ON THE QUANTITATIVE ANALYSIS
OF ELECTRONIC ENERGY
TRANSFER/MIGRATION IN
PROTEINS STUDIED BY
FLUORESCENCE SPECTROSCOPY

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Title
On the Quantitative Analysis of Electronic Energy Transfer/Migration in Proteins Studied by Fluorescence Spectroscopy

Abstract
Two recently developed theories of electronic energy transfer/migration were for the first time applied to real protein systems for extracting molecular distances. The partial donor-donor energy migration (PDDEM) is an extension to the previously developed donor-donor energy migration (DDEM, F Bergström et al PNAS 96, 1999, 12477) which allows using chemically identical but photophysically different fluorophores in energy migration experiments. A method based on fluorescence quenching was investigated and applied to create an asymmetric energy migration between fluorophores which were covalently and specifically attached to plasminogen activator inhibitor type 2 (PAI-2). It was also shown experimentally that distance information can be obtained if the fluorescence relaxation for photophysically identical donors, exhibits multi-exponential relaxation.

An extended Förster theory (EFT) that was previously derived (L. B.-Å. Johansson et al J. Chem. Phys., 1996, 105) has been developed for analysis of donor-acceptor energy transfer systems as well as DDEM systems. Recently the EFT was also applied to determine intra molecular distances in the protein plasminogen activator inhibitor type 1 (PAI-1) which was labelled with a sulfhydryl specific derivative of BODIPY. The EFT explicitly accounts for the time-dependent reorientations which in a complex manner influence the rate of electronic energy transfer/migration. This difficulty is related to the “κ²-problem”, which has been solved. It is also shown experimentally that the time-correlated single-photon counting (TCSPC) data is sensitive to the mutual configuration between the interacting fluorophores. To increase the accuracy in the extracted parameters it is furthermore suggested to collect the fluorescence data under various physico-chemical conditions. It was also shown that the Förster theory is only valid in the initial part of the fluorescence decay.

Keywords
Energy transfer, Energy migration, Homotransfer, Heterotransfer, EFT, Orientation factor, Time-resolved anisotropy, Fluorescence relaxation

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1. List of Papers

The thesis is based on the following Papers listed below and will be referred to in the text by their corresponding Roman numerals I-V.

I  M. Isaksson, S. Kalinin, S. Lobov, T. Ny and L. B.-Å. Johansson
An Environmental-Sensitive BODIPY®-Derivative with Bioapplication: Spectral and Photophysical Properties

II M. Isaksson, S. Kalinin, S. Lobov, S. Wang, T. Ny and L. B.-Å. Johansson
Partial donor-donor energy migration (PDDEM): A novel fluorescence method for internal protein distance measurements
*Phys. Chem. Chem. Phys.*, 2004, 6, 3001-3008

Extended Förster Theory for Determining Intraprotein Distances. 1. The $\kappa^2$-Dynamics and Fluorophore Reorientation

IV M. Isaksson, P. Hägglöf, P. Håkansson, T. Ny, and L. B.-Å. Johansson
Extended Förster Theory for Determining Intraprotein Distances: 2. BODIPY-Labelled Plasminogen Activator Inhibitor Type I, Submitted.

On the Quantitative Molecular Analysis of Electronic Energy Transfer within Donor-Acceptor Pairs
Accepted for publication in *Phys. Chem. Chem. Phys.*
2. Abbreviations

A = Acceptor of electronic energy
BD = Brownian Dynamics
DAET = Donor-Acceptor Energy Transfer
DDEM = Donor-Donor Energy Migration
D = Donor of electronic energy
EFT = Extended Förster Theory
ET = Energy Transfer
\( F(t) \) = time-dependent fluorescence intensity
FRET = Fluorescence/Förster Energy Transfer
IC = Internal Conversion
ISC = Inter-System Crossing
\( \kappa^2 \) = orientation factor
LED = Light Emitting Diode
MCA = Multi Channel Analyser
PMT = Photo-multiplier Tube
PDDEM = Partial Donor-Donor Energy Migration
\( R \) = distance between fluorophore groups
RET = Resonance Energy Transfer
\( r(t) \) = time-dependent anisotropy
\( S \) = order parameter
SLE = Stochastic Liouville Equation
SME = Stochastic Master Equation
TAC = Time-to-Amplitude Converter
TCSPC = Time Correlated Single Photon Counting
\( \tau \) = fluorescence lifetime
VR = Vibrational Relaxation
3. Introduction

Fluorescence spectroscopy is a versatile tool which is used in most varying applications, not least in the fields of bioscience. For instance, there are techniques such as the fluorescence imaging and the single-molecule fluorescence spectroscopy, which is continuously gaining interest for understanding biosystems. The extraordinarily high sensitivity in fluorescence spectroscopy is the main advantage with comparison to other spectroscopic techniques.

Fluorescence resonance energy transfer (FRET), or better RET, has frequently been used to gain structural information about biomacromolecules, such as DNA and proteins. The advantage with RET is its applicability under physiological conditions. Other techniques such as X-ray and multidimensional NMR usually give an atomic resolution, but then one needs perfect crystals for X-ray diffraction, and NMR exhibits a low sensitivity and is restricted to rather small proteins. Therefore, methods based on the electronic energy transfer process should be considered complementary to the X-ray and NMR methods.

RET depends on the possibility to specifically label a macromolecule by two chromophores, or to make use of intrinsic chromophores, e.g. the flavins. The low resolution of RET can be much ascribed to the uncertainty in the chromophores position and to the orientation factor ($\kappa^2$) that together with the distance between the chromophores determines the rate of energy transfer. The orientation factor has been extensively discussed in the literature and is referred to as the “$\kappa^2$-problem”. This thesis presents, on basis of recently developed theories of energy transfer, a solution to the “$\kappa^2$-problem” which overcomes the limitations of the hitherto used RET methods.

The importance of computer simulations is growing as a new research paradigm. A combination of simulation techniques and advanced theories of energy transfer allows one to relate the experimental data to molecular properties, such as intra protein distances, order parameters and rotational correlation times.
4. General Considerations

The basic concepts of fluorescence spectroscopy are introduced in this section.

4.1 Fluorescence

Fluorescence spectroscopy is a widely used method within biological sciences, which is likely due to its extraordinarily high sensitivity of detection. The phenomenon of fluorescence is defined as the spontaneous emission of light from an electronically excited state (usually the $S_1$ state) to different vibrational levels of the electronic ground state. This process is characterised by important properties like the emission spectrum ($F(\lambda)$, i.e. the intensity at a given wavelength), the fluorescence quantum yield ($\Phi$), the fluorescence lifetime ($\tau$) and the anisotropy ($r(t)$). These parameters contain information about the chromophore as well as its microenvironment, which can more or less indirectly be used to extract structural information about macromolecules.

4.1.1 Electronic Relaxation of Chromophoric Molecules

The different relaxation pathways of an electronically excited chromophore can be summarised in a famous Jabłoński diagram (Fig. 4.1). The overview given here is valid for a condensed phase which is the only phase considered in this thesis. Light absorption usually starts from the first singlet state ($S_0$) and its lowest vibrational level towards a vibrational level in $S_1$. This is indicated by a vertical line in Fig 4.1, which illustrates the fast transition rate that typically occurs within the femtosecond timescale. In the $S_1$ state the molecule rapidly ($10^{-12}$ s) relaxes to the lowest vibrational level (VR). There are several possible pathways of relaxation from the excited state and most of them are non-radiative. The most common pathways are internal conversion (IC) and fluorescence. Another important relaxation pathway not indicated in Fig 4.1 is the electronic energy transfer which is considered in detail below.
4.1.2 Radiative Lifetime and Quantum Yield

The radiative lifetime ($\tau_n$), sometimes also referred to as the intrinsic or natural lifetime, is the lifetime ($\tau$) in absence of other non-radiative processes like internal conversion and intersystem crossing. Since these processes compete with the fluorescence emission the observed lifetime is shorter than the radiative lifetime. The ratio between these lifetimes defines the fluorescence quantum yield ($\Phi$) which is given by:

$$\Phi = \frac{\tau_n}{\tau}$$  \hspace{1cm} (4.1)

Even if there exists methods to directly measure the quantum yield of a fluorophore (e.g. integrating spheres), one usually adopts a method whereby the fluorescence of the unknown compound is compared with a fluorescence standard. $\Phi$ is then given by:

$$\Phi = \phi_{\text{ref}} \frac{F[n - \exp(-A_{\text{ref}} \ln 10) n^2]}{F_{\text{ref}}[1 - \exp(-A \ln 10) n_{\text{ref}}^2]}$$  \hspace{1cm} (4.2)
Here $F$ is the integrated fluorescence intensity over the fluorescence band, when excited at a wavelength with absorbance $A[1]$. The radiative lifetime can also be calculated from the absorption and emission spectrum as well as the molar absorptivity according to the Strickler-Berg equation[2]:

$$\frac{1}{\tau_n} = 2.88 \times 10^{-9} n^2 \int \frac{F(\tilde{\nu})d\tilde{\nu}}{F(\tilde{\nu})\tilde{\nu}^3} \int \frac{\epsilon(\tilde{\nu})\tilde{\nu}^{-1}d\tilde{\nu}}{\tau_n}$$  \hspace{1cm} (4.3)$$

where $n$ is the refractive index of the solvent, $F$ is the fluorescence intensity, $\epsilon$ is the molar absorptivity and $\tilde{\nu}$ is the wave number. It is sometimes informative to compare the radiative lifetimes calculated from Eqs. 4.1 and 4.3. Deviations can indicate changes in the symmetry of the chromophore in the ground- and first excited state.

### 4.1.3 Fluorescence Quenching

Fluorescence quenching can be ascribed to intermolecular processes that decrease the fluorescence intensity. They are usually divided into two different mechanisms, static and dynamic quenching. Energy transfer between photophysically different fluorophores is an important quenching process which is discussed in detail below. A fluorescence quencher is a compound, which in orbital contact (i.e. within a few Å) to a fluorophore, in a radiation less process de-excites the $S_1$-state. Two examples of frequently used and efficient quenchers are acrylamide and iodide. The former is an electron deficient compound where a possible mechanism is an electron transfer from the excited fluorophore to acrylamide. Iodide is a heavy atom and the probable mechanism of the quenching is a spin-orbit coupling mechanism i.e. intersystem crossing (ISC). Fluorescence quenching is used in many applications in biosciences. For example it is frequently used to probe the surface of proteins.

The dynamic quenching is a diffusion controlled process that alters the observed lifetime of the excited state. The process is described by the well known Stern-Volmer equation:
where \( F \) and \( F_0 \) are the fluorescence intensities in presence and absence of quencher, \( \tau_0 \) is the lifetime without quencher, \( k_q \) is the bimolecular quenching constant and \( [Q] \) is the quencher concentration. A plot of \( F_0/F \) vs. \([Q]\) ideally gives a linear plot for which \( K_D \) determines the slope. Here it should be noticed that the static quenching also gives a linear plot. If one does not know what kind of quenching it is, then \( K_D \) equals \( K_{SV} \). The amount of static quenching present can be quantified by time-resolved measurements where:

\[
\frac{F_0}{F} = \frac{\tau_0}{\tau} \quad (4.5)
\]

should be fulfilled if dynamic quenching is the only contributing process.

The observation of an upward curvature of the Stern-Volmer plot can be explained by a simultaneous dynamic- and a static quenching and the Eq. 4.4 can then be written as:

\[
\frac{F_0}{F} = (1 + K_D [Q])(1 + K_S [Q]) \quad (4.6)
\]

where \( K_S \) stands for the static quenching constant. The observed upward curvature can be explained by the second order dependency on \([Q]\) in the Stern-Volmer equation. As a complement to the lifetime measurements, or if the lifetime measurement is not available, Eq. 4.6 can be rearranged so that:

\[
\left( \frac{F_0}{F} - 1 \right) [Q] = (K_D + K_S + K_D K_S [Q]) \quad (4.7)
\]
A plot of the left-hand side of Eq. 4.7 versus \([Q]\) corresponds to a straight line with a slope of \(K_D K_S\) and an intercept of \((K_D + K_S)\). The solution to the equation system gives the individual values of \(K_D\) and \(K_S\).

Frequently the Stern-Volmer plots of probe labelled proteins display a downward curvature. This is due to the different accessibility of the quencher to the fluorophore in the protein. If one assumes a two-state model, where a fraction of probes is fully accessible to the quencher and the other is totally shielded, one can show that\[1]\:

\[
\frac{F_0}{F_0 - F} = \frac{1}{f_a K_a [Q]} + \frac{1}{f_a}
\]

(4.8)

Here, \(f_a\) denotes the accessible fraction to the quencher and \(K_a\) is the quenching constant of this fraction. By plotting \(F_0/\Delta F\) versus \(1/[Q]\) one can calculate \(f_a\) and \(K_a\).

Diffusion-controlled quenching usually gives values of the bimolecular quenching constant (\(k_q\)) about \(1 \times 10^{10} \text{ M}^{-1}\text{s}^{-1}\). Steric shielding of fluorophores can give substantially lower values of \(k_q\) and this can be used to quench two fluorophores in a protein to different extent.

### 4.2 Electronic Energy Transfer and Migration

Resonance energy transfer (RET) is a non-radiative process that should not be directly linked to the fluorescence process. One frequently encounters the term fluorescence resonance energy transfer (FRET) in literature, but this terminology should be avoided since it can be confusing. The concept Förster resonance energy transfer is used in honour of Theodor Förster who originally gave the quantum mechanical description of energy transfer\[3\]. RET refers to a long-range weak dipole-dipole interaction between the electronic transition dipoles of the donor (D) and acceptor (A). The transfer can occur between identical as well as non-identical chromophores. If the chromophores are chemically and photo-physically identical, the term migration is to prefer, since the energy can jump backwards and forwards. In this thesis donor-donor energy migration (DDEM) and
donor-acceptor energy transfer (DAET) are studied and applied. Although the energy transfer/migration can be used for several applications, the focus is here on the distance measurements. The rate of energy transfer/migration (ET/EM) depends on the overlap between the donor emission spectrum and the acceptor absorption spectrum. The strength of the DA/DD-coupling can be characterised by the so-called Förster radius ($R_0$):

$$R_0^e = \frac{9000(\ln 10)\langle \kappa^2 \rangle \phi J}{128\pi^5 n}$$

(4.9)

which is a property of the chromophore pair used, and it typically ranges between 20 - 70 Å. This allows to determine distances of about 15 - 80 Å. The dynamic transfer rate can be calculated according to:

$$\langle \omega \rangle = \frac{3\langle \kappa^2 \rangle}{2\tau_D} \left( \frac{R_0}{R} \right)^6$$

(4.10)

In Eq. 4.10 the brackets denote an ensemble average, $\tau_D$ is the fluorescence lifetime of the donor, and $\langle \kappa^2 \rangle$ is the orientation factor or the averaged square of the angular part of the dipole-dipole interaction:

$$\kappa^2 = \left( \cos \delta - 3 \cos \beta_1 \cos \beta_2 \right)^2$$

(4.11)

Here $\delta$ is the angle between the transition dipole moments and $\beta_1$ and $\beta_2$ are the angles to a common vector ($\mathbf{R}$) interconnecting the two chromophores.

The most common method used to determine distances in macromolecules is based on fluorescence steady-state experiments[4]. In this method one obtains the efficiency ($E$) of energy transfer by measuring the fluorescence intensity of the donor in absence ($F_D$) and presence ($F_{DA}$) of an acceptor. The efficiency is calculated from:
\[ E = 1 - \frac{F_{DA}}{F_D} \]  \hspace{1cm} (4.12)

The energy transfer efficiency then depends on the distance according to:

\[ E = \frac{1}{1 + \left( \frac{R_0}{R} \right)^6} \]  \hspace{1cm} (4.13)

This method, however, may result in large errors in the distances determined and it is therefore recommended to perform time-resolved fluorescence measurements, especially in the limits where \( E \) is close to 1 or 0. The fluorescence decay of the donor, in the limit where \( t \to 0 \) (Paper IV), is given by:

\[ F_{DA}(t) = F_{DA}^0 \cdot e^{-t/\tau_D} \cdot e^{-t/\lambda} \]  \hspace{1cm} (4.14)

One also has the possibility to observe the rate of energy transfer through the rise time of the acceptor fluorescence intensity. This appears however, to be a less frequently used method. Possibly due to complication with direct excitation of acceptor and the requirement of a fluorescent acceptor. The quantitative treatment of energy migration between like molecules has hitherto been sparsely given in the literature although, some elaborate theories are available[5-9]. The obvious advantage with DDEM as compared to DAET is the incorporation of two identical fluorophores instead of the more cumbersome labelling procedure by using two chemically different fluorophores. The starting point of energy transfer as a tool for measuring distances in macromolecules is the well-known paper by Stryer and Haugland[4]. They introduced the concept “the spectroscopic ruler” as a description of how DAET can be used and they also demonstrated the validity of the six-root dependence between the rate of energy transfer and distance. To this date no experimental evidence of the square dependence of the orientation factor has been given.
4.3 Fluorescence Anisotropy

The photo-selection is an important characteristic of electronic absorption. Using linearly polarised light it is possible to create a uniaxial orientational distribution of excited molecules with respect to the electric field vector. This is because molecules absorb light with the highest probability if the electric transition dipole moment is parallel to the electric field vector of light. Reorientational motion of the fluorophore during the excited state depolarises the emitted light. Hence, by monitoring the degree of depolarisation of the emitted light, dynamic molecular information can be obtained as well as static information such as the local order of the system. Energy migration is in addition to molecular reorientation, a depolarising process which can be experimentally quantified. In order to measure the depolarisation the fluorescence intensity is monitored for two settings of the excitation- and emission polarisers. One then defines the fluorescence anisotropy as:

\[
r_{\text{exp}}(t) = \frac{I_{\text{VV}}(t) - I_{\text{VH}}(t)}{I_{\text{VV}}(t) + 2I_{\text{VH}}(t)} = \frac{D_{\text{exp}}(t)}{S_{\text{exp}}(t)}
\]

(4.15)

where V and H denotes the vertically and horizontally polariser settings, respectively. In Eq. 4.15 the denominator only depends on the fluorescence relaxation and is usually called summation curve or sum curve while the nominator only depends on the fluorescence depolarisation, if there is no energy transfer present, and is referred to as the difference curve or diff curve.

In terms of the absorption and emission transition dipole moments the anisotropy can be expressed as a change in direction of the transition dipole between the times of excitation and emission. The time-resolved anisotropy is then given by:

\[
r(t) = r_0 \langle P_2[\hat{\mu}(0) \bullet \hat{\mu}(t)]\rangle
\]

(4.16)
(4.17) where $\eta$, $V$, $R$ and $T$ are the viscosity, the molecular volume, the gas constant and the temperature, respectively. In general it is difficult to accurately determine the rotational correlation time if $\phi$ is multiples times longer than the fluorescence lifetime. The correlation times of the local fluorophore reorientation and that of the macromolecule are usually considered to be separable. It can sometimes in an energy transfer/migration experiment be necessary to account for the overall tumbling of the macromolecule, although this reorientation does not directly influence the rate of energy transfer/migration.

4.4 Experimental Techniques

4.4.1 Steady-State Fluorescence Experiments

The steady-state fluorescence experiments are a widely used method to study biomolecules. Our laboratory is equipped with a SPEX Fluorolog 112 with a stationary excitation light produced by a xenon arc lamp. The fluorescence intensity of the chromophore is recorded as a function of emission or excitation wavelength. The excitation wavelength is selected by a single diffraction grating monochromator and the emission wavelength by a double diffraction grating monochromator for increased resolution. The excitation light can also be polarised by an excitation polariser and the fluorescence detected through a second emission polariser in order to measure the
depolarisation of the sample. The detection is performed by a cooled photomultiplier tube (PMT).

4.4.2 Time-Resolved Fluorescence Experiments

The time-correlated single-photon counting (TCSPC) technique was used to monitor the time-resolved fluorescence. Two experimental setups were used in this thesis. One modified PRA 3000 (Photophysical Research Associates Inc., Ontario, Canada) and a 5000U (HORIBA Jobin Yvon IBH). The light source was either a light emitting diode (LED) or a pulsed laser diode operating at about 0.5 - 1 MHz. The PRA equipment uses interference filters to select the excitation and emission wavelengths, while the 5000U is equipped with excitation and emission monochromators. In a TCSPC experiment the excitation pulse sends a start signal to the time-to-amplitude converter (TAC) at the time of excitation, and when the first emitting photon from the sample reaches the detector, the TAC is stopped. The amplitude of output TAC voltage is proportional to the time delay between the start and stop pulse. The time delay is stored in a multi-channel analyser (MCA) where separation between channels represents a well defined and known time. Each photon detected adds the value of unity to the corresponding channel, and the result is displayed in a histogram.
5. Theoretical Considerations

In this section a recent theory will be presented which hitherto is not available in ordinary textbooks about energy transfer. Most of these theories were developed in the last decade. In 1948 Förster derived Eq 4.10[3] and until the early 1990’s there was not much theoretical development of the theory. Especially, the theory lacks the possibility to quantitatively account for the effect of the correlation between reorienting chromophores and the electronic energy transfer.

Electronic Energy transfer processes can be divided into three categories, namely: 1. Irreversible energy transfer from a donor to an acceptor. 2. Reversible energy migration where the energy is transferred with equal probability in both directions. 3. Partially reversible energy migration of which energy is transferred back and forth between a donor and acceptor. In the following sections these theories will be presented and followed by an extended Förster theory which allows to exactly describe the energy transfer/migration between reorienting and weakly dipole-dipole interacting chromophores.

5.1 Donor-Acceptor Energy Transfer (DAET)

DAET refers to a chromophore pair where there is no overlap between the donors emission spectrum and acceptors absorption spectrum. The application of the DAET theory is rather straightforward and traditionally is described by Eq. 4.14[1]. Two experiments are needed were the lifetime is first determined in a separate experiment and then using this information, the rate of energy transfer \(\langle \omega \rangle\) is extracted in an experiment of donor and acceptor interaction. There is however, a factor left undetermined in Eq. 4.14 namely, \(\langle \kappa^2 \rangle\). What value to use has been the subject of debate in the literature[10, 11]. In a paper by Dale et al[10] a method based on time-resolved fluorescence anisotropy measurements was presented where the local order is calculated from the residual anisotropies, which is then used to calculate the maximum and minimum values of \(\langle \kappa^2 \rangle\) according to:
\[
\left< \kappa^2 \right>_{\text{max}} = \frac{2}{3} \left( 1 + S_D + S_A + 3S_D S_A \right) 
\] (5.1)

\[
\left< \kappa^2 \right>_{\text{min}} = \frac{1}{3} \left( 2 - S_D - S_A \right) 
\] (5.2)

where \( S_i \) \((i = D, A)\) means the order parameter which can be calculated from the initial-\((r_0)\) and residual \((r_\infty)\) anisotropies by:

\[ S = \pm \sqrt{\frac{r_\infty}{r_0}} \quad -0.5 \leq S \leq 1 \] (5.3)

One can further decrease the uncertainty in \( \left< \kappa^2 \right> \) by adding knowledge about the residual anisotropy from the coupled system. In this thesis \( \left< \kappa^2 \right>_{\text{max}} \) and \( \left< \kappa^2 \right>_{\text{min}} \) were solved numerically for the cases where residual anisotropies from the coupled systems \((S_\delta \text{ cf. Eq. 5.6})\) were included. Eqs. 5.1 and 5.2 mean that the accuracy in the extracted distances depends on the local order of the chromophores in the experimental system. It is common that fluorophores which are covalently bonded to macromolecules exhibit highly restricted reorientation with order parameters about \( \sim 0.8 \). In most publications over the years and to this day the isotropic dynamic average \((\left< \kappa^2 \right> = 2/3)\) is used which might correspond to errors, in the extracted distance, of about 30 – 35 %.

### 5.2 Donor-Donor Energy Migration (DDEM)

In a special case of RET the chromophore pair considered constitutes chemically identical molecules. The energy then freely migrates with equal probability between the two. One important implication in the case of DDEM is that the fluorescence relaxation remains invariant to the rate of energy migration and the EM process can only be revealed by depolarisation experiments. An analytical model to quantitatively determine distances between two donor molecules were previously developed[5, 12, 13] and is briefly presented below.
In Eq 5.6 the subscript 1 and 2 refers to the two donors, \( f \) is the degree of labelling and \( \phi \) is the rotational correlation time. \( p(t) \) is probability that the initially excited donor is still excited after a time \( t \) and is related to the rate of DDEM (\( \langle \omega \rangle \)) according to:

\[
p(t) = \frac{1}{2} \left[ 1 + \exp(-2\langle \omega \rangle t) \right]
\] (5.7)

The analytical model of DDEM accounts to some extent for the dynamic effects in \( \omega \) induced when the rate of energy migration and reorienting motion is on the same timescale. The model was compared with an exact theory (see section 5.4) in a paper by Edman et al[14]. It was found to work quite well which can be explained by using a pre-exponential factor, \( \rho_0 \), as a floating parameter in the data analysis. The \( \rho_0 \)-value is defined as the maximum contribution to the anisotropy from the secondary excited donor. However, even though this effect might be approximately modelled analytically, there are other uncertainties such as \( \langle \kappa^2 \rangle \) that still can give large errors in the extracted distances. In the DDEM model an approach was presented to calculate \( \langle \kappa^2 \rangle \) other than that of 2/3 (dynamic isotropic average) which is most commonly used in literature. The assumption means that one of the donor orientational distributions is collinear with the common axis \( \mathbf{R} \) interconnecting the centre of mass between the two fluorophores. Further, the out of plane angle, \( \alpha \), is assumed to be zero. \( \delta \) in this
specific case is equal the $\beta_1$-angle. The angles are defined in Fig 5.1. In this model the $\langle \kappa^2 \rangle$ is given by:

$$\langle \kappa^2 \rangle = \frac{2}{3} \left\{ 1 + S_1 + S_2 S_\delta + 3 S_1 S_2 S_\delta \right\}$$

(5.8)

In this way the parameter $S_\delta$ effectively determines the orientation factor since $S_1$ and $S_2$ are fixed and usually known. There is no direct correlation between the $\delta$-angle and $\langle \kappa^2 \rangle$-value so whether this approach gives any higher accuracy in the extracted distances should be further investigated.

### 5.3 Partial Donor-Donor Energy Migration (PDDEM)

PDDEM occurs when back transfer is possible from the acceptor molecule to the donor and is present if there is sufficient overlap between the acceptor emission spectrum and the donor absorption spectrum. For PDDEM a theoretical description has been derived previously[15]. The special case, in which the donor and acceptor are of the same chemical species while they exhibit different fluorescence relaxation rates or multi-exponential fluorescence relaxation, will be considered here. The initial excitation probabilities and spectral shifts are assumed to be the same for this case. The above properties can be obtained for a fluorophore covalently attached to a protein at different positions where the rate of quenching is different. It is usually observed that a fluorophore with a mono-exponential fluorescence relaxation in solution show multi-exponential fluorescence relaxation when labelled to a macromolecule.

In a recent paper by Kalinin et al[16] it was shown that modelling of multi-exponential decays through discrete or continuous distributions of lifetimes had little or no impact on the energy migration rates in PDDEM. In this thesis discrete distributions will be assumed. Consider the following scheme of the kinetics of a coupled system:
Here $\alpha$ and $\beta$ denote the labelling site of each donor. Furthermore we assume that $\omega_{\alpha\beta} = \omega_{\beta\alpha} = \omega$. The master equation for such a system is well known and the observed fluorescence relaxation for this system is given by:

$$
F_{ab}(t) = \left\{ \left( -2\lambda_2 - \tau_\alpha^{-1} - \tau_\beta^{-1} \right) \exp(\lambda_1 t) + \left( 2\lambda_1 + \tau_\alpha^{-1} + \tau_\beta^{-1} \right) \exp(\lambda_2 t) \right\} / (\lambda_1 - \lambda_2) \tag{5.10}
$$

where

$$
\lambda_{1,2} = \frac{1}{2} \left[ -\tau_\alpha^{-1} - \tau_\beta^{-1} - 2\omega \pm \sqrt{\left(\tau_\alpha^{-1} - \tau_\beta^{-1}\right)^2 + 4\omega^2} \right] \tag{5.11}
$$

The modelling of multi-exponential fluorescence relaxation assumes that:

$$
F_a(t) = \sum_i f_a^i \exp(-t/\tau_a^i) \tag{5.12}
$$

$$
F_b(t) = \sum_i f_b^i \exp(-t/\tau_b^i) \tag{5.12}
$$

and for the coupled system the photophysics is:

$$
F_{ab}(t) = \sum_{ij} f_a^i f_b^j F_{ab}\left(t, \tau_a^i, \tau_b^j\right) \tag{5.13}
$$

If the lifetimes are not too different, the depolarisation experiments are necessary in order to determine the rate of EM. The fluorescence anisotropy in presence of PDDEM is given by:
The fluorescence anisotropies $r_\alpha(t)$ and $r_\beta(t)$ is given by the first two rows in Eq. 5.6 with the substitution $\alpha = 1$ and $\beta = 2$. The contribution from the energy migration ($r_{\alpha\beta}(t)$) to $r(t)$ is given by the three last rows in Eq. 5.6. From Eq. 5.14 it follows that when the lifetimes of the two donors become similar or when $\omega >> |\tau_\beta - 1 - \tau_\alpha - 1|$ the last term in Eq. 5.14 vanishes and one obtains the expression valid for DDEM (cf Eq 5.6).

For rapid energy migration, the fluorescence anisotropy therefore becomes independent of the fluorescence lifetimes.

Finally the excitation probability, $p(t)$, is given by:

$$p(t) = 1 - \frac{2\omega[\exp(\lambda_1 t) - \exp(\lambda_2 t)]}{(\lambda_1 - \lambda_2) F_{\alpha\beta}(t)}$$

(5.15)

### 5.4 Extended Förster Theory (EFT)

As the equation of the rate of energy transfer stands (Eq. 4.10) it is assumed that two molecules interact in the static or dynamic limit (i.e. $\kappa^2$ is a constant). The generalisation of Eq. 4.10 to include stochastic changes in $\kappa^2$ (i.e. $\kappa^2(t)$) is not trivial. In a paper by Westlund and Wennerström[17] a Liouville formalism was developed for treating energy transfer processes within a common framework of relaxation processes. In this work the results originally derived by Förster (Eq. 4.10) was generalised to also include reorientational dynamics of the donor and acceptor between two stationary molecules and then solved the problem analytically when one of the molecules is stationary and the other one is allowed to undergo a change between two orientations. Later an algorithm was introduced to solve the Stochastic Liouville equation (SLE) describing energy migration between a pair of
reorienting fluorophores[18]. In this approach one needs to account for the stochastic motions of the interacting fluorophores. This is possible by generating trajectories from the Brownian dynamics or molecular dynamics simulation techniques. Later a stochastic master equation (SME) of energy migration between two donors has been derived from the SLE and its formal solution was given by Johansson et al.[19]. The validity of SME was tested by Håkansson and Westlund[20] and found to accurately describe DDEM under most experimental conditions. The probability that the initially excited donor is still excited after time $t$ in DDEM is given by:

$$\chi(t) = \left\{ \frac{1}{2} \left[ 1 + \exp\left( -2\omega(t) t' \right) \right]\right\} \quad (5.16)$$

and for DAET the excitation probability is the same with the exception that the a factor of 2 in the exponent is excluded. In order to calculate the total excitation probability one needs to multiply Eq. 5.16 by the fluorescence relaxation of the uncoupled system. Eq 5.16 is referred to as the Extended Förster Theory (EFT). The EFT can be approximated by a cumulant series expansion when truncated at the first and second order gives the excitation probabilities:

$$\chi^{(1)}(t) = \frac{1}{2} \left[ 1 + \exp(-2\langle \omega \rangle t) \right] \quad (5.17)$$

$$\chi^{(2)}(t) = \frac{1}{2} \left[ 1 + \exp(-2\langle \omega \rangle t) \right]$$

$$\times \exp\left( + 2 \int_0^t \left( t - t' \right) \left[ \langle \omega(0) \omega(t') \rangle - \langle \omega \rangle^2 \right] dt' \right) \quad (5.18)$$

Eq. 5.17 is identical to the result derived by Förster. Implications of the second order cumulant approximation were studied by Edman et al.[21]. The correlation function, $\langle \omega(0) \omega(t) \rangle$ in Eq 5.18 was worked out in Paper IV. To simplify Eq. 4.10 we can rewrite it according to:
\[ \omega(t) = \Lambda \kappa^2(t), \quad (5.19a) \]

\[ \Lambda = \frac{3}{2\tau_0} \left( \frac{R_0}{R} \right)^6 \quad (5.19b) \]

This means that the time independent part, \( \Lambda \), in the energy transfer is separated from the dynamical part, \( \kappa^2(t) \). The latter is described by the stochastic fluctuations of the rate of energy migration. \( \Lambda \) is referred to as the coupling strength between the electronically interacting chromophores. The dynamic contribution to the excitation probability can be characterised by a dimensionless parameter (\( \vartheta \)), referred to as the Kubo number[22], which is given by:

\[ \vartheta = \Lambda \tau \kappa \sqrt{\langle \kappa^4 \rangle - \langle \kappa^2 \rangle^2} \quad (5.20) \]

Here, \( \tau \kappa \) is the integral correlation time of the correlation function \( \langle \kappa^2(0) \kappa^2(t) \rangle \) and it can be calculated numerically from the simulations. The last term in Eq. 5.20 is the variance of the stochastic fluctuations of \( \kappa^2(t) \) and its value squared will hereafter be denoted by:

\[ \Delta \kappa^4 = \langle \kappa^4 \rangle - \langle \kappa^2 \rangle^2 \quad (5.21) \]

In a DAET experiment the excitation probability is a direct observable, which decreases the donor fluorescence intensity due to energy transfer. Since the fluorescence relaxation is invariant to energy migration, the excitation probability is only observable through the rate of the depolarisation. The fluorescence anisotropy is composed of the following contributions:

\[ r(t) = \frac{r(0)}{2} [\rho_1(t) + \rho_2(t) + \rho_{12}(t) + \rho_{21}(t)] \quad (5.22) \]

where \( \rho_i \) (\( i = 1,2 \)) is the contribution from each donor in the absence of energy migration and given by:
Furthermore, $\rho_{ij}$ (i,j = 1, 2; i \neq j) is the contribution to $r(t)$ caused by energy migration:

$$\rho_{i}(t)=\langle P_{2}[\hat{\mu}_{i}(0) \cdot \mu_{i}(t)]\chi(t)\rangle \quad (5.23)$$

$$\rho_{ij}(t)=\langle P_{2}[\hat{\mu}_{i}^{R}(0) \cdot \hat{\mu}_{j}^{R}(t)](1-\chi(t))\rangle \quad (5.24)$$

The superfix \( R \) denotes a transformation from the molecular frame to a coordinate system which is fixed in the macromolecule (see Fig. 5.1). It is noteworthy that the excitation probability is inside the brackets in Eqs. 5.23 - 5.24, which means that the reorientational motions and energy migration can not be treated as independent processes.

Fig. 5.1. The configuration angles $\alpha, \beta_{1}, \beta_{2}$ describe the mutual orientation of the director frames with respect to the common $R$-frame. The $\delta$-angle is indicated as the angle between the two donor molecules.
6. Computational Methods

6.1 Brownian Dynamics Simulation

To calculate the excitation probability as it appears in the EFT one needs to create trajectories of the reorienting motion of the chromophores. Thereby one can model the stochastic behaviour of $\kappa^2(t)$ and the reorientational correlation functions in the anisotropy decay (Eqs. 5.23 - 5.24). For this we have used Brownian Dynamics simulations (BD). A model is then chosen that describes the reorientational motion in a satisfactory manner for the donor and acceptor molecule. This is tested by checking its ability to fit the anisotropy decays. A complex reorientational motion of the donors is usually described by a sum of two or more exponential functions. Even though Eq. 5.6 becomes more complex it can still be applied straightforwardly. In BD simulations the multi-exponentiality is determined by the choice of model and properties of the system such as the order parameter and the diffusion rate of the chromophores. Two different types of models/potentials were used and tested in this thesis (Paper III).

6.1.1 The Cone Potential

The cone potential is a frequently used model to mimic the restricted molecular motions of molecules in isotropic and anisotropic systems. In this model, a unit vector is undergoing restricted diffusion within the boundaries of a cone defined by the semi-cone angle ($\theta_c$). The simulation algorithm used here follows that of Fedchenia et al[23]. The unit vector representing the transition dipole moment of the fluorophore evolves according to:
\[ x_{i+1} = x_i + \xi_x \sqrt{D_k h} \]
\[ y_{i+1} = y_i + \xi_y \sqrt{D_k h} \]
\[ z_{i+1} = z_i + \xi_z \sqrt{D_k h} \]

where \( D_k \) denotes the diffusion constant, \( h \) is the time-step and \( \xi_j (j = x, y, z) \) are the Gaussian random numbers with a zero mean value and a standard deviation of one. The diffusing vector is normalised to unity in every time-step and if the vector in a diffusion step moves outside the barrier of the cone defined by the cone angle, it is reflected back in the tangential plane of the crossing point. The reflection requires that the incident and reflected angles are equal and the length of the diffusion step to be conserved.

### 6.1.2 Maier-Saupe Potential

Quite often one observes multi-exponential decays of the rotational correlation functions of a fluorophore attached to a macromolecule. The Maier-Saupe potential allows for a more flexible description of the reorientational dynamics of the chromophores. Furthermore the Maier-Saupe potential lacks the hard wall in the cone potential and it may therefore be more physically tractable. The potentials are defined by the Wigner rotation matrix elements\[24\]. The first two are given by:

\[ U_1(\beta) = \phi_1 P_1(\cos(\beta)) = \phi_1 \cos(\beta) \]
\[ U_2(\beta) = \phi_2 P_2(\cos(\beta)) = \frac{\phi_2}{2} (3\cos^2 \beta - 1) \]
\[ U(\beta) = U_1(\beta) + U_2(\beta) \] (6.2)

The \( \beta \)-angle is the angle between the unit vector describing the transition dipole and the symmetry axis of the potential. The unit vector is diffusing on a sphere influenced by a force determined by \( U(\beta) \).
6.1.3 Combined Potentials

A highly flexible model for reorientational motions can be achieved by combing two independent diffusion processes. For this purpose two trajectories $\{\hat{\mu}_1(t)\}$ and $\{\hat{\mu}_2(t)\}$ are created with the $U_i = \phi_i \cos(\beta)$ potential with different potential strength ($\phi$) and diffusion constants. The final trajectory is given by $\hat{\mu}(t) = \tilde{A}(\hat{\mu}_1(t))\hat{\mu}_2(t)$ where $\tilde{A}$ is the Euler orientational transformation matrix[25]. Even though a combination of different potentials have the ability to describe very complex reorientational motions, the combined potential method is preferable since it is simpler to incorporate in a minimising procedure.

6.2 Data Analysis

Following the procedure of the simulations any correlation function can be computed and the ensemble averages can be calculated over the number of trajectories in the simulations. Then $F_{VV}(t)$ and $F_{VH}(t)$ (or possibly $F_{MV}(t)$) are calculated. The major difference between DDEM and DAET/PDDEEM is that the summation curve (Eq. 4.15) is invariant to energy migration in DDEM. In DDEM $F_{VV}(t)$ and $F_{VH}(t)$ are given by:

$$F_{VV}(t) = f(t)[1 + 2r(t)]$$
$$F_{VH}(t) = f(t)[1 - r(t)]$$ \hspace{1cm} (6.3)

where $f(t)$ is the fluorescence relaxation curve obtained from a deconvolution of the summation curve determined in a separate experiment. Construction of the difference curve $\{d(t) = F_{VV}(t) - F_{VH}(t)\}$ is followed by a convolution with the instrumental response function:

$$D(t) = \int_0^t E(t - t')d(t')dt'$$ \hspace{1cm} (6.4)
$D(t)$ is then compared to the experimental difference curve \( \{D_{\text{exp}}(t) = I_{\text{vv}}(t) - I_{\text{vh}}(t)\} \) and a statistical reduced-$\chi^2$ test is performed:

$$\chi^2_r = \sum_{i=1}^{n} \left[ \frac{[D_{\text{exp}}(t) - D(t)]^2}{\sigma_i^2} \right]/(N - p + 1)$$

(6.5)

where $\sigma_i$ is the standard deviation of point (channel) $i$, $N$ is the number of data points and $p$ the number of parameters. Ideally, for a perfect fit, $\chi^2_r$ should be equal to one.

In a DAET experiment analysed using the EFT the fluorescence relaxation can not be separated as in Eq. 6.3. The difference curve for the donor (D) is actually given by:

$$d(t) = r(0)F_D \langle \chi(t) \rangle P_2 \langle \mu_D^g(0) \bullet \mu_D^g(t) \rangle$$

(6.6)

and the summation curve is:

$$s(t) = F_D \langle \chi(t) \rangle$$

(6.8)

where $F_D$ is the fluorescence relaxation of the donor alone. Since $\chi(t)$ is within the brackets of Eq 6.6, the anisotropy of the donor must depend on the rate of energy transfer. Note that we are only deriving the observables (or quantities constructed from observables) for the donor molecule. Additional information about the configuration could be obtained from the acceptor anisotropy. A particular interest is the correlation effect between the initially excited donor and the secondary excited acceptor (analogous to Eq. 5.24) which is collected in a separate depolarisation experiment on the acceptor fluorophore. In DDEM this effect in the anisotropy is mixed with each contribution from the directly excited donors. Therefore in a DAET experiment one has four different observables reporting on the same rate of energy transfer which opens the possibility to determine the configuration of the system together with the distance between the fluorophore.
7. Results and Discussion

7.1 Environmental Sensitive Probes Suitable for PDDEM

In the search for quantitative molecular information by means of energy transfer experiments, the choice of fluorescent molecules is important. In DDEM experiments the most appropriate fluorophores should be insensitive to the local physico-chemical conditions [26]. The probe should therefore ideally be chemically and photophysically invariant to the labelling position. While the latter usually is true for many fluorophores in solution, it appears less good for a fluorophore labelled to a macromolecule. If one extends the DDEM model to also include different photophysical properties, it turns out that the fluorescence rate of the total system contains distance information. Even if the photophysical difference must be great (with respect to the rate of energy migration) to extract a correct distance [15], it would lead to an error in the DDEM model which could be accounted for. In order to increase the sensitivity one can choose an environmental sensitive fluorophore. Such a fluorophore was studied in Paper I. N-(4,4-difluoro-1,3,5,7-tetramethyl-4-boro-3a,4a-diaza-s-indacene-2-yl) iodo-acetamid (BODIPY® 597/545 IA; NBDY) was characterised with respect to its photophysical properties. The results show that NBDY is more sensitive to different polar solvents than other sulfhydryl specific BODIPY derivatives. For example the Stokes shifts for NBDY in water and DMSO ranges between 29 nm and 51 nm. This implies different overlap integrals and Förster radii depending on the direction of energy transfer (\(\omega_{\alpha\beta} \neq \omega_{\beta\alpha}\)). However, the measured shifts for the four studied mutants of PAI-2 in 50 % w/w glycerol were not that different. One can here safely use the approximation that (\(\omega_{\alpha\beta} \approx \omega_{\beta\alpha}\)). It still remains uncertain whether the effects seen in different solvents can be observed also for NBDY in proteins.
7.2 Quenching of BODIPY in Proteins

The difference in fluorescence lifetimes of SBDY and NBDY at different labelling positions in plasminogen activator inhibitor type 2 (PAI-2) are not large enough to enable distance determinations by PDDEM. However, the fluorophores at various positions in proteins often display different accessibility to external quenchers like $\Gamma$\cite{1}. In Paper I and II, the labelled PAI-2 mutants were investigated whether the quenching enabled a quantitative PDDEM analyses. The Stern-Volmer plots of the SBDY labelled PAI-2 proteins in Fig. 7.1, show that the three mutants are quenched to quite different extent. The most difficult pair to use in a PDDEM experiment is 171cys/347cys, since both are effectively quenched by $\Gamma$. The optimal concentration is around 0.20 - 0.23 M $\Gamma$. The main disadvantage with the quenching method is that the observed fluorescence lifetime becomes shorter as the concentration of $\Gamma$ increases whereby the time-window for analyses is shortened (cf. Table 2 in Paper II).

![Fig. 7.1 Stern-Volmer plots showing $\Gamma$ quenching of three SBDY single labelled PAI-2 mutants. $F_0$ and $F$ is the fluorescence intensity before and after addition of quencher. The mutants are: 171cys PAI-2 (○), 347cys PAI-2 (□), 79cys PAI-2 (△).](image)

The negative curvature of 171cys and 79cys in Fig 7.1 is typically observed for fluorophores attached to proteins\cite{1}. SBDY covalently bonded to 171cys is effectively quenched by $\Gamma$ and this effect can be explained by the surface charge of PAI-2. A closer inspection of the amino acids in the vicinity of 171cys reveals two nearby positively charged lysine groups on each side of the labelled 171cys, which leads
to a locally higher concentration of \( \Gamma \). Quenching studies of NBDY labelled PAI-2 were also carried out under the same conditions as for SBDY. The result shows a similar behaviour for the three mutants as for SBDY but with the important exception of a much lower quenching efficiency. This effect might be explained by the shorter linker length of NBDY as compared to SBDY. The structure of NBDY and SBDY is displayed in Fig. 7.2.

Fig. 7.2. The chemical structure of NBDY and SBDY together with the structure of plasminogen activator inhibitor type 2. The three labelling positions are indicated by filled circles.

The mutated positions in PAI-2 were selected in order to position the CD-loop. Two double labelled mutants of PAI-2 (171cys/79cys, 347cys/79cys were constructed. These were analysed together with the single labelled mutants with the PDDEM model and PDDEM-depolarisation model. To avoid contribution to the anisotropy from reorientational motion of the protein 50 % w/w glycerol was added for the depolarisation experiments. The results are summerised in Table 7.1. NBDY gives shorter distances as is expected from the linker length and the distance 171cys/79cys is longer than 347cys/79cys. The isotropic dynamic average approximation was used for the PDDEM
model and $\langle \kappa^2 \rangle$ is calculated from Eq. 5.8 for the depolarisation measurements. The uncertainties in the absolute distances can be about 20%.

<table>
<thead>
<tr>
<th>System</th>
<th>Model</th>
<th>$R$ (Å)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SBDY in 79cys/171cys PAI-2</td>
<td>PDDEM</td>
<td>55.6</td>
</tr>
<tr>
<td>NBDY in 79cys/171cys PAI-2</td>
<td>PDDEM</td>
<td>51.7</td>
</tr>
<tr>
<td>NBDY in 79cys/171cys PAI-2</td>
<td>PDDEM depolarisation</td>
<td>48.5</td>
</tr>
<tr>
<td>SBDY in 79cys/347cys PAI-2</td>
<td>PDDEM</td>
<td>47.5</td>
</tr>
<tr>
<td>NBDY in 79cys/347cys PAI-2</td>
<td>PDDEM depolarisation</td>
<td>39.3</td>
</tr>
</tbody>
</table>

Table 7.1 The extracted distances from the PDDEM model obtained from between different mutations in plasminogen activator inhibitor type 2 (PAI-2). In the depolarisation experiments 50 % w/w glycerol was used.

7.3 Multi-exponential Relaxation of Photophysically Identical Donors is a Special Case of PDDEM

As mentioned earlier the DDEM model is only valid if the donors are photophysically identical with mono-exponential relaxation. Consider two donor molecules that couple with bi-exponential relaxation. The cross-coupling between the long and short lifetime fractions must then result in a shorter observed average lifetime. For two donors with mono-exponential relaxation, but with different fluorescence lifetimes, the total relaxation rate can not be faster than any of the individual ones. In Paper II the average lifetime of the unquenched SBDY labelled 171cys and 79cys is 5.5 ns for both. The corresponding double labelled PAI-2 had a markedly shorter average lifetime of about 4.8 ns.

This effect could potentially be misinterpreted as other self-quenching mechanisms[9] but it is nicely described by the PDDEM model. The effect was also observed for the second double labelled mutant. Because the 347cys has a relatively mono-exponential decay the effect is however not as pronounced as for the previous example (cf Table 2, Paper II). It is interesting to note that the quenching pattern and the fluorescence relaxation of 347cys are compatible.
7.4 Extended Förster Theory

The extended Förster theory addresses two important questions, which are related to energy transfer/migration experiments. The first, and maybe most important one, concerns the $\kappa^2$-problem which has frequently been discussed in literature. Upon analysing the experimental data, the extracted rate of energy transfer needs to be correctly weighted with the orientation factor in order to determine the distance between the chromophores. The second question has been less highlighted, namely the correlation between the reorientational dynamics and the energy transfer process. Consider for example a system where $\langle \kappa^2 \rangle$ is known (e.g. a true isotropic system where $\langle \kappa^2 \rangle = 2/3$). The parameter $\langle \omega \rangle$, determined from the experimental data, can be erroneously interpreted since it is only valid, which we have shown, in the initial part of the fluorescence relaxation decay of the donor in the coupled system. These two questions are studied in the Papers III-V and the results are presented in the following sections together with implications of the EFT. The questions raised are relevant for DDEM as well as DAET experiments, but their influence differs (Eqs. 5.16 and 5.24).

7.4.1 Approximations of EFT

The first and second order cumulant approximation of EFT is given by Eqs. 5.17 and 5.18. If one assumes that the $\kappa^2$-correlation function can be described by a single exponential function Eq. 5.18 reads as[19]:

$$\chi^{(2)} = \exp\left(\chi^2 \Delta \kappa^4 \tau_\kappa \{t + \tau_\kappa \left[\exp(-t/\tau_\kappa) - 1]\}\right)$$  

(7.1)

It is easy to test the validity of the two approximations by a comparison with Eq. 5.16. In the Paper IV synthetic data that mimics TCSPC experiment for a DAET system were generated using the EFT, these data were reanalysed with the EFT for 10 systems with high order ($S = 0.86$) and an isotropic system with the first- as well as the second order cumulant approximation. Each system was also studied at three different coupling strengths. The configurations were chosen to
cover most interesting cases. The results obtained for the systems are presented in Table 7.2.

<table>
<thead>
<tr>
<th></th>
<th>EFT All decay</th>
<th>1:cumulant VariableDecay</th>
<th>2:cumulant VariableDecay</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( A_{\text{true}} )</td>
<td>( \langle \kappa^2 \rangle )</td>
<td>( \Lambda_{\text{fit}} )</td>
</tr>
<tr>
<td>Isotropic</td>
<td>1.0 100</td>
<td>0.93</td>
<td>0.501</td>
</tr>
<tr>
<td></td>
<td>0.5 50</td>
<td>1.10</td>
<td>0.308</td>
</tr>
<tr>
<td></td>
<td>0.1 10</td>
<td>1.05</td>
<td>0.084</td>
</tr>
<tr>
<td>( \beta_1 = 25^\circ )</td>
<td>1.0 1.2</td>
<td>1.14</td>
<td>1.18</td>
</tr>
<tr>
<td>( \beta_2 = 40^\circ )</td>
<td>0.5 1.2</td>
<td>0.50</td>
<td>0.98</td>
</tr>
<tr>
<td>( \alpha = 0^\circ )</td>
<td>0.1 1.2</td>
<td>0.11</td>
<td>1.05</td>
</tr>
<tr>
<td>( \beta_1 = 85^\circ )</td>
<td>1.0 0.5</td>
<td>1.04</td>
<td>0.95</td>
</tr>
<tr>
<td>( \beta_2 = 79^\circ )</td>
<td>0.5 0.5</td>
<td>0.48</td>
<td>1.01</td>
</tr>
<tr>
<td>( \alpha = 135^\circ )</td>
<td>0.1 0.5</td>
<td>0.09</td>
<td>0.96</td>
</tr>
</tbody>
</table>

**Table 7.2.** Results recovered for three different DAET systems with three models. Each system was studied under three coupling strengths. The time-correlated single photon counting data was constructed with the extended Förster theory and a method proposed by Chowdhury et al.[27] to generate synthetic data that mimics a TCSPC experiment. In the variable decay the fitting range was decreased until a satisfactory fit was achieved (\( \chi^2 < 1.2 \)) or at least 1 ns. In the calculation of \( \Lambda \) for the two approximate models a value of 2/3 was used for \( \langle \kappa^2 \rangle \). \( \Lambda \) is in units of ns\(^{-1}\). The fluorescence relaxation was taken to be a single exponential with a lifetime of 10 ns.

For all systems studied the EFT theory successively recovers the correct coupling strengths. The first cumulant approximation (Eq. 5.17) usually gives an overall bad fitting and large errors. An approach where the fitting range were decreased until a good fit was obtained or at least 1 ns long was applied (variable decay) and the results improved to some extent. The reason for this can be assigned to the fact that the first cumulant is only valid in the initial fluorescence relaxation decay of the donor. The remaining error can be assigned to the erroneously chosen value of \( \langle \kappa^2 \rangle = 2/3 \). Obviously the goodness of the fits is correlated with a more correctly determined value of \( \Lambda \). The second cumulant approximation shows better results than the first cumulant. Especially for the case of isotropic order the model is promising. This can be explained by the fact that \( \langle \kappa^2 \rangle, \tau_{\kappa} \) and \( \Delta \kappa^4 \) are all known and fixed parameters in the analysis if the reorientational rate was previously determined for the donor and acceptor in separate
experiments. For ordered systems the error originates from an inherent inability of the models to correctly account for the \( \langle \kappa^2 \rangle \)-factor. Since the approximation of \( \langle \kappa^2 \rangle = \frac{2}{3} \) is so frequently used, the question arises how representative this approximation is. Fluorophores labelled to proteins usually exhibit high local order with order parameters of about 0.7-0.9[5, 13, 28]. Consequently there conditions are far from isotropic. But can \( \langle \kappa^2 \rangle = \frac{2}{3} \) still be used? To further investigate this question, the \( \langle \kappa^2 \rangle \)-distribution function was analysed. For spherically symmetric distributions the dynamic average \( \kappa^2 \)-value can be calculated by:[10, 12]:

\[
\langle \kappa^2 \rangle = \frac{2}{3} + \frac{2}{3} S_d S_D S_A \\
+ \frac{2}{6} \left[ (3 \cos^2 \beta_D - 1) S_D + (3 \cos^2 \beta_A - 1) S_A \right] \\
+ \frac{1}{3} \left( 3 \cos^2 \beta_D - 1 \right) S_D \left( 3 \cos^2 \beta_A - 1 \right) S_A \\
- 6 \sin \beta_D \cos \beta_D \sin \beta_A \cos \beta_A S_D S_A \cos \alpha
\]

\( P(\langle \kappa^2 \rangle) \) was then constructed for several systems with different local order, assuming that all configurations have equal probability. The result is displayed in Fig 7.3. Interestingly one finds that the probability has a rather sharp peak that moves towards higher \( \langle \kappa^2 \rangle \) as the order decreases. Overall the probability is about 50 % to either be above or below \( \frac{2}{3} \) for all systems. Hence having a configuration that corresponds to \( \langle \kappa^2 \rangle \approx \frac{2}{3} \) is very low for highly ordered fluorophores.
Fig. 7.3. The dynamic $\kappa^2$-distribution function is shown for several systems with different order. In the insert a magnification from 0.0 – 0.8 is displayed. The figure assumes equal probability for all configurations. The limits can be calculated through Eqs 5.1-5.2.

7.4.2 EFT Analysis of TCSPC Data

To model the stochastic behaviour of $\kappa^2(t)$ in the EFT, the reorientational motion of the fluorophores is needed in the form of trajectories. In order to determine the stochastic trajectories from experimental data we have used Brownian dynamic simulation, which is described in section 6. Therefore fluorescence depolarisation data for each fluorophore is necessary. The parameters to extract from the depolarisation data on the coupled system (i.e. the macromolecule labelled with two fluorophores) to describe the energy transfer process are the coupling strength ($\Lambda$) and the three configuration angles ($\beta_1, \beta_2, \alpha$). In the simulation minimising routine these are used as
floating parameters. Since four parameters usually are more than enough to fit a single decay we also applied a different method in Paper IV and V where the whole the configuration space is investigated for a range of $\Lambda$ values. To decrease the computer power necessary in the analysis independent information was obtained from the residual anisotropies for the individual fluorophores, as well as from the coupled system from which the $\delta$-angle can be calculated. In a DAET experiment the dynamic average rate of energy transfer ($\langle \omega \rangle$) can also be obtained from an analytic fit to the initial slope of the fluorescence relaxation decay of the donor. An alternative approach in the analyses of DDEM data would be to perform a short simulation of the initial decay for each configuration tested and then calculate the goodness of fit. If an acceptable fit is obtained, the simulation is continued for the whole decay. By combining the $\langle \omega \rangle$-value, with Eq. 7.1 and the relation:

\[
\cos \delta = \cos \alpha_{DA} \sin \beta_D \sin \beta_A + \cos \beta_D \cos \beta_A
\]

\[
\left| \beta_D - \beta_A \right| \leq \delta \\
\left| \beta_D + \beta_A \right| \geq \delta
\]  

(7.2)

the allowed configuration space can be reduced considerably. The lowest obtained $\chi^2$-value in the configuration space is then plotted as a function of the coupling strength ($\Lambda$) i.e. the distance ($R$). This procedure was applied to the synthetic data defined in Table 7.2, and the results are displayed in Figs 7.4-7.5. By including independent information about the $\delta$-angle a narrower range of possible solutions is observed. Panel B in Fig 7.4 also show a second acceptable solution. This difference between A and B can be explained by the higher Kubo number in A ($\vartheta = 4.2$) as compared to B ($\vartheta = 1.5$). Even though both configurations correspond to the same coupling strength, the values of the $\kappa^2$-correlation time ($\tau_\kappa$) and the variance ($\Delta \kappa^2$) are different. If the $\delta$-angle is unknown a unique solution can not be obtained. The range of uncertainty in the distance in Fig 7.4 A and B typically correspond to about 5 – 7 Å. One should notice that the maximum and minimum values of $\Lambda$ calculated by the method proposed by Dale et al[10] (Eqs.
5.1 and 5.2) are in the range of 12 - 0.36 ns\(^{-1}\) (A) and 5.0 – 0.15 ns\(^{-1}\) (B).

Fig. 7.4. The photophysics decay for the donor molecule in a DAET experiment for the two ordered systems presented in Table 7.2. were analysed where the lowest \(\chi^2\) obtained in the configuration space is plotted as a function of \(\Lambda\). In Panel A the true configuration is \(\beta_1 = 25^\circ, \beta_2 = 40^\circ, \alpha = 0^\circ, \delta = 15^\circ\) and B corresponds to \(\beta_1 = 83^\circ, \beta_2 = 79^\circ, \alpha = 135^\circ, \delta = 132^\circ\) and the correct value of \(\Lambda\) is 1.0 ns\(^{-1}\). For the line with solid triangles (▲) the \(\delta\)-angle is assumed to be unknown and known for the line with empty circles (●). 1.000,000 trajectories were used in the generation of synthetic data and 100,000 in the re-analysis. A resolution of 1\(^\circ\) was used to span the configuration space.

The information content in the fluorescence relaxation decays obtained for two coupling chromophores, effectively decreases as the \(\vartheta\) number decreases. For \(\vartheta << 1\) the first cumulant approximation is valid. This is illustrated in Table 7.2 The systems with the lowest Kubo number (\(\vartheta = 0.15\)) gives a fit with \(\chi^2 = 1.28\) (Fig. 7.5 D). The effect of decreasing the Kubo number is also seen in Figs. 7.4 and 7.5. In Fig. 7.5 the corresponding configurations as in Fig. 7.4 is shown for weaker coupling, and the assumption that the \(\delta\)-angle is known. The effect of a decreased Kubo number on the information content is even more evident in Fig 7.5 B where the second “false” solution in Fig 7.4 A,
Fig. 7.5. The coupling strength is shown as a function of the lowest obtainable $\chi^2$ value that can be found when all of the configurational space investigated. The donor fluorescence decay were analysed in a DAET experiment. Panel A and B corresponds to the configuration $\beta_1 = 25^\circ$, $\beta_2 = 40^\circ$, $\alpha = 0^\circ$, $\delta = 15^\circ$ and $\beta_1 = 83^\circ$, $\beta_2 = 79^\circ$, $\alpha = 135^\circ$, $\delta = 132^\circ$, respectively. The true value of the coupling strength were for A and C, $\Lambda = 0.5$ and for B and D, $\Lambda = 0.1$.

reaches an acceptable fit. One can easily imagine the difficulties in applying a normal minimising routine to a system with several minima’s or with a range of equally low $\chi^2$. Applying the minimising method proposed here opens the possibility not only to determine the distance, but also to test the uniqueness of the extracted distance. An alternative interpretation is the ability of the configuration to adapt for an erroneously chosen value on $\Lambda$. The traditional Förster theory has no potential to separate the product $\Lambda \langle \kappa^2 \rangle$. This is the so called $\kappa^2$-problem which now can be solved using the EFT. However, when applying the EFT on real experimental data, the accuracy in the analysis might be blurred due to experimental errors beyond the Poisson statistics which is characteristic for a TCSPC measurement.
To further improve the information content in an experiment, it was suggested in Paper III that one should collect experimental data under various physico-chemical conditions and to analyse the data in a global manner to help in stabilising the results. The main idea is to change the rate of reorientational motions of the fluorophores and perhaps also the local order, while the configuration and coupling strength is assumed to be the same. Hence, one would have more experimental data reporting on the rate of energy transfer without having more floating parameters in the analysis. The key factor in such an approach is the Kubo number (Eq. 5.20) which consist of $\Lambda$, $\tau_\kappa$ and $\Delta \kappa^d$. Since $\Lambda$ is constant, $\tau_\kappa$ and $\Delta \kappa^d$ are the only parameters left to modulate the energy transfer rate. $\Delta \kappa^d$ is a static parameter property that depends only on the local order.

In Fig 7.6 $(\Delta \kappa^d)^{0.5}$ is shown as a function of the order parameter ($S$) for three different configurations. For some configurations the effect can be large for a small change in the local order if the order is high. For some configurations, a change of the physico-chemical conditions would then further stabilise the analysis. The last term in the Kubo number that needs to be considered is $\tau_\kappa$. In Fig 7.7, values of $\tau_\kappa$ are calculated for some selected configurations which correspond to the possible range in $(\Delta \kappa^d)^{0.5}$ for an order parameter of $S = 0.75$. 

![Graph showing variance in stochastic fluctuations of $\kappa^2(t)$ calculated by $\{\langle \kappa^4 \rangle - \langle \kappa^2 \rangle^2\}^{0.5}$](image)

**Fig. 7.6.** The variance in the stochastic fluctuations of $\kappa^2(t)$ calculated by $\{\langle \kappa^4 \rangle - \langle \kappa^2 \rangle^2\}^{0.5}$. The configurations are: $\beta_1 = 25^\circ$, $\beta_2 = 25^\circ$, $\alpha = 0$ (●), $\beta_1 = 25^\circ$, $\beta_2 = 70^\circ$, $\alpha = 65$ (■), $\beta_1 = 90^\circ$, $\beta_2 = 90^\circ$, $\alpha = 90$ (▲).
There seems to be a correlation between $\tau_\kappa$ and $(\Delta \kappa^4)^{0.5}$ implying that the effect of $(\Delta \kappa^4)^{0.5}$ on the Kubo number is further increased by $\tau_\kappa$. The success of an analysis where the physico-chemical conditions are changed will be dependent on the true configuration which of course is not known a priori.

### 7.4.3 Application of the EFT on a Protein System

The EFT was for the first time applied to a real protein system in Paper V. Mutant forms of the protein plasminogen activator inhibitor type 1 (PAI-1) were labelled at three different positions with the environmentally insensitive fluorophores (SBDY). These position form the corners of a triangle, which side lengths are known from the X-ray structure of PAI-1[29]. Six different mutants (three double labelled and three single labelled) were analysed at nine different physico-chemical conditions (50, 25 and 0 w/w % glycerol at 277, 288 and 298 K). A combination of two Maier-Saupe potentials (section 6.1.2-6.1.3) was used to determine the reorientational potential for each mono-labelled mutant (V106C, H185C and M266C). The double labelled proteins were then analysed individually by the EFT-DDEM model (Eqs. 5.22 – 5.24) for each dataset and in a global manner. The configuration and...
the coupling strength were assumed to be the same for all nine different conditions. The diffusion and order parameters for V106C and H185C showed a small dependency on the different viscosity and temperature conditions. Therefore one should not expect too much of the global analysis, compared to the individually analysed data sets, for the corresponding double labelled mutant. The extracted distances (Table 1, Paper V) were all in agreement with the distances from X-ray structure for both individual and global analyses. The larger uncertainty in the extracted configuration compared to the distance that were seen in the reanalysis of synthetic data in Paper III was also observed between the extracted parameters for the different physico-chemical conditions. By applying the analysis method discussed in the previous section a single solution was obtained in the analysis that agreed with the results from the minimising routine based on the Levenberg-Marquardt algorithm. An example of such an analysis is shown in Fig 7.8. Even though a minimum is found, the variation of $\chi^2$ with distance is rather small within the limits determined by the order parameters (Eq. 5.1-5.2). However we believe that the variation is sufficient enough to more accurately determine the distance as compared to the order parameter method for which the uncertainty is shown as the limits on x-axis in the inset of Fig 7.8.

**Fig. 7.8.** The lowest $\chi^2$-value is plotted as a function of distance when all the configurations were investigated. A magnification over the limits determined by the order parameters is shown in the inset. Data corresponds to V106C-M266C at 277 K without Glycerol.
8. Conclusions and Future Aspects

Recent developed theories such as the PDDEM and the EFT-DDEM have been applied on real protein systems. A generalisation of the DDEM model to include also photophysically different fluorophores and molecular reorientation on the same timescale as the rate of energy migration energy migration, increases the informational contents in the experimental data. Numerical simulations of energy transfer based on the EFT shows that the long-lived “$\kappa^2$-problem” is solved. The shape of the fluorescence relaxation of a coupled system between two fluorophores was shown to provide information about the mutual configuration in addition to the energy transfer rate. To further stabilise the analysis it was suggested to collect data under various physico-chemical conditions in order to change the effect of reorientational motion and local order on the rate of energy transfer. The particular systems studied here showed little dependence on the physico-chemical conditions. Therefore further studies on others systems where the fluorophores are more sensitive to a change in physico-chemical conditions needs to be carried out.

The reorientational motions of the donors were not included in the theoretical treatment of PDDEM. This can be done in a similar way as for EFT-DDEM to gain EFT-PDDEM. Hereby many of the approximations used in the previous theories can be overcome but more important also used to strengthen the models.

Throughout this thesis the distance between the fluorophores was considered to be constant. Models of static and dynamic distributions of distances can be included in the EFT. The question as to whether this information can be separated from $\kappa^2(t)$ still remains to be answered and needs further studies. The effect of distance distributions in the experimental systems studied here is effectively accounted for by $\kappa^2(t)$. 

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10. References


