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Chiral switch of enzymatic ketone reduction by addition of γ-cyclodextrin

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ABSTRACT

We report a chiral switch in the configuration of 1-(*p*-methoxyphenyl)-propan-2-ol, synthesized in aqueous media by ketoreductase in the presence of high concentration of γ-CD. NMR, ECD and fluorescence spectrometry were used in the effort to explain this unexpected effect. A comparison has been made between the catalytic activity of the enzyme and α-helix content in the conformation and it has been observed that enzyme is most active at the maximum α-helix content. The β-sheet content and random coil conformation in the enzyme were found to be dependent on cyclodextrin concentration.

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1. Introduction

Stereoselective reduction of ketones leading to the formation of chiral alcohols is very useful transformation in organic synthesis. Chiral alcohols are present in a variety of pharmaceutical compounds and they are also used as intermediates for a chiral synthesis. Beside, chiral reduction with variety of chemical reducing agents, there are many example using enzymes for such transformation. Alcohol dehydrogenases (ADHs) are enzymes that catalyze reversible processes generally leading to the corresponding alcohol with enantioselectivity, usually higher than 99%.[1,2] It has been reported that secondary Alcohol dehydrogenase from *Thermoanaerobium brockii* (TbADH)[3] and a library of commercially available ketoreductases (KREDs) have a very broad substrate range and accept different aliphatic, aromatic, halogenated or heterocyclic ketones.[1,4–8] However, an important characteristic is their dependence of a cofactor, NAD(P)H, that is consumed during the process of reduction. This makes the synthetic process expensive due to the high cost of the respective cofactor. Hence, appropriate regeneration procedures for the reactions carried out in aqueous media have been described. In practice, either isopropanol and ADH itself—in (i) substrate-coupled reaction, or a second enzyme (formate dehydrogenase (FDH), or glucose dehydrogenase (GDH)) and formate/*d*-glucose—in (ii) enzyme-coupled reaction, are used. The only side products in these processes are acetone, gluconic acid and carbon dioxide.[9–11]

Enzymatic reduction of the poorly water-soluble ketones in aqueous-buffer system can lead to low reaction rate as a consequence of deficient substrate availability. The addition of a suitable organic solvent into the system present one viable strategy to overcome the difficulties related to the limited solubility of the substrate. However, some proteins become unstable and inactive on the presence of organic solvents.[12,13]

The other strategy is based on the optimization of the reaction conditions by application of special additives. Their presence can improve various properties like solubility and stability of reactants or, on the other hand, they can act as inhibitors of the enzyme–substrate complex formation.[14,15] It has been reported that cyclodextrins (CDs) can play a role of suitable additives. Naturally occurring cyclodextrins are cyclic oligomers of six (α), seven (β) or eight (γ) α-(1,4)-linked glucopyranose units. Each cyclodextrin has a toroidal structure. With an internal slightly hydrophobic cavity and hydrophilic outer surface the CDs can act as complexing agents with wide range of guest compounds with suitable hydrophobic group that can hold within the cavity. Additional advantages of cyclodextrin complexation are the increased solubility of the guest compounds in water.[14,16–19] CDs can interact as ligands in the formation of inclusion complexes with amino acid residues of proteins. Numerous experimental data demonstrating the influence of the inclusion effect on the conformation of proteins have been reported.[20–25]

The bio-reduction of 1-(*p*-methoxyphenyl)-propan-2-one carried out with TbADH and twelve ketoreductases is present, Scheme 1. In addition, the native α-, β- and γ-CDs were used as additives to enhance enantioselectivity and reaction rate in enzymatic reduction of 1-(*p*-methoxyphenyl)-propan-2-one.

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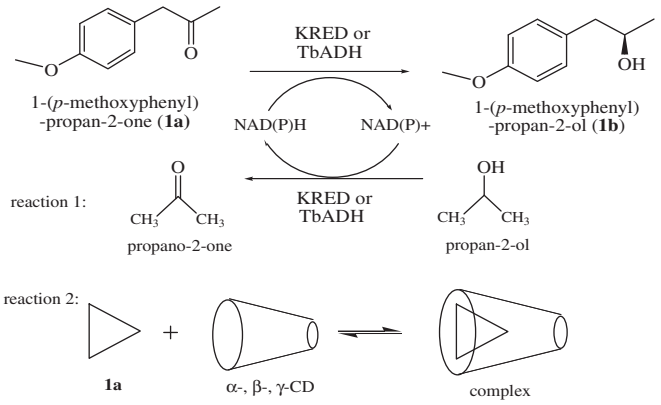

Scheme 1. Enzymatic reduction of 1-(*p*-methoxyphenyl)-propan-2-one catalyzed by ADH in aqueous media.

2. Results and discussion

2.1. Synthesis of (*R*)-1-(*p*-methoxyphenyl)-propan-2-ol

The asymmetric reduction of 1-(*p*-methoxyphenyl)-propan-2-one (**1a**) to (*R*)-1-(*p*-methoxyphenyl)-propan-2-ol ((*R*)-**1b**) was used as a model in the screening reduction reactions. ADH from *T. brockii* and twelve ketoreductases were used to reduce **1a** to (*R*)-**1b** in buffer solution by using 2-propanol as hydrogen donor for NAD(P)H regeneration (Table 1).

When TbADH and ketoreductases (KRED 101, 112, 115, 116, 117, 122 and 123) were used in phosphate buffer, (*R*)-**1b** was produced with good to very high yield (8–99%) and enantioselectivity (26–98%). Although, when ketoreductases (KRED 102, 106, 121, 124 and 127) were used for reduction of **1a** in Tris–HCl buffer, a low activity was found against substrate **1a**.

2.2. Effect of cyclodextrins on reaction rate and stereoselectivity of 1a

In substrate-coupled reaction system catalyzed by KRED 115 and KRED 122 enantiomeric purity of (*R*)-**1b** was found 84% and 34%, respectively. Next, the effect of α-, β- and γ-CDs on reaction rate and enantioselectivity of **1a** catalyzed by both enzymes was studied. The ketone **1a** was reduced in buffer solution in the presence of molar excess of α-, β- and γ-CDs.

Table 1
Comparison of conversion and selectivity for bio-transformation of 1-(*p*-methoxyphenyl)propan-2-one catalyzed by TbADH and ketoreductases

Enzyme catalyst	Time[a] (h)	Conv. (%)	ee[b] (%)
KRED-101	1	85	98
KRED-112	2	99	26
KRED-115	8	62	84
KRED-116	7	77	72
KRED-117	0.5	99	98
KRED-122	48	14	34
KRED-123	0.5	98	98
KRED-102[c]	0.5	>99	90
KRED-106[c]	24	<1	—
KRED-121[c]	24	<1	—
KRED-124[c]	24	<1	—
KRED-127[c]	24	<1	—
ADH from *Thermoanaerobium brockii*	48	8	98

<1 means trace of product alcohol being detected by HPLC in 24 h.
[a] The time when the maximal yield was reached.
[b] The alcohol was in (*R*)-configuration in all cases.
[c] Reduction process carried out in Tris–HCl, pH 7.0.

The use of cyclodextrins in asymmetric bio-reduction of **1a** was accompanied with decrease in conversions and a slight increase in the enantioselectivity of the formed product with the respect to the reduction carried out in the absence of the CDs (Table 2). When the processes were carried out by KRED 115 (α-, β- and γ-CD) and KRED 122 (β-CD) the configuration of the product alcohol **1b** is the same—(*R*). However, one extra effect was realized and verified in repeated experiments. The reduction carried out by KRED 122 in the presence of γ-CD led to decrease in the enantiomeric purity of the formed (*R*)-**1b** with increasing γ-CD concentration; although, up to ratio 1/3.8 **1a/γ**-CD provided (*S*)-enantiomer of the alcohol. However, it was observed that at a ratio 1/3.8 **1a**/γ-CD KRED 122 demonstrated a slight increasing in the activity and enantioselectivity against **1a** (Table 2 and Fig. 1).

Hunt et al.[26] have found that by the replacing of *d*-glucose with 2-propanol in the regeneration process of NAD(P)H a chiral inversion in the enantioselectivity of the product could happen. In addition, a possibility for complete inversion of enantioselectivity was observed via site-directed mutagenesis—where the new mutants showed better activity and enantioselectivity toward the wide-type enzymes.[27,28]

Various factors are needed to explain observed switch in the configuration of the formed alcohol. There are two possibilities for cyclodextrin complex formation: (i) complexation with a guest molecule (**1a**) or (ii) interaction with the enzyme.

2.3. NMR measurements

NMR spectroscopy is increasingly used to elucidate interactions of cyclodextrins with aromatic compounds.[29] The ^{1}H NMR signal changes of the phenyl signals of 1-(*p*-methoxyphenyl)-propan-2-one recorded in the absence and presence of γ-CD were studied to monitor their interaction.

The ^{1}H NMR spectra of **1a** and γ-CD in D_2O showed a downfiled shift of 13 Hz (24 equiv) of the **1a**, which suggests for a too small inclusion of the phenyl ring in the complexation with γ-CD. The binding constant (*K*) was 25.13 (Fig. 2).

2.4. Enzyme activity and circular dichroism measurements

Electron circular dichroism (ECD) has been widely used for studying the conformation and conformational change of proteins and polypeptides in solution.[30] The ellipticity band at 220 nm region is a standard use for estimate the content of secondary structure in a protein molecule.

Table 2
Comparison of conversion and selectivity of KRED 115 and KRED 122 for bio-transformation of 1-(*p*-methoxyphenyl)propan-2-one without and with cyclodextrins

1a: CDs		KRED 115		KRED 122	
		Conv.[a] (%)	ee[b] (%)	Conv.[c] (%)	ee (%)
CD absent		62	84	14	34 (*R*)
α-	1: 1.5	45	92	—	—
	1: 3.1	45	94	—	—
	1: 5.1	49	94	—	—
β-	1: 1.3	34	79	8	50 (*R*)
	1: 2.6	24	81	6	50 (*R*)
	1: 4.4	19	88	6	54 (*R*)
γ-	1: 1.1	69	92	7	44 (*R*)
	1: 2.3	49	92	8	35 (*R*)
	1: 3.1	—	—	8	20 (*R*)
	1: 3.8	41	90	16	40 (*S*)
	1: 4.6	—	—	8	20 (*S*)

— Means the processes were not provided.
[a] The reactions were terminated after 8 h.
[b] The alcohol was in (*R*)-configuration.
[c] The reactions were terminated after 24 h.

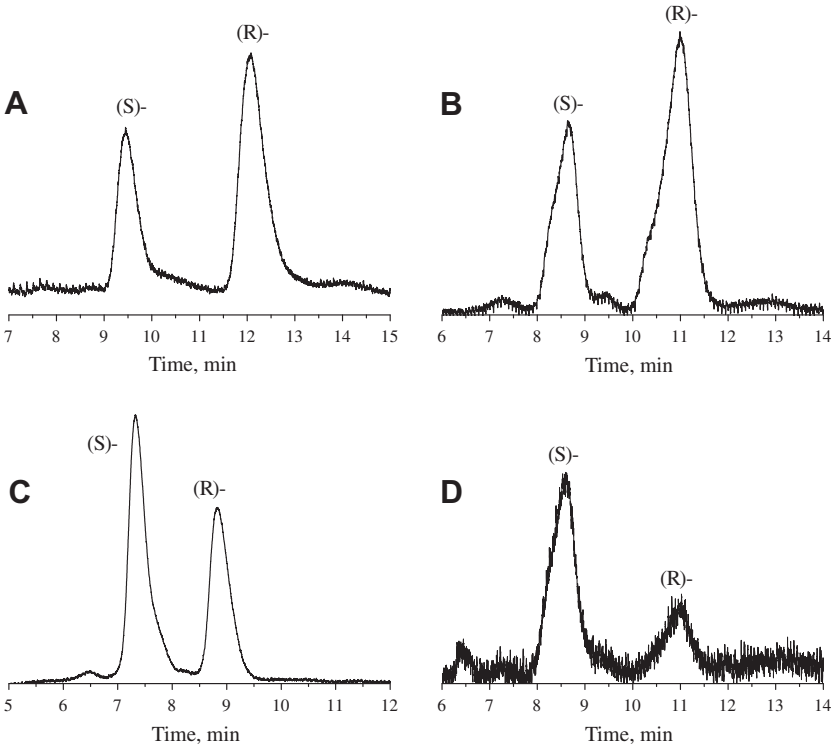

Figure 1. Chiral HPLC chromatograms of 1-(*p*-methoxyphenyl)-propan-2-ol in the absence (A) and in the presence of γ-CD (**1a**/γ-CD: 1:2,3 (B); 1:3,8 (D) and 1:4,6 (C)).

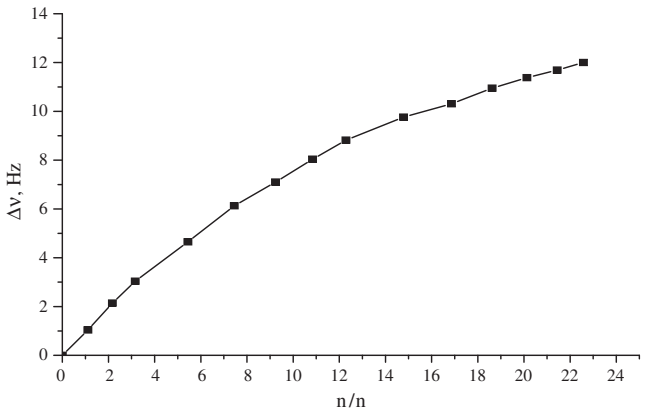

Figure 2. Chemical shift of 1-(*p*-methoxyphenyl)-propan-2-one as a function of concentration of the γ-CD (concentration of 1-(*p*-methoxyphenyl)-propan-2-one (2.03×10^{-3} M) was held constant; concentration of γ-CD (0.223–4.8×10^{-2} M)).

We first studied the ECD spectra for our systems of KRED 122 in the presence of CDs. The far-UV ECD spectra of KRED 122 at various β- and γ-CD concentrations are present in Figure 3 (A and B, respectively). In solution the enzyme shows negative ECD bands at 209 nm and 222 nm typical of α/β secondary structure.[30–32] Analysis of the secondary structure of enzyme by the method of Chen et al.[30] indicated that the enzyme contain 16% α-helical structure (Fig. 4).

With increasing γ-CD content in the system there is a qualitative change in ECD spectra of KRED122, as shown in Figure 3B. In the absence of γ-CD the molar ellipticity value is the highest. This suggests that KRED 122 is in more rigid conformation and a maximum ordered structure is maintained. With increasing γ-CD concentration, the molar ellipticity peaks show identical minima at 220 nm. However, there is a gradual reduction in the intensity. Up to 3.8×10^{-2} M γ-CD the intensity of the ellipticity peak reflected more than 50% with respect to the intensity in the absence of γ-CD. Although, the α-helix content also was reduced with the increasing amount γ-CD (Fig. 4). This suggests that the enzyme is in more rigid environment in systems when CD content increased. The CD supposed a more hydrophobic environment for enzyme molecule, so that the ordered structure is disturbed.

This conformational change probably indicates for the formation of an intermediated state, which is usually characterized by an increased β-sheet content.

The conformational change of the KRED 122 is strongly influenced by the presence of γ-CD. α-Helix content is maximum in the absence of γ-CD, corresponding of the maximum catalytic activity. The enzyme shows more random conformation and the fraction of β-sheet conformation increases with increasing γ-CD concentration (Fig. 4). The results indicate that the enzyme assumes more β-sheet and random conformations at high concentrations of γ-CD.

However, enzyme catalytic activity is dependent on native conformation of the proteins and structural changes can enhance the activity of enzyme.[33] As shown in Figure 4, at least 55–81% of the enzyme activity was retained in all studied systems. The activity decreased with the increasing γ-CD concentration, which is confirmed with the reduced structure observed by ECD measurements. However, more α-helix content is lost at higher γ-CD concentrations. As the α-helix content decreased, enzyme activity decreased such that about 80% of the native enzyme activity is retained when one-third of the initial α-helix content remains. As the α-helix content decrease further, there is a progressive loss in enzyme activity (Fig. 5). Comparison of the data in Figures 4 and 5, suggest for a greater structural perturbation with increasing γ-CD concentration. These data confirm the dependence of the enzyme activity of the native enzyme structure.[23,33,34] On the other hand, the reason for such a drop in α-helix content may be a function of deeper and tighter penetration of the amino acids of the

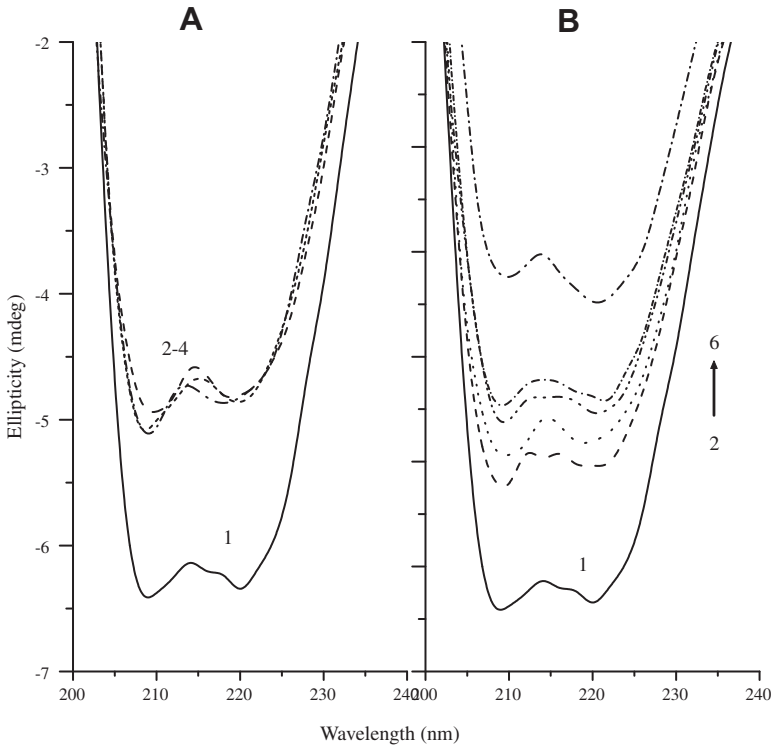

Figure 3. Far-UV ECD spectra of KRED 122 (10 μg mL^{-1}) (curve 1) and in the presence of (A) β-CD (2–4: 1.3×10^{-2}; 2.6×10^{-2} and 4.4×10^{-2} M) and (B) γ-CD (2–6: 0.038×10^{-2}; 1.1×10^{-2}; 2.3×10^{-2}; 3.8×10^{-2} and 4.6×10^{-2} M) in phosphate buffer at pH 6.8.

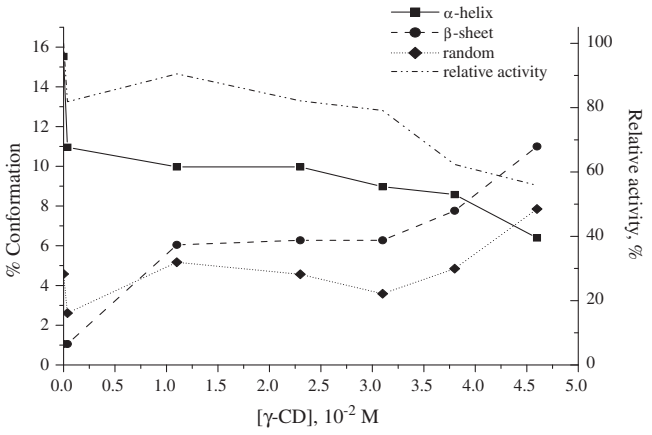

Figure 4. Percentage conformation of KRED 122 with γ-CD and correlation between the relative activity and α-helix content of KRED 122 (the dashed line is provided to guide the eye only).

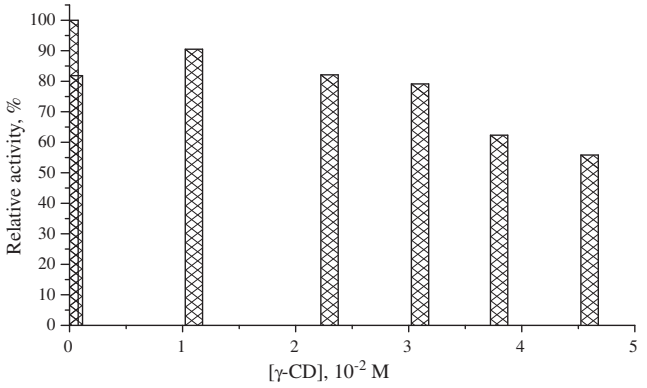

Figure 5. Residual KRED 122 activity in the presence of different concentrations of γ-CD.

enzyme within the CD cavity, with additional stabilization of hydrogen binding between peptide backbone of the enzyme and hydroxyl group on the outer rim of the CD cavity.[24] It is demonstrated[34,35] that the differences in the shape and in the size of α-, β- and γ-CD cavity influence the activity of the enzyme in enzyme/CD complex due to the change in the conformation of some amino acids in the enzyme catalytic site.

Similar experiments with β-CD (Fig. 3A) led to very different results demonstrate no change in the ellipticity with the addition of β-CD was obtained. The α-helix content remains almost constant (~10%) for all three studied β-CD concentrations.

The interpretation is supported by comparing their fluorescence emission spectra.

2.5. Fluorescence spectroscopy

The protein fluorescence has been used for the conformational change studies in the ternary protein structure. It is known that the fluorescence of aqueous tryptophan-containing proteins arises from their tryptophan residues. The fluorescence emission spectrum of KRED 122 at excitation of 282 nm shows λ_{max} at 340 nm.

Figure 6 shows effect of β- and γ-CDs on the fluorescence intensity of KRED 122. The fluorescence intensity was enhanced by the addition of CDs. The binding to γ-CD resulted in reduced fluorescence intensity—about 20%. On the other side, it is known that the fluorescence intensity of tryptophan is quenched by the presence of polar solvents or compounds. This corresponded to a change in polarity around the chromophore residues.[24] The decrease in the intensity indicated that the environment of the tryptophan was altered. These results can be correlated to our ECD

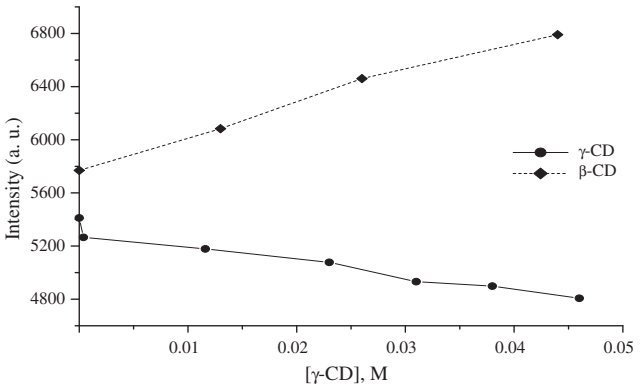

Figure 6. Relation of KRED 122 fluorescence intensity and concentration of β- and γ-CD.

measurements suggested that γ-CD binding resulted in a tertiary structural change, so that local rearrangement of secondary structure was occur in KRED 122.

The opposite trend was observed for the addition of β-CD to the enzyme solution (Fig. 6). Upon increasing β-CD concentration the fluorescence intensity increased, about 20%. This may suggest for the unfolding of the enzyme.

3. Conclusion

Here, we report a simple asymmetric reduction of 1-(*p*-methoxyphenyl)-propan-2-one by using ADH from *T. brockii* and twelve ketoreductases, utilized 2-propanol for the cofactor (NAD(P)H) regeneration. The bio-catalytic processes led to formation of (*R*)-1-(*p*-methoxyphenyl)-propan-2-ol in high conversion (>98%) and very good stereoselectivity (>98%). The former approach using 2-propanol was further extended by the participation of α-, β- and γ-CDs in the reduction process. It showed up

(i) a lower conversion of the substrate;
(ii) a slight improvement on the enantioselectivity.

For all types (α-, β-, γ-) and concentrations of CDs the (*R*)-form of the generated alcohol prevailed with the exception for γ-CD up to 3.8×10^{-2} M. In that cases the (*S*)-enantiomer was formed.

Consequently, this unexpected effect was studied based on the interaction between ketoreductases with the CDs and formation of the complex between substrate (1-(*p*-methoxyphenyl)-propan-2-one) molecule and CDs, on the basis of ^{1}H NMR, ECD and emission fluorescence spectroscopy. From the analysis can be concluded that:

(i) there exists parallelism between the ordered structure of the enzyme in terms of its α-helicity and enzyme activity;
(ii) the inactivation of the enzyme may be caused due to transformation of α-helix into either β-sheet structure or random coil conformation or their combinations;
(iii) as a result of the conformational rearrangement, fluorescent intensity continuous decreased that reflect of change in tryptophan environment in the presence of γ-CD;
(iv) the addition of β-CD had no effect on the α-helix content of the ketoreductase; the fluorescent intensity increased, that suggest for the unfolding of the enzyme;
(v) for the stoichiometry, there was a very small interaction of 1-(*p*-methoxyphenyl)-propan-2-one with the γ-CD.

In regards to the results of our NMR, ECD and fluorescence measurements can not simply be explained observed chiral switch in the configuration of the 1-(*p*-methoxyphenyl)-propan-2-ol. However, the observed results suppose explanation for the observed chiral switch in the arrangement of the alcohol formed in the enzymatic transformation.

4. Experimental section

4.1. Materials

Alcohol dehydrogenase (EC 1.1.1.2) from *T. brockii*; NADH; NADPH; TRIS (tris(hydroxymethyl)aminomethane); α- (M_w = 972.86 g mol^{-1}), β- (M_w = 1135.01 g mol^{-1}), γ- (M_w = 1297.15 g mol^{-1}) CDs were purchased from Sigma–Aldrich (Czech Republic). KRED reductases (KRED 28,000, Ketoreductase Screening Set, BioCatalytics Inc., Pasadena, CA, USA)) and 1-(*p*-methoxyphenyl)-propan-2-one were donated from Zentiva, part of Sanofi-Aventis group, Prague, Czech Republic. All other reagents and solvents were of analytical grade purchased from commercial suppliers.

4.2. General procedure

^{1}H and ^{13}C NMR spectra were recorded at room temperature in $CDCl_3$ solution on a Varian Mercury instrument operating at 300.08 and 75.46 MHz, respectively. Optical rotation data were collected on Autopol IV Polarimetr in $CHCl_3$. The UV–vis absorption spectra were recorded at room temperature on a Cary 400 UV–vis spectrophotometer (Varian, USA) with 1 cm quartz cuvette. The far-UV ECD measurements were performed in the range of 200–300 nm on a JASCO 400 spectrophotometer (JASCO, Japan) with 1 cm light path length quartz cuvette. The scan speed was 50 nm min^{-1} at 2 s integration time. The average of 4 scan was calculated. Fluorescence measurements were carried out with a fluorescence instrument (Fluoro Max 2, USA) with 1 cm quartz cell. The excitation wavelength was at 282 nm and emission wavelength at 340 nm. The average of four scans was used. The HPLC analyses of the reaction products were recorded with LC 5000 HPLC system (INGOS, Czech Republic). All measurements were carried out at 25 °C. Ketone to alcohol conversion was performed on reversed phase chromatography with Nucleosil C18 (Watrex, Czech Republic). Mobile phase was 60% acetonitril/47% water with flow rate 0.5 ml min^{-1}. Chiral analysis for ee determination for **1b** was performed with Cyclobond I™ 2000 SN column (Supelco, USA) using 60% methanol/40% water at 0.8 ml min^{-1}. Chromatograms were recorded at 286 nm.

4.3. General procedure for asymmetric reduction of 1a with TbADH and KRED enzymes

4.3.1. Activity assay

The activity of TbADH and ketoreductases was determined spectrophotometrically by measuring the oxidation of NAD(P)H at 340 nm (ε_{340} = 6.22 mM^{-1} cm^{-1}) in the presence of 10 mM **1a** dissolved in 0.5% (v/v) 2-propanol. The reaction mixture containing 100 mM phosphate buffer (pH 6.8) or Tris–HCl (pH 7.0) with 1 mM NAD(P)H. The reaction was started by adding the 20 μL (containing 20–30 μg) enzyme. The reaction kinetics were monitored over a 4 min period and one unit ADH activity was defined as the amount of enzyme that converted 1 μmol of NADH per minute.

4.3.2. Synthesis of the (*R*)-1-(*p*-methoxyphenyl)-propan-2-ol in aqueous system

The reactions were run at a volume of 10 mL and tempered at 30 °C. For TbADH the reaction mixture contained 100 mM phosphate buffer (pH 7.25) and 0.025 mM NADPH. For KRED reductases: 100 mM phosphate buffer (pH 6.8—for KRED 101, 112,

115, 116, 117, 122 and 123) or 100 mM Tris–HCl (pH 7.0—for KRED 102, 106, 121, 124 and 127) with 0.05 mM NADH. Ketone **1a** 10 mM was added as liquid, dissolved in 5% (v/v) 2-propanol. The reaction was initialized by the addition of 1 mg mL^{-1} enzyme. Then, 0.1 mL of the reaction mixture was periodically withdrawn and the reaction progress monitored by HPLC. Variable reaction times were required to reach moderate or good conversion. Once the reduction process reached a completion the reaction was terminated by extraction with 2×5 mL $CHCl_3$. Then the solvent was evaporated and the sample resuspended in methanol for a chiral HPLC analysis.

4.3.3. Enzymatic reduction of ketone 1a in the presence of various concentrations of α-, β- or γ-CDs

The reaction process followed the same protocol as Section 4.3.2. In the experiments the molar ratios of **1a** to CDs were: for α-CD—1/1,5; 1/3,1 and 1/5,1; for β-CD—1/1,3; 1/2,6 and 1/4,4; for γ-CD—1/1,1; 1/2,3; 1/3,2; 1/3,8 and 1/4,6. 10 mM ketone dissolved in 5% (v/v) 2-propanol was added to solution of desired concentration CDs and left under stirring for 10 min. Afterward, NADH and 1 mg mL^{-1} KRED 115 or 122 were added.

4.3.4. Circular dichroism

The experiments were performed with KRED 115 and 122 in 100 mM phosphate buffer (pH 6.8) in a 2 mL volume and arranged as series of independent measurements. In a general procedure, to β- or γ-CD solution was added 20 μg enzyme. After 10 min incubation time, the ECD spectra were recorded. In the experiments amount of CDs to enzyme was: 1:1; 30:1, 60:1; 80:1; 100:1 and 130:1 (w/w) (0.02; 0.6; 1.2; 1.6; 2 and 2.6 mg, respectively).

4.3.5. Fluorescence spectroscopy

The samples were prepared and the experiments arranged as Section 4.3.4. Duplicate fluorescence intensity measurements were carried out.

4.3.6 ^{1}H NMR measurements

^{1}H NMR spectra of 1-(*p*-methoxyphenyl)-propan-2-one were recorded in the presence and absence of γ-CD. The stock **1a** and γ-CD solutions were freshly prepared by dissolving **1a** in D_2O. The concentration of the **1a** was 2.03×10^{-3} M and that of γ-CD was varied from 0.223 to 4.8×10^{-2} M.

4.3.6.1 1-(*p*-Methoxyphenyl)-propan-2-ol *rac*-1b.

^{13}C NMR: 22.7; 45.0; 56.0; 69.3; 114.2; 116.2; 130.5; 158.5. ^{1}H NMR: 1.2 (3H, CH_3, d, J = 6.3 Hz); 2.6 (2H, CH_2, m); 3.0 (1H, OH, s); 3.8 (1H, OCH_3, s); 3.9–4.1 (1H, CH, m); 6.9 (2H, Ar_{2-6}-H, d, J = 9 Hz); 7.14 (2H, Ar_{3-5}-H, d, J = 8.7 Hz), Refs. 36–38.

4.3.6.2. (*R*)-1-(*p*-Methoxyphenyl)-propan-2-ol.

Yield: 45%; ee = 92% (HPLC); $[\alpha]_D^{20}$ −21.0 (c = 0.5 in $CHCl_3$), Refs. 36–38.

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