existence of rather different k_q values for collisional solution quenchers, that can be correlated to different exposure of different microstates (rotamers of Trp or alterations in its immediate environment). These situations are not so frequently encountered but do occur. Colicin [7] is one example and others exist. We have explored the existence of tryptophan-rotamers with a new computational method and linked the lifetime to the rotamer via the correlation between exposure and k_q values. In this way we were able to collect much more data than before [21] and have more reasonable values for our parameters:

$$k_{ET}(R) = k_0 \exp(-\beta(R - R_0))$$

where k_{ET} is the rate constant for electron transfer to the peptide carbonyl, k_0 the rate constant at Van der Waals contact R_0 (assumed to be 0.3 nm) and β expresses the exponential decay of the rate constant with distance. In our new data set we found $k_0 = 5$ ns^{-1} and $\beta = 1.5$ nm^{-1} from a collection of 26 data points. Already in the analysis of the fluorescence of HIV-1 integrase a similar low value of k_0 (3.7 ns^{-1}) was necessary to fit the time resolved fluorescence [60]. This equation is, however, a pragmatic description because it has to be elaborated for electron transfer through bond and through space and for orientational effects.

MEMBRANE-BOUND α-HELICES AS A SOURCE OF INFORMATION ABOUT TRYPTOPHAN ROTAMERS

Clayton and Sawyer [61,62] studied 5 different peptides of 18 amino acids long, which are able to form an amphipathic α-helix in the presence of lipid vesicles, but are unstructured in the absence. In the absence of vesicles the time dependence is tri-modal and the amplitude frac-

tions correlate nicely with the fractions of occurrence of the different rotamers observed in the statistical study of the protein databank of McGregor et al. [56] resp. g−, g+ and t rotamers. Again a coupling with the secondary structure is observed, since for Trp in helices only a bimodal distribution of Trp- positions is found: t and g− and also the fluorescence lifetimes show only two lifetimes. In the helix structure, g+ does not occur. (Note that in their paper these authors use the opposite definition of g+ and g−). These results follow the same line as the evidence for constraints imposed by the secondary structure on the rotamer collection of tryptophan shown by Willis et al. [63]. However, since the relative amplitudes are wavelength dependent this correlation fits at their emission wavelength of 335 but may not necessarily fit at other wavelengths. A review of the possibilities of fluorescence studies for membrane binding is given by Clayton and Sawyer [64].

Another example of a peptide with membrane binding properties is Penetratin, a 16-amino acid peptide, derived from the homeodomain of Antennapedia, a *Drosophila* transcription factor. This peptide is very interesting because it can be used as a vector for the intracellular delivery of peptides or oligonucleotides. To study the relative importance of the Trp residues in the WT-Penetratin peptide two analogues, the W48F and the W56F variant peptides were synthesized. A decrease in quantum yield and a blue shift of the maximal emission wavelength were observed by intrinsic tryptophan fluorescence emission upon interaction of the three peptides with negatively charged phosphatidylserine [65]. The W56F-Penetratin shows a larger blue shift than the W48F-Penetratin upon binding to vesicles containing 20% phosphatidylserine. No change in Trp fluorescence was observed after interaction with phosphatidylcholine. Upon binding to negatively charged phospholipids, the Trp mean lifetime decreased for the three peptides, and the Trp residues were shielded for acrylamide and iodide quenching. CD measurements indicated that the three peptides are random in buffer, and become α-helical upon association with negatively charged mixed PC/PS vesicles, but not with PC vesicles. These data show that WT-Penetratin and the two analogues interact with negatively charged phospholipids, and that this is accompanied by a conformational change from random to α-helical structure.

The lifetime data for the Penetratin peptides in random conformation correspond fairly well with the lifetimes and amplitude fractions observed by Clayton and Sawyer [61,62]. The three lifetimes are attributed to the classical three rotamers of chil (Cα−Cβ). It should be noted that the average lifetime of the lipid free WT-Penetratin can be calculated from the average lifetimes of the individual tryptophan residues assuming pure additivity [65]. This indicates that there are no interactions between the two Trp-residues, neither directly by energy transfer, nor indirectly by conformational effects. The observations in Penetratin, where the amplitude of the long lifetime component is strongly reduced (to a few percent) and all the lifetimes are reduced, do not correspond with the life time effects observed by Clayton and Sawyer [61,62]. In 100% TFE, the mean lifetimes of the tryptophan residues in the WT-, W48F-, and the W56F-Penetratin also decreased but less than two-fold instead of four- to seven-fold upon interaction with the negatively charged lipid vesicles. The 3Pro variant also showed a two-fold reduction of its lifetime when dissoved in 100% TFE, although this peptide does not form an alpha-helix structure. These results indicate that the conformational change of the peptide backbone from random to α-helix is not the major factor in the tryptophan quenching. Therefore other factors determining the lifetimes are probably involved.

The fluorescence intensity decrease might be due to quenching of the tryptophan indole moiety by side chains of the basic residues present in the Penetratin peptides [25]. A conformational change, upon lipid binding of the peptide with the negatively charged vesicles might bring one or more lysines close to the Trp48 and 56 residues. A similar quenching of Trp158 by Lys165 in the extracellular domain of Human Tissue Factor (sTF) was observed by Hasselbacher et al. [66]. The arginine residues of the Penetratin peptides might also contribute to tryptophan quenching [31,32]. Tryptophan quenching could also be induced by interaction with the PS headgroup.

An extensive time resolved fluorescence study has been performed on the calcium-induced binding of annexins to membranes [67,68] correlating tryptophan fluorescence to membrane binding and structure.

THE FUTURE

We can imagine that the day will come that fluorescence measurements help to model a protein from its primary structure. Such an attempt was made by Bosch et al. [69]. The primary structure of the 142 residue Glossoscolex paulistus d-chain hemoglobin was determined and Tryptophan was found to occupy positions 15, 33, and 129. Homology modeling allowed the authors to assign the positions of these Trp residues relative to the heme and its environment. The reference coordinates of the indole rings for W15 and W129 were 1.68 and 1.85 nm, respectively, from the geometric center of the heme, and W33 was located in close proximity to the heme

group at a distance which was approximately half of that for W15 and W129. It was possible to identify three rotamers of W33 on the basis of electrostatic and Van der Waals energy criteria. The calculated distances from the center of the heme were 0.83, 0.84, and 0.91 nm for Rot1, Rot2, and Rot3, respectively. Radiationless energy transfer from the excited indole to the heme was calculated on the basis of Forster theory. For W33, the distance was more important than the orientation factor, κ^2 due to its proximity to the heme. However, based on κ^2, Rot2 ($\kappa^2 = 0.945$) was more favorable for the energy transfer than Rot1 ($\kappa^2 = 0.433$) or Rot3 ($\kappa^2 = 0.125$). In contrast, despite its greater distance from the heme, the κ^2 of W129 (2.903) established it as a candidate to be more efficiently quenched by the heme than W15 ($\kappa^2 = 0.191$). With the average lifetime, $\langle\tau\rangle = 3$ ns, measured for the apo monomer as the reference, the lifetimes calculated for each emitter were: W33-1 (1 ps), W33-2 (2 ps), W33-3 (18 ps), W129 (100 ps), and W15 (600 ps). Experimentally, there are four components for oxymonomers at pH 7:two long ones of 4.6 and 2.1 ns, which contribute approximately 90% of the total fluorescence, one of 300 ps (4%), and the last one of 33 ps (7.4%). It is clear that the equilibrium structure resulting from homology modeling is able to explain the sub-nanosecond fluorescence lifetimes, but not the nanosecond range lifetimes which resemble the apo molecule and that the fluorescence studies here reveal that the modeling is not complete.

Is the tryptophan puzzle going to be solved by other residues? The use of derivatives of tryptophan is extensively discussed [70] but a new Aladdin's wonder lamp is being announced [71,72]: Aladan, a molecule that can replace tryptophan, and that possesses a much bigger Stokes shift and that is therefore very sensitive to the polarity and the dynamics of its environment. Unfortunately it is much bigger than tryptophan itself, although it has been successfully introduced in proteins. It certainly seems to be a very interesting probe for the dynamic properties of proteins, but whether it will ever be better than the real thing is the question.

REFERENCES

1. K. Willaert, R. Loewenthal, J. Sancho, M. Froeyen, A. Fersht, and Y. Engelborghs (1991) *Biochemistry* **31**, 711–716.
2. K. De Beuckeleer, G. Volckaert, and Y. Engelborghs (1999) *Proteins* **36**, 42–53.
3. Y. Engelborghs and A. Fersht (2000) In J. R. Lakowicz (Ed.) Topics in Fluorescence Spectroscopy, Vol 6: *Barnase: Fluorescence Analysis of a Three Tryptophan Protein*, Plenum Press, New York, pp. 83–102.
4. J. Hennecke, A. Sillen, M. Huber-Wunderlich, Y. Engelborghs, and R. Glockshuber (1997) *Biochemistry* **36**, 6391–6400.
5. A. Sillen, J. Hennecke, D. Roethlisberger, R. Glockshuber, and Y. Engelborghs (1999) *Proteins: Struct. Funct. Genet.* **37**, 253–263.
6. A. Sillen, J. Hennecke, R. Glockshuber, and Y. Engelborghs (2000) In J. R. Lakowicz (Ed.) Topics in Fluorescence Spectroscopy, Vol 6: *Fluorescence Study of the DsbA Protein from Escherichia Coli*, Plenum Press, New York, pp. 103–122.
7. R. Vos, Y. Engelborghs, J. Izard, and D. Baty (1995) *Biochemistry* **34**, 1734–1743.
8. T. C. Doyle, J. E. Hansen, and E. Reisler (2001) *Biophys. J.* **80**, 427–434.
9. E. Bombarda, A. Ababou, C. Vuillemier, D. Gérard, B. P. Roques, E. Piémont, and Y. Mély (1999) *Biophys. J.* **76**, 1561–1570.
10. G. B. Porter (1972) *Theor. Chim. Acta* **24**, 265–270.
11. P. Woolley, K. G. Steinhäuser, and B. Epe (1987) *Biophys. Chem.* **26**, 367–374.
12. S. Verheyden, A. Gils, A. Sillen, P. De Clerck, and Y. Engelborghs (2002) submitted.
13. Y. Engelborghs (2001) *Spectrachim. Acta Part A* **57**, 2255–2270.
14. J. R. Lakowicz: Principles of Fluorescence Spectroscopy, second ed. Kluwer Academic/Plenum Press, Boston, Dordrecht, London, Moscow, 1999.
15. E. A. Burstein, N. S. Vedenka, and M. N. Ivkova (1973) *Photochem. Photobiol.* **18**, 263–279.
16. E. A. Burstein and V. I. Emelyanenko (1996) *Photochem. Photobiol.* **64**, 316–320.
17. E. A. Burstein, S. M. Abornev, and Y. K. Reshetnyak (2001) *Biophys. J.* **81**, 1699–1709.
18. Y. K. Reshetnyak and E. A. Burstein (2001) *Biophys. J.* **81**, 1710–1734.
19. Y. K. Reshetnyak, Y. Koshevnik, and E. A. Burstein (2001) *Biophys. J.* **81**, 1735–1758.
20. Y. K. Reshetnyak, O. A. Andreev, D. D. Toptygin, L. Brand, and E. A. Burstein (2000) *J. Biomol. Struct. Dyn.* **18**, 113–125.
21. A. Sillen, F. Diaz, and Y. Engelborghs (2000) *Protein Sci.* **9**, 158–169.
22. E. Bismuto, P. L. Martelli, R. Casadio, and G. Irace (2000) *Protein Sci.* **9**, 1730–1742.
23. S. D'Auria, M. Rossi, I. Gryczynski, and J. (2000) In J. R. Lakowicz (Ed.) Topics in Fluorescence Spectroscopy, Vol 6: *Fluorescence of Extreme Thermophilic Proteins*, Plenum Press, New York, pp. 285–306.
24. E. Bismuto and G. Irace (2001) *FEBS Lett.* **509**, 476–480.
25. Y. Chen and M. D. Barkley (1998) *Biochemistry* **37**, 9976–9982.
26. S. J. Strickler and R. A. Berg (1962) *J. Chem. Phys.* **37**, 814–822.
27. J. A. Tuszynski, J. M. Dixon, and Y. Engelborghs (2001) *Chem. Phys. Letters* **350**, 509–514.
28. S. E. Webber (1997) *Photochem. Photobiol.* **65**, 33–38.
29. R. F. Chen, J. R. Knutson, H. Ziffer, and D. Porter (1991) *Biochemistry* **30**, 5184–5195.
30. H. Mach, C. R. Middaugh, and R. V. Lewis (1992) *Anal. Biochem.* **200**, 74–80.
31. P. L. Clark, Z. P. Liu, J. Zhang, and L. M. Gierasch (1996) *Protein Science* **5**, 1108–1117.
32. A. Sillen, S. Verheyden, L. Delfosse, T. Braem, G. Volckaert, and Y. Engelborghs (2002) submitted.
33. V. Nanda and L. Brand (2000) *Proteins, Struct. Funct. Genet.* **40**, 112–125.
34. R. W. Ricci and J. M. Nesta (1976) *J. Phys. Chem.* **80**, 974–980.
35. M. C. Chang, J. W. Petrich, D. B. McDonald, and G. R. Fleming (1983) *J. Am. Chem. Soc.* **105**, 3819–3824.
36. C. Goldman, P. G. Pascutti, P. Piquini, and A. S. Ito (1995) *J. Chem. Phys.* **103**, 10614–10620.
37. P. S. Antonini, W. Hillen, N. Ettner, W. Hinichs, P. Fantucci, S. M. Doglia, J.-A. Bousquet, and M. Chabbert (1997) *Biophys. J.* **72**, 1800–1811.
38. P. D. Adams, Y. Chen, K. Ma, M. G. Zagorski, F. D. Sönnichsen, M. L. McLaughlin, and M. D. Barkley (2002) *J. Am. Chem. Soc.* **124**, 9278–9286.

39. A. Ababou and E. Bombarda (2001) *Protein Sci.* **10**, 2103–2113.
40. P. C. M. Weisenborn, H. Meder, M. R. Egmond, T. J. W. G. Visser, and A. van Hoek (1996) *Biophys. Chem.* **58**, 281–288.
41. J. J. Prompers, C. W. Hilbers, and H. A. M. Pepermans (1999) *FEBS Letters* **456**, 409–416.
42. M. T. Neves-Petersen, Z. Gryczynski, J. Lakowicz, P. Fojan, S. Pedersen, E. Petersen, and S. B. Petersen (2002) *Protein Science* **11**, 588–600.
43. A. Vanhooren, B. Devreese, K. Vanhee, J. Van Beeumen, and I. Hanssens (2002) *Biochemistry* **41**, 11035–11043.
44. P. Gauduchon and Ph. Wahl (1978) *Biophys. Chem.* **8**, 87–104.
45. A. G. Szabo and D. M. Rayner (1980) *J. Am. Chem. Soc. USA* **102**, 554–563.
46. R. A. Engh, L. X.-Q. Chen, and G. R. Fleming (1986) *Chem. Phys. Letters* **126**, 365–372.
47. J. R. Lakowicz, B. P. Maliwil, H. Cherek, and A. Balter (1983) *Biochemistry* **22**, 1741–1752.
48. J. R. Lakowicz (2000) *Photochem. Photobiol.* **72**, 421–437.
49. W. R. Laws, J. B. A. Ross, H. R. Wyssbrod, J. M. Beechem, L. Brand, and J. C. Sutherland (1986) *Biochemistry* **25**, 599–607.
50. J. B. A. Ross, H. R. Wyssbrod, R. A. Porter, G. P. Schwartz, C. A. Michaels, and W. R. Laws, (1992) *Biochemistry* **31**, 1585–1594.
51. J. Malicka, M. Groth, J. Karolczak, C. Czaplewski, A. Liwo, and W. Wiczk (2001) *Biopolymers* **58** 447–457.
52. J. Janin, J. Wodak, M. Levitt, and B. Maigret (1978) *J. Mol. Biol.* **125**, 357–386.
53. T. N. Bath, V. Sasisekharan, and M. Vijayan (1979) *Int. J. Protein Res.* **13**, 170–184.
54. N. L. Summers, W. D. Carlson, and M. Karplus (1987) *J. Mol. Biol.* **196**, 175–198.
55. M. J. McGregor, S. A. Islam, and M. J. E. Sternberg (1987) *J. Mol. Biol.* **196**, 295–310.
56. H. Schrauber, F. Eisenhaber, and P. Argos (1993) *J. Mol. Biol.* **230**, 592–612.
57. T. E. S. Dahms, K. J. Willis, and A. G. Szabo (1995) *J. Am. Chem. Soc. USA* **117**, 2321–2326.
58. M. C. Moncrieffe, N. Juranic, M. D. Kemple, J. D. Potter, S. Macura, and F. G. Prendergast (2000) *J. Mol. Biol.* **297**, 147–163.
59. B. Ullrich, M. Laberge, F. Tölgyesi, Z. Szeltner, L. Polgár, and J. Fidy (2000) *Protein Sci.* **9**, 2232–2245.
60. C. Laboulais, E. Deprez, H. Leh, J. F. Mouscadet, J. C. Brochon, and M. Le Bret (2001) *Biophys. J.* **81**, 473–89.
61. A. H. A. Clayton and W. H. Sawyer (1999) *Eur. Biophys. J.* **28**, 133–141.
62. A. H. A. Clayton and W. H. Sawyer (1999) *Biophys. J.* **76**, 3235–3242.
63. K. J. Willis, W. Neugebauer, M. Sikorska, and A. G. Szabo (1994) *Biophys. J.* **66**, 1623–1630.
64. A. H. A. Clayton and W. H. Sawyer. (2002) *Eur. Biophys. J.* **31**, 9–13.
65. B. Christiaens, S. Symoens, S. Vanderheyden, Y. Engelborghs, A. Joliot, A. Prochiantz, J. Vandekerckhove, M. Rosseneu, and B. Vanloo (2002) *Eur. J. Biochem.* **269**, 2918–2926.
66. C. A. Hasselbacher, E. Rusinova, E. Waxman, R. Rusinova, R. A. Kohanski, W. Lam, A. J. Du Guha, T. C. Lin, and I. Polikarpov (1995) *Biophys. J.* **69**, 20–29.
67. J. Gallay, J. Sopkova, and M. Vincent (2000) In J. R. Lakowicz (Ed.) Topics in Fluorescence Spectroscopy, Vol 6: *The conformational flexibility of Domain III of Annexin V is Modulated by Calcium, pH and Binding to Membrane/water interfaces*, Plenum Press, New York, pp. 123–174.
68. J. Sopkova, C. Raguenes-Nicol, M. Vincent, A. Chevalier, A. Lewit-Bentley, F. Russo-Marie, and J. Galay (2002) *Protein Science* **11**, 1613–1625.
69. C. C. Bosch, H. Imasato, J. C. Rosa, H. J. Laure, C. H. da Silva, M. Tabak, R. C. Garratt, and L. J. Greene (2002) *Biophys. Chem.* **97**, 139–157.
70. J. B. A. Ross, R. Rusinova, L. A. Luck, and K. W. Rousslang (2000) In J. R. Lakowicz (Ed.) Topics in Fluorescence Spectroscopy, Vol 6: *Spectral Enhancement of Proteins by in vivo Incorporation of Tryptophan Analogues*, Plenum Press, New York, pp. 17–42.
71. B. E. Cohen et al (2002) *Science* **296**, 1700–1703.
72. M. Sundd and A. D. Robertson (2002) *Nature Struc. Biol.* **9**, 500–501.