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Enantioselective oxidation of secondary alcohols by quinohaemoprotein alcohol dehydrogenase from *Comamonas testosteroni*

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Abstract

Purified and reconstituted quinohaemoprotein alcohol dehydrogenase (QH-EDH) from *Comamonas testosteroni* is shown to oxidize secondary alcohols enantioselectively. The products formed during the oxidation of secondary alcohols were positively identified as the corresponding ketones. In the oxidation of chiral secondary n-alkyl alcohols a preference of the enzyme for the S(+)alcohols was found. The apparent kinetic parameters (K_m and V_{max}) for a range of n-alkyl alcohols depend on the length of the alcohol chain and the location of the hydroxyl function in the chain. The enzyme is stable up to a temperature of 37°C. Above this temperature the activity is irreversibly lost. The pH optimum of the enzyme in the conversion of secondary alcohols is 7.7.

Keywords: Quinohaemoprotein alcohol dehydrogenase; Enantioselectivity; Alcohol oxidation; Secondary alcohols

1. Introduction

The biological (physiological) activity of natural or synthetic substances (for example, drugs, food ingredients or agrochemicals) is often related to their absolute configurations. This is mainly caused by the fact that the biological (macro)molecules involved in molecular recognition are often chiral. The consequence is that if a particular molecule interacts with such a (macro)molecule one of the two enantiomers may be a potent drug whereas the other may be poisonous. The production of chiral compounds from non-chiral precursors is of economic interest because the products can find application in food, feed or can serve as intermediates in the synthesis of therapeutics.

There are a number of ways to produce homochiral compounds (as for example optically pure alcohols) enzymatically. First of all, optically pure natural substances can be used as precursors for synthesis, for example through the enantioselective hydrolysis of esters using lipases [1]. Secondly, redox enzymes can be used, for example in the enantioselective reduction of carbonyl compounds or in resolution procedures. The enzymes used are NAD(P)H

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dependent alcohol dehydrogenases, examples of processes are provided by Hummel and Kula [2], Davies et al. [3] and Fang et al. [4].

A drawback using NAD(P)H dependent dehydrogenases for preparative purposes is the diffusing free coenzyme which has to be regenerated continuously by either coupled enzymatic reactions [5] or electrochemically [6]. Alcohol dehydrogenases that contain a covalently bound cofactor are an interesting alternative. Examples of these enzymes are the group of quinoprotein alcohol dehydrogenases. A representative of this group is the quinohaemoprotein alcohol dehydrogenase (QH-EDH) from *Comamonas testosteroni*. This enzyme contains one haem c and one molecule of pyrroloquinoline quinone (PQQ) as cofactors. The enzyme has a molecular weight of 71 kD and a p*I* of 9.3. Ca^{2+} and PQQ are essential to obtain the active enzyme from the apoenzyme [7]. The enzyme has been shown to oxidize primary alcohols and aldehydes [7,8] and shows enantioselectivity towards a number of chiral primary alcohols and aldehydes. In these studies no activity with secondary alcohols was observed [7,8]. Its application in the kinetic resolution of a number of C_3-synthons and solketal is described [9,10]. In addition, electrochemical regeneration of the cofactor and the enzyme in a redox-polymer network has been achieved [11,12].

Quinohaemoproteins are also produced by a number of acetic acid bacteria, for example by *Acetobacter* and *Gluconobacter* [13]. They differ from the enzyme of *Comamonas testosteroni* in their specificity and in biochemical properties like molecular weight, isoelectric points and molecular composition. For example, QH-EDH isolated from the cytosol of a *Gluconobacter suboxydans* strain is known to oxidize a number of hexoses and pentoses, aldehydes and several primary and secondary alcohols [14].

In this paper the activity of the purified and reconstituted enzyme QH-EDH from *Comamonas testosteroni* with (linear) secondary alcohols will be shown. Data on apparent kinetic constants are provided which show that the enzyme is enantioselective towards the *S*(+)-enantiomers of these substrates. The affinity and oxidation rate appears to depend on the length of the alkyl chain and the location of the hydroxyl group in the chain.

2. Materials and methods

2.1. Materials

The alcohols used here were of analytical grade and obtained from commercial suppliers. *R*- and *S*-2-butanol, *R*- and *S*-2-octanol and 3-octanol were from Acros Chimica. 1-Propanol, 1-butanol, 1-octanol, 2-propanol, 2-butanol and 2-octanol were from Baker. 3-Nonanol and 4-heptanol were from Fluka. *R*- and *S*-2-hexanol, *R*- and *S*-2-heptanol, 2-pentanol, 3-pentanol, 3-heptanol 4-decanol *trans*-1,2-cyclohexanediol, 1,3-cyclohexanediol and 1,4-cyclohexanediol were from Aldrich. 1-Pentanol was from BDH Chemicals. Buffer salts were from Sigma. All other chemicals were from Merck. Wursters Blue and quinohaemoprotein alcohol dehydrogenase (QH-EDH) from *Comamonas testosteroni* were a gift from Professor J.A. Duine (Delft Technical University, Delft, The Netherlands). The enzyme was purified and reconstituted with PQQ essentially according to Groen et al. [7] and De Jong et al. [8]. The protein content was determined according to Bradford [15].

2.2. Electrophoresis

Electrophoresis was performed using Biorad II equipment, a 5.5% T and 3.5% C gel and 50 mM MES (pH 6.5) as electrophoresis buffer. The reconstituted enzyme was sampled on the anode side and electrophoresis carried out during 2 h at 175 V. An overlay experiment was performed by soaking the gels with either 1-octanol or 2-octanol in a MOPS/$CaCl_2$ buffer (pH 7.7) containing Wursters Blue as the elec-

tron acceptor. Subsequently, the gels were stained by silver colouring reagent to determine the location of the protein in the gel.

2.3. Determination of substrate conversion and product formation

Ferricyanide (final concentration 10 mM), substrate (5 mM) and a fixed amount of QH-EDH (25 μg ml^{-1}) were mixed together in a 20 mM MOPS buffer (pH 7.5) also containing 10 mM $CaCl_2$. Blanks consisted of either enzyme and ferricyanide, enzyme and substrate or ferricyanide and substrate in the same buffer. The progress of the oxidation was monitored at 25°C using a Perkin Elmer Lambda 2 spectrophotometer at 452 nm (molar extinction of the ferricyanide 0.20 l/mol cm [9]. The oxidations were continued until no change in extinction was observed. The amount of substrate and product present in the reaction mixture was determined by HPLC. The pump used was an LKB 2249 gradient pump, the column a Biorad Aminex HPX-87H (300 × 7.8 mm) and the eluent 5 mM H_2SO_4 in milli Q (0.5 ml min^{-1}). Detection was performed using a LCD/Milton Roy spectro monitor D (270 nm) and a Spectra-Physics SP 8430 RI detector.

2.4. Determination of enantioselectivity of the enzyme

The oxidation of 2-octanol was performed as described above. Samples of 1 ml each were taken and 10 μl 37% hydrochloric acid was added to stop the reaction. The sample was saturated with ammonium sulphate and extracted with 1 ml of toluene. The extract was used for derivatisation. 2 μl of *R*-1-(1-naphthyl)ethyl isocyanate was added and the mixture was heated to 100°C for 5 h [16]. Subsequently, 400 μl of the reaction mixture was injected on a HPLC system to separate the enantiomers. The system consisted of a Chrompack Lichrosorb Si-60-5 (250 × 4.6 mm) column and the pump and UV detector were as described previously. Dichloromethane was used as the liquid phase at a flow rate of 1 ml min^{-1}.

3. Results and discussion

3.1. Determination of secondary alcohol activity

The purified quinohaemoprotein alcohol dehydrogenase apoenzyme does not show any activity towards primary or secondary alcohols. After reconstitution with PQQ, the enzyme solution oxidizes primary alcohols, aldehydes and secondary alcohols, showing that the alcohol dehydrogenase activity is PQQ-dependent. Both the apoenzyme and the cofactor PQQ do not show oxidative activity in combination with any of the substrates mentioned. The ferricyanide linked activity at 25°C of the reconstituted QH-EDH is 6.6 U mg^{-1} for 1-butanol and 5.3 U mg^{-1} for 2-octanol.

Polyacrylamide gel electrophoresis of the reconstituted QH-EDH shows a single band with an overlay of either 1-octanol or 2-octanol as the substrate. Protein (silver) staining shows that the bands, observed with the overlay technique, coincide with the band in the protein staining, proving that the activity on secondary and primary alcohols originate from one and the same protein (i.e. the quinohaemoprotein alcohol dehydrogenase).

3.2. Kinetic parameters of the enzyme

Fig. 1 shows that the activity of the enzyme with 2-octanol has an optimum at pH 7.7. This optimum coincides with the pH optimum for the oxidation of primary alcohols [11]. Up to 37°C the enzyme is stable under the reaction conditions chosen, at elevated temperatures increasing instability of the enzyme was observed (Table 1). The specific activity is temperature dependent increasing from 6.6 U mg^{-1} at 25°C to 10 U mg^{-1} at 37°C with 1-butanol as the substrate.

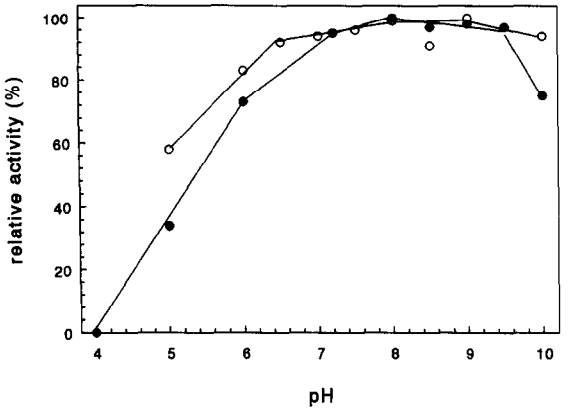

Fig. 1. pH profile of QH-EDH of *Comamonastestosteroni*in the oxidation of 1-propanol (●) and 2-octanol (○). Conditions: 1 mM ferricyanide, 10 mM propanol and 1 mM 2-octanol, respectively. Buffers used were acetate (pH 4.0–5.0), MES (pH 5.5–6.5), MOPS (pH 6.5–7.5), TRIS (pH 7.5–8.5), TAPS (pH 8.5–9.0), BIS TRIS–propane (pH 9.0–9.5) and CAPS (pH 9.5–11.0). All buffers also contained 10 mM $CaCl_2$.

The apparent kinetic constants of QH-EDH for a number of linear alcohols are summarized in Table 2. The enzyme is inhibited when high concentrations of alcohol are used, especially for larger aliphatic alcohols. In addition to linear alcohols the enzyme shows also activity towards some cyclic secondary alcohols, diols and aldehydes (Table 3). The enzyme is not active with glucose.

A relation was found between the V_{max} and K_m of the enzyme and the chain length of the alcohols and the position of the hydroxyl function in the chain (Table 2). For primary alcohols increasing oxidation rate and decreasing K_m is observed in the range of ethanol up to 1-pentanol [7,8]. For the series of 2-alcohols a relatively low activity was observed with 2-propanol (see Table 2). Increasing velocity and affinity was found with an increasing chain length of the alcohol with a V_{max} and K_m of 5.3 U mg^{-1} and 0.05 mM respectively for 2-octanol. The affinity of the enzyme for 2-octanol is a factor 10 lower than that for 1-butanol and

Table 1
Half-life of QH-EDH at various temperatures

Temperature (°C)	$t_{1/2}$ (h)
0	> 25
25	> 25
37	25
45	3.3
50	1.4
60	0.1

During incubation, the mixtures were frequently sampled at time intervals varying from 6 to 30 min, depending on the incubation temperature. The samples were quickly cooled in ice water and immediately assayed at 25°C to determine the remaining activity. Maximum incubation time: 6 h. The activity was determined using 1 mM 2-octanol and 1 mM ferricyanide in 20 mM MOPS buffer, pH 7.5 containing 10 mM $CaCl_2$.

Table 2
K_m(app) and V_{max}(app) of QH-EDH

Substrate	K_m(app) (mM)	V_{max}(app) [a] (%)
ethanol	2.2	75
1-propanol	0.06	90
1-butanol	0.006	100
1-pentanol	0.005	100
1-octanol	0.005	95
2-propanol	30	6
2-butanol	8	10
2-pentanol	0.6	34
2-octanol	0.05	80
3-pentanol	30	1.4
3-hexanol	5.3	9
3-heptanol	2.9	1
3-octanol	0.9	10
3-nonanol	0.8	4.5
4-heptanol	0.7	10
4-decanol	0.4	3.6

The activity of QH-EDH was determined spectrophotometrically at 420 nm and 25°C using 1 mM ferricyanide as the electron acceptor in 20 mM MOPS buffer (pH 7.5) containing 10 mM $CaCl_2$ and the alcohols as the substrate.
[a] 100% = 6.6 U mg^{-1}.

Table 3
Oxidation of other secondary alcohols and aldehydes by QH-EDH

Substrate	K_m(app) [a] (mM)	V_{max}(app) [b] (%)
cyclohexanol	0.83	9
1,2-cyclohexanediol	n.d.	< 0.1
1,3-cyclohexanediol	50	6.3
2-methylcyclohexanol	81	1.4
3-methylcyclohexanol	0.11	45
glucose	n.d.	< 0.1
acetaldehyde	0.7	80
1-butanol	0.005	100

Experimental conditions: see Table 2.
[a] n.d. = not detectable.
[b] 100% = 6.6 U mg^{-1}.

Table 4
Enantioselective oxidation of secondary alcohols by QH-EDH

Substrate	K_m(app) [a] (mM)	V_{max}(app) [b] (%)	E [c]
S-2-butanol	4.5	13	
R-2-butanol	28	6	13.5
S-2-hexanol	0.09	71	
R-2-hexanol	1.2	9	105
S-2-heptanol	0.04	63	
R-2-heptanol	1.1	5.5	315
S-2-octanol	0.05	80	
R-2-octanol	n.d.	< 0.1	> 800

Experimental conditions: see Table 2.
[a] n.d. = not detectable.
[b] 100% = 6.6 U mg^{-1}.
[c] E = enantiomeric ratio, defined as $(V_{max,A}/K_{m,A})/(V_{max,B}/K_{m,B})$ where A and B are the two enantiomers, A being the preferred one.

1-pentanol. The same phenomenon was observed in the series of 3-alcohols where K_m decreases with increasing chain length (Table 2), the best of the tested substrates were 3-octanol and 3-nonanol ($K_m = 0.8$ mM). A less uniform picture for the V_{max} values in the oxidation of 3-alcohols was found.

The results suggest that the optimal substrate, that is for which the enzyme has the highest affinity and reaction rate, should contain a large alkyl chain of at least four C-atoms. In addition, the oxidation rate and affinity decreases when the hydroxyl group shifts along the chain from C-1 to C-2 and so on. So it appears, that with an increase in the short alkyl chain of the secondary alcohol a less optimal fit of the substrate in the enzymes active site occurs.

From Table 4 it becomes clear that the enzyme has a preference towards the *S*(+)-alcohols, the affinity for the S(+)-enantiomer becoming more absolute with increasing chain length. The selectivity of the enzyme (expressed as an *E*-value, see Table 4) in the conversion of 2-butanol increases by a factor 30 compared with 2-heptanol. In the conversion of 2-octanol an even higher selectivity was found.

In this sense the properties of the enzyme are comparable with the behaviour, observed by Keinan et al. [5] and Pham and Phillips [18], for the alcohol dehydrogenases from *Thermoanaerobium* species. The reaction mechanism behind the selectivity can be explained with a model, incorporating the presence of two hydrophobic pockets in the active site in which the substrate should be accommodated during the oxidation. The two pockets differ from each other in size and in affinity towards the alkyl groups. The 'small alkyl pocket' then can accommodate alkyl groups as large as propyl (i.e. 4-decanol is a substrate (Table 2)), but the fit becomes less optimal with increasing chain length, which forces the longer alkyl chains into the 'larger alkyl pocket' and thus enhances the selectivity. The fact that for QH-EDH from *Comamonas testosteroni* the V_{max} increases with the chain length up to at least seven C-atoms, suggests that the 'large alkyl-binding pocket' is not limited, in contrast to the 'small pocket'. In addition, the increasing selectivity with increasing chain length then might originate from an improved fit in the 'large alkyl-binding pocket' as is the case with the other alcohols tested. Due to the orientation of the 'small alkyl pocket', the fit of *R*-alcohols with increasing chain length in the enzyme's active site becomes less favourable resulting in less favourable kinetic parameters.

Recently two distinct quinohaemoprotein alcohol dehydrogenases that show activity towards secondary alcohols were reported by Toyama et al. [17]. The kinetic parameters of one of these enzymes, ADH IIB originating from *Pseudomonas putida* grown on 1-butanol, are comparable to the values obtained for QH-EDH from *Comamonas testosteroni*. For both QH-EDH and ADH IIB long-chain primary and secondary alcohols are the preferred substrates. No data however are available on the enantioselectivity of ADH IIB for secondary alcohols.

3.3. Determination of substrate conversion and product formation, and resolution of a racemic secondary alcohol

Conversion experiments were performed with *S*-2-hexanol and *S*-2-heptanol. In all experiments the product formed was positively identi-

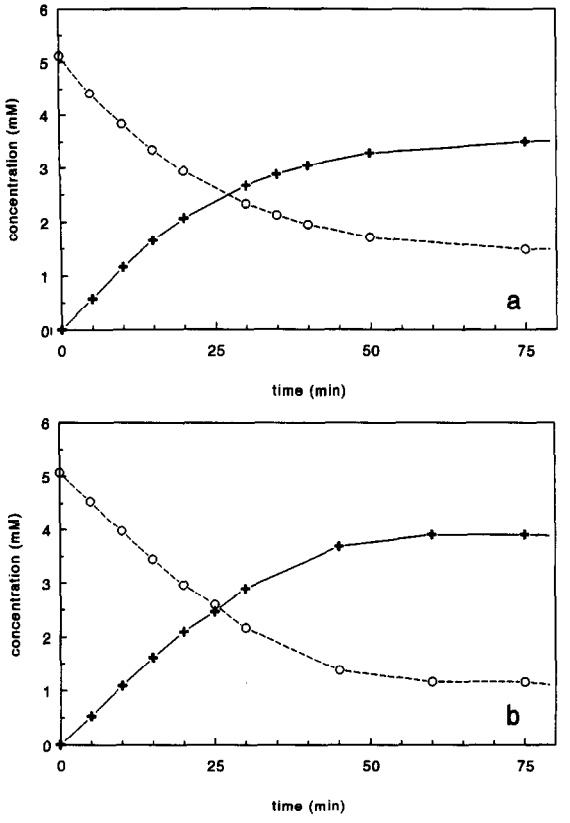

Fig. 2. The concentrations of alcohol (○) and ketone (+) in the oxidation of *S*-2-hexanol (a) and *S*-2-heptanol (b). Conditions are as described in 'Materials and methods'.

fied as the corresponding ketone. The decrease in the substrate concentration and the increase in the product formation are stoichiometrically coupled. Blanks were negative. Fig. 2 shows the concentrations of substrate and product in time for the oxidation of *S*-2-hexanol and *S*-2-heptanol respectively. The oxidation is not complete under the reaction conditions chosen. For the formation of 1 μmol 2-heptanone from 1 μmol *S*-2-heptanol 2 μmoles of ferricyanide were converted to ferrocyanide.

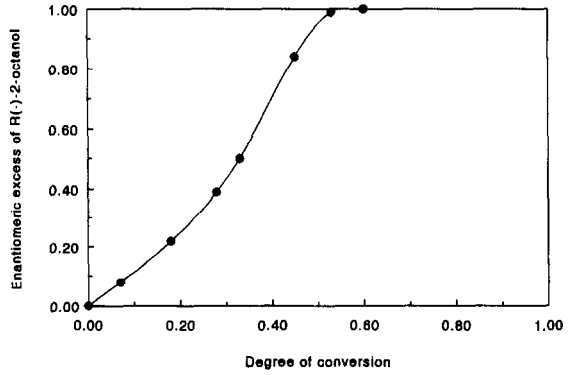

Fig. 3. Resolution of a racemic mixture of 2-octanol using QH-EDH. Conditions are as described in 'Materials and methods'.

As the enzyme shows a clear preference for the *S*-enantiomer of especially the larger secondary alcohols (see Table 4), resolution of a racemic mixture is achieved by oxidation of one of the enantiomers. Fig. 3 shows the enantiomeric excess of *R*(−)-2-octanol during the enzymatic conversion. As expected, the enantiomeric excess is larger than 99% and is almost complete when half of the racemic substrate is converted.

4. Conclusion

Quinohaemoprotein alcohol dehydrogenase (QH-EDH) from *Comamonas testosteroni* oxidizes a broad range of secondary alcohols into ketones, in addition to oxidizing primary alcohols and aldehydes (De Jong et al. [8]). In addition to aliphatic secondary alcohols, substituted and cyclic secondary alcohols are also substrates. Oxidation reactions of chiral secondary alcohols show a clear preference for the *S*-alcohol. This provides a means for the (kinetic) resolution of racemic 2-alcohols as was shown for 2-octanol.

5. List of symbols and abbreviations

c	concentration (mmol l^{-1})
E	enantiomeric ratio (−)
K_m(app)	apparent Michaelis–Menten constant (mmol l^{-1})
pI	isoelectric point
PQQ	pyrroloquinoline quinone
QH-EDH	quinohaemoprotein alcohol dehydrogenase

$t_{1/2}$	half life time (h)
U	unit of enzyme activity (μmol substrate min^{-1})
V_{max}(app)	apparent maximum velocity (U mg^{-1})

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