A2 Alexiev

Investigation of conformational changes at the K-channel entrance of cytochrome *c* oxidase using the fluorescent molecular rotor Sulfo-Cy3-maleimide and site-directed labeling

Jacqueline Gottwald, Jens Balke & Ulrike Alexiev

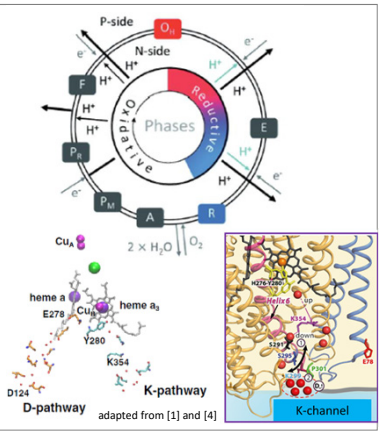
Freie Universität Berlin, Institut für Experimentalphysik, Arnimallee 14, D-14195 Berlin, Germany, E-mail: j.gottwald@fu-berlin.de

Introduction

As part of the respiratory chain, cytochrome *c* oxidase (CcO), a redox-coupled proton pump, catalyzes the reduction of molecular oxygen to water thereby establishing the transmembrane proton gradient that fuels ATP synthesis. The catalytic cycle is composed of two partial reactions, the reductive and oxidative phase. A-type CcOs translocate a proton from the N-side to the catalytic center through two uptake pathways named the D- and the K-channel after their essential residues K354 (Helix 8) and D124 (*P. denitrificans* CcO amino acids numbering). The K-channel delivers the first two protons in the reductive phase for the chemical reaction. In contrast to the D-channel the transient opening of the K-channel is a concerted event that involves restructuring of the extended H-bonding network upon electrostatic changes of the binuclear center, and conformational changes propagating to the K-channel entrance [1]. This also involves helix 6 (contains catalytic Y280) which becomes more flexible upon reduction and shows higher water diffusivity at the helix-loop border and around loop 6-7.

Goal of the current work:

1. Test whether the read-out of the fluorescence molecular rotor (FMR) Sulfo-Cyanin-3-maleimide (Cy3), which is sensitive to nano viscosity/-friction, changes at the K-channel entrance with position (helix (S295), border to loop (K299), loop (P301)) and in the future upon enzyme reduction.
2. The reductive phase comprises the intermediates O → E → R. O is supposedly in the resting state when the enzyme is isolated. Only the O_H-state, a metastable oxidized form (pulsed state, recently oxidative state), is able to pump protons [2]. Until now, it is not really clear how the two states differ in structure/conformation [3]. If subtle changes are present outside the binuclear center they might be missed in the conventional experiments. We therefore also tested the ability of the FMR sensor to detect such changes at the entrance of the K-channel.



Methods

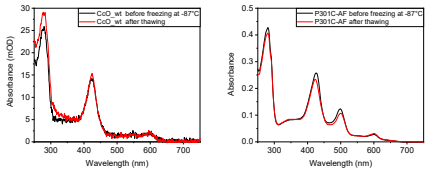
1. Site-specific labeling of the single reactive cysteine mutants S295C, K299C and P301C in CcO from *Paracoccus denitrificans* according to established protocols in the 2-subunit(2-SU) preparation [5]. The labeling stoichiometries with Sulfo-Cyanin-3-maleimide (Cy3) amount to 77%, 44% and 52%, respectively. The labeling of P301C with Iodoacetamidofluorescein (IAF) resulted in a labeling stoichiometry of 93%.
2. UV/Vis spectroscopy and oxidation/reduction of CcO. UV/Vis spectroscopy was performed using a Shimadzu UV2450. The detergent solubilized CcO (0.05% β-dodecyl maltoside, 20mM potassium phophate buffer, 20mM sodium chloride) was reduced with sodium dithionite and oxidized with potassium ferricyanide [1, 4]
3. Time-correlated single photon counting (TCSPC)-based picosecond time-resolved (tr) fluorescence spectroscopy was performed in a home built setup as described previously [6]. Excitation of Cy3 was at λ_{exc} = 485.5 nm; λ_{em} > 515nm (LP OG 515) and 20°C.

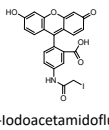
Results

Freeze-thawing damage of the solubilized membrane protein CcO upon molecular rotor labeling

Wild type and pH-indicator dye labeled P301C (loop 6-7) as control

Wild type | P301C-AF (Loop 6-7)





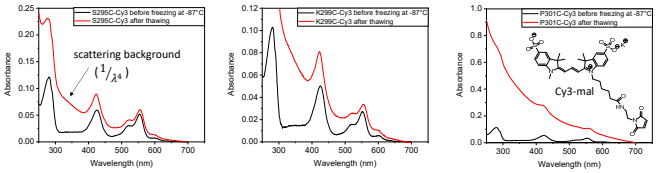
5-Iodoacetamidofluorescein (5-IAF)

➢ The 2-SU preparation of CcO is stable upon freeze-thawing cycles. The labeling with the large 5-IAF does not affect the stability of the protein as shown here for the labeling site P301C.

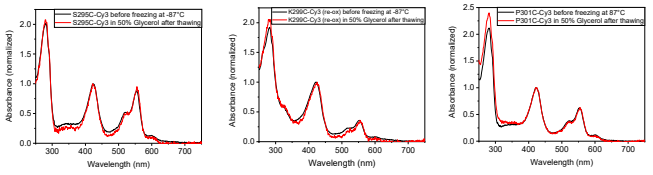
Molecular rotor labeled CcO in positions at helix 6, the border between helix 6 and protein surface, and in the loop 6-7

S295C-Cy3 (Helix 6) | K299C-Cy3 (Helix/Loop) | P301C-Cy3 (Loop 6-7)

w/o glycerol



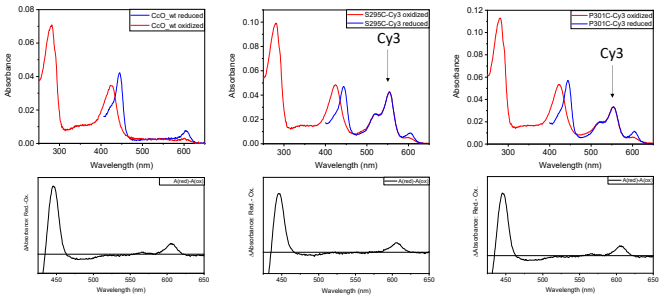
w/ glycerol



➢ Freeze-thaw inactivation of CcO was observed only after labeling with the molecular rotor Cy3, which results in aggregation of the protein. The rotor at the labeling position in the loop leads to the highest aggregation. Interestingly, a similar large dye as Cy3 but not a FMR does not affect protein stability. Glycerol prevents protein aggregation upon thawing and the mechanism is supposed to be a preferential exclusion effect and an interaction with the hydrophobic regions of the protein (hydration of the protein surface), thereby leading to stabilization of the protein structure. It seems that the molecular rotor at loop 6-7 at the N-side of CcO destroys specific interactions that are necessary for protein stability.

Effect of Cy3 labeling on the oxidized and reduced state

Wild type | S295C-Cy3 (Helix 6) | P301C-Cy3 (Loop 6-7)

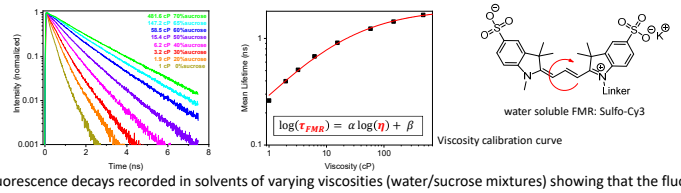


➢ Cy3 labeling does not or only marginally affect the oxidized and reduced state of the enzyme. The Soret band of the oxidized form is around 423-424 nm. The characteristic band of the reduced state for heme a is at 605 nm.

Correlation of Cy3 fluorescence lifetime to nanoviscosity and local steric restrictions

Fluorescent molecular rotors (FMRs) are fluorophores that have a fluorescence quantum yield that depends upon intermolecular rotation/twisting motion. Thus the fluorescence lifetime of the FMRs varies as a function of nanoviscosity/nanofriction, with high viscosities inhibiting molecular rotation and thus increasing the lifetime.

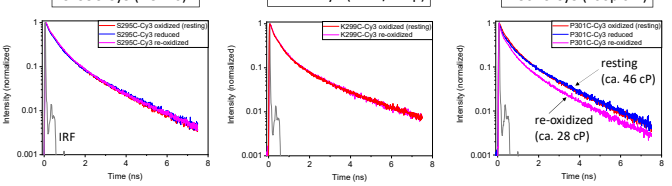
Viscosity sensing by the FMR Cy3



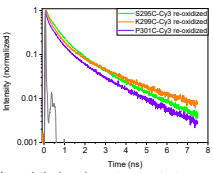
➢ Fluorescence decays recorded in solvents of varying viscosities (water/sucrose mixtures) showing that the fluorescence lifetime increases with viscosity. The Förster-Hoffmann equation (inset right plot) correlates viscosity with fluorescence lifetime in a log-log plot.

Cy3 fluorescence decay read-out at the entrance of the K-channel

S295C-Cy3 (Helix 6) | K299C-Cy3 (Helix/Loop) | P301C-Cy3 (Loop 6-7)



Comparison of physical properties in the re-oxidized state



Position	Lifetime (ns)	Viscosity (cP)	Steric restriction (%) from tr anisotropy	Polarity from λ_{max} of BADAN fluorescence (cm^{-1})
S295	1.14	38	0.768	20900
K299	1.41	117	0.645	21120
P301	1.06	28	0.704	21200

➢ While local steric restriction and polarity change in the order K299 < P301 < S295 (low to high steric restriction) and P301 < K299 < S295 (low to high polarity) [5, 1], the translation into nanoviscosity reveals the following order P301 < S295 << K299 (low to high viscosity).

Conclusion and Outlook

- ➢ Cy3 was successfully labeled at positions 295, 299 and 301 at the K-channel entrance.
- ➢ Attaching the FMR Cy3 to the CcO surface induces aggregation upon freeze-thawing that can be prevented by glycerol. Whether surface regions offer than the K-channel entrance are also sensitive to the FMR attachment needs to be determined.
- ➢ The read-out of the FMR sensor, ie fluorescence lifetime as a function of position, was translated into nanoviscosities and does not correlate with data for local steric restriction (from time resolved anisotropy) or polarity (BADAN fluorescence) from previous work [5].
- ➢ The FMR read-out differs between the resting and re-oxidized O-state in position 301 in the loop 6-7, indicating that the environment (nanoviscosity) of this loop changes from resting to the re-oxidized O-state.

References

[1] A. Wolf, J. Dragelj, J. Wonneberg, J. Stellmacher, J. Balke, A. Woelke, M. Hodoscek, E. W. Knapp,, and U. Alexiev, Chem. Sci. 11 (2020), 3804–3811, doi: 10.1039/c9sc06463j

[2] D. Bloch, I. Belevich, A. Jasaitis, C. Ribacka, A. Puustinen, M- I. Verkhovsky, and M. Wikström, PNAS 101 (2004) 529-533 doi: 10.1073pnas.0306036101

[3] Gorbikova E and Kalendar R, Front. Chem. 8 : 387. (2020) doi: 10.3389/fchem.2020.00387

[4] K. Kirchberg, H. Michel, U. Alexiev, Biochim. Biophys. Acta 1827 (2013) 276-284, doi: 10.1016/j.bbabio.2012.10.014

[5] A. Wolf, J. Wonneberg, J. Balke and U. Alexiev, FEBS J, 2019 doi: 10.1111/febs.15084

[6] U. Alexiev, I. Rimke, and T. Pöhlmann, J. Mol. Biol. 328, 705-719 (2003), doi: 10.1016/s0022-2836(03)00326-7

Acknowledgements

Financial support by the Deutsche Forschungsgemeinschaft (DFG) through collaborative research center 1078 (Project A2) is gratefully acknowledged.