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# Mediated electron transfer with monooxygenases—Insight in interactions between reduced mediators and the co-substrate oxygen

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ABSTRACT

One of the most important obstacles to overcome in biocatalysis with monooxygenases is the enzyme's dependency on the costly redox cofactor NAD(P)H. Electrochemical regeneration systems, in which an electrode serves as electron donor, provide an alternative route to enzymatic redox reactions. Mediators are often used to accelerate electron transfer between electrode and enzyme. We investigated the mediated bioelectrochemical conversion of *p*-xylene to 2,5-dimethylphenol (2,5-DMP) by a P450 BM3 variant and were able to produce 2,5-DMP electrochemically. Due to the fact that mediator reduction is limited by the electrode surface a scale-up was performed. However, increasing the electrode surface area to reactor volume ratio led to a drastic increase in cathodic oxygen reduction, causing a drop in product formation. It was shown that reduced cobalt sepulchrate reacts with the co-substrate oxygen. Furthermore, the reportedly oxygen stable mediator $[Cp^*Rh(I)(bpy)H]^+$ was compared to cobalt sepulchrate. While its turnover frequency is of comparable magnitude to cobalt sepulchrate when transferring the electrons between electrode and enzyme, using $NADP^+$ as intermediary between the mediator and the enzyme significantly increased the mediator's turnover frequency. The rhodium mediator $[Cp^*Rh(I)(bpy)H]^+$ does not appear to be significantly more oxygen stable.



## 1. Introduction

Cytochrome P450 monooxygenases are heme containing redox proteins found in human liver, but also in other mammals, plants, bacteria and fungi. With their ability to hydroxylate saturated carbon atoms one of their main purposes in nature is to metabolize exogeneous molecules. Typically, they catalyze oxidation reactions such as hydroxylations or epoxidations, but also reductions and rearrangements of oxygenated species or even isomerization [1,2]. The flavocytochrome P450 BM3 fatty acid hydroxylase from *Bacillus megaterium* is one of the best understood P450 monooxygenases [3]. The soluble, 119 kDa enzyme is a fusion protein of a P450 and a P450 reductase, containing FAD and FMN [4]. Its natural substrates are fatty acids [3]. One of the challenges in biocatalysis with P450 monooxygenases is the dependence on the expensive NAD(P)H cofactor used to activate the molecular oxygen [5]. This has led to the development of several strategies to reduce the amount of employed cofactor by either replacing or regenerating it. In most cases, cofactors are regenerated with the help of additional enzymes, but electrochemical methods are an attractive alternative to this approach [6]. Due to its favourable characteristics, P450 BM3 has served as a model monooxygenase in several electrochemical experiments. Mediated electron transfer was employed, just like direct electron transfer or photo-induced electron transfer with a fusion protein [7–14]. Different authors described the direct electron transfer from the electrode to the enzyme by immobilizing P450 BM3 with a polymer matrix on the electrode, however only in one case, biocatalytic activity was reported [9,11]. Immobilizing just the heme domain of P450 BM3 on an electrode apparently also resulted in a loss of catalytic activity in most cases [15,16]. Only Fantuzzi et al. [17] were able to immobilize the heme domain of a P450 BM3 under retention of its catalytic activity. As electron transfer rates from the electrode to the P450 BM3 tend to be slow given that the electron accepting heme is buried deep in

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the protein, the use of a mediator can increase electron transfer rates [8]. Mediators can transfer electrons between the electrode and the enzyme in order to accelerate the electron transfer. Cobalt sepulchrate, also known as [(1,3,6,8,10,13,16,19-octaazabicyclo-[6.6.6]eicosane)cobalt(III)]$^{3+}$, has found broad applications as a mediator in electrochemical biocatalysis [18,19]. It acts as an one electron shuttle [7] between electrode and monooxygenase, thereby cobalt switches between the oxidation states Co(II) and Co(III). The metallorganic compound pentamethylcyclopentadienyl rhodium 2,2′-bipyridin ([Cp*Rh(bpy)($H_2O$)]$^{2+}$) is one of the few molecules showing an actual catalytic activity with a TTN of more than 10. So far the rhodium complex was not used in combination with a P450 monooxygenase, but it was shown to react with FAD dependent styrene monooxygenase, making it a promising candidate for electrochemical biocatalysis with FAD dependent P450s such as P450 BM3. The rhodium complex has proven to be stable under biocatalytic conditions and shows catalytic activity towards the reduction of several compounds, such as NAD(P)$^+$, protons, FAD and FMN [20–22]. The reduction of NAD(P)$^+$ has furthermore been shown to selectively yield enzymatically active 1,4-NAD(P)H [23,24]. Unfortunately, the oxidized form of the complex tends to deactivate itself and also enzymes by binding to several amino acids and replacing the coordinated water molecules. In order to reduce deactivation, the use of "coordinating buffers", like TRIS or ammonia buffers, has been suggested [25]. Oxygen plays an important role as a substrate in P450 catalysis, but also as a reactant with the mediators discussed above. It is therefore necessary to understand its behaviour in an electrochemical cell. The amounts of hydrogen peroxide that are produced during the electrochemical processes due to the reduction of oxygen and also by P450 itself require methods to eliminate it, as it has been shown to deactivate the enzyme. Deactivation occurs as a result of oxidative damage to the heme and the protein. To eliminate hydrogen peroxide, there are several methods, such as shielding the working electrode with an additional Pt-mesh or adding catalase [12,26]. Unfortunately, so far the undesired uncoupling reaction has not been addressed very much and a general awareness of this oxygen dilemma is missing [27].

Phenol and its derivatives, e.g. cresols or dimethylphenols are mainly used for the production of polymers, herbicides and fungicides, but also as disinfectants, plasticizers and solvents [28,29]. Phenol is synthesized through the cumene process that gives acetone as a byproduct [28,30] and alkylphenols are produced by phenol methylation or chlorination, sulfonation, alkylation and oxidation of toluene or xylene [28]. The production of phenols is very energy intensive; furthermore, the annual growth rate of acetone demand is lower than that of phenol. In consequence alternative routes for production of phenols are required in order to avoid unwanted acetone yields and reduce production costs. Direct aromatic oxidation so far has been ruled out because of low yields and high production costs [30]. A biocatalytic approach presents therefore an interesting alternative to the conventional production of phenols due to its high selectivity and low energy demands. There have been various reports on direct aromatic ring hydroxylations with P450 monooxygenases [31]. The mutant P450 BM3 M2 (R47S, Y51W, I401M) employed in our experiments has been described by Dennig et al. [32] to convert *p*-xylene efficiently into 2,5-dimethylphenol (2,5-DMP) with a selectivity of 98% (Fig. 1).

Here we describe the electro-enzymatic conversion of *p*-xylene to 2,5-DMP. The reaction product is of high interest for the production of temperature stable polymers and as a building block for "next-generation" plastics [29].

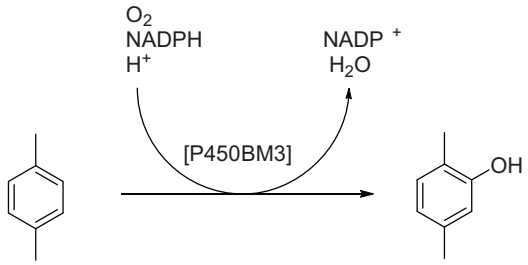


**Fig. 1.** Conversion of *p*-xylene to 2,5-dimethylphenpol by P450 BM3 monooxygenases.

## 2. Experimental

### 2.1. Expression of P450 BM3

Lysogeny broth (LB) medium was prepared with 1.0 g peptone, 0.5 g yeast extract, 1.0 g NaCl and 100 ml double-distilled water (dd$H_2O$) and sterilized by autoclaving. Terrific broth (TB) medium consisted of two solutions prepared separately, one containing 12 g peptone, 24 g yeast extract 4 ml glycerol in 800 ml dd$H_2O$, the other containing 2.31 g $KH_2PO_4$, 12.54 g $K_2HPO_4$ in 200 ml dd$H_2O$. The solutions were combined after autoclaving.

Precultures (*Escherichia coli* BL21 (DE3) Gold pET28a(+) carrying the gene, cloned by NcoI/EcoRI for P450 BM3 R47S/Y51W/I401 M also called P450 BM3 M2) were cultivated for 16 h (37 °C, 180 rpm), containing 5 ml LB medium and 50 μl kanamycin solution (35 mg/ml, dd$H_2O$). 100 ml of TB medium containing 1 ml kanamycin solution were then inoculated with 1 ml of preculture and cultivated (37 °C, 180 rpm). After reaching an $OD_{600}$ of 0.8–1.0, P450 expression was induced by adding 500 μM aminolevulinic acid, 200 μl IPTG (0.5 M, dd$H_2O$). Harvesting occurred 20 h after induction by centrifugation (3220 rcf, 4 °C, 20 min). The pellets were resuspended in potassium phosphate buffer (50 mM, pH 7.5) and centrifuged again (3220 rcf, 4 °C, 20 min). The buffer solution was removed and cell pellets were stored at −20 °C. To disrupt the cells, they were resuspended in potassium phosphate buffer (50 mM, pH 7.5) and treated with ultrasound (10% amplitude, 1 s sonication, 2 s pause, 2 min total sonication time). Cell debris was separated by centrifugation (3220 rcf, 4 °C, 30 min) and the supernatant containing the P450 BM3 was sterilized by filtration (PVDF, 22 μM). The concentration of P450 was determined according to the method described by Omura and Sato [33].

### 2.2. 2,5-Dimethylphenol detection

Defined volumes of the reaction mixtures were extracted in glass vials with known amounts of methyl *tert*-butyl ether (MTBE) containing 1 mM phenol as internal standard. To achieve phase separation, the vials were centrifuged (5290 rcf, 20 °C, 2 min). The reaction product was detected via gas chromatography, using an Agilent J&W DB-WAXetr column (see SI). Phenol had a retention time of 14.8 min, while 2,5-DMP showed a retention time of 15.8 min. Product concentrations were calculated from a calibration curve.

### 2.3. Electrode cleaning and preparation

All platinum electrodes were cleaned by placing them in a mixture of hydrogen peroxide and sulfuric acid (1:1) for 1 min, rinsing with double-distilled water (dd$H_2O$) after which they were stored in ethanol. A Luggin capillary containing the Ag/AgCl reference electrode (3 M KCl) was rinsed with dd$H_2O$ and stored in 3 M KCl. The