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Rationalisation of the substrate concentration dependent diastereoselectivity of a *Saccharomyces cerevisiae* short-chain dehydrogenase

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Abstract—The diastereoselectivity of the carbonyl reduction of bicyclo[2.2.2]octane-2,6-dione, catalysed by the purified yeast cytosolic short-chain dehydrogenase Ymr226cp, was shown to be substrate concentration dependent. The changing selectivity was attributed to two distinct binding configurations of the substrate in the active site, each yielding a distinct hydroxy ketone diastereomer. By applying individual K_M and V_{max} values for each binding configuration, the concentration dependence could be modelled with Michaelis–Menten kinetics and the apparent K_M and V_{max} values for the generation of each diastereomer determined. This is to the best of our knowledge the first rationalisation of a substrate dependent stereoselectivity for a pro-chiral substrate with an isolated enzyme.

1. Introduction

Stereoselective carbonyl reduction of xenobiotic compounds is of importance since the chiral alcohols obtained constitute as valuable building blocks in the synthesis of fine chemicals and pharmaceuticals.[1] The stereoselectivity of carbonyl reductions catalysed by whole-cells or crude cell extracts have often been observed to be substrate concentration dependent. This has traditionally been attributed to the presence of multiple enzymes converting the same substrate, but possessing different stereoselectivity, maximum reaction rate (V_{max}) and substrate affinity (K_M).[2–4] However, at least two examples of isolated enzymes with substrate dependent stereoselectivity have been reported: the Baeyer–Villiger oxidation of racemic bicyclo[3.2.0]hept-2-en-6-one with a monooxygenase from *Acinetobacter calcoaceticus*[5] and the carbonyl reduction of sigma-symmetrical 1,3-cyclopentadiones and 1,3-cyclohexadiones with a baker's yeast enzyme.[6] The substrate dependent enantioselectivity of the monooxygenase was rationalised by the presence of a second binding site closely spaced to the active site, which, when occupied, prevented the binding of one of the substrate enantiomers,[5] whereas the substrate dependent diastereoselectivity of the carbonyl reductase could not be explained.[6] In a recent study,[7] we detected a substrate dependent diastereoselectivity in the reduction of the prochiral diketone bicyclo[2.2.2]octane-2,6-dione **1** to (1*R*,4*S*,6*S*)-6-hydroxy-bicyclo[2.2.2]octane-2-one (−)-**2** (major isomer) and (1*S*,4*R*,6*S*)-6-hydroxy-bicyclo[2.2.2]octane-2-one (+)-**3** (minor isomer) (Fig. 1), catalysed by a recombinant *Saccharomyces cerevisiae* TMB4091 strain expressing the reductase gene YMR226c (Fig. 1). At a substrate concentration of 5 g/L, (−)-**2** was produced in high excess, corresponding to a diastereomeric excess (de) of 90%. However, when the initial concentration was raised to 20 g/L, increasing amounts of (+)-**3** were obtained while the de dropped to 84%. For comparison, the de only dropped from 99% and 98% to 97% and 95%, respectively, when two other known **1** reductase genes (*YPR1* and *GCY1*) were overexpressed in the same parent strain.[8]

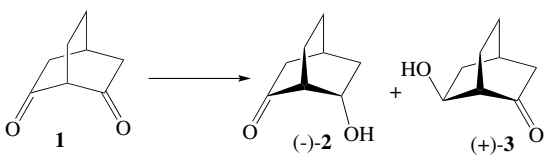

Figure 1. Reduction of bicyclo[2.2.2]octane-2,6-dione **1** to (1*R*,4*S*,6*S*)-6-hydroxy-bicyclo[2.2.2]octane-2-one (−)-**2** and (1*S*,4*R*,6*S*)-6-hydroxy-bicyclo[2.2.2]octane-2-one (+)-**3**, by yeast Ymr226cp protein.

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2. Results and discussion

Since the overexpression of *YPR1* and *GCY1* yielded higher selectivity, the YMR226c gene product (Ymr226cp) was suspected to cause the decreased selectivity in TMB4091. To confirm this hypothesis, the GST-tagged Ymr226cp reductase was isolated from a strain obtained from the *S. cerevisiae* Exclone collection and purified to homogeneity as previously described.[8] The isolated enzyme was used for in vitro reduction of **1**, using $NADP^+$ and glucose-6-phosphate dehydrogenase for NADPH co-factor regeneration (according to Katz et al.[9] but downscaled to 1 mL). A clear substrate dependent diastereoselectivity was observed for the isolated enzyme, starting at 86% de at 2.5 g/L **1** and dropping below 80% de when the substrate concentration approached 20 g/L (Fig. 2).

We hypothesise that the changing diastereoselectivity was caused by an alternative binding of the substrate to the active site. Since the substrate lacks bulky side-groups and has similar features when viewed from different angles (Fig. 1), a rotation of the molecule horizontally or vertically, might allow an alternative binding to the active site that would still permit the reduction. Whereas the first configuration positions the molecule so that the carbonyl carbon is attacked from the *exo*-side forming (−)-**2**, the second configuration would expose the *endo*-side of the other carbonyl carbon, hence generating (+)-**3**.

The binding of the substrate in the two configurations would occur with different affinity. Hence, it is possible to consider the reduction kinetics analogously to a system with two enzymes with different selectivity, K_M and V_{max} and the formation of the two diastereomers can be described as separate single-substrate Michaelis–Menten type reactions (Eq. 1):

$$v^n = \frac{V^n_{max} \times S}{K^n_M + S} \quad (1)$$

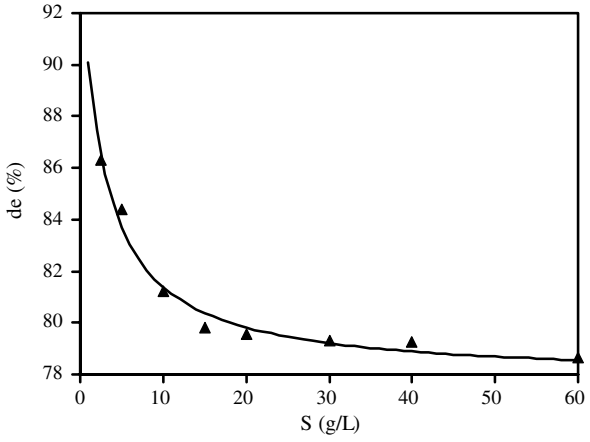

Figure 2. Diastereoselectivity at different substrate concentrations for isolated GST-tagged Ymr226cp (▲), and Michaelis–Menten model (full line) adapted after the Ymr226cp data. Standard deviations of triple measurements were determined at 5 and 30 g/L to ±0.6% and ±0.1%, respectively.

where n = (−)-**2** or (+)-**3**, v = reaction rate, V_{max} = maximum reaction rate, K_M = affinity constant and S = substrate concentration.

Moreover, the de can be described as a function of the rate of formation for the two diastereomers (Eq. 2).

$$\text{de} = \frac{[(-)\text{-}\mathbf{2}] - [(+)\text{-}\mathbf{3}]}{[(-)\text{-}\mathbf{2}] + [(+)\text{-}\mathbf{3}]} = \frac{v^{(-)\text{-}\mathbf{2}} - v^{(+)\text{-}\mathbf{3}}}{v^{(-)\text{-}\mathbf{2}} + v^{(+)\text{-}\mathbf{3}}} \quad (2)$$

By combining Eqs. 1 and 2, the de can be described as a function of the substrate concentration. This relationship was used to calculate the apparent K_M and V_{max} constants in Eq. 1 for (−)-**2** and (+)-**3**, by fitting Eq. 2, using the sum of the least squares minimisation method, to the experimental de data obtained from Ymr226cp reductions at initial reaction conditions ($R^2 = 0.98$).

The adapted model (Fig. 2) gave $K_M = 0.64$ g/L for (−)-**2** and $K_M = 2.92$ g/L for (+)-**3** and the V_{max} ratio $V_{max}(-)\text{-}\mathbf{2}/V_{max}(+)\text{-}\mathbf{3} = 7.94$. For the modelling, both apparent affinity constants were important, whereas only the ratio of the V_{max} values had an influence on the substrate dependency. The high V_{max} for (−)-**2** formation was expected since it was the major isomer formed, while the high K_M of (+)-**3** formation reflects its increased influence at higher substrate concentrations.

3. Conclusion

To the best of our knowledge, this is the first rationalisation of a substrate dependent stereoselectivity of a pro-chiral substrate with an isolated enzyme. The understanding of the substrate concentration dependent stereoselectivity can aid in the design of processes with improved selectivity. However, for better understanding of the underlying mechanism and improvement of selectivity with rational protein engineering, crystallisation of the enzyme together with the bound substrate will be required.

4. Experimental

4.1. Chemicals

Substrate **1** was synthesised as described previously.[10] Crude **1** was purified by silica gel chromatography (MATREX, 25–70 μm), re-crystallised and estimated to be of high purity on a DRX400 NMR spectrophotometric system (Bruker, Fallanden, Switzerland) with solvent as internal reference [$CHCl_3$ (^1H, 7.27 ppm), $CDCl_3$ (^{13}C, 77.23 ppm)].

4.2. Enzyme, co-factor and co-substrate

Glucose-6-phosphate dehydrogenase (EC 1.1.1.49, torula yeast, NADPH dependent), D-glucose-6-phosphate and $NADP^+$ were purchased from Sigma Aldrich (St Louis, Missouri).

4.3. Expression and purification of GST-tagged fusion protein from *S. cerevisiae*

The GST-tagged *S. cerevisiae* reductase Ymr226cp was isolated from the Exclone collection [Resgen, Invitrogen Corporation (UK)] and purified as described previously.[10] The purity of the protein was investigated with SDS-PAGE. The protein was run on a 12% acrylamide gel (Biorad ready gels, Biorad Laboratories, Hercules, California, USA) with 120 V for 1 h (Fig. 2). The staining solution contained acetic acid/distilled water/coomassie blue (200/1800/0.5) and destaining solution contained acetic acid/methanol/distilled water (50/50/400).

4.4. In vitro bioreduction of 1 with NADPH regeneration

Bioreductions of BCO2,6D using isolated Ymr226cp were performed in phosphate buffer (100 mM, pH 7.0) on a 1 mL scale by mixing 2 U/mL glucose-6-phosphate dehydrogenase, 10 mg/mL glucose-6-phosphate, 0.75 mg/mL $NADP^+$ and 2.5–60 mg/mL **1**. The bioreductions were incubated at 30 °C and mixed on a rocking table. Samples were taken in 100 μL aliquots and analysed on GC.

4.5. Analyses of substrates and products

Diastereomeric purity was analysed on a Perkin Elmer Autosystem XL gas chromatography system (GC), equipped with a Flame Ionisation Detector (FID). The samples were run on an α-Dex 120 column (Supelco, 30 m × 0.25 mm, 0.25 μm film, injector and detector kept at 300 °C), at 1 mL/min and 130 °C using He as carrier gas. The retention times of the peaks were as follows: diketone **1** 25.2 min, the two enantiomers of **3** 39.4 min and 39.9 min, respectively, (+)-**2** 41.8 min and the major hydroxy ketone product (−)-**2** 42.2 min.

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