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Identification of *Candida tenuis* xylose reductase as highly selective biocatalyst for the synthesis of aromatic α-hydroxy esters and improvement of its efficiency by protein engineering

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Supporting Information

Materials

Ethyl pyruvate, ethyl (*R*)-lactate, ethyl (*S*)-lactate, ethyl benzoylformate, ethyl (*R*)-mandelate, ethyl (*S*)-mandelate, ethyl 4-cyanobenzoylformate, were purchased at Sigma-Aldrich, Vienna, Austria, ethyl 2-cyanobenzoylformate and ethyl 3-cyanobenzoylformate were bought from Rieke Metals, Inc., Nebraska, U.S.A. Racemic ethyl 4-cyanomandelate was from Synthon Chemicals GmbH & Co. KG, Wolfen, Germany. The substrates ethyl 2-chlorobenzoylformate, ethyl 3-chlorobenzoylformate, ethyl 4-chlorobenzoylformate along with the corresponding alcohols ethyl (*R*)-2-chloromandelate, ethyl (*S*)-2-chloromandelate, racemic ethyl 3-chloromandelate, ethyl (*R*)-3-chloromandelate, ethyl (*R*)-4-chloromandelate and racemic ethyl 4-chloromandelate were synthesized by DSM Fine Chemicals Austria GmbH, Linz, Austria. Other materials were reported elsewhere [1].

Site directed mutagenesis, enzyme production and purification

The mutations W23F and W23Y were introduced using reported methods [2, 3, 4]. Recombinant wild-type CtXR and the mutants thereof were produced in *E. coli* and purified to apparent homogeneity as described recently [4, 5]. Purified W23F and W23Y mutants migrated in SDS/PAGE as single protein bands to exactly the same position as the wild-type (data not shown).

Steady state assays

Initial rate measurements were carried out using reported protocols [3]. All experiments were performed at 25 °C in 50 mM potassium phosphate buffer, pH 7.0. To enhance the solubility of hydrophobic α-keto esters 5 % ethanol was added to the buffer. Kinetic parameters for enzymatic xylose reduction by NADH were not affected by the added ethanol. The initial rates were recorded immediately after preparation of the substrates to avoid their non-enzymatic decomposition in aqueous solution [6]. They were obtained under conditions in which the substrate concentration was varied and the NADH concentration was constant and saturating (230 μM NADH). The enzyme concentration in the assays was in the range of 0.2-1.5 μM, depending on the activity towards the respective substrate. Appropriate controls containing enzyme and coenzyme, or the substrate and coenzyme were determined under conditions otherwise exactly identical to the enzymic assay. Data processing and statistical analysis were carried out as reported elsewhere [3]. The limited solubility of aromatic α-keto esters prevented saturation of the enzymes. The catalytic efficiency was obtained from the part of the Michaelis-Menten plot where under conditions of [substrate] «

K_m, the reaction rate is linearly dependent on [substrate] with a slope that equals (k_{cat}/K_m) divided by the molar concentration of enzyme.

Enzymatic reduction of α-keto esters and stereochemical analysis of reduction products

Aromatic α-keto esters (ethyl benzoylformate and ethyl 4-cyanobenzoylformate 5 mM, ethyl 2-chlorobenzoylformate and ethyl 4-chlorobenzoylformate 1.5 mM) were dissolved in ethanol prior to dilution into 50 mM potassium phosphate buffer, pH 7.0 to a final ethanol concentration of 5 %. 1.5 mM ethyl pyruvate was directly dissolved in 50 mM potassium phosphate buffer, pH 7.0. The α-keto ester was incubated at 25 °C in the presence of 1 mM excess [NADH] and enzyme (wild-type, W23F or W23Y). The reaction mixtures (2 mL) contained about 0.4 – 2.4 units of enzyme activity, measured with the substrate to be reduced. After ketone reduction of ~ 80 % (< 5 – 25 min), determined as the depletion of NADH, the solutions were centrifuged prior to chromatographic analysis. The stereochemical outcome of ethyl pyruvate reduction was determined by chiral GC. The enzyme was separated by ultrafiltration, the analytes extracted from the filtrate with 2 mL CH_2Cl_2 and the organic phase dried with Na_2SO_4. The samples were analyzed on a HP 5890 Series II gas chromatograph equipped with a HP 6890 series injector and a chiral column (Chrompack Chirasil-DEX CB capillary column, dimensions 25 m x 0.25 m x 0.25 µm). Helium was used as carrier gas with a split ratio of 1:10. The temperature program started with 40°C for 1 min followed by a ramp to a final temperature of 200°C at 15°C min^{-1}. Ethyl (*R*)-lactate and ethyl (*S*)-lactate were separated on the column and detected with a

flame ionization detector (FID). HPLC analysis of the aromatic α-hydroxyesters was performed with a reversed phase CHIRALPAK AD-RH column from Daicel (purchased at VWR International, Vienna, Austria) on a LaChrom HPLC system (Merck-Hitachi) equipped with an L-7400 UV-detector (280 nm). Best resolutions of the (*R*)- and (*S*)-alcohols were obtained with acetonitrile and water (20:80, by volume) as eluent at a flow rate of 0.5 mL/min and a temperature of 40°C.

References

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