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Suitability of the hydrocarbon-hydroxylating molybdenum-enzyme ethylbenzene dehydrogenase for industrial chiral alcohol production

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ABSTRACT

The molybdenum/iron-sulfur/heme protein ethylbenzene dehydrogenase (EbDH) was successfully applied to catalyze enantiospecific hydroxylation of alkylaromatic and alkylheterocyclic compounds. The optimization of the synthetic procedure involves use of the enzyme in a crude purification state that saves significant preparation effort and is more stable than purified EbDH without exhibiting unwanted side reactions. Moreover, immobilization of the enzyme on a crystalline cellulose support and changes in reaction conditions were introduced in order to increase the amounts of product formed (anaerobic atmosphere, electrochemical electron acceptor recycling or utilization of ferricyanide as alternative electron acceptor in high concentrations). We report here on an extension of effective enzyme activity from 4 h to more than 10 days and final product yields of up to 0.4–0.5 g/l, which represent a decent starting point for further optimization. Therefore, we expect that the hydrocarbon-hydroxylation capabilities of EbDH may be developed into a new process of industrial production of chiral alcohols.

1. Introduction

Chiral alcohols are important building blocks for the synthesis of fine chemicals and pharmaceuticals. Many of them are produced biotechnologically *via* enantioselective reduction of the respective ketones by various alcohol dehydrogenases (Höffken et al., 2006; Huisman et al., 2010; Hummel, 1997; Kroutil et al., 2004). The available enzymes exhibit a wide range in substrate and coenzyme specificities, reaction rates and enantiomeric excesses of the products, and economically feasible processes have been implemented with a number of model enzymes, particularly by coupling the ketone reduction reaction with cost-efficient regeneration techniques for the required NAD(P)H cofactors (Breuer et al., 2004; Hasegawa et al., 2010; Patel, 2008). However, the process is limited to ketones contained within the substrate range of the used enzymes and their compatibility with the NAD(P)H regeneration process. A fundamentally new process for chiral alcohol production may be derived from the recent discovery that the first enzyme of the anaerobic ethylbenzene metabolic pathway, ethylbenzene dehydrogenase (EbDH), produces (almost) exclusively (*S*)-alcohols from ethylbenzene and many alternative substrates (Dudzik et al., 2013; Knack et al., 2012; Szaleniec, 2012; Szaleniec et al., 2014, 2009).

EbDH is a periplasmic heterotrimeric molybdenum enzyme of the DMSO reductase family and contains a Mo-*bis*-molybpdopterin guanine dinucleotide (MGD) cofactor in its active site, which is responsible for the stereospecific hydroxylation of the substrates with water – Fig. 1A (Johnson et al., 2001; Kloer et al., 2006; Kniemeyer and Heider, 2001). The two electrons retrieved during this reaction are channeled through the enzyme *via* five Fe-S-clusters to a heme b cofactor, which releases them to an external electron acceptor like cytochrome c or the easily measurable redox dye ferrocenium ($[Fe(cp)_2]BF_4$). EbDH is known from a few ethylbenzene- or *n*-propylbenzene-degrading denitrifying bacteria (Johnson et al., 2001; Kniemeyer and Heider, 2001; Rabus and Heider, 1998), catalyzing the initial reaction of the degradation pathway (Fuchs et al., 2011; Heider, 2007; Heider and Schühle, 2013). The enzyme is strictly regulated in these bacteria and is only present in cells growing anaerobically on ethylbenzene or *n*-propylbenzene (Kniemeyer and Heider, 2001; Kühner et al., 2005).

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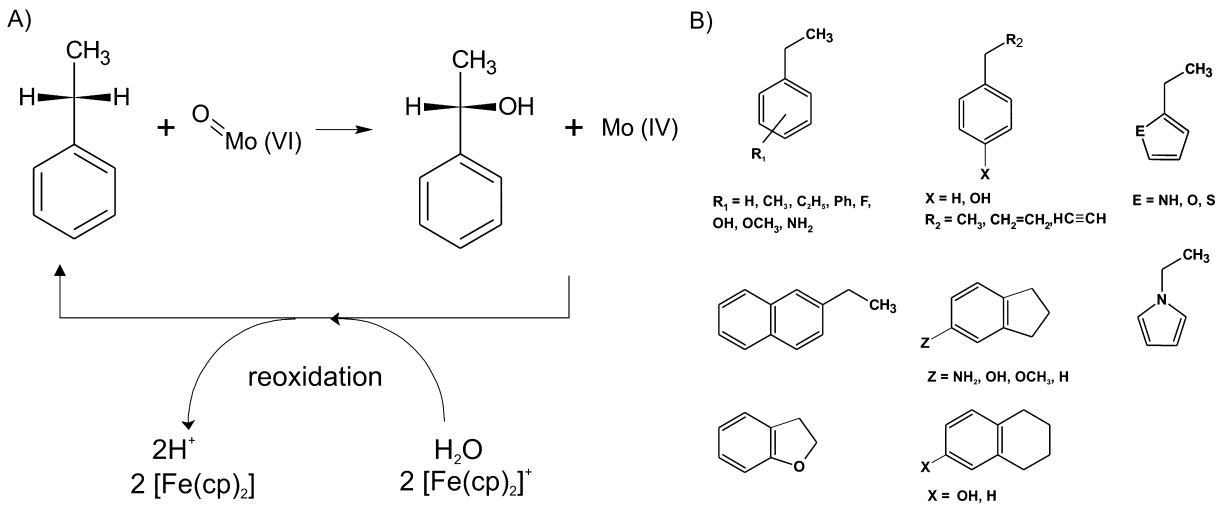

Fig. 1. A) Schematic representation of the reaction catalyzed by EbDH; B) substrates hydroxylated by EbDH to secondary alcohols (Dudzik et al., 2013; Knack et al., 2012, 2013).

Besides ethylbenzene and *n*-propylbenzene, many other aromatic substrate analogues are accepted by EbDH and converted to the corresponding alcohols at the C1-atom of the side chain – see Fig. 1B (Knack et al., 2012; Szaleniec et al., 2009, 2007). For most substrates, this reaction occurs with almost absolute stereospecificity to the (*S*)-alcohols (Dudzik et al., 2013; Knack et al., 2012; Szaleniec et al., 2014, 2009). The mechanistic details of the hydroxylation reaction and the molecular prerequisites for its stereospecificity have been deduced by molecular modeling approaches on the basis of the structure of reduced EbDH (Kloer et al., 2006; Knack et al., 2013; Szaleniec et al., 2010, 2014, 2012).

The extremely large substrate range (Fig. 1B) and the strongly stereospecific hydroxylation reaction (Dudzik et al., 2013; Szaleniec et al., 2009) endorse a possible biotechnological application of EbDH for the production of chiral alcohols. This would be a completely novel route to this group of fine chemicals. Although similar hydroxylation reactions are also catalyzed by monooxygenases, e.g. members of the cytochrome P450 enzyme family, these enzymes mostly are not as region- and stereospecific as EbDH (Adam et al., 2001; Filipovic et al., 1992; Ichinose, 2012, 2013; Schrewe et al., 2013; van Beilen et al., 2003). Moreover, they require molecular oxygen and a reducing agent in defined stoichiometries for their activity, which results in challenging problems for devising a biotechnological turnover process as well as methods avoiding oxygen transfer limitations from gas to liquid phases (Leak et al., 2009).

On the other hand, EbDH offers a very strong region- and stereospecificity combined with a very large substrate range which may even be further extended by the use of similar enzymes from other anaerobic hydrocarbon degraders or by mutagenesis studies on EbDH. However, although EbDH can principally be handled under aerobic conditions, its potential use is still limited by its nature as a primarily anaerobic enzyme. In particular, the enzyme has only a half-life time of 7 min in air in the reduced form, while it is more stable in the oxidized form (Kniemeyer and Heider, 2001; Szaleniec et al., 2007). A biotechnological process involving EbDH has therefore to account for this oxygen sensitivity, but also for the low solubility of the currently used artificial electron acceptor – ferrocenium. EbDH is also a highly complex enzyme containing several different metal cofactors in three separate subunits and is currently only available from the wild type strain, but promising attempts of producing the enzyme recombinantly are underway (our unpublished results).

In this communication, we present data on electrochemical recycling of the electron acceptor ferrocenium and on coupling the EbDH-catalyzed substrate hydroxylation to ferricyanide ($K_3[Fe(CN)_6]$), an alternative low-cost electron acceptor that can be used in sufficiently high concentrations to obtain economically worthwhile products yields. Moreover, we present the results of the first attempts to immobilize EbDH to convert the enzyme into a better controllable biocatalyst, especially regarding the need to stabilize its activity and to facilitate the recovery of the alcohol products.

2. Materials and methods

2.1. Enzyme preparation

Ethylbenzene dehydrogenase (EbDH) was purified from "*Aromatoleum aromaticum*" EbN1 grown anaerobically on ethylbenzene (Rabus and Widdel, 1995). Growth of bacteria, crude extract preparation and the purification steps where done as described previously (Szaleniec et al., 2007). In order to enhance EbDH stability and to reduce the costs of enzyme purification, a shortened purification protocol was established. This protocol differs from the full procedure described previously in omitting the last chromatography step and collecting the active fractions already after the first column, DEAE-Sepharose. The fractions are then concentrated by ultracentrifugation (50 kDa membrane in Centricon system) and stabilized by addition of 10% glycerol and ferrocenium (end concentration 150 μM). The obtained enzyme preparation contains EbDH along with a range of additional heme proteins and bacterial cytochromes which seem to additionally stabilize the enzyme. Protein concentrations were determined by the Coomassie dye binding assay (Bradford, 1976).

2.2. Activity assay and kinetic measurements

Apparent kinetic parameters for ferrocenium and ferricyanide were established with photometric enzyme activity assays described before (Knack et al., 2012; Szaleniec et al., 2007). Purified EbDH was added to the reaction mixture (100 mM Tris/HCl, pH 7.5 with 50–200 μM ferrocenium or 0.56–2.3 mM ferricyanide as electron acceptors), and the photometric test was started by addition of the organic substrate in enzyme-saturating concentration (assay concentration of 100 μM for ethylbenzene, 3-phenyl-1-propene