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Probing entry point and histidine residues at the K-channel entrance of Cytochrome *c* oxidase for protonation dynamics

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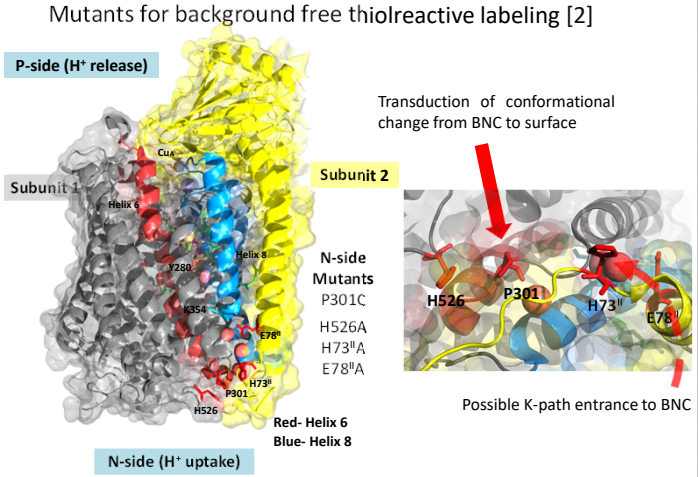
Introduction

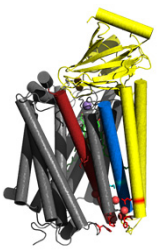
As a part of the respiratory chain cytochrome c oxidase (CcO) is essential for both eukaryotes and most aerobic prokaryotes. The redox-coupled proton pump CcO translocates protons which are probably collected by a proton collecting antennae, through two proton channels, named the D and the K-channel after their essential residues K354 and D124, form the N-side to the catalytic center. The necessary energy for this transport is provided by reducing oxygen to water while oxidizing cytochrome *c* at the positive side of the membrane. This increases the trans-membrane proton gradient, the driving force for ATP synthesis by ATP synthase [1]. However, protonation dynamics, coupling by electron transport, and conformational changes associated with CcO's function are not yet fully explained. Site-directed labeling with a pH-sensitive fluorescent dye allows to map the surface of CcO regarding H-bond networks, electrostatics, conformational changes and polarity [2, 3, 4]. Recently, we have shown by absorbance spectroscopy of the labeled enzyme a significant pK_a and polar shift of the bound pH-indicator dye fluorescein [2, 3] upon reduction of the enzyme, indicating changes in the H-bond network around the K-channel entrance. So far, no evidence was found for a proton collecting antennae around the K-channel entrance [4]. Since negatively charged amino acids such as glutamatic acid or aspartic acid and histidines as buffer residues are part of a proton colleting antennae [5], we exchanged the residue $E78^{II}$ to alanine as this residue is thought to be the entry point of the K-channel [6]. The two histidines in direct vicinity of the K-channel entrance, H526 and $H73^{II}$, we also exchanged to alanine. To test the impact of H → A mutation on hydrogen bond networks, we constructed double mutants with the labeling site P301C and performed titration of bound fluorescein.

In addition, we constructed double and triple mutants of the H → A mutations with the $E78^{II}A$ mutation. The UV/VIS spectroscopic characterization and the determination of the enzyme activity is shown. Both histidine mutations and the $E78^{II}A$ mutation affect enzyme activity. The triple mutant shows the lowest activity, indicating a concerted mechanism that involves H-bonding networks at the K-channel entrance.

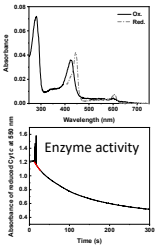
Mutants for background free thiolreactive labeling [2]



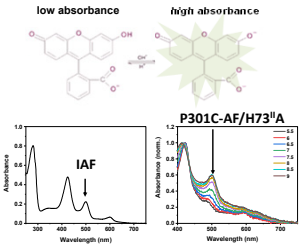
Experimental methods



Characterization of the mutants using UV-Vis spectroscopy [3]



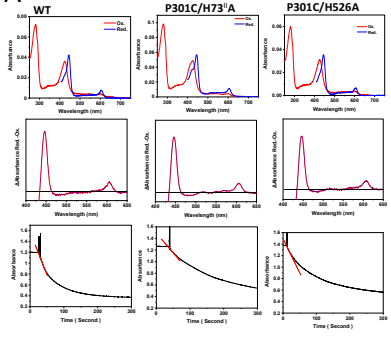
Site-directed labeling with fluorescein for proton affinity determination [2,3]

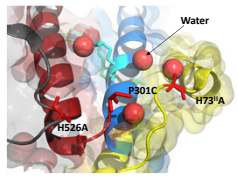


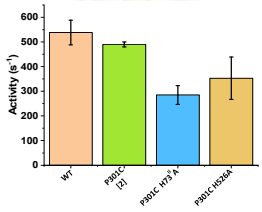
Mutagenesis

For the construction of single ($H73^{II}A$, H526A, $E78^{II}A$), double (P301C/$H73^{II}A$, P301C/H526A, $E78^{II}A/H73^{II}A$) and triple ($E78^{II}A/H73^{II}A/H526A$) mutants according to established protocols [2].

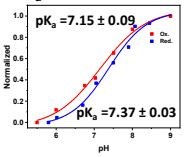
Cysteine mutants for site-specific labeling







pK_a determination of P301C-AF/$H73^{II}$A

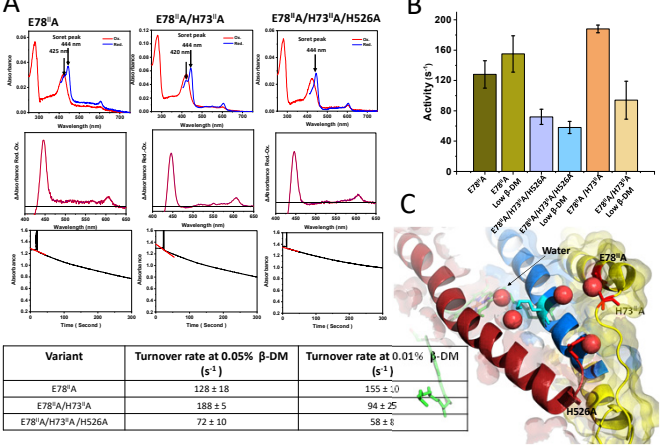


$$\Delta A = \frac{A_{max}}{[1 + 10^{n(pK_a - pH)}]}$$

Variant	Turnover rate (s^{-1})
WT	538 ± 50
P301C	490 ± 10
P301C/ $H73^{II}$A	285 ± 38
P301C/ H526A	353 ± 86

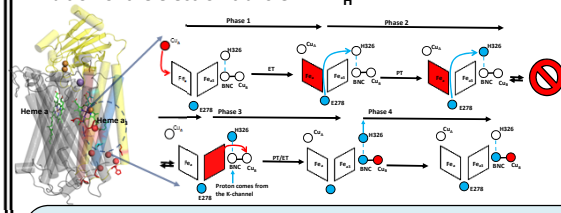
Both histidine → alanine mutations moderately affect the enzyme activity. The pK_a of fluorescein in position 301 changes only marginally upon $H73^{II}A$ mutation from 7.09 ± 0.06 to 7.15 ± 0.09 in the oxidized state and from 7.25 ± 0.08 to 7.37 ± 0.03 in the reduced state. Thus, $H73^{II}A$ affects enzyme activity but not the H-bond network around loop 6-7.

Exchange of putative proton collecting antennae residues to alanine



Variant	Turnover rate at 0.05% β-DM (s^{-1})	Turnover rate at 0.01% β-DM (s^{-1})
$E78^{II}A$	128 ± 18	155 ± 10
$E78^{II}A/H73^{II}A$	188 ± 5	94 ± 25
$E78^{II}A/H73^{II}A/H526A$	72 ± 10	58 ± 8

Inhibition of the electron transfer in $O_H \longrightarrow R$



Partial retention of electrons at Heme a by $E78^{II}A$ and $E78^{II}A/H73^{II}A$. In the double mutant the amount of retention is higher.

$E101^{II}A$ from *Rhodobacter sphaeroides* (equivalent to $E78^{II}A$) was completely inhibited at high but not at low β-DM concentration. This is in contrast to our results where the rates are similar. Surprisingly, in the double mutant $E78^{II}A/H73^{II}A$ low β-DM concentration even reduced the activity by 50 %. But in both mutants a partially reduced Soret band was found, indicating a reduced Heme a and partially oxidized Heme a_3 due to partial electron retention on Heme a. Thus, the K-path entrance in CcO from *P. denitrificans* differs from the one in *Rh. sphaeroides* with respect to the steroid ligand binding site or the inhibitory ligand β-DM.

Conclusions

- Histidine and glutamic acid residues seem to contribute to an extended H-bonding network as indicated by the change in enzyme activity.
- The single and the double mutants of $E78^{II}A$ partially block the K-path entrance by electron retention on Heme a.
- The triple mutant $E78^{II}A/H73^{II}A/H526A$ shows the lowest enzyme activity but the inhibitory mechanism seems to be different compared to the single and double mutant as we do not observe electron retention at Heme a.

References

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Acknowledgements

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