Ph.D. THESIS

Functional dynamics of proteins revealed by fluorescence and femtosecond transient absorption spectroscopy

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1. Dynamics of actomyosin complex revealed by fluorescence spectroscopy

Introduction

In the conceptual framework of the molecular events during muscle contraction[1] the cyclic interaction of actin and myosin drives the sliding of thin and thick filaments past one another. The interaction of these two proteins is powered by the hydrolysis of ATP that occurs in the catalytic domain of the myosin head. Although this "sliding filament hypothesis" gained wide acceptance, many questions remained unanswered regarding the mechanism of energy transduction and the intra-molecular conformational changes within the myosin head (and possibly within the actin filament).

Myosin

Myosin is a large asymmetric molecule containing a long tail and a globular head.

In the presence of strong denaturing solutions (5 M HCl or 8 M urea) the myosin dissociates into six polypeptide chains: into two heavy chains (200 000 Da each) and four light chains (two with molecular weight of 20 000 Da, one with 15,000 and another with 25,000). The two heavy chains form a double helix, at one end both are folded into separate globular structures forming the two heads of the myosin.

Exposed to proteolytic enzymes the myosin molecule can be decomposed into further subcomponents. Using chymotrypsin the myosin dissociates into heavy meromyosin (HMM), with molecular weight of 350 kDa and light meromyosin (LMM) with the molecular weight of 150 kDa. HMM can be split into further parts –
S1 (subfragmentum 1) és S2 (subfragmentum 2) – using papain. Among the mentioned fragments, HMM and S1 have a distinguished role: both of them can bind actin, have ATPase activity and can move actin filaments.

Further digestion of the myosin molecule revealed clarified that S1 is the smallest active component of the myosin molecule which can move the actin filament[2].

The regulatory and essential light chains wrap around the 20-kDa fragment of S1, which forms an 85Å-long a helix that spans much of the S1. It was suggested that

the conformational changes in the nucleotide-binding pocket could be transmitted to the 85Å-long a helix. This structure may play the role of a lever arm, which magnifies small conformational changes to larger movements.

Actin

Actin monomer is a 43 kDa weight globular protein composed by two main domains, containing two further subdomains each.

The junction of the two main domains is the cation- and nucleotide-binding cleft in which Mg^{2+} or Ca^{2+}, ATP, ADP.P_i, or ADP can be found. In nucleotide free buffers the actin denatures quickly.

In the presence of physiological salt concentrations the actin monomers polymerizes easily forming a double helix. Coexisting with the actin filaments there is always an actin monomer population. In equilibrium the concentration of the monomers is the critical concentration, bellow which the monomers cannot polymerize. The critical concentration is a function of environmental factors, e.g. the nucleotide and cation concentrations.

Actomyosin interaction. The contraction cycle.

According to the swinging lever arm hypothesis - which is the current explanation for the force generation mechanism - during the ATP-hydrolysis the subfragment 1 undergoes subtle conformational changes and as a result the lever arm moves through 10 nm along the actin filaments.

When no nucleotide is bound to S1 the myosin head is in tight binding with the actin (rigor state) forming a 45° angle with its axes. The nucleotide-binding pocket of S1 is opened in this case. After binding an ATP molecule, the myosin head dissociates from actin filament and the nucleotide-binding pocket of S1 turns to the closed state. In the ADP-P_i–bound state, the catalytic domain binds weakly to actin. Actin docking causes phosphate release from the active site. The lever arm then swings to the post-stroke position in the ADP-bound state, which moves the actin filament by 10 nm. After completing the stroke the ADP dissociates from myosin and the next ATP binds to the active site, which rapidly reverts the catalytic core to its weak-binding actin state. The lever arm will then recock back to its pre-stroke state.

Aims

Conformational changes in the actomyosin complex play a distinguished role during muscle contraction. Taking into account this property of the actomyosin complex it is important to characterize the dynamical properties of different regions of the complex in the individual states of the contraction cycle. The general main of this work was to characterize the flexibility of the catalytic- and light-chain-binding domains of myosin-S1 in the actomyosin complex in the nucleotide free (rigor) state.

As a first step it was important to find suitable methods to characterize the flexibility and the dynamics of these proteins. In our special case the choice was based on the fluorescent labelling of the highly reactive cysteine residues, which can be found on the catalytic- and the light-chain-binding domains. We were able to label specifically the mentioned residues with fluorophores, thus we could use fluorescence resonance energy transfer methods.

As a first step of the investigation the aim was to developed special FRET methods to describe the dynamical properties of the interacting proteins.

After the elaboration of the fluorescent spectroscopic methods we attempted to characterise the dynamic properties of the catalytic- and light-chain-binding domains in the actomyosin complex.

Methods

In order to investigate the dynamic properties of the actomyosin complex we used two fluorescence spectroscopic methods, based on the phenomena called fluorescence resonance energy transfer. The term fluorescence resonance energy transfer (FRET) is commonly used to describe singlet-singlet energy transfer via a mechanism based on long-range dipole-dipole resonance coupling.

The efficiency of the process (i.e., what percentage of the excited molecules were relaxed trough energy transfer):

$$E = 1 - \frac{\tau_{DA}}{\tau_D} = 1 - \frac{F_{DA}}{F_D},$$ (I.1)

where τ_{DA} and F_{DA} stands for the lifetime and the intensity of the donor in the presence τ_D and F_D the lifetime and the intensity of the donor in the absence of the acceptor.

FRET in the case of systems with helical symmetries

The first applied FRET method uses the helical symmetry of the molecules, and using it one can determine distance distributions and their modifications characteristic for structural changes of the molecules. The method was developed for the actomyosin complex[3] but it can be used for any system with helical symmetries.

In the case of one donor – on acceptor system the FRET efficiency can be calculated as follows:

$$E = \frac{(R_0 / R)^6}{1 + (R_0 / R)^6},$$ (I.2)

where R stands for the donor-acceptor distance, R_0 that specific donor-acceptor distance, for the transfer efficiency is 0.5 (50%).

In the case of the actomyosin complex the donor was attached to the myosin subfragment-1, the acceptors were attached to the actin protomers. In this more complex case the calculation of the FRET efficiency is modified as follows[4]:

$$E = \frac{\sum_{i=1}^{N}(R_0 / R_i)^6}{1 + \sum_{i=1}^{N}(R_0 / R_i)^6},$$ (I.3)

where the R_i values are the individual donor-acceptor distances and N is the number of the acceptors.

For the simplicity during the calculation of the transfer efficiency we take into account the contribution of the five closest acceptors.

Calculating the individual R_i distances we take into account the helical symmetry of the actin filament: F-actin is a helix having a 13/6 geometry (in 13 adjacent monomers are placed in 6 turns). Thus the geometry of a filament is described by the radial coordinate of the labelled residue (r), and also by the pitch of the actin monomer in the actin filament, and the relative rotation of the neighbouring monomer along the genetic helix, which can be taken to be 55 Å and 166 Å, respectively. The coordinates (x, y, and z) of the labelled actin residues on the five actin monomers considered can be then calculated by using the following equations:

$$x_i = r\ cos((3-i)166^o) \tag{I.4a}$$
$$y_i = r\ sin((3-i)166^o) \tag{I.4b}$$
$$z_i = (3-i)27.5\ \text{Å}, \tag{I.4c}$$

where i takes the integer value from 1 to 5 and refers to individual actin monomers. The value of r (~ 25 Å) was known from crystallographic data.

If the acceptor molar ratio (g) is known, the probability (p_k) of the individual arrangements could be obtained from the binomial distribution as follows:

$$p_k = g^k (1-g)^{5-k} \tag{I.5}$$

Knowing the positions of the potential acceptor labelling sites on the five actin monomers, the cumulative transfer efficiency (E_g) at a given acceptor molar ratio (g) can be calculated using Eqs. (I.4) and (I.5) as follows:

$$E_g = \sum_{k=1}^{5} p_k E_k \tag{I.6}$$

where E_k is the FRET efficiency between the donor and the acceptors in the kth arrangement of actin monomers, whereas p_k is the probability of the kth arrangement.

By applying these equations one can compare simulated curves with the experimental data and determine the physically veritable position of the donor in an actin-binding protein.

With a more complex analysis the method can also give information regarding the conformational heterogeneity of the protein matrix. To accommodate conformational distribution the donor-acceptor distance distributions were taken into account. It is assumed in the analysis that the transitions between the conformational states are slow on a nanosecond time scale. The distance distribution characteristic for a donor-acceptor system ($\omega(R)$) can be approximated with a Gaussian function, with a mean value of R_c and the full width at half-maximum of σ:

$$\omega(R) = \frac{1}{\sigma\sqrt{2\pi}}\exp\left[-\frac{(R-R_c)^2}{2\sigma^2}\right],$$ (I.7)

In this case the summation of the individual transfer efficiencies is carried out as follows:

$$E_g = \sum_{jk} p_k \omega(R_j) E_{jk} .$$ (I.8)

Flexibility of proteins revealed by FRET

In order to characterize the flexibility of proteins we used another FRET-method. The method is based on the assumption that the value of the rate constant characteristic for the energy transfer $<k_t>$ is an appropriate parameter for monitoring local fluctuations in a macromolecule[5]. To determine its value experimentally the FRET parameter $f\,'$ was introduced as:

$$f' = \frac{\langle E \rangle}{\langle F_{DA} \rangle}$$ (I.9)

It should be noted that the measured normalized energy transfer parameter of the system having a single donor that interacts with more than one acceptors is the sum of the normalized energy transfer efficiencies that characterize the individual donor-acceptor systems[6].

8

Results and discussion

Distance distributions in the actomyosin complex.

The cyclic interaction between actin and myosin is thought to provide the molecular basis for muscle contraction. In the presented experiments we determined the distance of the catalytic domain of S1 and the light-chain-binding domain from the actin filament in the nucleotide free (rigor) state of the actomyosin complex[7].

In order to characterize the distances we use our FRET method developed for systems with helical symmetry. For the determination of the catalytic domain – actin filament distance the Cys-707 residue of myosin-S1 was labelled with IAEDANS, which served as FRET donor. Acceptors were attached to the Cys-374 residue of the actin protomers. To determine the light-chain-binding domain – actin distance the donor (IAEDANS) was attached to the Cys-177 residue of the essential light-chain (ELC) of the subfragment-1.
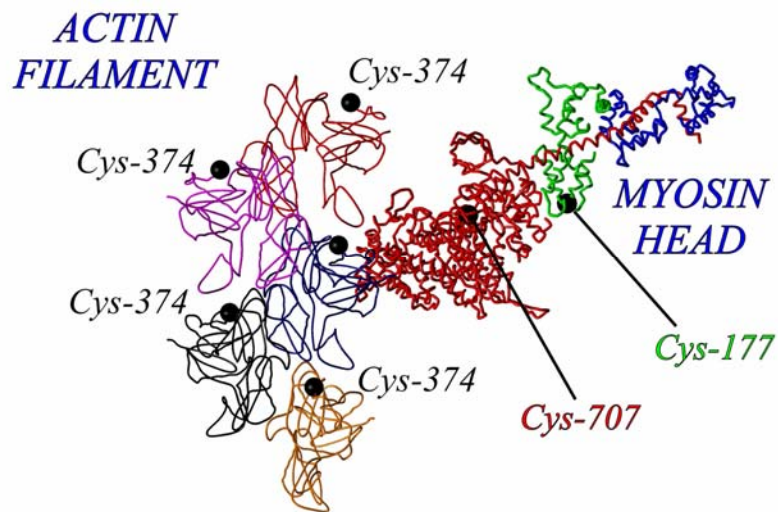

Figure. I.1. Schematic representation of the atomic model of the actomyosin complex. The Cys-707 and Cys-177 residues which were covalently modified with donor in fluorescence experiments are labeled in the catalytic domain and in the essential light-chain. The positions of potential acceptor labeling sites (Cys-374) on the actin filament are also indicated.

When the donor was attached to the Cys-707 of the S1 the measured transfer efficiency increased proportionally to the acceptor molar ratio, which is evidenced by a good linear fit (Fig. I.2a). Considering that such linear relationship is typical for a single donor - single acceptor system, it seems to be likely that the donor on the Cys-707 of S1 probably transferred energy predominantly to the acceptor on the closest actin monomer. In contrast, the dependence of transfer efficiency on the acceptor molar ratio deviated from the linear in the case of transfer between the donor on Cys-177 of ELC and acceptors on the actin filament.
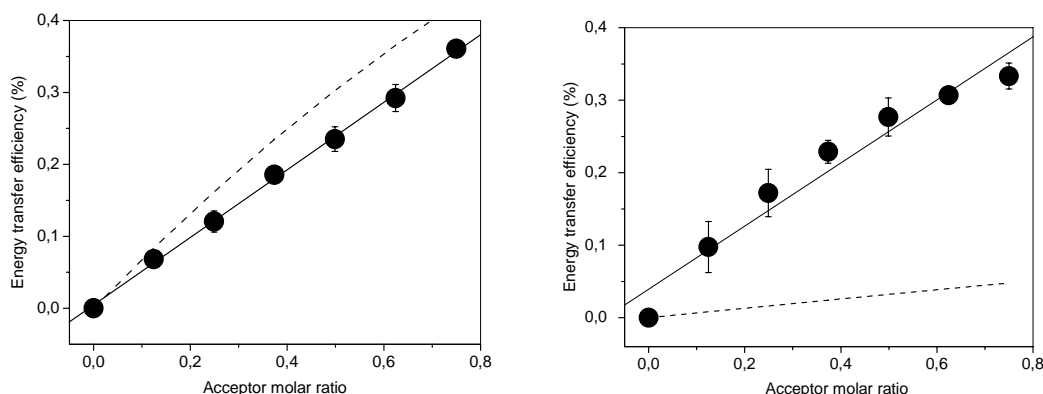

Figure I.2. The measured transfer efficiency as a function of acceptor molar ratio in the case of energy transfer between the catalytic domain (Cys-707) and actin (*a*), or between the light-chain binding domain and actin (*b*). The linear fits, which would be expected for a single donor – single acceptor system (*continuous lines*), and the transfer efficiencies calculated using the atomic model of the acto-S1 complex (*dashed lines*)

The analysis of the measured transfer efficiency data was carried out by assuming either homogenous S1 population, where all the S1's are in identical conformation, or by considering a conformational distribution of the labelled protein resulting in a donor-acceptor distance distribution. In the former case it was assumed that the donor position could be described with single set of x, y and z coordinates for the entire S1 population. This approximation resulted in a good fit for the donor on Cys-707 (Fig. I.3a). The value of x, i.e., the distance of Cys-707 from the filament axis was 77 ± 3 Å. The values of y and z were 5 ± 5 Å and 3 ± 4 Å, respectively. The x, y and z coordinates correspond to 52 ± 3 Å distance between the donor and the acceptor on the nearest actin monomer by considering the 25 Å consensus radial coordinate of Cys-374 in the actin filament.

In the case of transfer between the donor on Cys-177 and acceptors on actin, the assumption of homogenous S1 population failed to give a good fit (dotted line in Fig. I.3b).
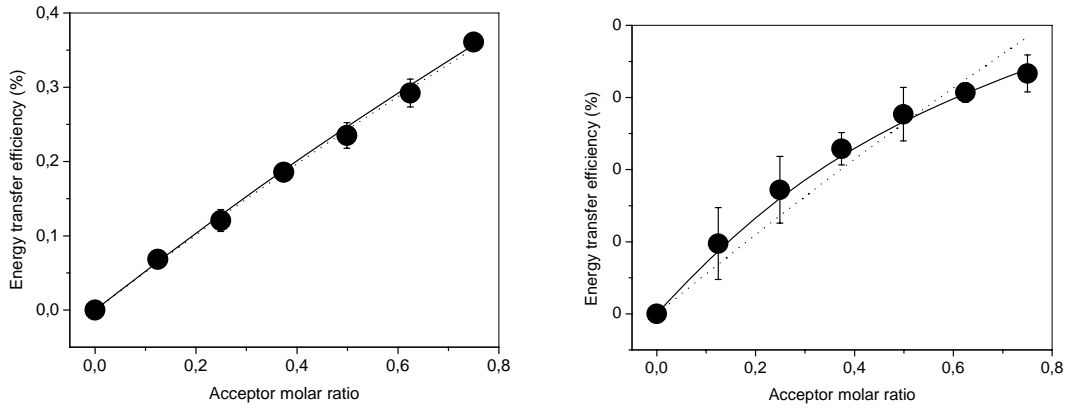

Figure I.3.Best fits to the experimental data in the case of resonance energy transfer between the acceptors on actin and donor on Cys-707 (*a*), or Cys-177 (*b*) of S1. The fits were performed assuming either homogeneous (*dotted lines*), or heterogeneous (*continuous lines*) S1 populations.

Deviation from the linear fit could be in principle explained by nonrandom actin polymerization resulting in an inhomogeneous acceptor distribution[8]. To estimate the effect of nonrandom filament assembly, we polymerized the actin in the presence of phalloidin. Such preparation was shown to result in a random monomer assembly[8]. Because the results obtained in the presence and absence of phalloidin were indistinguishable from each other, the effect of nonrandom actin assembly was excluded. The consideration of these results and the inability of the simulations assuming homogeneous S1 population to approximate the experimental results in the case of ELC suggested that the conformation of the light-chain-binding domain of S1 was heterogeneous.

To test the effect of heterogeneous S1 population, the distance calculations were carried out with the assumption that the S1 population could adopt a wide range of conformations characterized by a donor-acceptor distance distribution (Fig. 3. a,b). The inter-conversion rate among these conformations was assumed to be slow on a nanosecond time-scale. In the case of donor on the catalytic domain (Cys-707) the assumption of homogeneous S1 population resulted in good fit of the simulated data to the experimental ones. Thus, it might be expected that the positional distribution of the

S1 population is narrow even if such a distribution is assumed. The distribution of distances between Cys-707 of S1 and the z axis was centered at 77 ± 2 Å. Considering the radial coordinate of Cys-374 of actin the distance between Cys-707 of S1 and Cys-374 on the closest actin monomer was calculated to be 52 ± 2 Å, which is in good agreement with calculations that assumed a homogeneous S1 population. The width of this distance distribution was 5 ± 3 Å. The relatively small width indicates that the positional distribution between Cys-707 in S1's catalytic domain and the actin filament is narrow.

In the case of energy transfer between the donor on Cys-177 of the ELC and the acceptors on actin, the simulation gave a remarkably good fit if heterogeneity of the S1 population was assumed (Fig. 3 b). The distance distribution between Cys-177 of the ELC and the z axis of the actin filament was centered at 98 ± 3 Å with a width of 102 ± 4 Å. Accordingly, the mean distance between Cys-177 of ELC and Cys-374 on actin was 73 ± 3 Å. The wide positional distribution of the ELC, and therefore probably the light-chain binding domain of S1, reflects either the large flexibility of the protein matrix, or the presence of a large number of distinct conformations of a relatively rigid protein matrix. Further experiments were required to distinguish between the alternative explanations (see below).

When a conformational distribution accompanied with a distance distribution of the light-chain-binding domain of S1 was considered in the analysis of the FRET data, the mean distance was 73 Å between the donor on Cys-177 and acceptors on actin. The distance resolved in the present analysis (73 Å) is longer than those (50-60 Å) published previously by other laboratories[9, 10] and correlates better with the distance (c.a. 89 Å) calculated from the atomic model of the actomyosin complex. The nonzero size of the probes applied in the FRET experiments (5-15 Å) may explain[11] the perishing difference between determinations based on FRET data and the atomic model.

These experiments did not resolve which part of the S1 was responsible for the emergence of elastic strain. FRET experiments could detect the swinging motion of the light-chain-binding domain and provided evidence for the close coupling between the isomerization of myosin head and the phosphate-release step[12]. The ability of the catalytic and light-chain-binding domain to rotate relative to each other was also shown

previously by using conventional EPR spectroscopy[13]. In accordance with this observation, saturation transfer-EPR measurements suggested that the catalytic and light-chain-binding domains were connected by a flexible hinge in the myosin head[14]. In good correlation with these observations, our results indicate that the dynamic properties of the protein matrix of S1 allow the independent rotation of the light-chain-binding domain relative to the catalytic domain, which is fixed in a unique conformation to the actin filament under rigor conditions. Interestingly, in saturation transfer-EPR measurements, the difference between the mobility of the catalytic and light-chain-binding domains existed only in the filament form of myosin[14], but disappeared in either the monomeric form or when the myosin was bound to actin. In the light of this result it seems to be important to determine by other experimental methods whether the large flexibility of the protein matrix was responsible for the wide distance distribution of the light-chain-binding domain in our experiments, or a relatively rigid protein experienced a number of distinct conformations separated by relatively high free energy barriers.

Flexibility of the actomyosin complex

To further characterize the dynamic properties of myosin S1 in the actomyosin complex we investigated the inter-protein flexibility of the acto-S1 complex in the nucleotide-free, i.e. rigor, state by using the other described FRET – based method. The experiments were designed to provide information about the flexibility of the protein matrix between the Cys-374 residues of the actin and either the catalytic domain (Cys-707) or the light-chain-binding domain (Cys-177) of S1.

The temperature profile of parameter f' provides information about the changes in the flexibility of the protein matrix and about the possible temperature induced conformational transitions[5].

The value of f' was insensitive to temperature changes between 5 and 35 $^{\circ}$C when the donor was on the catalytic domain (Cys-707) of S1 and the acceptors were attached to the actin filament (Cys-374) (Fig. 2). In contrast, the value of f' was strongly temperature dependent when measured for the donor in the essential light-chain of S1 (Cys-177) and acceptors on Cys-374 of the actin filament (Fig. I.4).
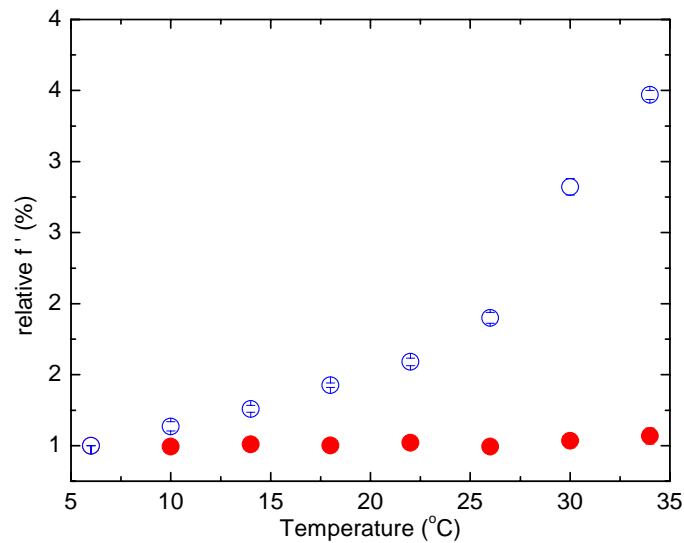
Figure I.4. The temperature dependence of the relative f′ in the case of energy transfer between acceptors on the actin filament and donor on the Cys-707 of S1 (filled circles), or donor on the Cys-177 of the essential light-chain and (empty circles).

The FRET data presented here demonstrated that the protein matrix between Cys-707 of S1 and acceptors on the actin filament in the rigor complex is substantially rigid. This result is what one would expect from the observation that the distance distribution between these points is narrow[7]. On the other hand, the wide positional distribution reported for the essential light-chain in the rigor acto-S1 complex[7] is due to the large flexibility of the protein matrix connecting the catalytic and light-chain-binding domains of S1.

The flexibility of the connection between the catalytic and light-chain-binding domains could contribute to the reduction of the energy barrier to inter-conversion between neighbouring states of the contraction cycle. On the other hand, consideration of the difference in the helical symmetry of the actin and myosin filaments suggests that the flexibility of the light-chain-binding domain might be important in accommodating the symmetry difference and allowing the formation of cross-bridges between the two filament systems. Our data also show that the protein region between the actin filament and the Cys-707 of S1 is rigid, in good accordance with the assumption of the 'rotating lever arm' model[15-17].

Summary

A FRET based method was developed to measure the distance distributions in macromolecule systems with helical symmetries.

The applicability of the method was proved in the case of the actomyosin complex. Using the method we were able to determine the distance between the catalytic or light-chain-binding domains of myosin-S1 and the actin filament.

Assuming a distance distribution we were able to characterize quantitatively the flexibility of the catalytic and light-chain-binding domain of myosin-S1.

By measuring the temperature profile of the f' parameter a qualitative picture of the meaning of distance distributions was obtained: the protein matrix between Cys-707 of S1 and acceptors on the actin filament is substantially rigid in the rigor complex. This result is what one would expect from the observation that the distance distribution between these points is narrow. We also showed that the wide positional distribution reported for the essential light-chain in the rigor acto-S1 complex is due to the large flexibility of the protein matrix connecting the actin and light-chain-binding domain of S1.

Our results corroborated previous findings that the catalytic domain and the light-chain-binding domain can rotate relative to each other.

2. Photoactivation of *E. coli* photolyase as revealed by femtosecond transient absorption spectroscopy

Introduction

Photolyase

UV light induces two major lesions in DNA: the cyclobutane pyrimidine dimers(Pyr<>Pyr) and the pyrimidine-pyrimidone (6-4) photoproduct (Pyr [6-4] Pyr). The subject of our experiments was the enzyme called photolyase which catalyzes the repair of UV-induced lesions in DNA[18].

Flavoproteins are ubiquitous proteins in which the flavin cofactor plays the role of electron transfer intermediate in various biochemical reactions. Flavins display rich redox chemistry as they can adopt three different redox states: oxidized, semi-reduced (radical) and fully reduced, and in addition the redox changes can be accompanied by protonation changes. The different forms of the flavin chromophore have characteristic absorption spectra in the visible and near UV, but the physiological functions of most flavoproteins are light independent.

DNA photolyase is able to repair far-UV damaged DNA, using light-energy to cleave the cyclobutane ring of the Pyr<>Pyr dimers[18, 19]. After binding DNA, repair mechanism is initiated by the absorption of a blue or near-UV photon, followed by an electron transfer from photoexcited FADH– to the Pyr<>Pyr dimer which breaks the dimerization bond. After the cleavage step the electron is transferred back to flavin cofactor.

Photolyases are monomeric proteins of 450-550 amino acids and two noncovalently bound chromophore cofactors. One of the cofactors is always FAD, and the second is either methenyltetrahydrofolate (MTHF) or 8-hydroxy-7,8-didemethyl-5-eazariboflavin (8-HDF).

Flavin is one of the most commonly used cofactors in nature, and FAD is the most common form of flavin found in enzymes. At least 151 enzymes use FAD and/or FMN as cofactors. Flavin can be reduced and oxidized by one- and twoelectron-transfer reactions and the active form of flavin in photolyase is the two-electron-reduced form[18].

In isolated photolyase from many organisms, the flavin is in the neutral radical form (FADH$^\bullet$), and the active fully reduced form can be generated by another photochemical process (photoreduction). The aim of our experiments was to elucidate the electron transfer process trough which results in the reduction of the flavin cofactor.
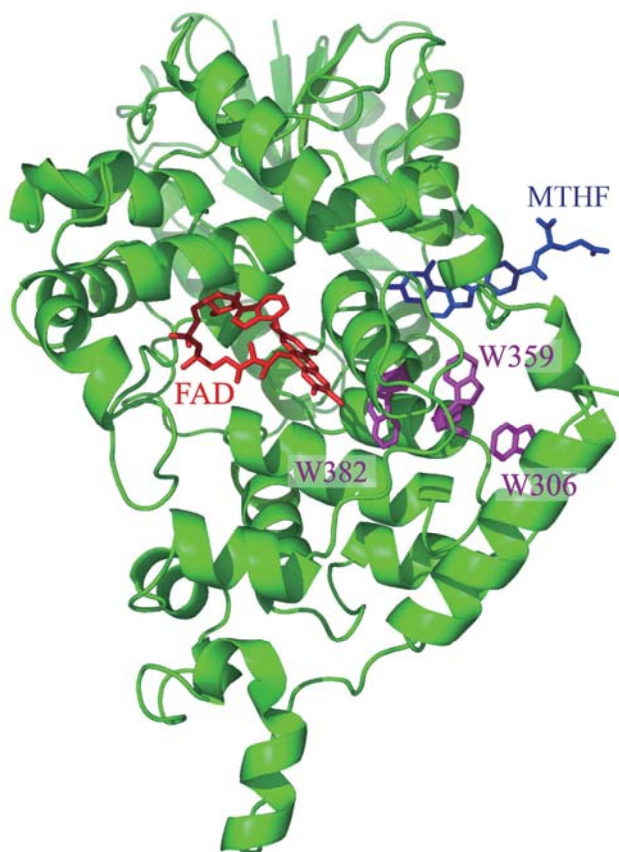

Figure II.1. The structure of *E. coli photolyase*, with the antenna chromophore (MTHF), the flavin cofactor (FAD) and the tryptophan triad involved in the electron transfer.

Photolyase contains a second chromophore (MTHF or8-HDF) which is not necessary for catalysis and has no effect on specific enzyme-substrate binding[20, 21], but it can increase the rate of repair 10-100-fold[18] depending on the wavelength used to effect catalysis. This is because the second chromophore has a higher extinction coefficient than FADH$^-$ and an absorption maximum at longer wavelength relative to that of the two-electron-reduced flavin that is the active form.

Photoreactivation and photoactivation in E. coli photolyase

In photolyase two electron transfer processes take place: photoreactivation, responsible for the DNA repair and photoactivation which is the process leading to the reduction of the flavin cofactor.

During photoreactivation the enzyme binds a Pyr<>Pyr in DNA, the folate (or 8-HDF) then absorbs a near-UV/blue-light photon and transfers the excitation energy (via fluorescence rezonance energy transfer) to flavin, which then transfers in ~170 ps an electron to the Pyr<>Pyr[22]; the 5-5 and 6-6 bonds of the cyclobutane ring are now in violation of Hückel rules, and therefore, the Pyr<>Pyr is split to form two pyrimidines. After splitting the bond an electron is transferred back to the nascently formed FADH$^\bullet$ to regenerate the FADH$^-$ form.

In isolated photolyase from many organisms, the flavin is in the neutral radical form (FADH$^\bullet$), and the active fully reduced form can be generated by another electron transfer process called photoreduction. In *E. coli* photolyase the reduction of the catalytically neutral FADH$^\bullet$ is realized by an electron transfer trough tryptophan triad (W382-W359-W306) close to the flavin cofactor[23]. The terminal electron donor is the W306 tryptophan, located at 15 Å from the flavin[24]. Reduction of W306 with an external electron stabilizes the FADH$^-$ state of flavin. In the absence of external electron donor flavin cofactor relaxes to its FADH$^\bullet$ state with charge recombination[23, 25].

Aims

The purpose of my work was to give deeper understanding of the electron transfer process (trough the W382-W359-W306 tryptophan triad) behind the photoactivation of *E. coli* photolyase.

In their earlier work my colleagues replaced tryptophan W382 with the redox inert phenylalanine, and they proved that as a first step of the electron transfer process, after excitation an electron jumps to the flavin cofactor[25]. Thus the aim of this work was to answer the following questions:

1) Can be the flavin cofactor permanently reduced if the tryptophan is replaced by a redox inert phenylalanine?

2) Does the W359→W382 electron transfer step exist during photoactivation ?

3) If yes, how fast is the W359→W382 step?

Methods

The presented experiments were realized using pump-probe spectroscopy. The principle of pump-probe measurements can be easily understood from the following picture (Fig II.2): after excitation (pump) at specific delay times the sample is illuminated by a (probe) beam and the instrument detects the transmission. The presented pump-probe setup is able to detect changes of the absorption on the femtosecond scale.
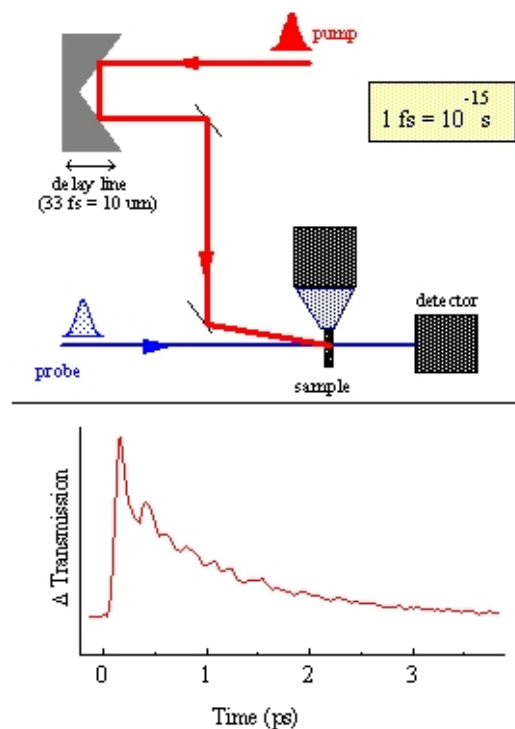

Figure II.2. Shematic picture of a pump-probe setup.(Vos M H, 1999[26].)

The detected absorption changes can be explained by the following processes:
1. Bleaching of the sample after the excitation.
2. Stimulated emission.
3. Excited state absorption.

Results and discussion

In order to examine the role of the tryptophan triad in the electron transfer process, the tryptophans were replaced by site directed mutagenesis one by one with redox inert phenylalanine. Earlier work of my colleagues elucidated the role of the closest tryptophan (W382) in the electron transfer process and the time constant of the relaxation of $FADH^{\bullet *}$ state[25]. In the case of wild type photolyase the rate constant is about $(24 \text{ ps})^{-1}$, in the case of the mutant the rate constant is $(80 \text{ ps})^{-1}$ on the whole spectral range. The longer rate constant observed in the case of the mutant hints that no electron transfer takes place, which means that the initial electron donor is the tryptophan W382. The measured 80 ps is the time constant of the relaxation of $FADH^{\bullet *}$ state. The time constant observed in the case of wild type is the result of two competitive processes: relaxation of $FADH^{\bullet *}$ with and without electron transfer. The initial electron transfer step takes place in 38-45 ps.

The experiments were performed on two spectral range: 420-590 nm (with pump wavelength at 620 nm) and 630-700 nm (pump wavelength set at 550 nm). Comparing the transient kinetics on the 630-700 nm spectral range one can observe that both wild type and mutant photolyase relax with the same time constant, indicating that the excited flavin is relaxed trough electron process in the case of the mutant as well.

Fitting the transients it was found that the time constant of on the 420-470 nm spectral range is ~30 ps too. Comparing the kinetics of the mutant and the wild type photolyase it can be easily observe, that while in the case of wild type after 150 ps the absorption change does not relax to zero, in the case of the mutant it diminish (Fig II.3).
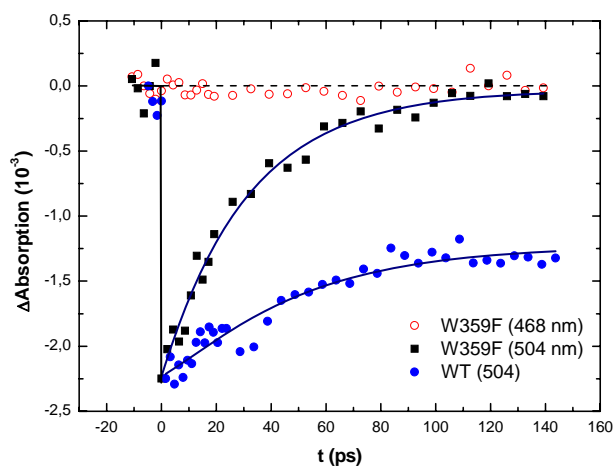

Figure II.3. Kinetics of absorption change of wild type (filled circles), and W359F mutant (squares) at the main bleaching, at 504 nm and at the 468 nm isosbestic point (open circles).

Analyzing the transient spectra it can be seen that the wild type photolyase relaxes to its resting spectra (which can be associated to the $FADH^-W^{\bullet+}$ state) during the time window of the experiments. However, in the mutant the spectrum relaxes to 0 (Fig. II.4) This suggests that in the mutant, where the second (W359) tryptophan is replaced, the initial radical donor W382 is not significantly oxidized at any time. This implies that the back reaction to the neutral ground state is rapid compared to the decay of the excited state $FADH^{\bullet*}$ As this is presumably also the case for WT, the forward radical transfer $W382^{\bullet} \rightarrow W359^{\bullet}$ must also be substantially faster than $W382^{\bullet}$ formation to obtain the observed sizeable yield of $FADH^-W^{\bullet+}$. This means that the tryptophan radical observed in the picosecond WT product state is located on the second (W359) or possibly even third (W306) tryptophan in the chain rather than the first (W382).
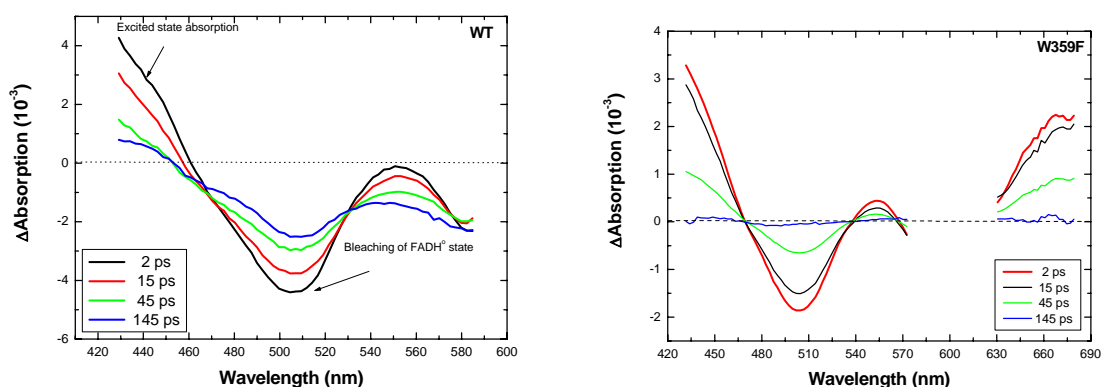

Figure II.4. Transient absorption spectra of wild type (WT) photolyase and the mutant photolyase (W359F) at various delay times.

Solving the differential equation system of the reaction scheme we were able to estimate the upper limit of the rate constant of the charge recombination in 4 ps.

Summary

Summarizing the results of the performed experiments I could prove that:

1) Replacing the tryptophan W359 with a redox inert phenylalanine prohibits build-up of long lived charge pairs

2) In W359F photolyase, flavin excited state is quenched by very short-lived oxidation of aromatic residues as in many other flavoproteins

3) Charge recombination of the primary charge separation state FADH-W382•+ and (in WT) electron transfer from W359 to W382•+ occur with time constants <4 ps

4) Strong indication for < 4 ps ET among the tryptophans, suggesting low ΔG, low λ reactions

5) Phenylalanine does not act as an ET intermediate

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