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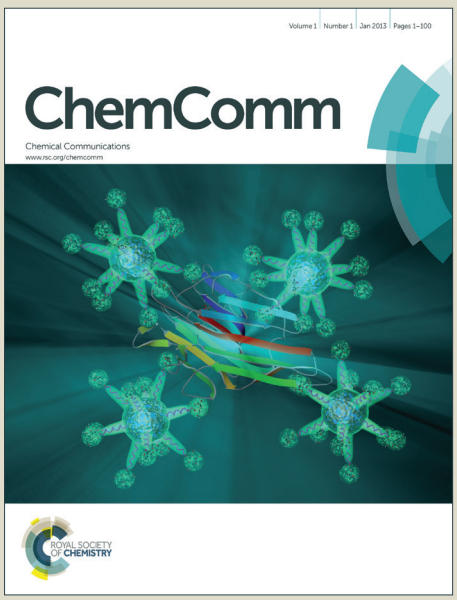

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Electrocatalytic Interconversion of NADH and NAD^+ by *Escherichia coli* Flavohemoglobin

Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

www.rsc.org/

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***E. coli* flavohemoglobin, oriented at electrodes via amphiphilic polymyxin B, electrocatalytically interconverts NADH and NAD^+ at potentials of its heme operating as an electron transfer relay between the electrode and protein's FAD, where NADH/NAD^+ is transformed. Results are crucial for development of NAD^+-dependent bioelectrodes for biosynthesis, biosensors and biofuel cells.**

Biocatalytic recycling of NAD(P)H/$NAD(P)^+$, a soluble electron accepting cofactor of more than 400 $NAD(P)^+$-dependent dehydrogenases,[1, 2] represents one of the most intriguing chemistries and a true biotechnological challenge both in industrial biosynthesis[3, 4] and electrochemical biosensor and biofuel cell development.[5, 6] Despite of a quite low potential of the NAD(P)H/$NAD(P)^+$ couple (-0.52 V *vs.* Ag/AgCl, at pH 7), $NAD(P)^+$ and NAD(P)H transformations proceed irreversibly and with overvoltages of 1 V and more.[5] A substantial overpotential, interference from other redox species and electrode fouling with reaction intermediates make direct electrochemical oxidation of NAD(P)H impractical,[7] thus calling for the search of advanced catalysts for NAD(P)H/$NAD(P)^+$ recycling.[5] In this connection, metal oxides and complexes-, conductive polymer- and quinone/phenothiazine derivative-modified electrodes were extensively explored.[5, 6] They can provide long-term stability and high efficiency of catalysis but may be disadvantageous, among other things, due to extra modification steps, non-specific catalysis of interfering reactions, and not the least, their unsustainability and toxicity. Biological catalysts can be considered as a green and sustainable alternative for NAD(P)H/$NAD(P)^+$ recycling. Hitherto, just a few proteins were shown to directly, specifically and at low overpotentials electrochemically convert NAD(P)H into $NAD(P)^+$.[8-11] Diaphorase[8] and the isolated Iλ subcomplex of bovine mitochondrial NADH: ubiquinone oxidoreductase[10] and diaphorase fragment of NAD^+-reducing [NiFe]-hydrogenase from *Ralstonia eutropha*[12] are notable examples. Of those, the two latter systems enable a reversible interconversion of NADH and NAD^+, though, being isolated fragments of biological systems of a higher complexity, they may not immediately reflect the biological ET pathways or mechanisms of catalysis regulation. This and other issues related to the search of new cost-effective biocatalysts for NAD(P)H/$NAD(P)^+$ transformation guided us to focus on bacterial flavohemoglobin from *E. coli* (HMP).

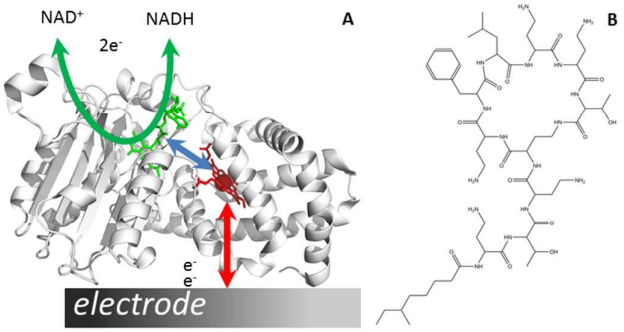

Figure 1. (A) Schematic representation of HMP (PDB file ID: 1gvh[13]). Heme is denoted in red and FAD in green. The protein is oriented at the electrode via its heme domain thus enabling a reversible catalytic transformation of NADH/NAD^+ at the FAD site upon applying a proper potential. (B) The structure of polymyxin B sulphate (the counter anion is not shown) used as a promoter of orientation.

HMP is a complex 43 kDa two-domain protein containing a *b*-type heme and an FAD as prosthetic groups in its N-and C-terminal domains,[13] respectively (Figure 1A). The heme domain possesses a classical globin fold, and the FAD domain with its NADH binding site shows similarity with ferredoxin-$NADP^+$ reductases.[14] In reaction with O_2 and NADH,[15-17] HMP demonstrates a genuine NO dioxygenase activity,[18-20] by reducing oxygen to superoxide at the heme site with a concomitant oxidation of NADH at the FAD site. Electrons from NADH are relayed by FAD to heme and further to the iron-

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Electronic Supplementary Information (ESI) available: [Materials and methods and additional data on electrocatalytic currents dependencies on substrate concentrations]. See DOI: 10.1039/x0xx00000x

bound oxygen via the intramolecular ET. Subsequently, formed superoxide is consumed in the reaction with NO.[21-23] Under anaerobic conditions, HMP reduces NO to N_2O. [24]

Recently, HMP electronically wired to electrodes via Os complex-containing polymers was shown to electrocatalytically oxidize NADH at potentials of the Os complexes used.[25] In the absence of ET mediators, no bioelectrocatalysis occurred, despite a strong signal from FAD. The absence of direct bioelectrocatalysis was ascribed to the orientation of HMP at the electrode excluding ET communication with HMP's heme and thus impairing electron flow to the FAD domain and catalysis at the NADH-binding site. Here, we studied direct bioelectrocatalytic transformation of NADH/NAD^+ by HMP under conditions where the electrode replaces O_2. To orient this complex enzyme properly for its immediate bioelectronic function, a promoter[26-28] of enzyme orientation at the electrode was used. We show that properly oriented HMP can reversibly electrocatalyse interconversion of NADH and NAD^+, thus paving the way for development of technologically relevant bioelectrocatalysts for cofactor regeneration.

HMP adsorbs on bare graphite (Gr) in the biocatalytically non-productive orientation, namely, through the FAD-domain, and exhibits two redox couples correlating with redox transformations of FAD and surface quinoid structures at -477 mV and around 62 mV, correspondingly.[25] Electrocatalytically active HMP films (Figure 1A) were obtained by inclusion of polymyxin B (PMB) as a co-adsorbate, previously shown to promote direct ET orientations of a number of proteins on Gr.[26, 29] PMB is a small polypeptide conjugated to a fatty acid (Figure 1B) and behaves as a cationic detergent at physiological pH. As such, it can productively interact both with the hydrophilic Gr surface and with the hydrophobic heme domain of HMP.[17] The complex between HMP and PMB was formed in solution, prior to adsorption on Gr. Specific binding of the PMB lipid tail in the heme active site can be inferred based on known lipid binding properties of HMP.[30] Accordingly, no orientation effects were achieved neither with another aminoglycoside neomycin, devoid of a lipid tail, nor on PMB-modified electrodes.

Cyclic voltammetry (CV) of HMP co-adsorbed with PMB demonstrated well-defined oxidation/reduction signals stemming from the heme active site of HMP (Figure 2, inset, $E^{0'}$ of -232±3 mV). The peak current dependence on the potential scan rate was characteristic of the surface-confined ET (ESI, Figure S1),[31] and the CV peak shapes were close to those predicted for a $1e^-$ transfer reaction (a $Fe^{2+/3+}$ couple) characterized by the heterogeneous ET rate constant, k_s, of 19.5±2.5 s^{-1} as estimated by the Laviron approach.[32] Integration of the CV peaks gave 6.0±0.5 pmoles of the adsorbed electroactive HMP, which corresponded to the 8.6 pmol cm^{-2} HMP surface coverage, Gr surface roughness being 10.[25] This value is close to the theoretical monolayer coverage of 7.5 pmol cm^{-2} if dimensions of HMP (12 mer-complex of a 16.4×16.4 nm^2 size divided by the number of the constituted monomer units)[13] and the protein vertical orientation through the heme domain are taken into account.

Electroenzymatic oxidation of NADH by HMP started from potentials of the $Fe^{2+/3+}$ couple of HMP's heme (Figure 2), oxidation currents being independent of the potential scan rate. Apparently, the NADH oxidation was reversible, since a reduction wave emerged at the same potentials, resulting from the reduction of NAD^+ produced during NADH oxidation (Figure 2). This wave disappeared upon rotation of the electrode removing the NAD^+ product from the electrode surface. General reversibility of the enzyme action is an established phenomenon for hydrolases and some other classes of enzymes, hydrolytic ester cleavage[33] and esterification by lipases[34] being a classic example. In contrast, bioelectronically governed reversible conversion of substrates by oxidoreductases counts just a few examples, including those reported by Armstrong on bioelectrocatalytic oxidation of succinate and reduction of fumarate by succinate dehydrogenase,[35, 36] and electrochemical interconversion of NADH/NAD^+ by the abovementioned isolated fragments of Complex I[10, 37] and NAD^+-dependent hydrogenase.[12] With the latter, electrons during bioelectrocatalytic transformations of NADH/NAD^+ were shuttled between the flavo-centers and electrodes via iron-sulfur complex clusters operating as an electronic relay,[10, 12] with the onset potential of bioelectrocatalysis roughly correlating with the potential of the flavo-centers, around -0.5 - -0.6 V. Interestingly, despite of different kinetics, bioelectrocatalytic current densities observed with HMP and these complexes were quite similar.

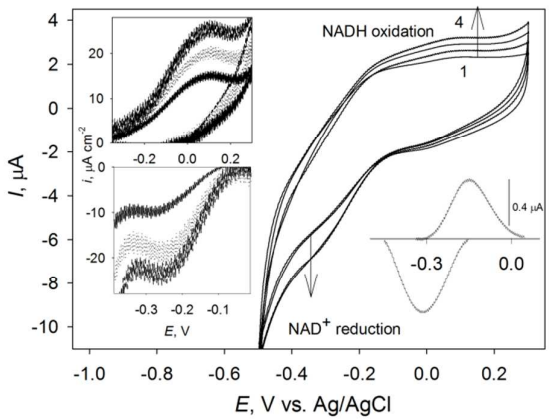

Figure 2. Representative CVs recorded with the HMP/PMB-modified Gr electrodes (1) in the absence and in the presence of (2) 1, (3) 2, and (4) 3 mM NADH, scan rate 30 mV s^{-1}. In (4) the electrode rotation rate is 960 rpm and the cathodic wave merges with the background curve. In the absence of rotation, the anodic wave is the same as in (4) and the cathodic one coincides with (2) and (3). Insets: Background corrected CV signals from HMP's heme, scan rate 300 mV s^{-1}; and electrocatalytic current densities of NADH oxidation and NAD^+ reduction (1, 2 and 3 mM), scan rate 70 mV s^{-1}. CVs were recorded in 20 mM Tris, pH 7.4, at a stationary electrode.

Rotating disk electrode experiments with HMP/PMB complex adsorbed on Gr demonstrated that the limiting catalytic currents of NADH oxidation, i_{lim}, were independent of the rotation rate, which implies a kinetic control of the enzymatic reaction. The obtained data fitted well the Michaelis-Menten dependence with a K_M of 1.68±0.22 mM at 70 mV and 2.28±0.32 mM at -200 mV (ESI, Figure S3), being three orders

of magnitude higher than 3.2 μM reported for NADH oxidation in solution, at pH 7,[18] and nevertheless lower than up to 10.8 mM shown with Os redox polymers as ET mediators.[25] The catalytic rate constant, k_{cat} ($k_{cat} = i_{lim}/(\{\text{amount of protein}\}nF)$, where F is the Faraday number and the number of electrons n=2) of 2.4±0.2 s^{-1} was essentially lower than 94 s^{-1} reported for the solution catalysis with O_2 as electron acceptor[18] and suggests a strong influence of enzyme immobilization and replacement of the natural electron acceptor by the electrode[38] on the catalytic performance of HMP.

In conclusion, we demonstrate that the HMP complex with a cationic aminoglycoside polymyxin B can be properly oriented at the electrode surface and then electrochemically catalyse interconversion of NADH and NAD^+ at potentials of HMP's heme, around -200 mV vs. Ag/AgCl, heme operating as an ET relay between the electrode and the FAD center of HMP. Considering that the formal potential of the NADH/NAD^+ couple is several hundred mVs more negative, bioelectrocatalytic reduction of NAD^+ by HMP involves an endergonic (uphill) electron tunneling from FAD to heme, governed by the overall energetics of the process (based on the relationship between the driving force and reorganization energy), distance between the redox centers, possibility of stabilization of intermediate reaction states, and some other factors.[39] This uphill tunneling should play a regulatory role, since biological activity of HMP does not imply reduction of NAD^+. That is of fundamental scientific importance and paves the way to direct monitoring of HMP's ET reactions and regulating mechanisms of the redox interconversion of the enzyme substrates. Hitherto, just a few biologically adequate, integrate enzymatic redox systems were reliably shown to bioelectrocatalytically interconvert their substrates in both reductive and oxidative ways, at the formal potentials of their substrate couples.[21,12, 37] The developed bioelectrode for regeneration of oxidised and/or reduced forms of pyridine nucleotides, displaying a high specificity for pyridine nucleotides, functions without mediators and can be used in a cascade enzymatic systems involving NAD^+-dependent dehydrogenases (Figure S4, ESI), both for industrial electroenzymatic biotransformation and in biosensors/biofuel cells.

Acknowledgement

The work was supported by the Danish Council for Independent Research, Natural Sciences (FNU), project number 11-107176

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