

Functional dynamics of two photolyase mutants revealed by ultrafast spectroscopy

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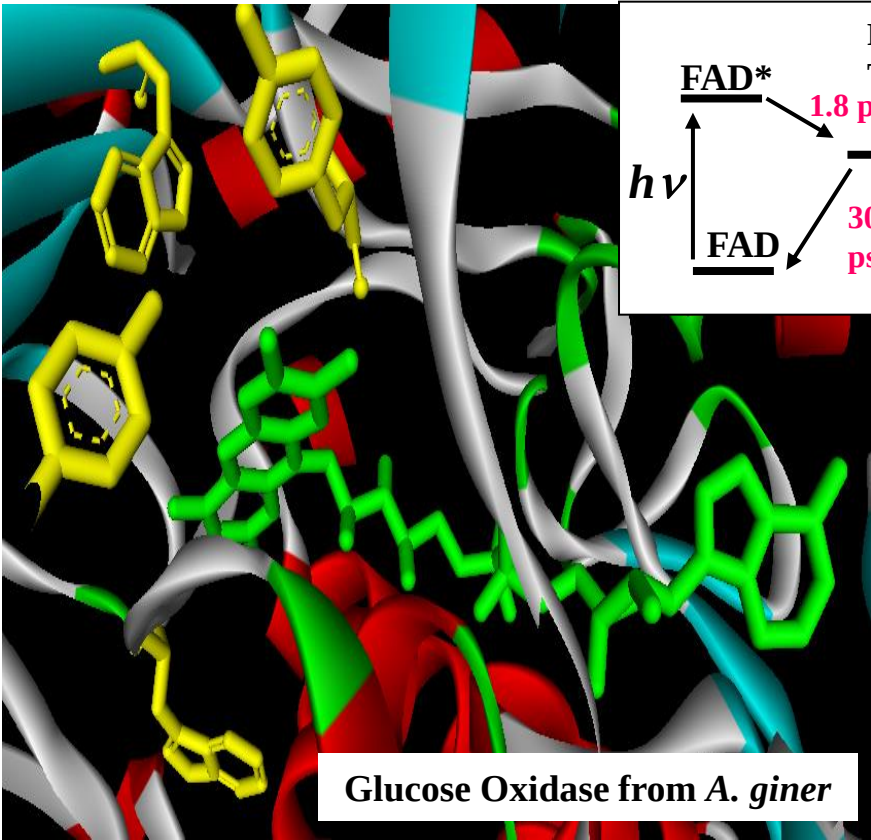
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Ultrafast Biologic Procedures

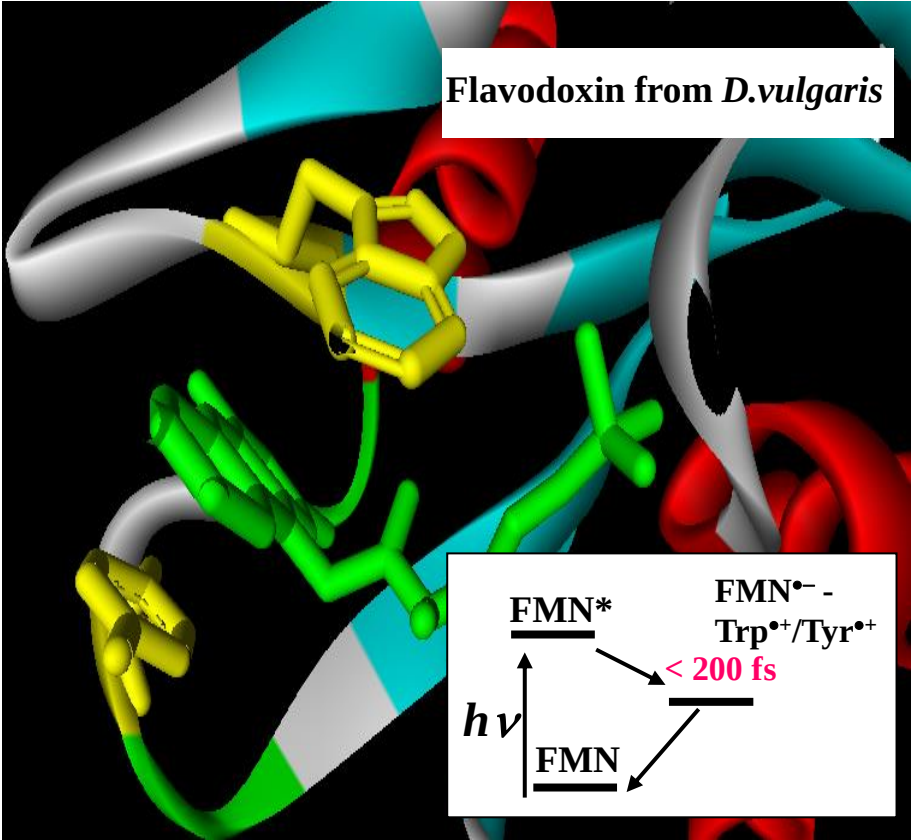
The fact that biologically relevant procedures may emerge in the range of femto- to picoseconds might sound astonishing. However it is true. Some outstanding examples can be read below:

- Rhodopsin: Retinal**, the prosthetic group of rhodopsin, goes through an all-trans conformational change within 200 fs due to the absorption of a photon. This results in the formation of **photorhodopsin** that is essential for proper vision
- Inside the **photosynthetic light-harvesting protein complex**, on the surface of the **chlorophyll** the electron transfer happens in less then 300 fs
- Flavoproteins**: electron transfer leading to flavin redox potential changes in Glucose-oxidase, Photolyase (Pho) and Cryptochrome (Cry)

Ultrafast Electron Transfer in Flavoproteins



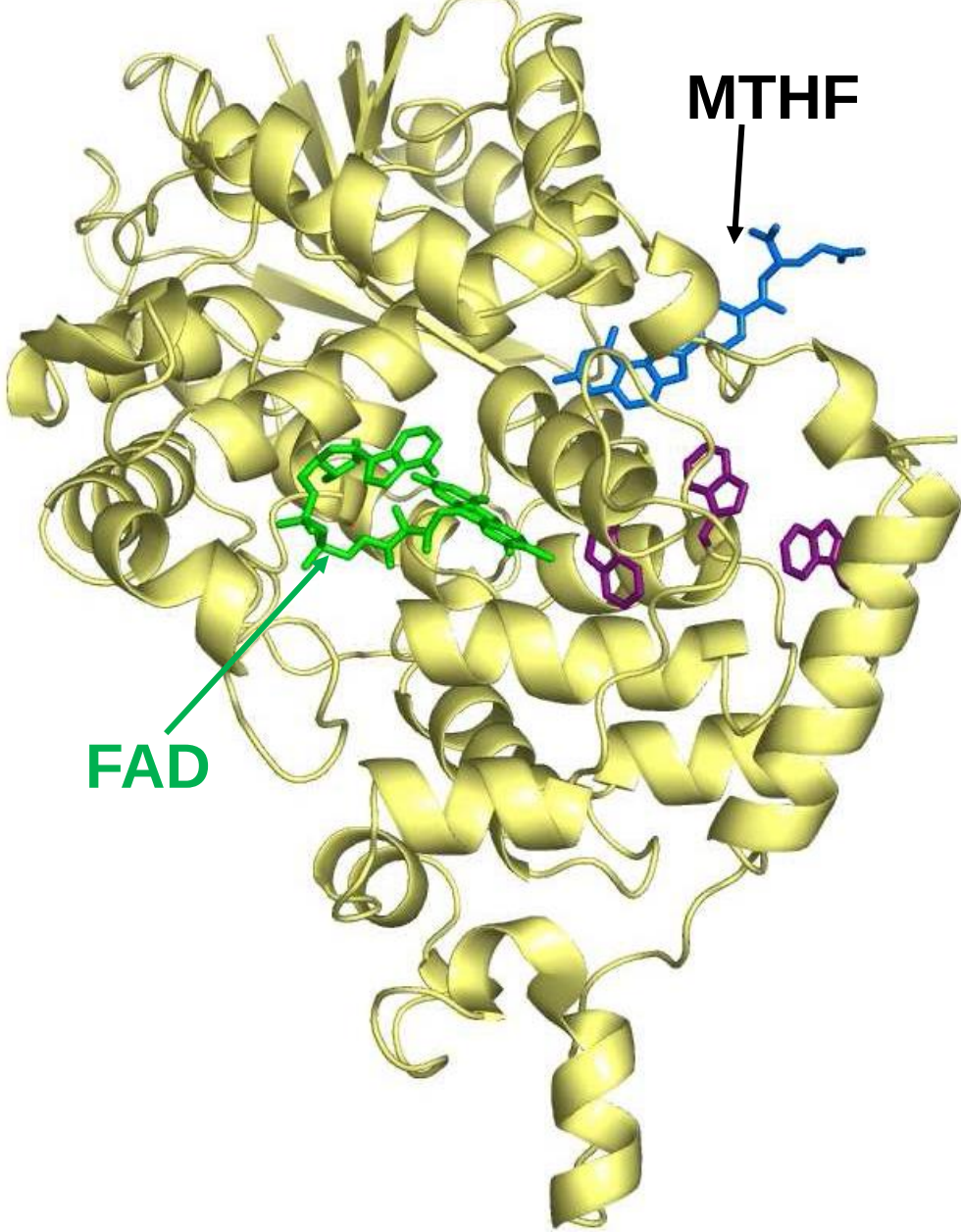
In the Glucose-oxidase the electron transfer resulting in the redox potential changes of the FAD occurs to be almost 2 ps [1,2].



In the Flavodoxine the same procedure regarding the redox state of the FMN requires less than 200 fs.

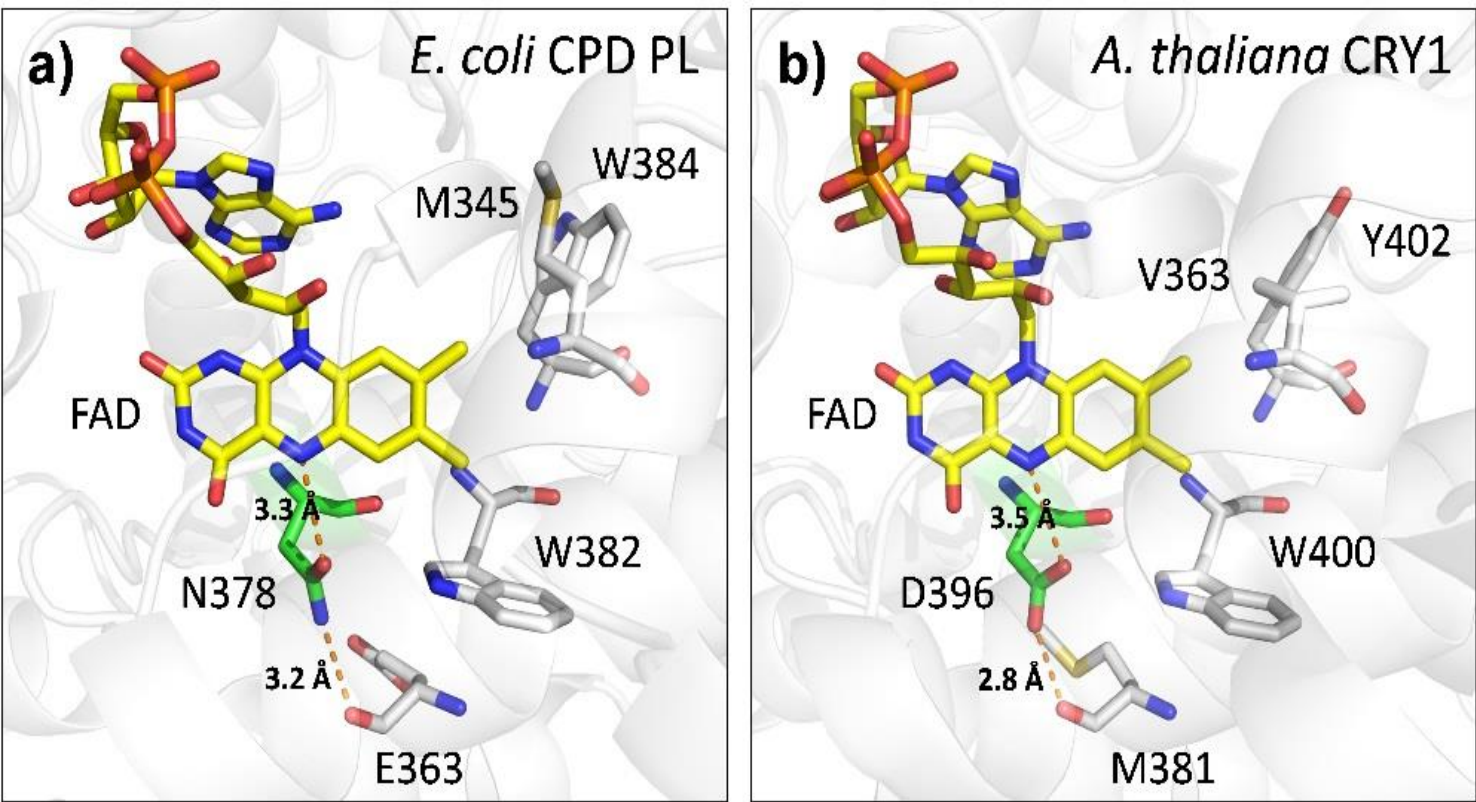
The only problem with these examples is that they are irrelevant from the aspect of functionality. However, this is not the case with the Photolyase.

Photolyase in General



Photolyase is a **DNA repair enzyme**. It corrects UV light induced DNA adducts such as Cyclobutane-pyrimidine and pyrimidine-pyrimidone dimers. The process is called **photo-reactivation** that requires the utilization of a blue or an UV photon. The enzyme was discovered by Claud S. Rupert and his colleges in 1958.

It can be found in prokaryots for example E.coli, in plants and even in some marsupials such as the rat kangaroo. The chromophore that is essential for the proper functioning of the protein is FAD. **The redox state of the FAD determines whether the enzyme is able to fulfil its duty or not.** On the surface of the macromolecule there is an other chromophore a **MTHF** the presence of which is not inevitable from the aspect of functionality, it merely **supports the efficacy of the light-harvesting in poorly enlightened circumstances via FRET**. There are three tryptophanes that are called the **Tryptophan-Nanowire**. They play a role in the electron-transfer to the FAD in

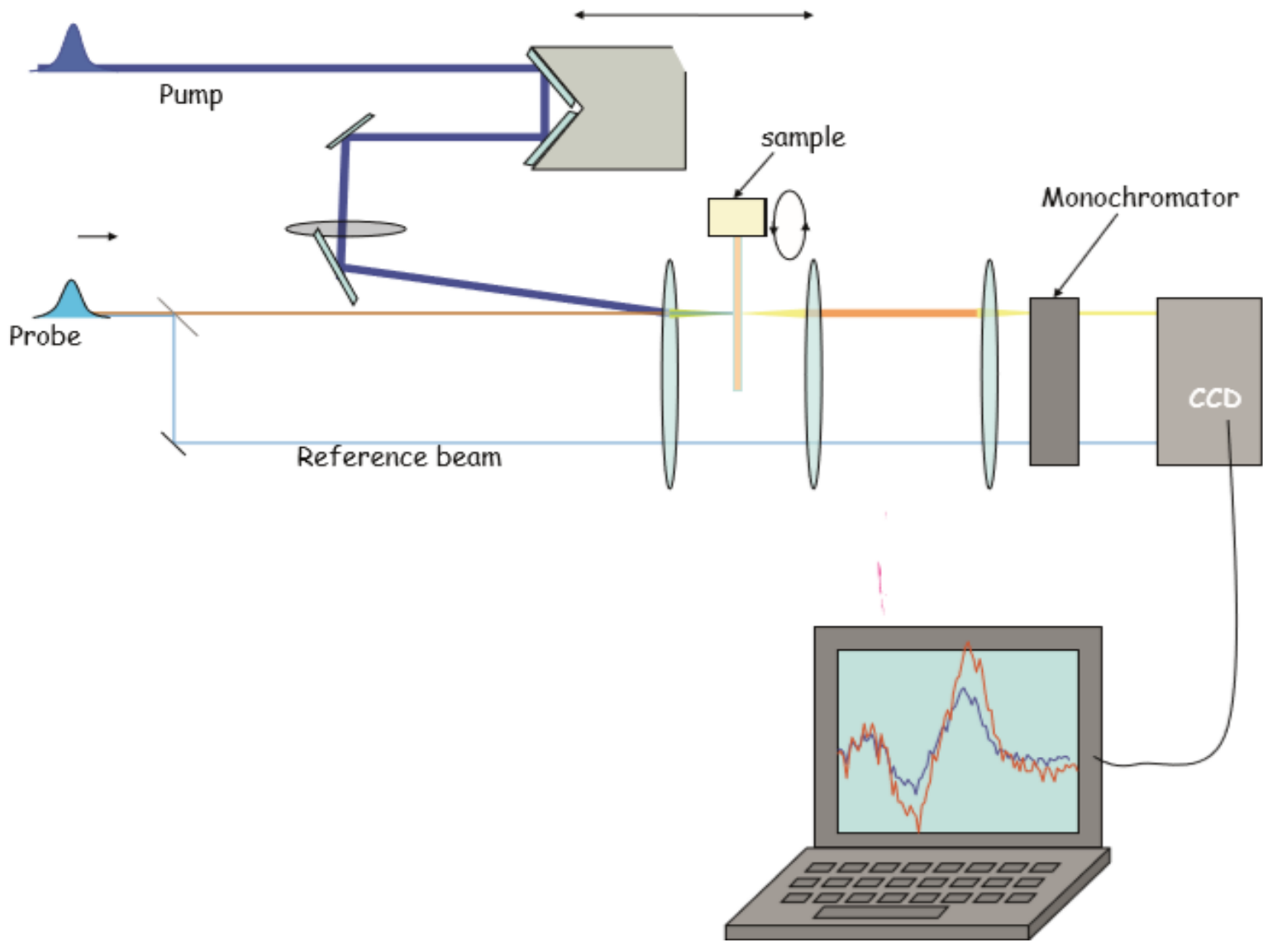


Although, the human body doesn't contain Pho it does comprise Cry, the sister protein of photolyase. Crys show significant resemblance to photolyase regarding the amino-acid sequence and third structure. In contrast to photolyase it is unable to repair DNA, however it plays an important role in winding the circadian clock both in plants and animals. What is more new studies suppose^[3] that it has magneto-receptor functions as well thus helping the navigation of birds. The main supposed difference between Pho and Cry is to be found in the **378th AA in Pho**, which is an **asparagine** in and the identical position in **Cry**, **396th** that is an **aspartate**^[5].

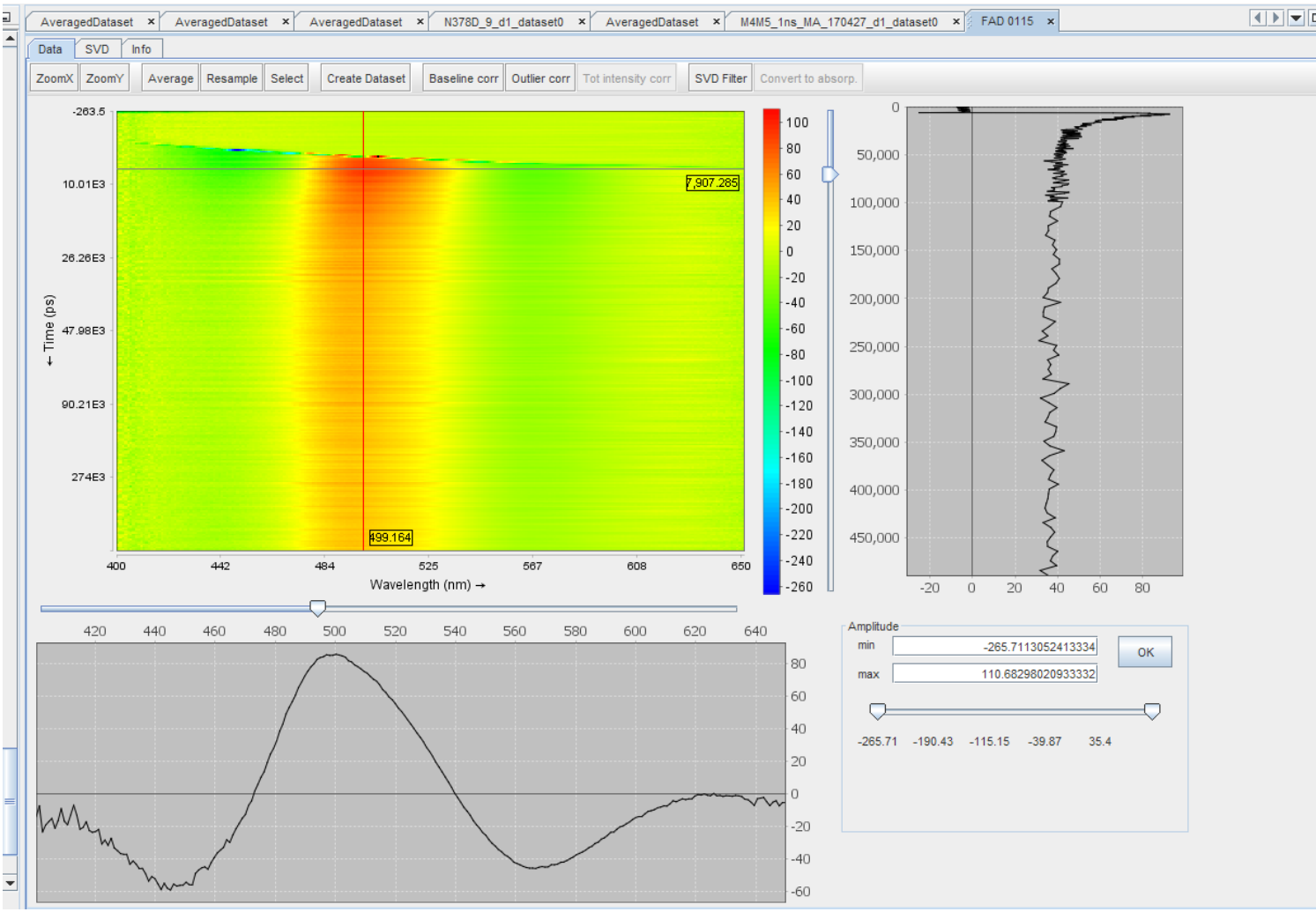
Goals and Hypothesises

- If the 378th asparagine is mutated to an aspartate the redox state of the FAD chromophore in Pho would change and the enzyme would lose its capability of repairing DNA. The removal of the antenna would terminate its interfering affect on the examination of the spectra of the FAD. (**N378DE109A mutant**)
- The Cry of animal cells have a cysteine in the analogous position of the Pho 378th AA. We supposed result regarding the alteration of the redox state of the FAD to be much alike of the first mutation ^[6] . (**N378CE109A mutant**)

Transient Absorption Spectroscopy



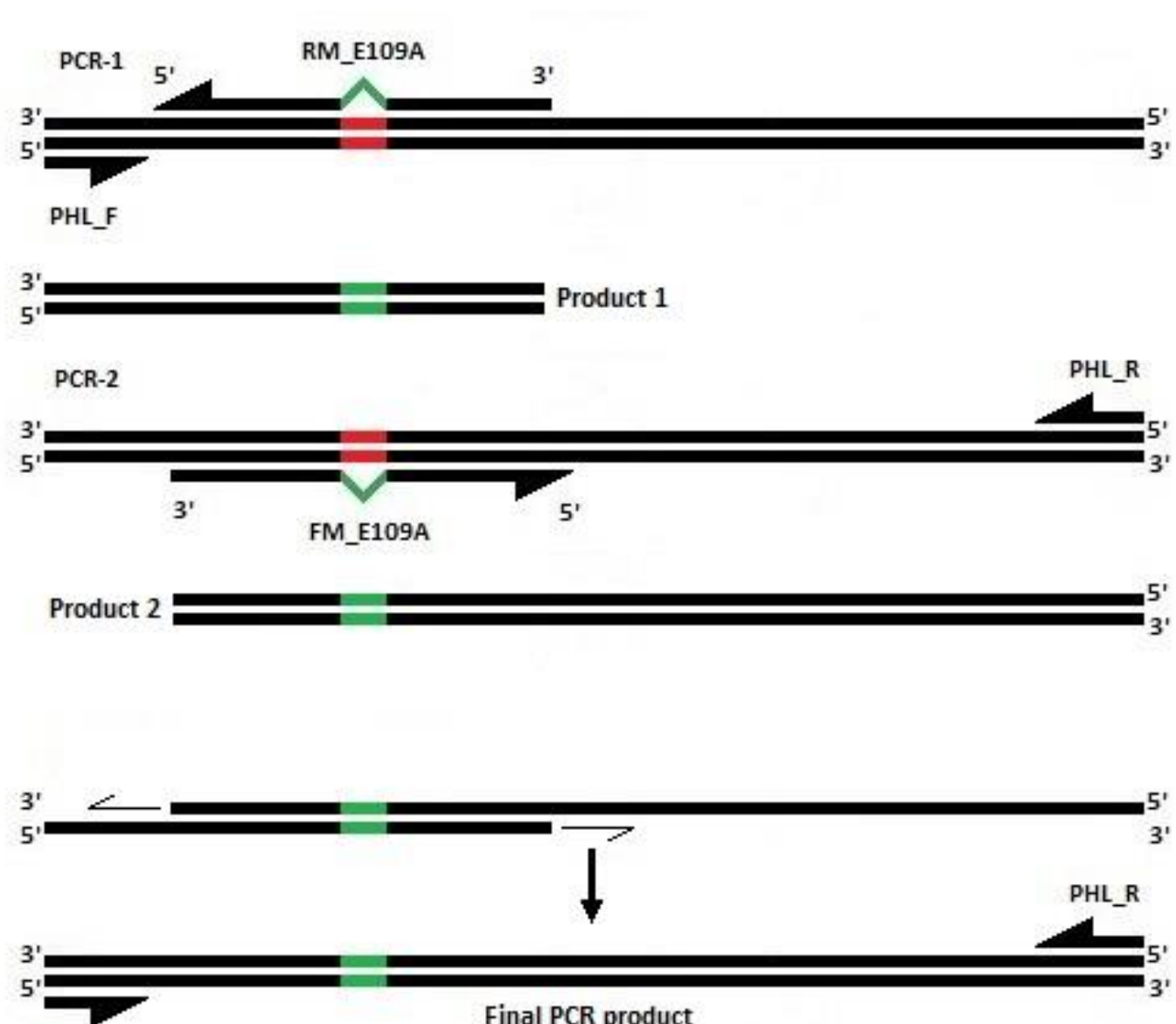
- For our measurements a **Mai Tai Spitfire titan:sapphire 800 nm** laser is used which **emits 1000 impulses per minute**. Exactly after the laser beam comes out of the amplifier we divide it into two beams. The first goes through a **Barium-borate crystal** out of which due to non-linear optical phenomena comes a **400nm blue beam**. This is perfect for exciting our sample.
- The second beam is lead through a **CaF₂ crystal**. The beam leaves the crystal as a white laser due to non-linear optical phenomenon and it contains all the wavelengths characteristic to visible light. **This works as a probe**.
- With the help of the **delayer** we can manipulate the exact time of how much earlier to excite the sample before the probe laser reaches it. **Thus we are able to detect the alteration of the absorption by time**.
- A **CCD is employed to detect the changes of the absorption**.



- This is how the primer data look like in the software we employ. On the vertical axis a desired time can be chosen in which all the changes of absorption in all the wavelengths may be seen.
- On the horizontal axis a desired wavelength can be selected. The graph then shows the alteration of the absorption by time regarding the given wavelength.

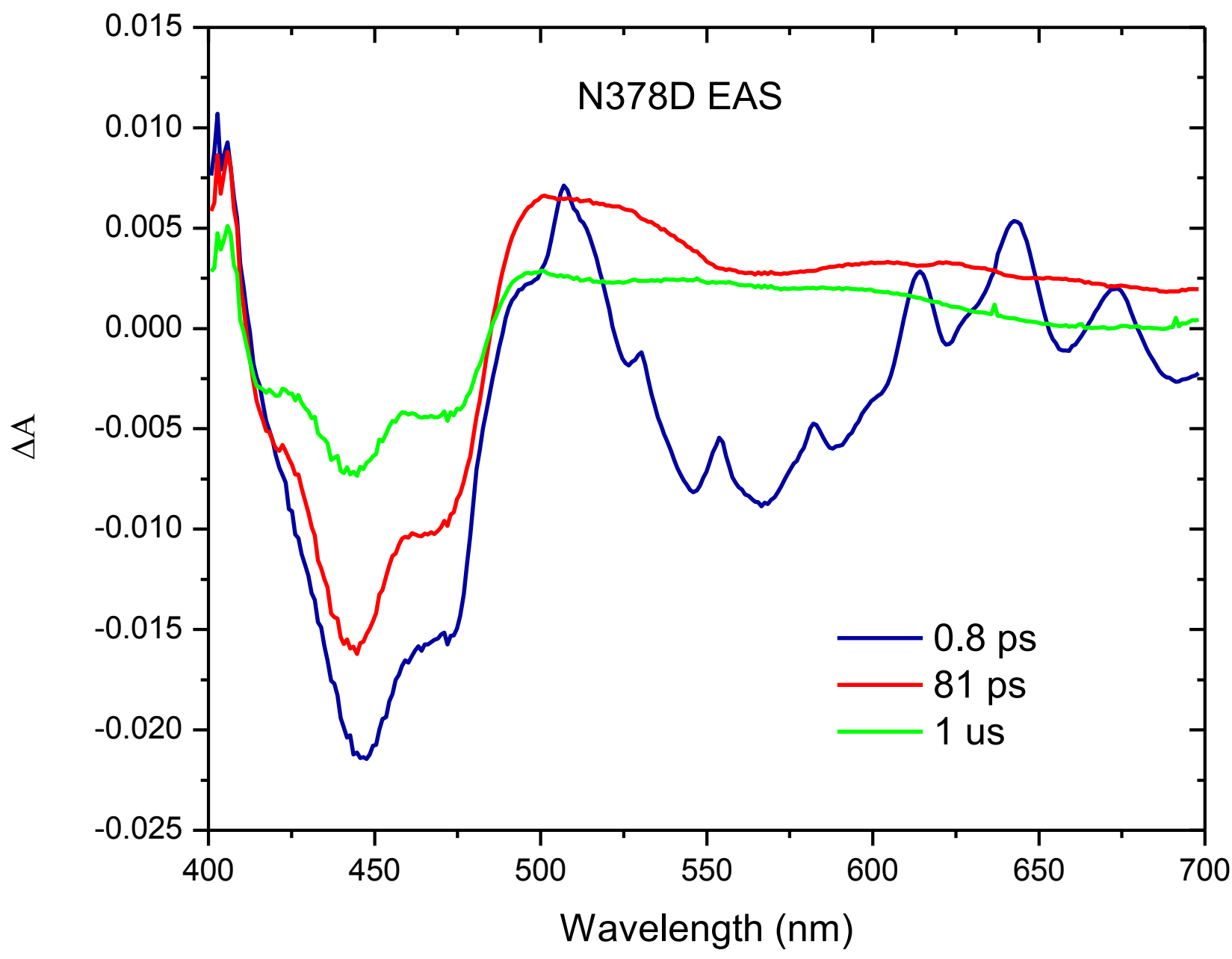
Mutagenesis and Expression

- Design of the mutation containing primers
- Overlap-extension mutagenesis (the figure on the right)
- Making of sticky ends (endonuclease)
- DNA ligation into pET-28 plasmid
- Transformation (TOP10)
- Purification of amplified DNA (Miniprep)
- Sequencation
- Transformation (BL21)
- Protein extraction and purification (his-tag column-chromatography)

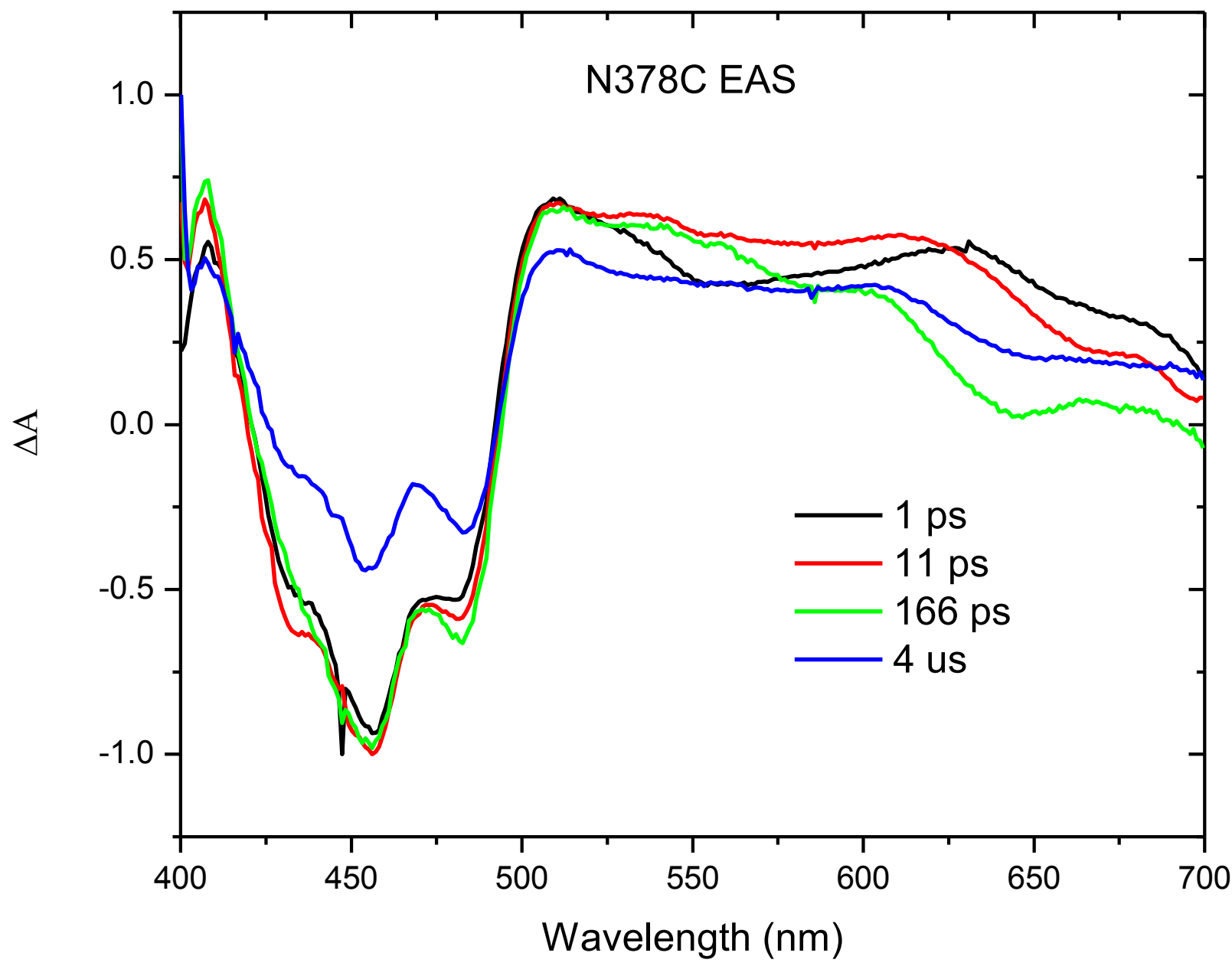


Transient Absorption Results

In these graphs the change of absorption by wavelength is showed. The different colours represent different times. These are the so-called **Evolution Associated Spectra**, that means the evolution of absorption by time. The first region of the spectra (400-450 nm) is the **Bleaching**, the second is the **Excited State Absorption (EAS)** (450-550 nm) and the third segment is the **Stimulated Emission**. So as to see these spectra the so-called Spectral Decomposition was made that is to distinguish the absorptions of the different substances in the protein and its solution.



In this graph the EAS of the N378DE109A can be seen. In the area of EAS the anionic FAD spectra might be noticed at 0.8 ps. This state changes to the oxidised form rather quickly, within almost 80 ps.



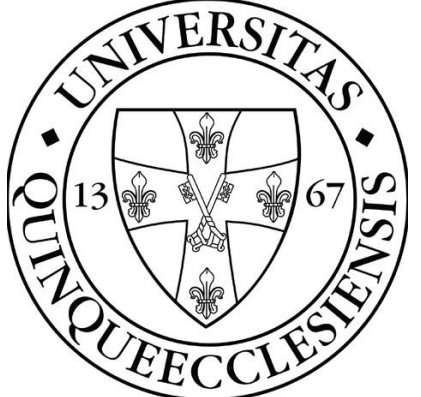
In this graph the EAS of the N378CE109A is shown. In the area of EAS the anionic FAD spectra might be also be seen at 1 ps. However, astonishingly in this mutant the FAD remains in the anionic state for more then 160 ps and would only reach the oxidised state in approximately 4 μs.

Conclusions

As the results of the mutations it can be concluded that the redox state of the FAD altered in both mutants and it became to be oxidized. After extinction the spectra showed that each mutant became anionic that can happen if the FAD is basically in the oxidized form. It can be stated that both Pho mutants became Cry-like.

Further Goals

- Mutation of the three tryptophanes of the Nanowire
- Cry D396N PHR region. We expect the FAD of this Cry to alter to be like Pho.



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