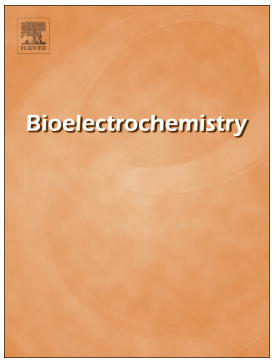

Modulating proposed electron transfer pathways in P450BM3 led to improved activity and coupling efficiency

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Modulating proposed electron transfer pathways in $P450_{BM3}$ led to improved activity and coupling efficiency

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Abstract: Electrochemical *in vitro* reduction of P450 enzymes is a promising alternative to *in vivo* applications. Previously we presented three engineered $P450_{BM3}$ variants for aniline hydroxylation, equipped with a carbon nanotube binding-peptide (CNT-tag) for self-assembly on CNT electrodes. Compared to wildtype $P450_{BM3}$ the NADPH-dependent activity was enhanced, but the coupling efficiency remained low. For $P450_{BM3}$ Verma, Schwaneberg and Roccatano [2014, Biopolymers 101, 197–209] calculated putative electron transfer pathways (eTPs) by MD simulations. We hypothesised that knockouts of these transfer pathways would alter the coupling efficiency of the system. The results revealed no improved system for the electrically-driven P450s. For the NADPH-driven P450s, however, the most active eTP-mutant showed a 13-fold increased activity and a 32-fold elevated coupling efficiency using NADPH as reducing equivalent. This suggests an alternative principle of electron transport for the reduction by NADPH and an electrode, respectively. The work presents moreover a tool to improve the coupling and activity of P450s with non-natural substrates.

Efficient cytochrome P450 monooxygenases are of great interest for the chemical and pharmaceutical industry[1–3] due to their ability to stereo- and regioselective oxidise non-activated carbons.[4–6] The bacterial P450 fusion construct from *Bacillus megaterium* ($P450_{BM3}$) ranges amongst the most active and stable P450 monooxygenases and consists of a haem- and a flavin domain.[7–10] $P450_{BM3}$ has been extensively studied and successfully modified by enzyme engineering in order to accept various substrate classes.[11–13]

A major limitation for P450 applications in cell-free systems is the dependency on the reducing equivalent NADPH.[14] The nicotinamide enters the specific binding site of the reductase domain and delivers a hydride ion to FAD. Subsequently, electrons are transferred to FMN, which further shuttles single electrons to the haem iron (Scheme 1).[15,16] One possibility to overcome the need for NADPH, is the electrochemical reduction of the enzyme.[17] Immobilisation of $P450_{BM3}$, electrochemical reduction of its cofactors (FAD, FMN and haem), as well as inter- and intramolecular electron transfer is extensively discussed in literature.[18–21] We recently developed an electrically-driven $P450_{BM3}$ system optimised for self-assembly at carbon nanotube (CNT) electrodes.[22] In this system, the enzyme was specifically anchored by a CNT-binding peptide (CNT-tag). Three full-length fusion construct $P450_{BM3}$ variants were engineered to be able to convert aniline as a model substrate since it can be detected electrochemically upon hydroxylation.

Both, in the NADPH- and electrically-driven P450 systems the reducing equivalents are not fully converted to the product. Undesired and toxic reactive oxygen species (ROS) are produced as side-products.[23] This so-called uncoupling is in particular dominant when working with non-native substrates and can be a major limitation in P450 efficiency and stability.

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