Electronic Energy Migration/Transfer as a Tool to Explore Biomacromolecular Structures

Therese Mikaelsson
Experience is a brutal teacher, but you learn. My God, do you learn!

/ C.S. Lewis
Abstract

Fluorescence-based techniques are widely used in bioscience, offering a high sensitivity and versatility. In this work, fluorescence electronic energy migration/transfer is applied to measure intramolecular distances in two types of systems and under various conditions.

The main part of the thesis utilizes the process of donor-acceptor energy transfer to probe distances within the ribosomal protein S16. Proteins are essential to all organisms. Therefore, it is of great interest to study protein structure and function in order to understand and prevent protein malfunction. Moreover, it is also important to try to study the proteins in an environment which resembles its natural habitat. Here two protein homologs were investigated; S16Thermo and S16Meso, isolated from a hyperthermophilic bacterium and a mesophilic bacterium, respectively. It was concluded that the chemically induced unfolded state ensemble of S16Thermo is more compact than the corresponding ensemble of S16Meso. This unfolded state compaction may be one reason for the increased thermal stability of S16Thermo as compared to S16Meso.

The unfolded state of S16 was also studied under highly crowded conditions, mimicking the environment found in cells. It appears that a high degree of crowding, induced by 200 mg/mL dextran 20, forces the unfolded state ensemble of S16Thermo to become even more compact. Further, intramolecular distances in the folded state of five S16 mutants were investigated upon increasing amounts of dextran 20. We found that the probed distances in S16Thermo are unaffected by increasing degree of crowding. However, S16Meso shows decreasing intramolecular distances for all three studied variants, up to 100 mg/mL dextran. At higher concentrations, the change in distance becomes anisotropic. This suggests that marginally stable proteins like s16Meso may respond to macromolecular crowding by fine-tuning its structure. More stable proteins like S16Thermo however, show no structural change upon increasing degree of crowding.

We also investigated the possibility of local specific interactions between the protein and crowding agent, by means of fluorescence quenching experiments. Upon increasing amounts of a tyrosine labelled dextran, a diverse pattern of fluorescence quantum yield and lifetime suggests that specific, local protein-crowder interactions may occur.

In a second studied system, electronic energy migration between two donor-groups, separated by a rigid steroid, was studied by two-photon excitation depolarization experiments. Data were analysed by using recent advances, based on the extended Förster theory, which yield a reasonable value of the distance between the two interacting donor-groups. To the best of our knowledge, this is the first quantitative analysis of energy migration data, obtained from two-photon excited fluorescence.


Huvuddelen av avhandlingen tillämpar elektronisk energiöverföring för att utforska delar av strukturen inom protéinen S16. Proteiner utgör en essentiell del av


Denna avhandling visar att elektronisk energiöverföring kan tillämpas för att studera proteiner i olika typer av miljöer. Man får då direkt information om hur avstånd förändras under olika förhållanden. Vi har även visat att crowding kan ge olika effekter hos proteiner och man bör därför ta hänsyn till miljön som det protein man studerar befinner sig i.
Table of Contents

List of Papers vii
List of Abbreviations ix
1. Introduction 1
2. Theoretical Considerations 3
   2.1 Absorption 3
   2.2 Emission 3
   2.2.1 Fluorescence Lifetime 4
   2.2.2 Fluorescence Quantum Yield 5
   2.2.3 Fluorescence Quenching 5
   2.2.4 Fluorescence Anisotropy 6
   2.3 Electronic Energy Transfer and Migration 8
   2.3.1 Donor-Acceptor Energy Transfer 8
   2.3.2 Donor-Donor Energy Migration 10
   2.4 Two-Photon Excitation 11
   2.4.1 TPE Anisotropy 11
   2.4.2 TPE and DDEM 11
   2.5 CD-Spectroscopy 12
   2.5.1 Equilibrium Unfolding Monitored by CD 12
   2.6 Macromolecular Crowding 13
3 Results and Discussion 15
   3.1 TPE Donor Donor Energy Migration 15
   3.1.1 Model Bisteroids 15
   3.1.2 Perylenyl Bisteroids 16
   3.1.3 Anthryl Bisteroids 18
   3.2 Donor Acceptor Energy Transfer 21
   3.2.1 Model System: Ribosomal Protein S16 21
   3.2.2 Tryptophan – BODIPY 22
   3.2.3 Thermostability of S16 23
   3.2.4 S16 in Presence of Crowding 26
      3.2.4.1 Equilibrium Unfolding 27
      3.2.4.2 Unfolded State Compaction 29
      3.2.4.3 Folded State Effects 32
      3.2.4.4 Fluorescence Anisotropy and Viscosity 33
      3.2.4.5 Quantum Yield Measurement 36
4 Conclusions 43
5 Future Perspectives 45
6 Acknowledgements 47
7 References 49
List of Papers

The thesis is based on the following papers:

I. Oleg Opanasyuk, Therese Mikaelsson, Linus Ryderfors, Emad Mukhtar and Lennart B.-Å. Johansson
   *On the analyses of fluorescence depolarization data in the presence of electronic energy migration. Part II: Applying and evaluating two-photon excited fluorescence.*
   Physical Chemistry Chemical Physics, 2012, 14, 1917 – 1922
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II. Marcus Wallgren, Jörgen Ådén, Olena Pylypenko, Therese Mikaelsson, Lennart B.-Å. Johansson, Alexey Rak and Magnus Wolf-Watz
   *Extreme Temperature Tolerance of a Hyperthermophilic Protein Coupled to Residual Structure in the Unfolded State.*
   Journal of Molecular Biology, 2008, 379, 845 – 858

III. Therese Mikaelsson, Jörgen Ådén, Lennart B.-Å. Johansson and Pernilla Wittung-Stafshede
   *Direct Observation of Protein Unfolded State Compaction in the Presence of Macromolecular Crowding*
   Biophysical Journal, 2013, 104, 694 – 704

IV. Therese Mikaelsson, Jörgen Ådén, Pernilla Wittung-Stafshede and Lennart B.-Å. Johansson
   *Effects of Macromolecular Crowding in Folded Protein Homologs: Global Versus Local Consequences*
   Submitted

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Therese Mikaelsson contributed to the above mentioned work according to:

Paper I: Performed sample preparations and one- and two-photon experiments.

Paper II: Performed steady-state and time-resolved fluorescence measurements together with distance experiments by means of DAET. Assisted in writing parts of the manuscript.

Paper III: Performed all fluorescence and CD experiments as well as the analyse, with the exception for kinetic experiments. Assisted in writing the manuscript.

Paper IV: Performed all experiments and analyse, except for NMR and chemical stability experiments. Wrote most of the manuscript.
## List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>A</td>
<td>Acceptor of electronic energy</td>
</tr>
<tr>
<td>BODIPY</td>
<td>N-((4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-yl)methyl)iodoacetamide</td>
</tr>
<tr>
<td>CD</td>
<td>Circular Dichroism</td>
</tr>
<tr>
<td>D</td>
<td>Donor of electronic energy</td>
</tr>
<tr>
<td>DAET</td>
<td>Donor-acceptor energy transfer</td>
</tr>
<tr>
<td>DDEM</td>
<td>Donor-donor energy migration</td>
</tr>
<tr>
<td>EFT</td>
<td>Extended Förster theory</td>
</tr>
<tr>
<td>FRET</td>
<td>Fluorescence resonance energy transfer</td>
</tr>
<tr>
<td>$F(t)$</td>
<td>Time-resolved fluorescence intensity</td>
</tr>
<tr>
<td>$\eta$</td>
<td>Viscosity</td>
</tr>
<tr>
<td>$\lambda$</td>
<td>Wavelength</td>
</tr>
<tr>
<td>$n$</td>
<td>Refractive index</td>
</tr>
<tr>
<td>OPE</td>
<td>One-photon excitation</td>
</tr>
<tr>
<td>$R$</td>
<td>Distance between interacting chromophores</td>
</tr>
<tr>
<td>$R_0$</td>
<td>Förster radius</td>
</tr>
<tr>
<td>$r(t)$</td>
<td>Time-resolved fluorescence anisotropy</td>
</tr>
<tr>
<td>$\sigma$</td>
<td>Variance</td>
</tr>
<tr>
<td>TCSPC</td>
<td>Time correlated single photon counting</td>
</tr>
<tr>
<td>TPE</td>
<td>Two-photon excitation</td>
</tr>
<tr>
<td>Trp</td>
<td>Tryptophan</td>
</tr>
<tr>
<td>Tyr</td>
<td>Tyrosine</td>
</tr>
<tr>
<td>UV</td>
<td>Ultra violet</td>
</tr>
<tr>
<td>Vis</td>
<td>Visible light</td>
</tr>
<tr>
<td>$Z_M$</td>
<td>Meso form of protein S16</td>
</tr>
<tr>
<td>$Z_T$</td>
<td>Thermo form of protein S16</td>
</tr>
</tbody>
</table>
1. Introduction

Today fluorescence spectroscopy and microscopy are among the most commonly used experimental techniques in bioscience. One of its advantages is the extraordinarily sensitivity, which even enables experiments at a single molecular level. Methods based on fluorescence are also widely used for the visualization of processes in biological tissues and cells. Frequently, fluorescence spectroscopy is used to determine inter- and intramolecular distances between interacting chromophores. This method is commonly referred to as fluorescence resonance energy transfer (FRET). It is based on a theory by Förster [1] and its potential as a “spectroscopic ruler” was first tested by Stryer and Haugland [2] in 1967. They showed that it is possible to determine distances between two chromophores attached to a rigid peptide. One of the chromophores (the donor) absorbs light and transfers this energy to the second chromophore (the acceptor). By measuring the rate of energy transfer, it is possible to estimate the distance between the donor and acceptor molecule.

The classical Förster theory [1] has been used in numerous studies. Unfortunately, this theory neglects the influence of reorientation and spatial motions of the interacting molecules. These approximations are in many cases invalid but can be overcome by an extended Förster theory (EFT) [3]. This approach however, is rather complex and involves time-consuming data analyses. The analyses involve a description of reorientational restrictions and dynamics of the interacting chromophores. To determine relevant molecular parameters, a general approach has been derived [4]. This includes the advantage of using fluorescence depolarisation experiments based on one-photon excitation (OPE), as well as the less common two-photon excitation (TPE). OPE and TPE experiments provide independent information about the reorienting motions [5, 6] and energy migration processes [7]. In this thesis, Paper I shows how to apply the analyses on experimental TPE depolarisation data.

The main part of the thesis aims at using donor-acceptor energy transfer (DAET) as a tool to investigate the intramolecular structure of the ribosomal protein S16. Proteins play an important role in bioprocesses. They are essential for all organisms and participate in basically every process in cells. Proteins are macromolecules that consist of long peptide chains of amino acids. These adopt specific three-dimensional structures to become biologically active proteins. The structure is crucial for the specific function of the protein [8]. Hence, if a protein structure is perturbed, the protein
activity and function may be influenced. For instance, many diseases are connected with protein misfolding and aggregation [9] (e.g. Alzheimer’s, Huntington’s and Parkinson’s disease). Hence, in order to prevent and understand protein misfolding, it is informative to study their structure and function. During the last three decades, significant progress has been achieved in understanding mechanisms and driving forces behind protein folding [10, 11]. However, most of these achievements are obtained from dilute buffer solutions. But proteins fold inside cells, where the environment is highly crowded due to the presence of large amounts of macromolecules, such as other proteins, nucleic acids, ribosomes and carbohydrates [12]. This means an increased viscosity and excluded volume, as well as the opportunity for specific and nonspecific interactions. To mimic crowded environments in \textit{in vitro} experiments, one may add so called macromolecular crowding agents. These are synthetic or natural non-charged, inert polymers of defined size which occupy space (e.g. dextrans and Ficoll).

One advantage of FRET is its applicability under physiological conditions [13, 14]. This makes it a useful tool for investigating changes in the structure of proteins. In Paper II – IV we applied intramolecular DAET to probe distances within two homologs of the model protein S16. Paper II demonstrate that the structural ribosomal protein S16, isolated from a mesophilic bacterium (S16\textsubscript{Meso}), show much lower thermal stability than that isolated from a hyperthermophilic bacterium (S16\textsubscript{Thermo}). Therefore we investigate whether the difference in thermostability can be traced to a difference in the persistent structure of the unfolded state ensemble. The unfolded state ensemble of S16 is further investigated in Paper III. Here, a large amount of the crowding agent dextran 20 was added to the samples, in order to mimic a naturally crowded environment. By means of DAET and far-UV circular dichroism (CD) we have studied the folded and unfolded state ensemble of S16 in presence and absence of macromolecular crowding. Finally, Paper IV is a continuation of studying the influence of crowding on the folded state of S16\textsubscript{Thermo} and S16\textsubscript{Meso}. This is achieved by probing several distances at increasing amounts of dextran 20. This study also investigate the possibility of specific interactions between the protein and the crowding agent, by means of fluorescence quenching experiments.
2. Theoretical Considerations

2.1 Absorption

The human eye detects white light as a uniform colour, even though the radiation is actually composed of a broad range of wavelengths e.g. covering the ultraviolet (UV), visible (Vis) and infrared (IR) region. When we observe different colours, we function as a spectrometer, analyzing the light which is reflected by a solid or passing through a liquid. The colour of a material is determined by its molecular composition and originates from electronic transitions between different electronic states in the molecule. If an external electromagnetic field can drive such transitions depends on a quantity called the electronic transition dipole moment. The transition dipole moment is a vector, which describes how electric charge within a molecule is redistributed during the excitation. If the energy in the radiation is sufficient to excite an electron to a higher energy state, the radiation is absorbed and corresponds to the energy difference between the two states. This event is commonly described by a Jabłoński diagram [15], Fig. 1, which illustrates some of the electronic states of a molecule and the possible transitions between them. The absorption of radiation (A) at a certain wavelength (λ) is related to the concentration, [C], of the absorbing species and the path length of the cell (l) according to Lambert-Beer’s law, see e.g. [16]:

\[ A(\lambda) = \log_{10} \frac{I_0(\lambda)}{I(\lambda)} = \varepsilon(\lambda)[C]l \] (1)

Here \( I_0 \) and \( I \) denote the incident and the transmitted radiation, respectively. The molar absorptivity coefficient (\( \varepsilon \)) depends on the wavelength of the incident radiation and reaches its maxima for the most probable transition. A molecule capable of absorbing light is called a chromophore.

2.2 Emission

After electronic excitation of a molecule (i.e. the absorption of electromagnetic radiation), the energy can be released by various inter- and intramolecular processes. Most commonly, energy is released as a disordered thermal motion into the surroundings, a process known as internal conversion. Another pathway is radiative emission, in which a photon is released when the electron returns to the ground state. In a condensed phase the emitted radiation is of longer wavelengths because energy is partly lost to
the surroundings. This process is known as vibrational relaxation and occurs typically within $10^{-12}$ s [17]. There are two principal types of radiative decay, fluorescence and phosphorescence. In fluorescence, the electron in the excited orbital is paired with the second electron in the ground-state orbital. The two electrons are of opposite spin and the return to ground state, by the emission of a photon, is rapid ($10^{-8}$ s) because it is spin-allowed. In phosphorescence, the molecule undergoes intersystem crossing to the triplet state and the electron in the excited state has the same spin as the electron in the ground-state. Here, the transition back to the ground-state orbital is less likely and the emission is much slower ($10^{-3} - 10^{2}$ s). Electronic relaxation can also occur through interaction with another molecule, a process known as quenching. Electronic energy transfer is one type of quenching and has been applied in this thesis.

![Figure 1. Jabłoński diagram, illustrating the electronic states of a donor and acceptor molecule, as well as the possible intra- and intermolecular transitions of the donor. Possible relaxation paths of the acceptor are also shown.](image)

### 2.2.1 Fluorescence Lifetime

The fluorescence lifetime describes an average residence time of a chromophore in the excited state (before emitting a photon). It is an intrinsic molecular property and is not affected by the concentration of the sample. The decay of the excited state of a molecule is described by:

$$F(t) = F_0 \exp(-t/\tau) \quad (2a)$$

where $F_0$ is the initial intensity and the fluorescence lifetime, $\tau$, is described by the rate constants according to:
where \( k_r \) and \( k_{nr} \) are the radiative and non-radiative rate constants. The above described fluorescence decay obeys first order kinetics, while in practice can be more complex. The lifetime in the absence of non-radiative processes defines the intrinsic or natural lifetime, \( \tau_n \).

### 2.2.2 Fluorescence Quantum Yield

The efficiency of fluorescence is described by the quantum yield (\( \phi \)). This is the ratio of the number of photon emitted to the number of photon absorbed. The quantum yield is frequently determined by the use of a reference substance (ref) with a known quantum yield according to:

\[
\phi = \phi_{ref} \frac{F}{F_{ref}} \frac{[1 - \exp(-A_{ref} \ln 10)] n^2}{[1 - \exp(-A \ln 10)] n_{ref}^2} \tag{3}
\]

Here \( F, A \) and \( n \) stands for the integrated fluorescence intensity, the absorbance at the wavelength of excitation and the refractive index of the medium, respectively.

### 2.2.3 Fluorescence Quenching

Fluorescence quenching means a process by which the fluorescence intensity is decreased. There are several processes that cause quenching, such as collisional quenching, excited state reactions, energy transfer and ground state complex formation. The quenching process may occur during the excited state lifetime (dynamical quenching), or due to the formation of a complex prior to excitation (static quenching). Both types of quenching require orbital contact between the two interacting species. Quenching data are usually presented in a Stern-Volmer plot. Then \( F_0/F \) is plotted versus \([Q]\), where \( F_0 \) and \( F \) are the fluorescence intensities in absence and presence of quencher and \([Q]\) is the concentration of quencher. The shape of the Stern-Volmer graph yields information about quenching processes. A linear Stern-Volmer plot indicates that all fluorophores are equally accessible to quenching. If there are two populations of fluorophores where one is not accessible to quencher, the Stern-Volmer plot deviates from linearity towards the x-axis (i.e. \([Q]\)). If the plot deviates towards the y-axis (\( F_0/F \)) on the other hand, the fluorophore most likely undergoes both dynamic and static quenching [17]. Dynamic and static quenching can be distinguished by
lifetime measurements or by their different dependence on temperature and viscosity.

### 2.2.4 Fluorescence Anisotropy

The emission from a sample of fluorophores, excited with linearly polarised light, is usually partly polarised. This is because molecules with their electronic transition dipole oriented along the direction of the electric vector of light are preferentially excited (photoselection). Therefore an anisotropic excited state population is formed, which is uniaxially oriented about the electric field vector. Depolarisation of the emission from this distribution may subsequently occur due to processes that occur during the lifetime of the excited state. These processes are e.g. molecular reorientation and electronic energy transfer. The degree of emission polarisation is frequently described in terms of the fluorescence anisotropy ($r$). The principal setup for measurements of anisotropy is illustrated in Fig. 2. The sample is excited by vertically polarised light and the following emission is monitored with the emission polariser oriented vertically ($F_{VV}$) and horizontally ($F_{VH}$). The anisotropy is defined by and calculated from:

$$r = \frac{F_{VV} - F_{VH}}{F_{VV} + 2F_{VH}}$$

(4)

The anisotropy is a product of photoselection (reducing $r$ by $2/5$) and the angular displacement between the absorption and emission dipoles ($\delta$) at the time of excitation and emission. In absence of any additional processes leading to depolarisation, the average anisotropy can be described by:

$$r = \frac{2}{5} \left( \frac{3\langle \cos^2 \delta(t) \rangle - 1}{2} \right)$$

(5)

The influence of reorientational motion can be resolved by time-resolved anisotropy, which is generally modelled by a multiexponential decay:

$$r(t) = \sum_i r_i \exp(-t/\theta_i)$$

(6)

Here, $r_i$ are the fractional amplitudes associated with the correlation times $\theta_i$. The shape of the anisotropy decay is determined by overall macromolecular rotation, as well as internal motions in the macromolecule.
Figure 2. Schematic of the experimental setup for fluorescence TCSPC measurements. Excitation polariser is set to vertical and emission polariser is set at magical angle (54.7 ° [17]) for lifetime measurements. For depolarisation experiments, the emission polariser is varied between vertical (Fvv) and horizontal (Fvh) settings.

The time-resolved fluorescence depolarisation data in Paper III were analyzed according to the following model:

\[ r(t) = r(0) \sum_i r_i \exp\left(-\frac{t}{\theta_i}\right) + r_\infty \exp\left(-\frac{t}{\Theta_{\text{slow}}}\right) \]  

Here the initial anisotropy is \( r(0) \leq r_0 \), where \( r_0 \) is the fundamental anisotropy [18, 19]. \( r_\infty \) denotes the local residual anisotropy, \( \Theta_{\text{slow}} \) describes the rotational correlation time of the protein and \( \theta_i \) are local, fast correlation times (internal motions) that together form one fast effective correlation time \( \Theta_{\text{fast}} \) :

\[ \Theta_{\text{fast}} = \frac{\sum_i r_i \theta_i^2}{\sum_i r_i \theta_i} \]
2.3 Electronic Energy Transfer and Migration

Fluorescence resonance energy transfer can occur between a donor (D) molecule in the excited state and an acceptor (A) molecule in the ground state. For this to occur, the emission spectra of the donor must overlap with the absorption spectra of the acceptor. The rate of energy transfer is dependent upon the radiative lifetime of the donor, the mutual orientation of the donor and acceptor transition dipoles as well as the distance between the donor and acceptor. This dependence on distance makes it possible to measure D-A distances and is the reason why resonance energy transfer has been described as a “spectroscopic ruler” [20]. The theory of electronic energy transfer was first described by Förster in 1948 [1]. Energy transfer is a non-radiative process, i.e. a process which doesn’t involve photons. The theory can be qualitatively explained by an oscillating dipole which exchanges energy with another dipole of similar frequency [21]. With two chemically and photophysically different chromophores, e.g. Trp and BODIPY, the process is referred to as Donor-Acceptor Energy Transfer (DAET). When the two chromophores are chemically and photophysically identical, it is called Donor-Donor-Energy-Migration (DDEM). Electronic energy transport between chromophoric molecules are commonly used to gain structural insights into e.g. proteins and lipids [22].

2.3.1 Donor-Acceptor Energy Transfer

In the case of DAET, the energy from an excited donor ($D^*$) is transferred to an acceptor molecule in the electronic ground state according to:

$$D^* + A \xrightarrow{\omega} D + A^*$$

(8)

The rate of energy transfer, $\omega$, is given by:

$$\omega = \frac{3 \langle \kappa^2 \rangle (R_{0D})^6}{2 \tau_D^* R}$$

(9)

The rate of energy transfer depends on the mean square average of the angular part of dipole-dipole interaction, $\langle \kappa^2 \rangle$, the radiative fluorescence lifetime of the donor, $\tau_D^*$, the Förster radius, $R_{0D}$, and the distance between the centres of mass of the donor and acceptor, $R$. The Förster radius is:
Here $n$ and $N_A$ stand for the refractive index of the medium and the Avogadro’s constant, respectively. $J$ is the overlap integral that describes the spectral overlap between the normalised donor fluorescence $F_D(\lambda)$ and the acceptor’s molar absorptivity $[\varepsilon_A(\lambda)]$:

$$J = \int F_D(\lambda) \varepsilon_A(\lambda) \lambda^4 d\lambda$$  \hspace{1cm} (10b)

The angular part of dipole-dipole coupling in Eq. 9 is given by:

$$\langle \kappa^2 \rangle = \left\langle \left( \hat{\mu}_D \cdot \hat{\mu}_A - 3(\hat{\mu}_D \cdot \hat{R})(\hat{\mu}_A \cdot \hat{R}) \right)^2 \right\rangle$$  \hspace{1cm} (10c)

Here $\hat{R}$ is the vector connecting the interacting molecules, $\hat{\mu}_D$ and $\hat{\mu}_A$ denotes the unit vectors of the donor and acceptor and their transition dipole moments.

The best accuracy of distance measurements is achieved by using time-resolved fluorescence experiments. In the DAET studies presented in this thesis, energy transfer experiments have been performed using the time correlated single-photon counting (TCSPC) technique [23]. The rate of energy transfer has been extracted from the fluorescence decay of the acceptor, which is excited via energy transfer from the donor. When using this method, it is important to take into account the relative probability of direct excitation of the acceptor molecule, $p_{ex}^A$, upon exciting the donor. The fluorescence decay of the acceptor molecule is then given by [24]:

$$F(t) = (1 - p_{ex}^A) \sum \frac{\alpha_D^i \alpha_A^j \omega}{\omega + 1/\tau_D^i - 1/\tau_A^j} \left[ -\exp \left( \frac{t}{\tau_D^i - \omega} \right) + \exp \left( \frac{t}{\tau_A^j} \right) \right] + p_{ex}^A F_A(t)$$  \hspace{1cm} (11)

A sum of discrete lifetimes describes the fluorescence decay of the donor $\tau_D^i$ and acceptor $\tau_A^j$ in the absence of energy transfer, according to

$$F_D(t) = \sum_i \alpha_D^i \exp \left( -t/\tau_D^i \right)$$  \hspace{1cm} (12a)

and
\[ F_\lambda(t) = \sum_j \alpha_j \exp\left(-t/\tau_\lambda^j\right) \] 

The presence of a distribution of D-A distances has been modelled by a Gaussian density function

\[
\rho(R) = \frac{1}{\sigma \sqrt{2\pi}} \exp\left(-\frac{(R - \bar{R})^2}{2\sigma^2}\right)
\]

where \( \bar{R} \) is the average distance between the centres of mass of the donor and acceptor group and the variance \( \sigma \) is related to the full width at half-maximum (FWHM) of the distribution according to:

\[
FWHM = 2\sigma \sqrt{2\ln 2} \approx 2.355\sigma
\]

### 2.3.2 Donor-Donor Energy Migration

Applications of DAET usually require specific labelling by two different chromophoric molecules. The problem with specific labelling can be circumvented by using DDEM. Then two identical chromophores are used for labelling. If the photophysical properties of the two donor-groups are identical at the two labelling sites, the energy transfer process is reversible.

\[
D^* + D \xrightleftharpoons{\phi} D + D^*
\]
2.4 Two-Photon Excitation

In most fluorescence experiments, the fluorophore is excited by a single photon. However, the fluorophore can also reach the same excited singlet state by simultaneously absorbing two or more long-wavelength photons. This is referred to as two- or multiphoton excitation. Two-photon excitation is a nonlinear process, i.e. the fluorescence intensity increase in proportion to the square of the excitation density power. Also, the selection rules for two-photon absorption are different from one photon excitation. This means that the excited state may differ between OPE and TPE and the absorption spectra show different shapes [26]. The emission spectra and fluorescence lifetimes however, are the same regardless of mode of excitation. TPE requires high illuminating intensities since the probability of simultaneous absorption of two photons is extremely low. This means that the laser beam must be tightly focused and the excitation is restricted to the small focal volume (typically femtolitres). This small and localized excitation volume is an advantage since it introduces specificity within the sample, reduces light scattering and provides less photo-damage outside the focal region [17, 27].

2.4.1 TPE Anisotropy

The anisotropies for OPE and TPE may be very different [28, 29]. This is because TPE induces a more strongly aligned population of excited fluorophores. Each interaction is proportional to \( \cos^2 \beta \) and hence, the photoselection function becomes proportional to \( \cos^4 \beta \), where \( \beta \) is the angle of the emission dipole relative to the electric field of the excitation radiation. As a consequence for collinear absorption and emission transitions, the fundamental anisotropy for TPE is 0.57, as compared to 0.4 for OPE.

2.4.2 TPE and DDEM

A fluorophore excited via TPE rapidly undergoes intramolecular processes and ends up in the lowest excited electronic state before one-photon emission may occur. Thus, as mentioned above, the emission spectrum and time-resolved fluorescence decay following TPE are the same as for OPE. Subsequently, a donor molecule excited by TPE will interact with another ground state donor according to the same mechanism as upon OPE. The benefit of using OPE and TPE simultaneously is that OPE and TPE experiments are independent, but still provide the same molecular information about reorienting motions [6] and energy migration processes [7].
2.5 CD-Spectroscopy

When circularly polarised light passes through an optically active and absorbing sample, the absorbances of left and right circularly polarised light are usually different. This difference in absorption is called circular dichroism (CD). Optical activity arises from the lack of symmetry within molecules, most importantly from asymmetrical carbon atoms and the effects these atoms may have on nearby chromophores. Many biological chromophores are optically active, which makes it possible to study them by CD. For proteins, the CD spectrum is generally divided into three regions; the far-UV (< 250 nm) which yields information about the secondary structure, the near-UV (250 – 300 nm) which is sensitive to the tertiary structure and finally, the near-UV-Vis region (> 300 nm), reflecting the environment of protein prosthetic chromophore groups [30]. The CD spectrum in the region of 190 – 230 nm is due to exciton coupling between the amide groups which form the polypeptide backbone [30]. This coupling makes the CD-spectra in this region very sensitive to the conformation of the backbone. Hence, CD can be used to monitor changes in protein secondary structure and is an important tool for estimating the proteins content of secondary structure.

2.5.1 Equilibrium Unfolding Monitored by CD

Equilibrium unfolding is the process of unfolding a protein by gradually changing its environment. Since equilibrium is maintained in all steps, the process is reversible and it is used to determine the conformational stability of proteins. In its simplest form, equilibrium unfolding assumes that the proteins only exhibit two thermodynamically stable states, the folded (F) and the unfolded (U) state:

\[ F \leftrightarrow U \]  \hspace{1cm} (15)

However, each state may contain an ensemble of conformations, especially the unfolded state. By gradually changing the fractions of folded and unfolded proteins by the addition of urea, the dependence of denaturation \((mF\) and \(mU)\) and free energy of unfolding in absence of denaturant \((\Delta G^0)\) can be extracted according to [31]:

\[ \Delta G^0 = -RT \ln \frac{m_U}{m_F} \]
\[
CD_{\text{obs}} = \frac{CD_d - mF[D] + (CD_U + mU[D]) \exp(\Delta G_U^0 - m_{\text{eq}}[D])}{1 + \exp(\Delta G_U^0 - m_{\text{eq}}[D])} / RT
\]

(16)

Here \(CD_{\text{obs}}\) denotes the observed CD signal; \([D]\) is the denaturant concentration and \(m_{\text{eq}}\) the denaturant dependence of \(\Delta G_U^0\) in the direction of unfolding. \(CD_U\) and \(CD_F\) are the CD-amplitudes of the unfolded and folded states, respectively. \(R\) is the gas constant and \(T\) the temperature.

Unfolding can also be induced by increasing the temperature. The midpoint of thermal unfolding (\(T_m\)) can be determined by fitting the experimental data to [32]:

\[
CD_{\text{norm}}(T) = \frac{S_F + aT + K_{\text{obs}}(S_U + bT)}{1 + K_{\text{obs}}}
\]

(17a)

where

\[
K_{\text{obs}}(T) = \exp \left( \frac{\Delta H_m}{RT_m} \left( 1 - \frac{T}{T_m} \right) \right)
\]

(17b)

Here \(CD_{\text{norm}}\) is the normalised CD signal, \(S_F, S_U, a\) and \(b\) are the CD signals for the folded and unfolded state and the slope of the folded and unfolded baselines, respectively. \(\Delta H_m\) is the enthalpy value at \(T_m\).

### 2.6 Macromolecular Crowding

Biochemical reactions in living systems take place inside cells, in which 10 – 40% (50 - 400 mg/mL) of the volume is occupied by macromolecules [33-35]. Since no single macromolecule is present in high concentration, but all species taken together occupy a significant fraction of the volume, such media are referred to as “crowded”. In a crowded system, significant nonspecific interactions between the macromolecules occurs, which depends upon global properties of the interacting macromolecules. Nonspecific interactions are (generally) considerably weaker than specific interactions between reaction partners and are either attractive (hydrophobic, electrostatic) or repulsive (steric, electrostatic). These fundamental interactions can considerably alter the kinetics and equilibrium of reactions.
Steric repulsion between macromolecules in a crowded solution yields an excluded volume effect to all solute molecules. However, to what extent the medium volume is unavailable to other macromolecules will depend on the size, shape and number of all molecules present. The actual volume available to the macromolecule defines an effective concentration that is higher than the actual concentration in the crowded medium resulting in an increased thermodynamic activity [36]. With a decrease in available volume, the entropy decreases and the free energy of the system increases. One way for the system to reduce its free energy would be to minimize the excluded volume, by e.g. compaction and association [37-39]. Protein folding is one example where crowding tend to favour the folded state due to more extended conformations in the unfolded state [40]. A large number of in vitro studies have shown that macromolecular crowding increases the stability of proteins [41-43] but the actual mechanism of stabilization is not fully understood. Several recent studies show however, that the excluded volume effect induces a more compact unfolded state, as compared to the dilute condition, which results in protein stabilization [44, 45]. The work in this thesis supports these finding. In Paper III we use DAET to obtain direct evidence of unfolded state compaction of the ribosomal protein S16 in the presence of dextran 20. A few studies also report on structural changes of the folded state in the presence of crowding [43, 46-48]. This issue has been addressed in Paper IV. Here we continue our study of the model protein S16, by probing selected distances in the folded state at increasing amounts of dextran 20. FRET have been used to study macromolecular crowding previously [44, 47, 49]. However, in these studies the effects of macromolecular crowding were not investigated directly. Ittah et al. [50] used a procedure similar to ours. By utilizing time-resolved FRET they can study effects on the conformational ensembles of flexible states of protein molecules upon molecular crowding.

To study the effects of macromolecular crowding experimentally, high concentrations of natural or synthetic macromolecules, so called crowding agents, are added to mimic a cell-like system. This addition however yields solutions far from ideal that may, in addition to the excluded volume effect, also exhibit (weak) associations between the crowder and molecule of interest [51-53].
3 Results and Discussion

3.1 TPE Donor Donor Energy Migration

The classical Förster theory [1] neglects the influence of reorientational and spatial motions of the interacting molecules which in many cases is invalid. To overcome this limitation, the extended Förster theory was derived [3]. Data analyses using the EFT involves a molecular description of reorientational restrictions and dynamics of the interacting chromophores. This makes the analysis complex and time-consuming. It demands modelling of anisotropic orienting potentials. The interaction depends on their mutual orientation (cf. \( \langle \kappa^2 \rangle \) in Eq. 10c). Time-resolved fluorescence depolarization measurements for each donor in the absence of energy migration can be used to model the potentials. In the recent work [4] a general approach is derived which can be used to determine relevant molecular parameters. The approach was tested by fitting simulated data to synthetic data that mimic true depolarization TCSPC experiments. In Paper I, this approach was used for the analysis of fluorescence depolarisation experiments with respect to DDEM within a \textit{bis}(9-anthrylmethylphosphonate) bisteroid.

Fluorescence depolarisation experiments can be performed by using one-photon and two-photon excitation. The advantage of using both of them would be to gain independent molecular information about the reorienting motions[5, 6] and energy migration process [7]. A combined treatment of OPE and TPE fluorescence depolarisation in presence of energy migration and molecular reorientation, using computer simulations have been developed [4] and it is in this thesis applied on real experimental data. To the best of our knowledge, this is the first time EFT is used for the analyse of TPE experiments and DDEM data.

3.1.1 Model Bisteroids

The energy migration between two chemically and photophysically identical chromophores were studied. These groups are covalently bonded to a bisteroid that forms a rigid spacer between the two interacting groups. The particular derivatives studied are: \textit{mono}(3-perylenylmethyphosphonate) bisteroid (Pe-BS) and \textit{bis}(3-perylenylmethyphosphonate) bisteroid (Pe-BSPe), as well as \textit{mono}(9-anthrylmethylphosphonate) bisteroid (An-BS) and \textit{bis}(9-anthryl-methylphosphonate) bisteroid (An-BS-An) (cf. Fig. 3) [54].
Figure 3. The chemical structure of A: bis(3-perylenylmethylphosphonate) bisteroid and B: bis(9-anthrylmethylphosphonate) bisteroid. In the mono bisteroid (structure not shown) one of the perylenylmethylphosphonate /anthrylmethylphosphonate is replaced by a H-atom.

### 3.1.2 Perylenyl Bisteroids

When dissolving the perylenyl bisteroids in several different solvents, they revealed spectral and photophysical behaviour strongly deviating from the monomeric form. The fluorescence emission spectrum showed in addition to the normal fluorescence spectra of perylenyl, a broad structure-less band at longer wavelengths. The fluorescence did not obey a single exponential decay and the relaxation rate was depending on the emission wavelength. This influence could be reduced, but not completely eliminated, by dilution and upon adding an acid or a base, depending on the solvent used. This kind of behaviour is typical for excimer formation [55], which means formation of an excited dimer that dissociates in its ground state. The mixture of chloroform/methanol (2 : 1) with the addition of trifluoroacetic acid (TFA) dissolved the perylenyl bisteroids without formation of excimers. However, in order to study energy migration, the overall motion of the bisteroids needs to be hampered and for this reason the bisteroids were solubilised in micelles, formed by the detergent Triton X-100. Pe-BS exhibited a mono-exponential fluorescence decay in the micelles with a lifetime of 5.8 ns and a steady-state fluorescence anisotropy of 0.2 for the $S_0 \leftrightarrow S_1$ transitions. Unfortunately, Pe-BS-Pe still exhibited a small, but significant contribution of excimer.
Finally, after testing several different solvents, a mixture of solvents was found to be working. Hexamethylphosphoramide (HMPA), a useful polar aprotic solvent, with the addition of TFA was found to dissolve both the perylenyl bisteroids without any trace of excimer emission. By adding different amounts of glycerol to this mixture, the overall motion of the bisteroids could be held back. Steady-state and time-resolved fluorescent experiments were performed at several different temperatures, yielding an average lifetime of 4.2 ns and a steady-state anisotropy value of maximum 0.35 for Pe-BS in 90 wt % glycerol at 10°C. Two-photon excited fluorescence measurements were also performed, at the Ångström laboratory in Uppsala. The steady-state spectra and time-resolved anisotropy decay for Pe-BS and Pe-BS-Pe in 80 wt % glycerol and 50°C is shown in Figs. 4 and 5. The time-resolved influence of energy migration is clearly seen at shorter times in Fig. 5, with Pe-BS-Pe loosing the initial anisotropy on a much faster timescale than Pe-BS. Fig. 5 also show that a higher initial anisotropy is achieved with TPE.

Unfortunately, the HMPA/TFA/glycerol mixtures of Pe-BS-Pe were not stable over time and excimers were reformed. The TPE-experiments showed traces of excimer formation which interfered with the energy migration analysis.
3.1.3 Anthryl Bisteroids

Due to difficulties in using the synthesised perylenyl bisteroids, two bisteroids similar to the perylenyl bisteroids, were tested; mono(9-anthrylmethylphosphonate) bisteroid (An-BS) and bis(9-anthrylmethylphosphonate) bisteroid (An-BS-An) (cf. Fig. 3). The energy migration takes place between the 9-anthrylmethyl groups of An-BS-An. To obtain information about the motions of the 9-anthrylmethyl groups, the An-BS was also studied. To hamper the overall tumbling motions of the two anthryl bisteroids, they were dissolved in the viscous solvent 1,2-propandiol. Their fluorescence emission showed very similar and structured spectra (Fig. 6), and the fluorescence relaxation was fitted to a bi-exponential decay. The relaxation was invariant to emission wavelengths and showed a dominating lifetime of 12 ns.

Figure 6. Steady-state excitation ($\lambda_{em} = 420$ nm) and emission ($\lambda_{ex} = 350$ nm) spectra (black) and anisotropy (grey) of An-BS and An-BS-An in 1,2-Propanediol at 10°C.
The anisotropy decay was determined for An-BS and An-BS-An at different temperatures. Two-photon excited fluorescence experiments were also performed and the influence of energy migration is clearly revealed from the anisotropy decay curves and the experimental depolarisation data in Fig. 7. For the anthryl bisteroids the initial anisotropy value was very similar for OPE and TPE.

**Figure 7. Left:** Time-resolved TPE fluorescence anisotropy decay of An-BS (black dots, red line) and An-BS-An (grey dots, blue line) in 1,2-propanediol at 10°C. **Right:** Measured fluorescence depolarisation intensity decays $F_{||}(t)$ (upper in red), $F_{\perp}(t)$ (lower in blue) and total intensity decay $F(t)$ (middle in green) for An-BS-An. The corresponding fitted decays are in solid black lines.

The TPE fluorescence depolarisation experiments were quantitatively analysed by using a recently developed approach [4]. The analyse yielded a distance between the 9-anthrylmethyl groups (25.7 Å) which is reasonable as compared to that calculated from chemical structure of the studied compound (24.2 Å). It is also consistent with the distance previously obtained [56].
3.2 Donor Acceptor Energy Transfer

In Paper II – IV, the ribosomal protein S16 was used as a model system to investigate effects on protein stability. DAET experiments were performed to determine intramolecular distances between an intrinsic tryptophan (Trp) residue and N-((4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-yl)methyl)iodoacetamide (BODIPY), which was covalently attached at selected positions within the protein.

3.2.1 Model System: Ribosomal Protein S16

Two homologs of the ribosomal protein S16 were isolated; S16_{thermo} from the hyperthermophilic bacterium *Aquifex aeolicus* and S16_{meso} from the mesophilic *Chlamydia pneumoniae*. The crystal structure of S16_{thermo} is illustrated in Fig. 8 and it shares 33 % sequence identity with S16_{meso}. The primary sequence is composed of 112 amino acid residues in S16_{thermo} (13.0 kDa) and 117 amino acid residues in S16_{meso} (13.9 kDa). Paper II demonstrates that S16_{thermo} shows an extremely high melting point (111 ± 8 °C) as compared to S16_{meso} (58 ± 0.6 °C).

In order to study electronic energy transfer within the proteins, several mutants were created (Table 1). The intrinsic Trp58 was used as donor and selected amino acids were replaced by cystein and labelled with the acceptor group BODIPY. In total, five mutants were created which enabled studies of three different intraprotein directions (as judged by the crystal structure).

Table 1. S16 mutants.

<table>
<thead>
<tr>
<th>Homolog</th>
<th>Mutant</th>
<th>Mutation 1</th>
<th>Mutation 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>S16_{thermo}</td>
<td>W74CT</td>
<td>Trp 74 → Cys 74</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>W74F/F10CT</td>
<td>Trp 74 → Phe 74</td>
<td>Phe 10 → Cys 10</td>
</tr>
<tr>
<td>S16_{meso}</td>
<td>W39F/Q10CM</td>
<td>Trp 39 → Phe 39</td>
<td>Gln 10 → Cys 10</td>
</tr>
<tr>
<td></td>
<td>W39F/K74CM</td>
<td>Trp 39 → Phe 39</td>
<td>Lys 74 → Cys 74</td>
</tr>
<tr>
<td></td>
<td>W39F/Y81CM</td>
<td>Trp 39 → Phe 39</td>
<td>Tyr 81 → Cys 81</td>
</tr>
</tbody>
</table>

Five S16 mutants used in energy transfer experiments. Mutation 1 is needed to remove the second Trp residue. Mutation 2 is performed in order to insert a Cys residue, to which BODIPY is connected.
Figure 8. Crystal structure of wild-type S16 thermo and the corresponding primary sequences for S16 thermo and S16 meso. The dashed lines indicate the intramolecular distances as determined by the X-ray structure, written in red. The donor Trp58 is coloured in green and the three possible acceptor sites are coloured in orange and marked with bold in the amino acid sequence. The secondary structure is indicated in the sequence and crystal structure.

### 3.2.2 Tryptophan – BODIPY

To determine intramolecular distances within the S16 mutants, we utilize one of the naturally present Trp residues (Trp58) in combination with BODIPY, conjugated to a Cys residue. This D-A pair has proven to be suitable for probing intra-protein distances [24]. BODIPY has a main absorption band centred around 500 nm ($\varepsilon \approx 80$ 000 mol$^{-1}$ dm$^3$ cm$^{-1}$) and an additional broad transition which is much weaker and centred at approximately 360 nm ($\varepsilon \approx 5$ 000 mol$^{-1}$ dm$^3$ cm$^{-1}$). The weaker band overlaps efficiently with the emission spectrum of Trp (Fig. 9), which is centred at approximately 280 nm ($\varepsilon \approx 15$ 930 mol$^{-1}$ dm$^3$ cm$^{-1}$).
Figure 9. The absorption spectra of Trp (—) and BODIPY (—) together with their corresponding corrected fluorescence spectra; Trp (—) and BODIPY (—·—·—·)

In paper II the Förster radius used for Trp-BODIPY was 34.6 Å [24]. In paper III and IV the Förster radius was determined according to Eq. 10a, where the radiative lifetime of Trp is 18.9 ns [57]. The overlap integral was calculated for each studied sample. The value of $\langle \kappa^2 \rangle$ was taken to be the isotropic dynamic average, $2/3$ [58, 59].

3.2.3 Thermostability of S16

There is a large interest in designing enzymes which are resistant to temperature and exhibit high activity over a wide temperature span. In order to explore how proteins can be thermally stabilized, several properties of S16Thermo and S16Meso were compared in Paper II. The intention in this work was to examine and compare the folded and denatured state ensembles of the two S16 homologs. Two S16Thermo mutants were used; W74C and W74F/F10C, together with the S16Meso mutant W39F/Q10C. Evidently, the two mutants W74F/F10C T and W39F/Q10C M both probe the distance between the positions 10 and 58. These mutations and the labelling were thoroughly investigated by means of CD to ensure that no structural changes within the folded protein occurred.
Time-resolved fluorescence experiments were performed to investigate distances and distance distributions in the folded, 50% folded/unfolded and unfolded states of S16. Unfolding was chemically induced by the addition of urea and fluorescence measurements were used to determine the fraction of unfolding.

Fluorescence relaxation data were collected for the donor and acceptor in the absence of energy transfer, as well as for the acceptor, excited via energy transfer from the donor. Fig. 10 illustrates the fluorescence relaxation of the acceptor excited directly and via energy transfer. In the case of excitation via energy transfer, it is apparent that the decay is shifted towards longer times, which is expected for a consecutive process. For all mutants, Trp and BODIPY in absence of energy transfer, experience a bi-exponential fluorescence decay. For Trp the average lifetime decreases with the fraction of unfolding, which is consistent with a changing environment surrounding the Trp residue.

Data were globally analyzed by assuming a single distance (Eq. 11) as well as a Gaussian distribution (Eq. 13a), from which an average distance and a variance is obtained. A single distance is only likely for the native protein since under denaturing conditions a range of Trp-BODIPY distances is expected.
Table 2. Trp-BODIPY distances as determined by DAET

<table>
<thead>
<tr>
<th>S16 Mutant</th>
<th>Fraction folded (%)</th>
<th>$R$ (Å)</th>
<th>$\bar{R}$ (Å)</th>
<th>$\sigma$ (Å)</th>
<th>$\chi^2$</th>
<th>D.W.</th>
</tr>
</thead>
<tbody>
<tr>
<td>W74Ct</td>
<td>100 ± 0</td>
<td>22.8</td>
<td>22.9</td>
<td>1.3</td>
<td>1.07</td>
<td>1.93</td>
</tr>
<tr>
<td></td>
<td>50 ± 6</td>
<td>-</td>
<td>24.7</td>
<td>2.3</td>
<td>1.02</td>
<td>1.96</td>
</tr>
<tr>
<td></td>
<td>10 ± 6</td>
<td>-</td>
<td>26.2</td>
<td>3.1</td>
<td>1.21</td>
<td>1.83</td>
</tr>
<tr>
<td>W74F/F10Ct</td>
<td>100 ± 0</td>
<td>-</td>
<td>20.5</td>
<td>2.6</td>
<td>1.17</td>
<td>2.01</td>
</tr>
<tr>
<td></td>
<td>50 ± 5</td>
<td>-</td>
<td>22.0</td>
<td>3.5</td>
<td>1.04</td>
<td>1.99</td>
</tr>
<tr>
<td></td>
<td>10 ± 5</td>
<td>-</td>
<td>22.7</td>
<td>4.9</td>
<td>0.99</td>
<td>1.85</td>
</tr>
<tr>
<td>W39F/Q10Cm</td>
<td>100 ± 0</td>
<td>-</td>
<td>19.8</td>
<td>3.6</td>
<td>1.04</td>
<td>1.95</td>
</tr>
<tr>
<td></td>
<td>50 ± 6</td>
<td>-</td>
<td>23.1</td>
<td>5.7</td>
<td>1.09</td>
<td>1.92</td>
</tr>
<tr>
<td></td>
<td>10 ± 6</td>
<td>-</td>
<td>24.1</td>
<td>6.9</td>
<td>1.15</td>
<td>1.78</td>
</tr>
</tbody>
</table>

Trp-BODIPY distances as determined from energy transfer experiments, analysed assuming one single distance ($R$) or a distribution of distances with an average distance ($\bar{R}$) and a variance ($\sigma$). The qualities of the fittings were judged by the statistical parameters $\chi^2$ and Durbin-Watson (D.W.). The fraction folded protein 50% and 10% assumes the remaining part is unfolded.

As can be seen in Table 2, only the folded W74Ct provides acceptable statistics when assuming a single distance. From the distribution model, all mutants show an increase in average distance and variance with the fraction of unfolded protein. The folded W74Ct exhibit a very narrow variance, which explains why this mutant can be fitted to a single distance. The distribution for W74Ct continues to be more narrow than for the other mutants at denaturing conditions.

W74F/F10Ct and W39F/Q10Cm show similar average distances in the folded state, but the variance is wider for W39F/Q10Cm. The increase in average distance and variance upon unfolding is also larger for W39F/Q10Cm than for W74F/F10Ct. Since the same distance (residue 58-10) is probed for the two mutants, this indicates a more compact denatured state of S16Themo than for S16Meso. Care should be taken in these interpretations however, since the denatured state ensemble produced under high concentrations of denaturant may differ from the denatured state ensemble present under native conditions [60].
3.2.4 S16 in Presence of Crowding

We continue the study of S16 by exploring the excluded volume effect, which was generated by macromolecular crowding. Theory predicts [61, 62] that any reaction resulting in a volume change will be affected by the excluded volume effect. Several studies suggest that in a crowded environment, the unfolded state of a protein will be destabilised, indirectly stabilising the folded state [40, 63]. Additional studies suggest that macromolecular crowding induces a more compact unfolded state of proteins [45, 64, 65] and may even induce structural changes in the folded state [43, 46-48]. Here we use time-resolved DAET, which provides direct distance information, to investigate whether macromolecular crowding cause structural changes within the model protein S16. Biophysical properties like protein stability, structure and folding dynamics of S16 were investigated in paper III and IV.

![Molecular structure of Dextran](image)

**Figure 11.** Molecular structure of Dextran. The straight chain consists of α-D-1,6 glucose linked glucan with side-chains of α-D-1,3 glucose linked to the backbone.

To mimic the crowded environment e.g. inside cells, large amounts of natural or synthetic macromolecules can be added to samples. In study III and IV we choose to work with dextran 20 (20 kDa). It is soluble at high concentrations, not interfering with our spectroscopic techniques and is comparable in size with the model protein (S16). Previously it has been proposed that the excluded volume effect increases when the protein and crowding agent are of similar size [66]. Dextran is a highly branched polysaccharide, composed of several glucose molecules as shown in Fig. 11. In study IV we gradually increase the concentration of dextran to monitor
how the thermal midpoint (S16Meso) and selected intramolecular distances are influenced. In study III the unfolded state is investigated and a concentration of 200 mg/mL dextran 20 was selected because it represents a biologically relevant concentration, which is still suitable for careful biophysical experiments.

3.2.4.1 Equilibrium Unfolding

In the study of conformational stability of the S16 mutants, we use the chemical denaturant urea for unfolding. It is then important to note that in dextran samples, there will be some volume excluded to the solvent due to the crowding agent. Therefore, the effective concentration of urea is somewhat higher than the concentration calculated by total volume. This issue have previously been addressed and corrections suggested [45, 66]. It is proposed to correct the denaturant concentrations based on the partial specific volume of the crowding agent. The addition of urea to the dextran samples also introduces another complication. It seems that the volumes of urea and dextran solutions are not additivative. To avoid this partial volume effect, one can choose to work with the unit molal, i.e. mole urea/kg water added. In this study we use both the unit of molal, as well as effective molar (corrected for the solvent exclusion effects). In Paper III we work with the two S16Thermo mutants W74C and W74F/F10C. By selecting these two S16 variants, according to the crystal structure (Fig. 8), we can probe two perpendicular distances within the protein.

By examining the equilibrium unfolding curves in Fig. 12, we conclude that the two mutants together with S16Thermo wild-type (WT) follow a two-state equilibrium unfolding process according to Eq 15. For all variants of S16 we find that the unfolding curves are shifted towards higher urea concentration in the presence of 200 mg/mL dextran. This implies that the proteins are stabilised by the addition of dextran. All samples showed reversible unfolding.

In Paper IV the thermal stability was tested for the three S16Meso mutants at different concentrations of dextran. Due to the high thermal stability of S16Thermo, similar experiments could not be performed for these variants. The thermal midpoints of unfolding for the three mutants of S16Meso are summarised in Table 3. The unfolding event was reversible for all concentrations of dextran up to 200 mg/mL. In presence of 300 mg/mL, only W39F/Q10CM showed reversibility, as judged by recovery of initial CD signal upon cooling.
Figure 12. Equilibrium unfolding curves for S16 wild-type, W74C, and W74F/F10C in the presence (○) and absence (●) of 200 mg/mL dextran 20. The normalised CD-signal at 220 nm ($\Phi_{220}$) is plotted as a function of urea concentration, expressed as effective mol/L (left) and mol/kg H$_2$O (right).
We find that there is a small increase in the thermal midpoint upon increasing amounts of dextran, indicating an increased thermal stability in presence of crowding.

**Table 3.** Thermal midpoints of unfolding for S16Meso

<table>
<thead>
<tr>
<th>[Dextran] (mg/mL)</th>
<th>Thermal Midpoint (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>W39F/Q10C</td>
</tr>
<tr>
<td>0</td>
<td>66.2 ± 0.1</td>
</tr>
<tr>
<td>100</td>
<td>67.9 ± 0.1</td>
</tr>
<tr>
<td>200</td>
<td>69.4 ± 0.1</td>
</tr>
<tr>
<td>300</td>
<td>69.9 ± 0.2</td>
</tr>
</tbody>
</table>

Thermal midpoints of unfolding for the three S16Meso mutants as determined by fitting the experimental CD changes at 220 nm to Eqs. 17.

### 3.2.4.2 Unfolded State Compaction

In paper III, DAET studies were performed for the two S16Thermo mutants W74C and W74F/F10C, in the folded state as well as in the unfolded state. Based on the unfolding curves (Fig 12) we selected two urea concentrations (in moles/kg H2O) on the folded baseline (0 and 2 m) and two concentrations on the unfolded baseline (8 and 10 m). Due to the increased stability of W74C in the presence of dextran, an additional point on the unfolded baseline was needed (12 m). As in paper II, the data were analysed assuming both one single distance and as a Gaussian distribution, yielding an average distance with a variance, summarized in Fig. 13. Actual values can be found in Paper III, Table 2. All Trp-BODIPY distances at 0 and 2 m urea can be described by a single distance. At higher urea concentrations however, a distribution of distances is needed in order to achieve a fit to the experimental data, which is statistically supported. At low concentrations of urea, the average distances are very similar in absence and presence of dextran. At higher concentrations of urea however, the average distance is always shorter in the presence of dextran. This implies that the folded states of the two S16Thermo mutants are similar in buffer and 200 mg/mL dextran, but that the unfolded state is slightly compacted by the presence of dextran.
For W74F/F10C and 8 m urea we find that the average Trp-BODIPY distance is much shorter in dextran than in buffer. In addition, the distribution is much narrower, which can clearly be seen in Fig. 13. This indicates a protein almost as compact as in the folded state. 8 m urea is an early point on the unfolded baseline and upon increasing the urea concentration further, the average distance increases and the distribution widens. However, the average distance remains shorter in presence of dextran than in absence.

**Figure 13.** Average Trp-BODIPY distances (circles) together with the Gaussian distance distribution (lines) for the two S16Therm mutants W74C (left) and W74F/F10C (right) in absence (black) and presence (grey) of 200 mg/mL dextran. Urea concentrations are indicated in the figure. The area of the Gaussian distributions has been normalized to unity in all cases.
An interesting correlation between distance data and Trp fluorescence spectra was observed. Tryptophan emission is known to shift in response to a change in the polarity of its surrounding environment. The peak fluorescence shifts from approximately 308 to 350 nm with increasing polarity [67]. The peak fluorescence from Trp58, which according to the crystal structure is located in the interior of the protein, shifts to longer wavelength with increasing concentration of urea (see Fig. 14). This indicates that Trp58 becomes more exposed to the polar solvent. At all denaturing conditions, the peak fluorescence is located at a shorter wavelength in the presence of dextran, supporting the compaction data. Also in agreement with the distance data is the Trp peak fluorescence at W74F/F10C at 8 m urea. It is found at considerably shorter wavelength in presence of dextran than in the absence.

![Figure 14](image.png)

**Figure 14.** Peak fluorescence wavelength of Trp58 at varying concentration of urea in the presence (o) and absence (●) of 200 mg/mL dextran.

To further support that the observed compaction is an effect of the polymeric nature of dextran, we performed both chemical unfolding and distance measurements of the two mutants in the presence of 200 mg/mL sucrose. Sucrose is a dimer consisting of one glucose and one fructose molecule and act as a control by mimicking the monomeric form of the polymer dextran. The results show that the two s16 thermo mutants and the wild-type are slightly stabilized against urea unfolding as compared to buffer, as expected for osmolytes [68]. Distance measurements at the extreme-values however, show no signs of compaction. Instead, the average Trp-BODIPY distances are always longer in the presence of sucrose, as compared to both buffer and dextran solutions.
3.2.4.3  Folded State Effects

In paper IV, we continue our structural study of S16. Here we examine the effects of macromolecular crowding on the folded state by adding varying amounts of dextran. All five S16 mutants were used. Far-UV CD spectra were recorded for three S16\textsubscript{Meso} mutants and at varying concentrations of dextran. However, no significant changes were detected. The Trp-BODIPY distances for all five mutants were determined at increasing concentrations of dextran, according to the same procedure as described earlier. One single distance describes all distance measurements well, except for the S16\textsubscript{Meso} mutant W39F/Y81C, which requires a distance distribution.

![Figure 15](image_url)

**Figure 15.** Change in average Trp-BODIPY distance (\(\Delta R = \overline{R_{\text{dextran}}} - \overline{R_{\text{buffer}}}\)) for the two S16\textsubscript{Thermo} mutants (upper) and three S16\textsubscript{Meso} mutants (lower), at varying concentrations of dextran.

The distances obtained for the two S16\textsubscript{Thermo} mutants (see Fig. 15) seems to be invariant to the degree of crowding. For S16\textsubscript{Meso} however, we detect a small decrease in the average distances up to 100 mg/mL dextran. At higher dextran concentrations, the three mutants respond differently. For W39F/K74C\textsubscript{M} and W39F/Y81C\textsubscript{M} the Trp-BODIPY distance continues to decrease at higher dextran concentrations. The distance in W39F/Q10C\textsubscript{M} on
the other hand, increases back to its initial value at 200 mg/mL dextran and continues to increase at 300 mg/mL. Thus, it seems that there is a small effect on the folded state of S16Meso upon macromolecular crowding. Up to 100 mg/mL we find a compaction of the protein but at higher degrees of crowding the effect is not similar in all directions (i.e. anisotropic).

It is evident that the variance for the different mutants is diverse (see Table 1 in Paper IV). Since the Trp-residue is in position 58 for all five mutants, the different variances must have its origin in the flexibility of the BODIPY-group. W39F/Y81C_M shows the largest variance, indicating a high flexibility of the region to which BODIPY is attached. Also, the variance decreases some upon increasing amounts of dextran, indicating that crowding reduces the flexibility. W39F/K74C_M on the other hand experiences a very narrow variance, suggesting that BODIPY is located in a well-defined location within the structure. In this case, the amount of crowding has no influence of the variance.

3.2.4.4 Fluorescence Anisotropy and Viscosity

The steady-state and time-resolved fluorescence anisotropy were determined for all samples in paper III and IV and are listed in Tables 4 and 5. The steady-state anisotropy is low, which indicates that the BODIPY group undergoes rapid internal rotational motions in the protein. This is also supported by time-resolved data, which were analyzed according to Eq. 7a assuming two fast correlation times ($\theta_1$ and $\theta_2$) in Eq. 7b.

Fig. 16 show an example of the time-resolved fluorescence anisotropy decay, where the fast initial decay is clearly seen. It is also apparent that the $r(t)$ curves in presence and absence of dextran are very similar, even though there is a large difference in the bulk viscosity.

The fast and slow rotational correlation times are similar for the two S16Thermo mutants in buffer and at low denaturant concentration (Table 4). The long correlation time of approximately 9 ns agrees well with the previously measured protein tumbling-rate constant, obtained from NMR experiments (Christoph Weise, personal communications). At higher urea concentrations, $\Theta_{\text{fast}}$ and $\Theta_{\text{slow}}$ decreases. This is consistent with protein unfolding, where the dynamics of the BODIPY-group increases. Due to increased fast motions, it is no longer possible to extract the tumbling motions of the protein.
Figure 16. The fluorescence anisotropy decay of BODIPY attached to W74C in buffer (black) and in 200 mg/mL dextran (red). Also shown are the weighted residuals which provide the statistical quality of the modeling.

Table 4. Anisotropy data for BODIPY attached to S16Thermo at varying concentrations of urea

<table>
<thead>
<tr>
<th>Solvent</th>
<th>[Urea] (m)</th>
<th>Viscosity (cP)</th>
<th>W74C</th>
<th></th>
<th>W74F/F10C</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>r&lt;sub&gt;ss&lt;/sub&gt;</td>
<td>Θ&lt;sub&gt;fast&lt;/sub&gt; (ns)</td>
<td>Θ&lt;sub&gt;slow&lt;/sub&gt; (ns)</td>
<td>r&lt;sub&gt;ss&lt;/sub&gt;</td>
</tr>
<tr>
<td>Buffer</td>
<td>0</td>
<td>1.2</td>
<td>0.05</td>
<td>1.2</td>
<td>9.0</td>
<td>0.08</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>1.4</td>
<td>0.05</td>
<td>1.3</td>
<td>9.8</td>
<td>0.07</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>1.6</td>
<td>0.03</td>
<td>1.1</td>
<td>8.1</td>
<td>0.04</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>1.7</td>
<td>0.02</td>
<td>1.1</td>
<td>8.3</td>
<td>0.03</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>1.8</td>
<td>0.02</td>
<td>1.2</td>
<td>6.7</td>
<td>-</td>
</tr>
<tr>
<td>Dextran</td>
<td>0</td>
<td>11.9</td>
<td>0.05</td>
<td>1.7</td>
<td>15.3</td>
<td>0.09</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>13.7</td>
<td>0.06</td>
<td>1.4</td>
<td>14.6</td>
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</tr>
<tr>
<td></td>
<td>8</td>
<td>20.0</td>
<td>0.05</td>
<td>1.2</td>
<td>11.8</td>
<td>0.07</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>21.7</td>
<td>0.04</td>
<td>1.1</td>
<td>8.2</td>
<td>0.04</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>22.6</td>
<td>0.05</td>
<td>1.2</td>
<td>7.0</td>
<td>-</td>
</tr>
</tbody>
</table>

Viscosity and steady-state anisotropies (r<sub>ss</sub>) together with fast (Θ<sub>fast</sub>) and slow (Θ<sub>slow</sub>) correlation times for BODIPY attached to S16Thermo in 200 mg/mL dextran and varying amounts of urea. The correlation times were obtained from time-resolved anisotropy data.
From Table 4 it is also evident that $\Theta_{\text{fast}}$ and especially $\Theta_{\text{slow}}$ are longer in presence of dextran. For globular proteins, the slow correlation time is related to the bulk viscosity of the sample ($\eta$) according to the Perrin equation:

$$\theta_{\text{slow}} = \frac{\eta V}{RT}$$

(18a)

Here $V$ corresponds to the hydrated volume of the protein and $R$ and $T$ is the gas constant and the temperature, respectively. In the case of S16 in presence of dextran however, the increase in protein correlation time is not linearly dependent upon the viscosity. The fact that large molecules mostly influence the macroscopic viscosity is an effect that has been known for many years [69] and have also been reported in presence of crowding agents [70].

**Table 5.** $\Theta_{\text{slow}}$ for BODIPY attached to S16\text{Thermo} and S16\text{Meso} at varying concentrations of dextran

<table>
<thead>
<tr>
<th>[Dextran] (mg/mL)</th>
<th>Viscosity (cP)</th>
<th>$\Theta_{\text{slow}}$ S16\text{Thermo}</th>
<th>$\Theta_{\text{slow}}$ S16\text{Meso}</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>W74F/F10C</td>
<td>W74C</td>
</tr>
<tr>
<td>0</td>
<td>1.2</td>
<td>8.4</td>
<td>8.8</td>
</tr>
<tr>
<td>50</td>
<td>2.2</td>
<td>10.5</td>
<td>10.3</td>
</tr>
<tr>
<td>100</td>
<td>3.8</td>
<td>11.5</td>
<td>11.3</td>
</tr>
<tr>
<td>200</td>
<td>11.4</td>
<td>14.1</td>
<td>14.9</td>
</tr>
<tr>
<td>300</td>
<td>33.7</td>
<td>18.7</td>
<td>17.1</td>
</tr>
</tbody>
</table>

The macroscopic viscosity of the samples at varying concentrations of dextran, together with the slow correlation times for BODIPY attached to different positions within S16.

Table 5 shows $\Theta_{\text{slow}}$ for all mutants upon increasing the concentration of dextran. As expected, the correlation time increases with the dextran concentration for all mutants, except W39F/K74C M. The BODIPY-group in W39F/K74C M most likely experiences a high dynamic, making it difficult to extract $\Theta_{\text{slow}}$, similar to the unfolding experiments previously discussed. Upon increasing the concentration of dextran, we keep the $RT$ term in Eq. 18a constant. For S16\text{Thermo} the distance measurements indicate that there is no change in protein volume with the dextran concentration. According to Eq. 18a this would yield the following expression:

$$\frac{\theta_{\text{slow}}}{\eta} = \frac{V}{RT} = \text{constant}$$

(18b)
However, Fig. 17 demonstrates that $\Theta_{\text{slow}}/\eta$ is not constant over the measured concentration range, again supporting the theory that the protein experiences a viscosity that is lower than the bulk viscosity, a so called microviscosity.

![Figure 17](image)

**Figure 17.** Perrin plots of two S16Thermo mutants (left); W74F/F10C (●) and W74C (●), and three S16Meso mutants; W39F/Q10C (▲), W39F/K74C (■) and W39F/Y81C (○).

### 3.2.4.5 Quantum Yield Measurement

In Paper IV, the fluorescence quantum yields ($\phi$) and average lifetimes ($\tau$) of BODIPY were determined for all samples. For all mutants, the average fluorescence lifetime of BODIPY decreases slightly with increasing concentration of dextran. This is consistent with dynamic quenching, induced by the increased concentration of dextran. The quantum yield changes differently for the five mutants, although in most cases, a small decrease with increasing degree of crowding is apparent. For the two S16Meso mutants W39F/Q10C and W39F/K74C, the quantum yield is high and invariant to the dextran concentration. On the other hand, W39F/Y81C and W74F/F10C show low quantum yields, which are also invariant to the concentration of dextran. This is most likely due to quenching by amino acids in close proximity to the BODIPY-group. Tyrosine and tryptophan are known quenchers of BODIPY [71, 72]. Fig. 18 shows the distribution of tyrosines in the protein S16Thermo, as well as the corresponding positions in S16Meso. As previously mentioned, the mutants W74F/F10C and W39F/Q10C are both labelled by a BODIPY-group in position 10. However, the amino acid sequence is not identical in S16Thermo and S16Meso. This might explain the different quenching effects observed.
Figure 18. Crystal structure of S16 thermo showing the tyrosine residues present within the thermophile (blue), mesophile (green) and in both variants (red). Yellow spheres indicate the three possible BODIPY sites.

W74C
 shows an unusual behaviour, it is namely unquenched under dilute conditions. However, with increasing dextran concentration, the quantum yield decreases. This suggests that the dynamics of the BODIPY-group and quencher is altered upon increasing amounts of dextran, thereby enabling quenching.

The quenching data suggest that there might be additional local interactions, in addition to steric repulsion between the protein and dextran. In order to gain more information regarding this, we performed additional quenching experiments with dextran, labelled with tyrosine (Tyr-dextran).

The Tyr-dextran contains on average 4.5 tyrosines per dextran molecule with a weight average molecular mass of 15.6 kDa. A concentration of 50 mg/mL Tyr-dextran corresponds to a Tyr-dextran – protein ratio of approximately 1000. This concentration is high enough to yield significant collisional quenching [71, 72], therefore we expected some quenching without implication of specific interactions. The two S16 meso mutants W39F/Q10C and W39F/K74C together with S16 thermo W74C were selected, mainly because they exhibit high quantum yields, and because W74C shows an interesting quenching pattern with dextran concentration. The quenching was monitored by steady-state and time-resolved fluorescence.
measurements as the Tyr-dextran concentration was increased. Interestingly, the three mutants respond to the added Tyr-dextran in three different ways, which is shown in Fig. 20.

Different outcomes are expected, depending on how the protein interacts with Tyr-dextran.

Case 1. If there are no specific interactions between solely protein and Tyr-dextran, the quenching effect on BODIPY will be due to collisions. In this case $\phi$ and $\langle \tau \rangle$ are expected to decrease by the same extent with increasing Tyr-dextran concentration.

Case 2. If there is a specific interaction between Tyr-dextran and the region surrounding BODIPY, the quenching effect will be larger than for collisional quenching and $\phi$ and $\langle \tau \rangle$ should not change linearly with Tyr-dextran concentration. Since there is on average 4.5 Tyr-residues in each Tyr-dextran, the probability of finding a tyrosine in proximity of BODIPY is random. If a tyrosine group is present in close proximity of the BODIPY-group, quenching is expected to occur faster than pure collisional quenching (I in Fig. 19). A local fast quenching may even appear like static quenching. If no tyrosine is present however, the BODIPY is shielded against other quenchers and hence unaffected (II in Fig. 19).

![Figure 19. Schematic illustration of the possible positions of BODIPY (B) and tyrosine (Y) upon a specific interaction between S16 and Tyr-dextran. I: BODIPY and Tyr are close enough for quenching to occur. II: No Tyr is close enough for BODIPY to be quenched. In this case dextran also acts to shield BODIPY from any other quenchers.](image-url)

For W39F/K74CM, the quantum yield and average lifetime decreases with the Tyr-dextran concentration to the same extent. This is consistent with pure collisional quenching, indicating the absence of specific protein-dextran interactions. This is also supported by the Stern-Volmer plot shown in Fig. 21.
In W39F/Q10CM, the average fluorescence lifetime decreases with the Tyr-dextran concentration in a similar manner as for W39F/K74CM. However, the quantum yield decreases to 0.7 with the first addition of Tyr-dextran, and stay approximately the same at higher concentrations. This is consistent with both dynamic and static quenching and Case 2 described previously. It seems the first addition of Tyr-dextran places a Tyr-Dextran in proximity of each BODIPY position, with approximately 30 % chance of Tyr-BODIPY interaction, leaving 70 % unquenched. Furthermore, increasing Tyr-dextran concentration cause no additional effect on BODIPY, since all the positions surrounding BODIPY are already occupied. Noteworthy is also the time-resolved fluorescence decay, which becomes highly biphasic in the presence of Tyr-dextran. The decay consist of one fast (0.4 ns) and one slow (5.8 ns) component (see Fig 22.). This kind of behaviour is rarely, if at all observed for collisional quenching in solution. It is however consistent with two populations of BODIPY; one which is subjected to a fast collisional quenching and another population which stay unaffected [73, 74]. This interpretation is also supported by the Stern-Volmer plot shown in Fig. 21.
The S16\textsubscript{Thermo} mutant W74C exhibits an unusual behaviour. One finds a drastic decrease of the quantum yield and average lifetime upon small additions of Tyr-dextran. But as the concentration of Tyr-dextran is gradually increased, $\phi$ and $\langle \tau \rangle$ starts to increase again. In addition, this mutant also reveals a biphasic BODIPY fluorescence decay, which is clearly seen in Fig. 22. As in the case for W39F/Q10C\textsubscript{M}, one fast and one slow component of the decay is obtained. The analyses of the fluorescence decay data yield one and the same long correlation time for all concentrations of Tyr-dextran. This supports the assumption of two fluorophore populations, of which one is not accessible to quenchers. With increasing Tyr-dextran concentration, the fraction of the fast component decreases, which explains why the average lifetime increases. The high degree of quenching at low concentration of Tyr-dextran suggests an interaction between regions surrounding BODIPY and Tyr-dextran. By increasing the concentration of Tyr-dextran, this interaction seems to decrease, which is puzzling. One explanation might be that at higher dextran concentration, the molecular shape of dextran changes, whereby the distribution of tyrosines changes.
The dynamical influence on the quenching process was tested for W74C by varying temperature at two concentrations of Tyr-dextran (Table 6). In 100 mg/mL Tyr-dextran, the quantum yield and lifetime decreases with increasing temperature, as would be expected for a dynamical process. On the other hand, in 12.5 mg/mL Tyr-dextran the quantum yield and lifetime increases with temperature. As in the case of increasing Tyr-dextran concentration, the increased lifetime is due to a decreasing fraction of the short lifetime component. This suggests that local interactions between the protein and Tyr-dextran decreases with increasing temperature.

<table>
<thead>
<tr>
<th>Temp (°C)</th>
<th>12.5 mg/mL</th>
<th>100 mg/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\phi$ ((\text{ns}))</td>
<td>$\phi$ ((\text{ns}))</td>
</tr>
<tr>
<td>10</td>
<td>0.56 5.4</td>
<td>0.89 5.7</td>
</tr>
<tr>
<td>20</td>
<td>0.55 5.3</td>
<td>0.84 5.7</td>
</tr>
<tr>
<td>30</td>
<td>0.66 5.6</td>
<td>0.86 5.6</td>
</tr>
<tr>
<td>40</td>
<td>0.59 5.7</td>
<td>0.76 5.5</td>
</tr>
<tr>
<td>50</td>
<td>0.65 5.7</td>
<td>0.78 5.4</td>
</tr>
</tbody>
</table>

The quantum yield ($\phi$) and average fluorescence lifetime ($\langle \tau \rangle$) for BODIPY in the S16\text{thermo} mutant W74C at 12.5 mg/mL and 100 mg/mL Tyr-dextran and at varying temperature.
As a control experiment, we measured quantum yields and fluorescence lifetimes of W74Cβ in the presence of 1.5 mM free tyrosine. This concentration represents the limit of solubility in presence of protein and buffer. It is comparable to 25 mg/mL Tyr-dextran, when assuming that one tyrosine per Tyr-dextran is able to act as quencher at the time. The quantum yields for BODIPY was 0.98 and 0.93 in absence and presence of 50 mg/mL dextran, respectively. This strongly suggests that the large quenching effects observed for this mutant cannot be described by collisions between S16 and high concentrations of Tyr-dextran.
4 Conclusions

In Paper I, an approach [4] is applied for the quantitative analysis of depolarisation data obtained from time-resolved two-photon excited fluorescence experiments. Energy migration between two 9-anthrylmethyl groups, which are separated by a rigid steroid spacer, was described by an extended Förster theory. Reasonable values of the distance between the donor-groups were found. This strongly suggests that this EFT approach can successfully be used for TPE data, preferably in combination with OPE data.

The three studies on the ribosomal protein S16 provide important information regarding the protein itself, as well as the influence of macromolecular crowding. Two homologs of the protein S16 were investigated, isolated from a hyperthermophilic bacterium (S16Thermo) and a mesophilic bacterium (S16Meso). DAET experiments in Paper II reveal that S16Thermo exhibits a more compact denatured state ensemble than that of S16Meso. An important conclusion from this study is that the unfolded state compaction may be one of the reasons for a higher thermal stability of S16Thermo relative to S16Meso.

Paper III showed that the addition of the crowding agent dextran 20 (200 mg/mL), forces the unfolded state of S16Thermo to become more compact. However, it appears that the unfolded state compaction is not symmetrical and seems to depend on preferences of the proteins as well.

In Paper IV intramolecular distances in the folded state were investigated for two mutants of S16Thermo and three mutants of S16Meso, at varying amounts of dextran 20. It was found that distances within S16Thermo are unaffected by the degree of crowding. Interestingly, S16Meso show decreasing distances up to 100 mg/mL dextran, whereas the change in distances becomes anisotropic at higher concentrations. This suggests that macromolecular crowding may cause marginally stable proteins (like S16Meso) to adapt to surrounding steric repulsive forces by fine-tuning its structure. More stable proteins (like S16Thermo) however, show no structural effect due to crowding.

Paper IV provide evidence of specific protein-crowder interactions. Quenching experiments indicate attractive forces between dextran and positions within the protein structure. However, these local effects seem small as compared to the dominating entropic effects, caused by steric repulsion.
5 Future Perspectives

Our results show that the developed approach for analysing TPE depolarisation data yields physically reasonable parameters. However, the method should be tested further on additional bichromophoric model systems. It would also be interesting to use it on biologically interesting systems, such as proteins. Since TPE excites the samples using light of lower energy, it is less photo-damaging and yield less background scattering to such an environment than OPE. TPE is of great value in e.g. biomolecular imaging and fluorescence correlation spectroscopy [75-77] since it provides an increased resolution and better three-dimensional depth as well as a reduced photobleaching and toxicity outside the focal volume. Thus, the same benefits are also valid in DDEM experiments.

I believe that the topic of macromolecular crowding is of great importance. It would be desirable to always design experiments as close to reality as possible. Since working within living cells still introduces many difficulties, we should focus on exploring other options rather than simply go to the other extreme, as in using pure buffer solutions. For instance, it would be interesting to study a mixture of crowding agents, with different properties, such as size and shape and to vary the mixture ratios of these. Preferably, highly concentrated extracts of cell contents could be used. DAET could be a useful tool in such an experiment, provided one choose to work with a donor-acceptor pair, where neither is naturally present within the cell. To circumvent the specificity in the labelling procedure, one can choose to work with two chemically identical chromophores and hence use the approach of DDEM previously described in this thesis.
6 Acknowledgements

So, I’m finally reaching the end of my journey! What normally takes a PhD-student 4-5 years took me almost 8 years. But I can honestly and truly say that it has been the best 8 years of my life and I wouldn’t want to have had it any other way. I don’t believe I will ever find a job more satisfying than this one, and I am very lucky to have experienced it. To be the first person to know the results of an experiment, a tiny piece of information as part of solving the mysteries of this world, is very exciting, and I hope I will be lucky enough to be able to continue this road in the future! But I have not walked this path by myself and it would not have been possible, or as much fun, without a lot of special people, who I now have the opportunity to give special thanks!!

On top of my list is of course Lennart. Thank you for giving me the opportunity to work in your group and in your lab, it has been a privilege. The projects we have been working on have been interesting and sometimes lead us to places we did not imagine to begin with. This is of course one of the upsides of being a scientist, you simply go wherever the experiments take you ☺ Thank you for all the guidance you have provided me with and for all the encouragements and helpful discussions we have had during the years.

Pernilla, you have been an important part of the last two years of my research and you introduced an interesting topic into my crowded world. Thank you for all the support and encouragement and for always transmitting positive energy.

Jörgen, thank you for always providing me with perfect protein samples, for all the help with CD-measurements, for being patient and always answering my questions regarding all sides of protein chemistry and for all the nice pictures you have provided me with. I have enjoyed our collaboration and I hope you have too!

Matteus and Roberth, thank you for being the perfect roommates during my first years at the department, I have truly missed you both since you left.

I would like to thank all the previous group-members; Denys, Mikael, Nils, Linus, Oleg, Erik and Radek for introducing me into the topic and for all the help in the lab. Special thanks to Linus for all the help in your lab in Uppsala and together with Radek and Oleg I also thank you for our collaborations. I also want to send a special thank you to Stanislav, for all the help with the MATLAB analysis program.
There are also some people in the corridor I would like to especially acknowledge; **Magnus** for introducing me to the perfect model-protein S16, **Per-Olof** for assigning me to a lot of teaching which I have both enjoyed and learned a lot from, **Gerhard** for all the positive energy you disperse early in the morning when the corridors are so empty, **Tobias** for always lending a helping hand, **Alexander** for the teaching we have endured together and for your steady hands when changing the light sources in the CD-instruments.

Thank you also to all the nice **colleagues** at the **department of chemistry** and in the **corridor**, for collaborations, always being willing to help each other and for all the nice times in the “fikaroom”.

For my friends and family, I switch to Swedish:

Speciellt stort tack till dig **Maria**, för att du är en sådan fantastisk vän och kollega. Vi kämpade tillsammans i 4 år på kemistprogrammet och hade även turen att landa i samma korridor under vår tid som doktorander. Du har betytt mycket för mig och kommer alltid att ha en plats på min ”hurts”, var den än må befinna sig i framtiden. Tack också till dig och **Daniel** för allt det roliga vi haft på fritiden ☺ och för att ni alltid ställt upp för oss och flickorna!

Tack **Vivvi**, för att du tar så otroligt bra hand om våra tjejer varje dag, så att jag istället kan fokusera på jobbet till 100 %. Du är den bästa dagmamman jag kan tänka mig och en fantastisk person!


Tack **IngaLill** och **Petra**, samt resten av **Mikaelsson-Dahlman** släktet, som med öppna armar välkomnade mig in som en del av familjen!

Till **min älskade familj**: STORT TACK för att ni finns där för mig varje dag och för att ni alltid får mig att tänka på andra saker. **Lars**, tack för att du alltid stöttat mig och respekterat mina val samt för att du alltid utan att beklaga dig en ende gång, skött alla morgonbestyr, så att jag kunnat åka iväg tidigt för att i slutet av dagen kunna komma hem till er i rimlig tid. Tack **Izabelle** och **Olivia** för att ni är helt underbara små tjejer!
7 References


55. Birks, J.B., Photophysics of Aromatic Molecules. 1970: John Wiley & Sons Ltd.


57. Privat, J.P., P. Wahl, and J.C. Auchet, Rates of deactivation processes of indole-derivatives in water-organic solvent mixtures - application to
54


55