ENGINEERING OF CARBOHYDRATE OXIDOREDUCTASES FOR SENSORS AND BIO-FUELCELLS

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Pyranose dehydrogenase (PDH) and pyranose 2-oxidase (POx) are flavoproteins that catalyze the oxidation of free, non-phosphorylated sugars to the corresponding ketosugars. Pyranose dehydrogenase has a broad substrate specificity for monosaccharides (and few disaccharides), but is limited to a narrow range of electron acceptors and reacts extremely slowly with dioxygen, whereas pyranose 2-oxidase shows pronounced specificity for glucose and displays high oxidase as well as dehydrogenase activity. For bio-fuelcell and sensor applications, oxygen reactivity is undesirable as it leads to electron "leakage" and the formation of damaging hydrogen peroxide; for biocatalytic applications, oxygen reactivity is advantageous, as oxygen is freely available and obviates downstream removal of undesired electron acceptors. Site-saturation mutagenesis libraries of eleven (POx) and twelve (PDH) residues around the active sites were screened for oxidase and dehydrogenase activities. In POx, variants T166R, Q448H, L545C, L547R and N593C displayed reduced oxidase activities (between 40% and 0.2% of the wildtype) concomitant with unaffected or even increased dehydrogenase activity, dependent on the electron acceptor used (DCPIP, 1,4-benzoquinone or ferricenium ion). Kinetic characterization showed that both affinity and turnover numbers can be affected. The switch from oxidase to dehydrogenase activity was also observed electrochemically using screen-printed electrodes. In this miniaturized set-up with a reaction volume of only 50 µL the dehydrogenase activity of variant N593C was entirely preserved in the presence of a suitable mediator, shuttling electrons from the FAD cofactor to the electrode surface. The oxidase activity, utilizing molecular oxygen as acceptor, is abolished in this variant. Of all variants of PDH that were produced by saturation mutagenesis, only variants of one position displayed increased oxygen reactivity to a minor degree. Histidine 103, carrying the covalently attached FAD cofactor, was substituted by tyrosine, phenylalanine, tryptophan and methionine. Variant H103Y displayed a five-fold increase of oxygen reactivity. Stopped flow analysis revealed that the mutation slowed down the reductive half-reaction whereas the oxidative half-reaction was affected to a minor degree. No alterations in the secondary structure were observed, but disruption of the FAD bond had negative effects on thermal and conformational stability. We also engineered PDH by systematically removing several N-glycosylation sites, in order to improve performance by reducing the distance of the active site to the electrode surface, improving accessibility for redox polymers and facilitate denser enzyme packing on the electrode. One glycosylation site, N^{319}, was found to be indispensable for functional expression and folding of the enzyme, as no active variants could be obtained. A variant with two sites, N^{75} and N^{175} near the active site entrance, exchanged against G and Q, respectively, showed significantly improved properties when used on electrodes with Osmium-based redox polymers (Mediated Electron Transfer) and a low level of Direct Electron Transfer. The lack of two glycosylation sites results in minor negative effects on expression yield and stability. Removal of a third site, N^{252}, on the opposite side of the active site entrance, does not bring further improvements in catalysis and electron transfer, but is detrimental to functional expression and stability. The bulk of hyperglycosylation of the recombinantly expressed enzyme (observed in both *Pichia pastoris* and *Saccharomyces cerevisiae*) is located only on this one glycosylation site.

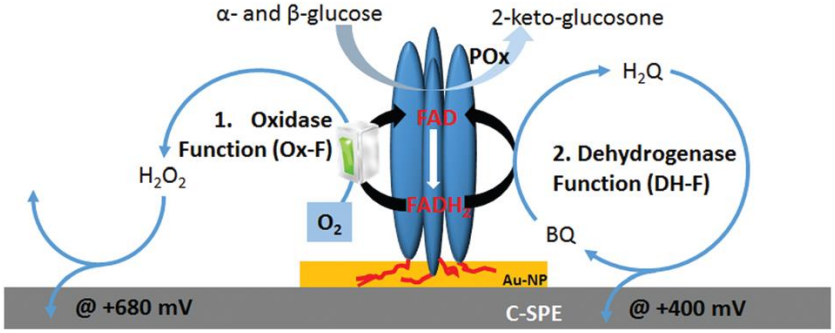

Figure 1 – Schematic representation of immobilized POx on gold-nanoparticle-SPE electrode and the main electrochemical processes at different potentials during oxidation of glucose