Dynamics of physical processes in proteins studied by kinetic absorption spectroscopy

Ph.D. Thesis

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Introduction

Inter- and intraprotein electron transfer is mostly known for its role in the bioenergetics of living organisms. Marcus theory relates the rate of electron transfer with its driving force (the free energy difference between the equilibrium states of the donor-acceptor pair before and after electron transfer) and the reorganisation energy. In addition, the rate of electron transfer decays exponentially with the distance between electron donor and acceptor. The corresponding distance decay constant depends on the medium between the donor and acceptor in a non-trivial way. A number of theories have been proposed to explain how the protein matrix controls the decay constant, and the collection of new experimental data may allow their refinement, and a better understanding of the process. Many electron transfer processes, resulting in redox state changes of molecules or cofactors, are reflected in their absorption spectrum, making kinetic absorption spectroscopy an appropriate tool of choice for their study. The introduction of TUPS (8-thiouredopyrene 1,3,6-trisulfonate) by Kotlyar et al. (1997a) provided an easily accessible, covalent photoactive redox label with the promise of the possibility to measure electron transfer processes both in the reductive and in the oxidative direction in non-photosynthetic systems. TUPS is a viable alternative to the widely used ruthenium complexes in the field due to its favourable properties: its long lived triplet excited state, its low redox potential and last, but not least, the relatively easy chemistry of its production from the precursor and of its attachment to either lysine or cysteine residues. After several publications by the inventors on the applicability of TUPS to induce protein reduction, our aim was to further study the photochemistry of TUPS in solution and investigate the possibility of using it in photoinduced protein oxidation. The natural choice for studies of the latter was mitochondrial cytochrome *c*, a simple, well characterized protein with a single heme as its redox center.

Protein dynamics is an omnipresent phenomenon which, in many cases, is strongly coupled to function. Kinetic absorption spectroscopy is a useful tool to study phenomena related to protein dynamics if the processes can be initiated by short laser pulses and followed using the absorption changes of chromophores. One of the best systems for such studies is photoactive yellow protein (PYP) with its p-coumaric acid chromophore. This protein is a robust, strongly coloured molecule which serves as a prototype for G-protein coupled receptor signal transduction. Three distinct processes take place after its photoexcitation: the isomerization of the chromophore, the internal proton transfer from the E46 donor to the chromophore and a major conformational change to the signalling state, which involves partial unfolding of the protein. The reversible partial unfolding is expected to result in a substantial transient change in the water-exposed surface area of PYP. According to the theory of Dér et al. (2007) explaining the Hofmeister effect (HE) of certain anions on protein

structural stability, such change of the hydrated protein surface must be affected thermodynamically by Hofmeister cosolutes. Therefore, the dynamic properties of PYP make it an excellent object to study experimentally the HE on the stability and reaction kinetics of proteins. In the second part of our work we were aiming to demonstrate the effective use of HE as a tool for the identification of crucial steps of protein function, involving major conformational changes.

Aims

- To investigate in detail the photochemistry and photoinduced electron transfer processes of TUPS in solution, in order to better characterize it as a photoinduced reductant/oxidant.
- To investigate the oxidative properties of TUPS triplet, and its application in photoinduced protein oxidation.
- To study the directional dependence of the electron transfer rate from the heme of cytochrome *c* to various positions on the surface of the protein and to investigate the changes in the electron transfer rate between TUPS and the heme of cytochrome *c* upon distance change by modification of the label linker.
- To determine the absorption spectra and the time-dependent concentrations of the PYP photocycle intermediates at close-to-physiological conditions (moderatly alkaline pH, high salt concentration).
- To obtain a sufficiently detailed reaction (photocycle) scheme which adequately describes the PYP photocycle under these conditions and to calculate the molecular rate constants connecting the intermediates, as well as their dependence on Hofmeister salts, by the global fit of the kinetic absorption data.
- Thereby, to demonstrate the efficient use of HE as a tool for the identification of crucial steps of protein function, involving major conformational changes.

Materials and methods

Recombinant cytochrome c expression and purification

Recombinant mutant cytochromes c were expressed in BL21 *E. coli* strain from pBAD24 plasmid. After cell destruction, the protein was purified from the cell debris free supernatant, using ammonium sulphate precipitation and ion-exchange chromatography on a Bio-Rex 70 cation-exchange resin. Wild type cytochrome was purchased from Sigma.

Preparation of TUPS-lysine and TUPS-cystamine derivatives

TUPS-lysine adduct preparation and purification was done by the

group of Alexander Kotlyar and described in Kotlyar et al. (1997a). TUPS-cystamine derivative was prepared via reaction of cystamine with 2-4 fold excess of IPTS; the non-reacted IPTS was neutralised by addition of lysine at the end of incubation.

Cytochrome c labelling

Cytochrome *c* was labelled with TUPS via the reaction of IPTS with surface lysines at Lys8 and Lys39 and purified using HPLC ion exchange chromatography as described in Kotlyar et al. (1997b). Cytochrome *c* labelling at genetically introduced cysteines was done via its SH-SS exchange reaction with TUPS-cystamine derivative.

UV-VIS stationary and kinetic spectroscopy

An Unicam UV4 spectrophotometer was used to measure stationary UV-VIS spectra. Kinetic flash spectroscopy was carried out on a custom made setup built around a HR-320 monochromator (ISA Jobin YVON). A 35W Xe-lamp served as a probe light source. To decrease the influence of the measuring light on the sample, it was chopped with a mechanical shutter. An Andor ICCD or a Princeton Instruments diode array where used as multiwavelength detectors. Single wavelength kinetic traces were recorded using a photomultiplier equipped with a homemade amplifier, connected to an oscilloscope PC card. TUPS containing samples were excited with the third harmonic of a Nd:Yag laser at 355 nm. The 450 nm laser pulse, for the excitation of photoactive yellow protein was generated with the Nd:Yag laser extended with an OPO.

Molecular dynamics (MD) and electron transfer pathway calculations

The MD calculations were carried out with the SYBYL 7.0 software (Tripos, Inc., St. Louis, MO). For the MD simulations, the Tripos force field and a dielectric constant value of 80 were applied, and the cutoff was set to 30 Å for non-bonding interactions. Optimal electron transfer pathways and electron transfer parameters were calculated by the HARLEM program (Kurnikov, 2001)

Mathematical data analysis

Statistical analysis of data, curve fitting and mathematical modelling (spectrotemporal model fit) were done in the MATLAB scripting language (Mathworks™). The initial seeding spectra for model fit of the PYP data were estimated as described in (Joshi et al. 2006). The distribution of exponential rate constants and the set of discrete rate constants were determined by the maximum-entropy/nonlinear-least-squares method using the program MemExp-3.0 (Steinbach et al., 2002).

Results and discussion

1. Using multichannel transient spectroscopy the interactions of TUPS in reductive and oxidative reactions were investigated. A new reaction for this dye has been characterised, where its excited triplet state serves as an oxidant, producing a reduced radical in the presence of a suitable electron donor. The characteristic difference and absolute spectra of the reduced radical of TUPS were determined, in addition to the previously known spectra of its triplet and positive radical. In anaerobic, redox neutral conditions in the presence of salt, the spectra recorded during the TUPS triplet decay reveal features of all three forms, as a result of the electron self-exchange between two TUPS molecules in their triplet excited states. Least-squares fits of the concentration profiles of the different forms with various models were tested in order to determine what processes are responsible for triplet decay and radical formation under such experimental conditions. The successful model includes the following processes: spontaneous triplet decay, triplet-triplet annihilation, electron transfer between triplets resulting in the formation of positive and negative radicals of TUPS, plus the reverse of the latter process, the recombination of the TUPS radicals. (Kotlyar et al. 2004)

2. The described oxidative properties of TUPS triplet suggested its applicability as a photoinduced covalent redox label, suitable for initiation not only reductive but also oxidative redox processes in biological systems. This has been demonstrated using the complex of TUPS with reduced cytochrome *c*, where the label was attached to a lysine at the 86th position. Upon photoexcitation of this complex, TUPS triplet rapidly decays, as a result of electron transfer from the heme of cytochrome *c* forming the reduced radical of TUPS and oxidized heme. The process is reversible. Detailed kinetic analysis of the forward and reverse electron transfer has shown that the re-reduction of the heme by the TUPS negative radical is faster than its initial oxidation, thereby preventing the accumulation of the transient products in large quantities. (Kotlyar et al. 2004)

3. To investigate the distance dependence of the electron transfer the TUPS label has been attached to two lysine residues (K8 and K39) at substantially different distances from the heme cofactor and on the opposite sides of horse heart cytochrome c. The photoinduced electron transfer between TUPS and the heme of cytochrome c proved to be more complicated than predicted on the basis of the Marcus theory. While, at the available signal to noise ratio, forward electron transfer appeared roughly monoexponential, the reverse electron transfer clearly did not. The multiexponential behaviour could be explained by sample heterogeneity, which is, in fact, corroborated by the results of MD simulations. MD results have shown that the TUPS label is likely to occupy several geometrically feasible equilibrium positions, with different effective distances from the heme, and thereby the transferred electron may also experience various

packing densities between the cofactors. Moreover, the calculated optimal electron transfer pathways do not involve the covalent link but proceed via through space jumps from the protein surface to the dye edge, effectively decoupling the length of the covalent link and the electron transfer rates. (Tenger et al. 2005)

4. Flash induced difference spectra mesured on PYP were analysed by a combination of chemometric methods, and five transitions were detected, providing evidence for five intermediates. The data allowed to distinguish two pR intermediates, which are spectrally rather similar but different kinetically. The presence of a red shifted shoulder in the pB_1 spectrum indicated its coexistence with its alkaline form. Visual inspection of the absolute spectra showed the dominance of the blue shifted intermediate(s) at the end of the photocycle, but also the presence of the early red shifted intermediate(s). This suggests that an equilibrium between intermediates was formed. Finally, to cope with the biexponential recovery of the dark state, a spectrally silent intermediate, pG_1 was required at the end of the photocycle. Taking into account these observations a sequential, generally reversible scheme of the PYP photocycle involving the pR_1, pR_2, pB_1, pB_2 and pG_1 intermediates has been proposed. This scheme was used in a global spectrotemporal model fit to the data obtained at different salt conditions, yielding the rate coefficients for the molecular transitions, the kinetic traces of intermediates and their final spectra.

5. Kosmotropic and chaotropic cosolutes influenced the formation and decay of, and the equilibria between photocycle intermediates in a systematic manner which follows the Hofmeister series of anions. Chaotropic salts prolong, kosmotropic salts shorten the duty cycle of the total pB form of PYP, as compared to the Hofmeister neutral NaCl. The spectrotemporal least squares model fit to our data taken under different salt conditions provided more quantitative information, allowing to decipher the HE on the individual molecular transitions of the photocycle. While an opening of the protein conformation during $pB_1 \rightarrow pB_2$ is in good agreement with our results, we have also pinpointed additional opening processes. These are hinted at by the monotonic acceleration of the rates while the salt cosolute changes from kosmotropes to chaotropes. The early $pR_1 \rightarrow pR_2$ transition already exhibits such behaviour but, surprisingly, far the biggest effect is associated with the $pR_2 \rightarrow pB_1$ transition. This transition coincides with the chromophore protonation and the hydratation of the chromophore pocket. Following Dér et al. (2007), the strongest effect of the Hofmeister salts on the rates of reactions is expected for processes involving large-scale water-exposed surface area changes. From this it could be concluded that in addition to the chromophore protonation, a conformational change associated with the exposure of hydrophobic residues of the protein should take place during this step. The recently observed increase of the diffusion coefficient of PYP in the same time domain fully supports this conclusion (Hoshihara et al., 2008). These

authors attributed the corresponding conformational change to the unfolding of the N-terminal domain. Notably, the free energy difference between pR_2 and pB_1 does not seem to change significantly from chaotropes to kosmotropes. This follows from the fact that the salt dependence of the rate of the back reaction follows that of the forward process, so the stabilization of pB_1 against pR_2 is probably due to stochastic fluctuations of the energy barrier under the influence of cosolutes, as described in (Neagu et al. 2001). In the $pB_1 \leftrightarrow pB_2$ reaction, on the other hand, the equilibrium shifts towards pB_2, in agreement with (Hoersch et al., 2007). (Khoroshyy et al. 2013)

6. The detailed study of the Hofmeister effects on the kinetics and transient equilibria in the PYP photocycle has proven that the HE is a useful tool in finding and characterizing large scale protein conformational changes. In particular, based on our results and relevant data of the literature, we can suggest the following scenario for the structural changes during the PYP photocycle (after the formation of pR_1) (Khoroshyy et al. 2013):

$pR_1 \rightarrow pR_2$: conformational relaxation (opening) after isomerization

$pR_2 \rightarrow pB_1$: large-scale conformational opening with exposure of hydrophobic residues to the water (unfolding of the N-terminal domain, coinciding with the protonation of the chromophore)

$pB_1 \rightarrow pB_2$: further conformational opening (changes in the PAS core and the β-scaffold)

$pB_2 \rightarrow pG_1$: conformational refolding (coinciding with the deprotonation of the chromophore)

$pG_1 \rightarrow pG$: reisomerization of the chromophore, final refolding to terminate the photocycle.

References

Dér, A., Kelemen, L., Fábián, L., Taneva, S.G., Fodor, E., Páli, T., Cupane, A., Cacace, M.G., and Ramsden, J.J. (2007) Interfacial water structure controls protein conformation. J Phys Chem B, 111, 5344-5350.

Hoersch, D., Otto, H., Joshi, C.P., Borucki, B., Cusanovich, M.A., and Heyn, M.P. (2007) Role of a conserved salt bridge between the PAS core and the N-terminal domain in the activation of the photoreceptor photoactive yellow protein. Biophys J, 93, 1687-99.

Hoshihara, Y., Imamoto, Y., Kataoka, M., Tokunaga, F., and Terazima, M. (2008) Conformational changes in the N-terminal region of photoactive yellow protein: a time-resolved diffusion study. Biophys J, 94, 2187-93.

Joshi, C.P., Borucki, B., Otto, H., Meyer, T.E., Cusanovich, M.A., and Heyn, M.P. (2006) Photocycle and photoreversal of photoactive yellow protein at alkaline pH: kinetics, intermediates, and equilibria. Biochemistry, 45, 7057-68.

Kotlyar, A.B., Borovok, N., and Hazani, M. (1997a) Use of thiouredopyrenetrisulfonate photochemistry for driving electron transfer reactions in aqueous solutions. Biochemistry, 36, 15823-7.

Kotlyar, A.B., Borovok, N., and Hazani, M. (1997b) Photoinduced electron transfer in singly labeled thiouredopyrenetrisulfonate cytochrome c derivatives.. Biochemistry, 36, 15828-33.

Kurnikov, I. (2001). HARLEM (HAmiltonians to Research LargE Molecules). software package. http://www.kurnikov.org.

Neagu, A., Neagu, M., and Dér, A. (2001) Fluctuations and the Hofmeister effect. Biophys J, 81, 1285-1294.

Steinbach, P., Ionescu, R., and Matthews, C. (2002) Analysis of kinetics using a hybrid maximum-entropy/nonlinear-least-squares method: application to protein folding. Biophys J, 82, 2244-2255.

List of publications

In extenso publications which are related to this thesis:

Khoroshyy, P. Dér, A., and Zimányi, L. (2013) Effect of Hofmeister cosolutes on the photocycle of photoactive yellow protein at moderately alkaline pH. J. Photochem. Photobiol. B, *in press* (http://dx.doi.org/10.1016/j.jphotobiol.2012.12.014) (IF 2.413)

Tenger, K., **Khoroshyy, P**., Leitgeb, B., Rákhely, G., Borovok, N., Kotlyar, A., Dolgikh D.A. and Zimányi L. (2005) Complex kinetics of the electron transfer between the photoactive redox label TUPS and the heme of cytochrome c. J. Chem. Inf. Mod. 45(6):1520-1526. (IF 4.675)

Kotlyar, A.B., Borovok, N., **Khoroshyy, P.**, Tenger, K. and Zimányi, L. (2004) Redox photochemistry of thiouredopyrenetrisulfonate. Photochem. Photobiol. 79(6):489-493 (IF 2.413)

Other in extenso publications:

Hajdu, K., Gergely, Cs., Martin, M., Cloitre, T., Zimányi, L., Tenger, K., **Khoroshyy, P**., Palestino, A., Agarwal, V., Hernádi K., Németh, Z., and Nagy, L. (2012) Porous silicon/photosynthetic reaction center hybrid nanostructure. Langmuir 28(32):1866–11873 (IF 4.186)

Zimányi, L., **Khoroshyy, P**., and Mair, T. (2010) A chemometric method to identify enzymatic reactions leading to the transition from glycolytic oscillations to waves. Physica D 239(11):866-872 (IF 1.594)

Tenger, K., **Khoroshyy, P**., Rákhely, G. and Zimányi, L. (2010) Maturation of a eukaryotic cytochrome c in the cytoplasm of Escherichia coli without the assistance by a dedicated biogenesis apparatus. J. Bioenerg. Biomemb. 42:125-133 (IF 2.813)

Tenger, K., **Khoroshyy, P**., Kovács, K.L., Zimányi, L. and Rákhely, G. (2007) Improved system for heterologous expression of cytochrome c

mutants in *Escherichia coli.* Acta Biol. Hung. 58:23-35 (IF 0.59)

Mair, T., Zimányi, L., **Khoroshyy, P.**, Müller, A. and Müller, S.C. (2006) Analysis of the oscillatory kinetics of glycolytic intermediates in a yeast extract by FTIR spectroscopy. Biosystems 83:188-194 (IF 1.784)

Conference abstracts which are related to this thesis:

Zimányi, L., **Khoroshyy, P**., and Dér, A. (2011) Hofmeister salt effects on the photocycle dynamics of photoactive yellow protein. 7th International Conference for Biological Physics, San Diego, USA, p.116.

Khoroshyy, P., Dér, A. and Zimányi, L. (2011) Kinetic effect of Hofmeister ions on the photocycle of photoactive yellow protein. Eur. Biophys. J. 40(S1):208.

Khoroshyy, P., Tenger, K. and Zimányi, L. (2006) Intra- and interprotein photoinduced electron transfer in respiratory chain redox proteins. Biochim. Biophys. Acta 1757(14):187.

Tenger, K., **Khoroshyy, P.**, Leitgeb, B., Rákhely, G., Borovok, N., Kotlyar, A. and Zimányi, L. (2005) Complex electron transfer kinetics between the photoactive label TUPS and the heme of cytochrome c. Eur. Biophys. J. 34(6):665.

Tenger, K., Rákhely, G., **Khoroshyy, P**., Leitgeb, B. és Zimányi, L. (2005) A fényindukált elektrontranszfer komplex kinetikája a fotoaktív redox jelölő, TUPS, és a citokróm c hem kofaktora között. A Magyar Biofizikai Társaság 22. Kongresszusa, Debrecen, 2005. június 26-29.

Other conference abstracts:

Tenger, K., **Khoroshyy, P.**, Rákhely, G. and Zimányi, L. (2008) Heterologous overexpression of eukaryotic cytochrome c and cytochrome c heme lyase to study the mechanism of cytochrome c maturation. Biochim. Biophys. Acta 1777:S90.

Khoroshyy, P., Tenger, K. and Zimányi, L. (2008) Tuning the electron transfer rate by the redox potential of cytochrome c in complex with cytochrome c oxidase. Biochim. Biophys. Acta 1777:S90.

Tenger, K., **Khoroshyy, P**., Kovács, K.L., Rákhely, G. and Zimányi, L. (2007) Heterologous overexpression of eukaryotic cytochrome c and cytochrome c heme lyase in Escherichia coli for biophysical studies. Regional Biophysics Conference, Balatonfüred, Hungary, p.92.

Khoroshyy, P., Tenger, K. and Zimányi, L. (2007) Tuning of the electron transfer rate by the altered redox potential of heme c in cytochrome c and in its complex with cytochrome c oxidase. Regional Biophysics Conference, Balatonfüred, Hungary, p.114.

Khoroshyy, P., Zimányi, L., Mair, T., Müller, A. and Müller, S.C. (2005) Chemometric analysis of the oscillatory kinetics of glycolytic intermediates in a yeast extract from data measured by FTIR-spectroscopy. Regional Biophysics Meeting, Zrece, Slovenia, p.89.

Tenger, K., Rákhely, G., **Khoroshyy, P**. and Zimányi, L. (2005)

Expression, purification and labeling of recombinant horse cytochrome c, and measurements of photoinduced intraprotein electron transfer. Regional Biophysics Meeting, Zrece, Slovenia, p.61.

Zimányi, L., Tenger, K., **Khoroshyy, P**., Dolgikh, D., Siletsky, N., Borovok, N. and Kotlyar, A. (2004) Photoinduced electron transfer in cytochrome c and cytochrome c oxidase. Biochim. Biophys. Acta 13:158.

Khoroshyy, P., Tenger, K., Borovok, N., Kotlyar, A., Siletsky, S. and Zimányi, L. (2003) Electron Transfer Steps in the Complex of Cytochrome c and Cytochrome Oxidase. 10th European Conference on the Spectroscopy of Biological Molecules, Szeged, Hungary, ISBN 963 482 614 8, p. 153.

Khoroshyy, P., Tenger, K., Borovok, N., Kotlyar, A. és Zimányi, L. (2003) Az elektrontranszfer lépései citokróm c és citokróm oxidáz komplexében. 33. Membrán-Transzport Konferencia, Sümeg, 2003. május 20-23.