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Enantioselective Oxidation of Secondary Alcohols at a Quinohaemoprotein Alcohol Dehydrogenase Electrode

WIM A. C. SOMERS,[1] EDWIN C. A. STIGTER,[2]
WIM VAN HARTINGSVELDT,*[,2] AND JAN PIETER VAN DER LUGT[2]

[1]Gist-brocades BV, P.O. Box 1, 2600 MA Delft, The Netherlands.
[2]TNO Nutrition and Food Research Institute, Division of Biochemistry and Gene Technology, P.O. Box 360, 3700 AJ Zeist, The Netherlands, E-mail: Vanhartingsveldt@roeding.tno.nl

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ABSTRACT

Quinohaemoprotein alcohol dehydrogenase from *Comamonas testosteroni* was co-immobilized with a redox polymer (a poly(vinylpyridine) complex functionalized with osmium bis(bipyridine) chloride) on an electrode. The enzyme electrode readily oxidizes primary alcohols and secondary alcohols with maximum current densities varying between 0.43 and 0.98 A m^{-2} depending on the substrate and the operation temperature. The affinity of the enzyme for aliphatic alcohols increases with the chain length of the substrate (i.e., 1-pentanol [K_m = 0.006 m*M*] is a much better substrate than ethanol [K_m = 2.2 m*M*]). The same property is observed for secondary alcohols in the series 2-propanol (K_m = 22 m*M*) to 2-octanol (K_m = 0.05 m*M*). The enzyme electrode is enantioselective in the oxidation of secondary alcohols. A strong preference is observed for the S-2-alcohols; the enantioselectivity increases with increasing chain length. The enantiomeric ratio (E) increases from 13 for (R,S)-2-butanol to approximately 80 for (R,S)-2-heptanol and (R,S)-2-octanol. This makes the enzyme electrode, potentially, a powerful tool for the preparation of a large range of alkanones and/or for the (kinetic) resolution of racemic alcohols.

*Author to whom all correspondence and reprint requests should be addressed.

Index Entries: Quinohaemoprotein alcohol dehydrogenase; enzyme electrodes; enantioselective oxidation; bio-electrochemistry; alcohols; ketones.

Abbreviations: BCA, bicinchoric acid; BIS-TRIS Propane, 1, 3-bis[tris (hydroxymethyl)methylamino] propane; BSA, bovine serum albumin; C, concentration (mmol l^{-1}); CAPS, 3-[cyclohexylamino]-1-propanesulfonic acid; I, current density (A m^{-2}); K_m, apparent Michaelis Menten constant (mmol l^{-1}); MOPS, 3[-N-Morpholino] propane sulfonic acid; PEGDE, polyethylene glycol diglycidylether; pI, isoelectric point; PQQ, pyrrolo-quinoline quinone; POs-EA, poly(vinylpyridine) complex with osmium bis(bipyridine) chloride; QH-EDH, quinohaemoprotein alcohol dehydrogenase; T, temperature (%C); TAPS, n-tris[Hydroymethyl] methyl-3-aminopropanesulfonic acid; U, Unit of enzyme activity (mol min^{-1}); V_{max}, maximum velocity (U mg^{-1}).

INTRODUCTION

Enzyme electrodes for analytical *(1)* and preparative purposes *(2)* have become more and more important as tools for the continuous use of redox enzymes. In these systems, the enzyme is oxidizing or reducing the substrate, whereas the electron(s) is (are) transferred to or from an electrode. Because direct electron transfer from an enzyme to an electrode is a rare event, often so-called mediators are used. These compounds can be readily oxidized and reduced by the electrode and the enzyme, alternately. Electrodes based on the application of a solute electron mediator often are characterized by a decrease in electrode performance owing to leakage or wash-out of the mediator *(3)*. This problem can be overcome by the use of immobilized mediators, for example, redox polymers. Redox mediators like ferrocene derivates and osmium-complexes have been attached to polymer backbones like polysiloxane *(4)*, polypyrrole *(5)*, and polyvinylpyridine *(6)*. This provides fast-responding electrodes in which both the redox mediator and enzyme are immobilized.

Quinoproteins *(7)* are attractive for incorporation in enzyme electrodes because they are active with a broad range of artificial electron acceptors while the quinone cofactor is firmly bound to the enzyme. In addition, oxygen is not an electron acceptor for these enzymes. Therefore, harmful or interfering influences of reaction products (oxidases will form hydrogen peroxide when oxygen is present) do not play a role in these electrodes. In addition the electrode response is not influenced by the presence of oxygen.

Quinohaemoprotein ethanol dehydrogenase (QH-EDH) from *Comamonas testosteroni* contains pyrrolo-quinoline quinone (PQQ) as well as haem C as cofactors *(8)*. From a preparative point of view, the QH-EDH

from *Comamonas testosteroni* is very attractive as it is highly enantioselective for certain C_3-alcohols, e.g., it can be successfully employed in the kinetic resolution of solketal *(9)*. Recently, it has been shown that the enzyme is also an attractive biocatalyst for the enantioselective oxidation of secondary alcohols to ketones *(10)*. Ketones (f.e. 2-alkanones) have particular properties as flavors (f.e. in cheese). The production of 2-alkanones from the corresponding alcohols may be an attractive route for the (bio-) synthesis of these flavours *(11)*.

Quinoprotein enzyme electrodes, containing methanol dehydrogenase (EC 1.1.99.8), in which electron transfer is achieved through the organic conducting salt TTF-TCNQ were reported *(12)*. Application of the membrane-bound QH-EDH from *Gluconobacter suboxydans*, which differs in molecular composition and substrate specificity from the quinohaemoprotein from *C. testosteroni (13)* has been described in alcohol sensors. These electrodes are characterized by both mediated *(14)* and direct *(15)* electron transfer. Enzyme electrodes containing the QH-EDH from *C. testosteroni* immobilized in a poly(vinylpyridine) complex with osmium bis(bipyridine) chloride were described *(16–18)*. This electrode can be efficiently used for the conversion of primary alcohols and C_3-synthons as solketal and glycidol *(18)*.

This article describes the use of a QH-EDH electrode in the enantio-elective oxidation of secondary alcohols. The characteristics of the QH-EDH electrode are compared with the properties of the soluble enzyme. A methodology for the continuous synthesis of ketones will be indicated, making use of the high efficiency and enantioselectivity of the enzyme electrodes.

MATERIAL AND METHODS

Materials

QH-EDH was a gift from Prof. J.A. Duine (Delft University of Technology, Delft, The Netherlands) and purified and reconstituted with (PQQ) as described elsewhere *(8,19)*. The enzyme preparation used had a specific activity of 6.6 U mg^{-1} with 1-butanol as the substrate. The redox-polymer, a poly(vinylpyridine) complex with osmium bis(bipyridine) chloride (POs-EA), was synthesized essentially according to Gregg and Heller *(16)*. Polyethylene glycol diglycidyl ether (PEGDE) was from Polysciences. The alcohols used were from 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, cyclohexanol, 4-methyl-

2-pentanol, 3-methyl-2-pentanol, and 5-methyl-2-hexanol were from Aldrich. 1-pentanol was from BDH Chemicals. The glutaraldehyde (25 v/v%), bovine serum albumin (BSA), MOPS, BIS-TRIS-propane, TAPS, and CAPS were from Sigma (St. Louis, MO). All other chemicals were from Merck. All chemicals were of analytical grade.

Enzyme Activity and Protein Determination

The activity of soluble QH-EDH was determined spectrophotometrically at 420 nm using 1 m*M* ferricyanide as the electron acceptor in 20 m*M* TRIS buffer, pH 7.7, containing 10 m*M* $CaCl_2$ and 1 m*M* 1-butanol as the substrate. The protein content was determined with a micro (BCA) protein assay kit (Pierce, Cleveland, OH).

Construction of the QH-EDH Electrodes

The electrodes were made by applying a mixture containing 5 μL poly(vinylpyridine) complex with osmium bis(bipyridine) (POs-EA) (8 mg cm^{-3} in deionized water), 2 μL PEGDE (2.3 mg cm^{-3} in deionized water), 5 μL of buffer (50 m*M*) and 5 μL QH-EDH solution (10 mg cm^{-3} in 5 m*M* MOPS buffer with 10 m*M* calcium chloride, pH 7.5 on a polished carbon disk (Amor, diameter 0.8 cm) and distributing the mixture over the electrode surface with a spatula. The electrodes were dried overnight (16 h) at 4°C over silicagel in a desiccator under vacuum. Subsequently the electrodes were crosslinked during 3 h at 4°C by immersing them in 10 mL glutaraldehyde of concentrations varying from 0–10% (v/v) in 100 m*M* MOPS buffer, pH 7.0, containing 10 m*M* calcium chloride. The reaction mixture was gently stirred. To remove all remaining glutaraldehyde, the electrodes were washed during 45 min in the same buffer without glutaraldehyde. Subsequently the electrodes were dried overnight at 4°C over silicagel in an desiccator under vacuum.

Electrochemical Measurements

Electrochemical experiments were performed using an Amor electrochemical flow-through cell (*see* Fig. 1) provided with a spacer with a surface of 6 mm^2. The electrode disk was placed in the cell with the uncoated surface on the working electrode. Buffer (20 m*M* Tris-HCl with 10 m*M* $CaCl_2$ and 100 m*M* NaCl, pH 7.7) was pumped through the cell by a peristaltic pump (flow: 1 mL min^{-1}). When a stable background current was obtained, a substrate solution in the same buffer was either pumped continuously through the cell or pulsed by injecting substrate in the buffer flow using a Pharmacia FPLC autoinjector (model Act-100). The cell potential in all experiments was set at 0.40 V (vs an Ag/AgCl electrode) using an Antec potentiostat. The response of the enzyme electrode was moni-

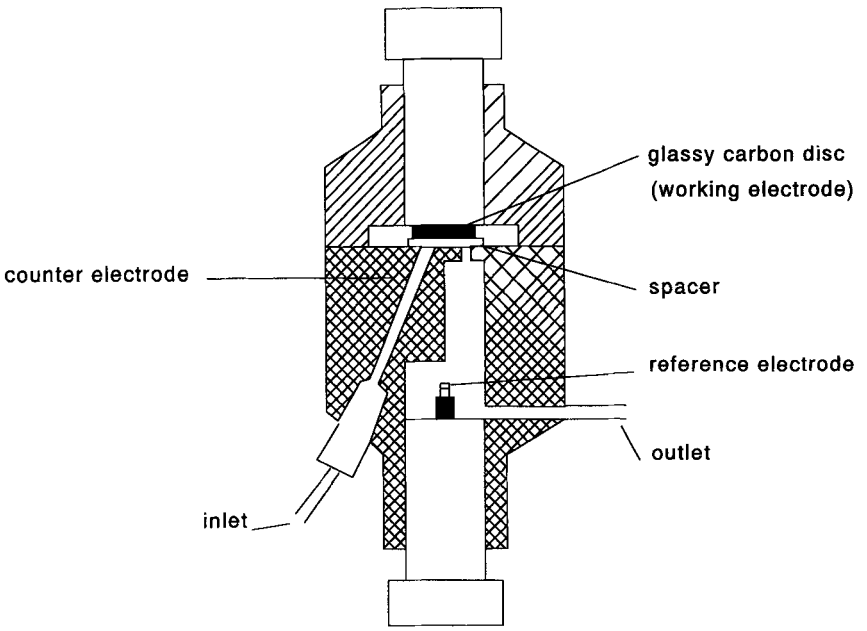

Fig. 1. Schematic representation of the electrochemical cell.

tored by measurement of the catalytic current. An electrode containing no enzyme was used as a reference electrode. Unless mentioned otherwise, all experiments were performed at 25°C by immersing the electrochemical cell in a water bath. The effect of the pH was studied using 20 m*M* buffers containing 10 m*M* calcium chloride and 100 m*M* sodium chloride. The following buffers were used: acetic acid, 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.

RESULTS AND DISCUSSION

Characteristics of the QH-EDH Electrode

QH-EDH electrodes have the ability to convert a broad range of primary alcohols *(17,18)*. The pH optimum and the temperature-dependent behavior of the electrodes were studied in the conversion of secondary alcohols. Figure 2 shows the pH optima of the soluble enzyme as well as the enzyme immobilized on the electrode. The pH optima for the oxidation of primary and secondary alcohols coincide. The pH optimum for the enzyme electrode is broader; especially below pH 5.0, the activity of the enzyme electrode is significantly higher than the activity of the soluble enzyme. Maximum reaction velocity is obtained around pH 7.7. The conversion of the substrate at the electrode is a function of temperature (Fig. 3). Previously it was shown that above 37°C, the enzyme is destabilized

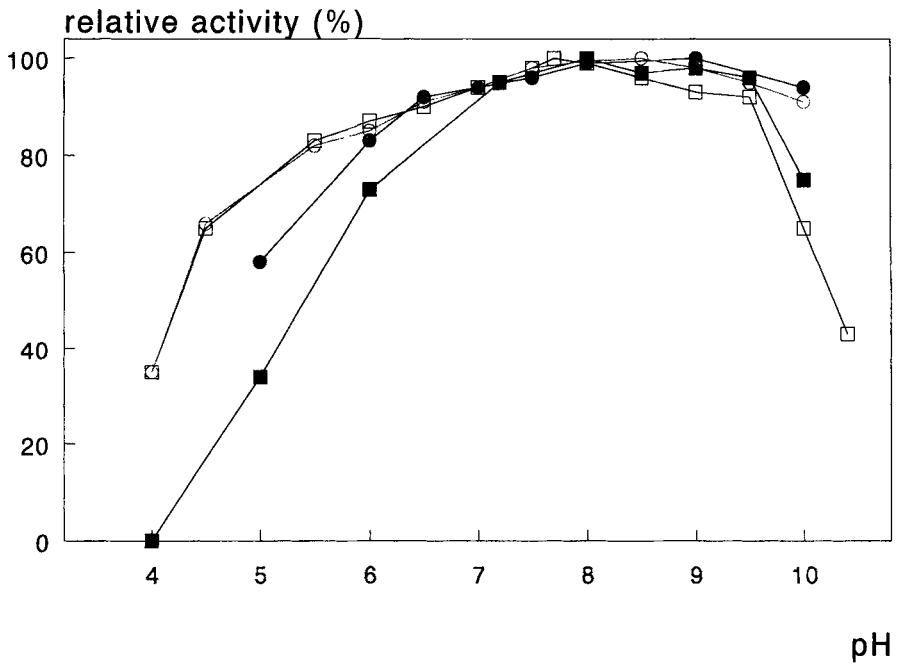

Fig. 2. pH profile of QH-EDH and QH-EDH electrodes in the conversion of primary and secondary alcohols. Substrates, 10 m*M* 1-propanol; (□, electrode; ■, soluble enzyme); 1 m*M* 2-(R,S)-octanol (○, electrode; ●, soluble enzyme); temperature, 25°C; other conditions, *see* "Materials and Methods."

(10,18). Up to this temperature, increasing current densities of the enzyme electrode may be observed (Fig. 2), the maximum being 0.98 A m^{-2} in the conversion of 2-octanol. The half-life time of the enzyme at 37°C was shown to exceed 25 h at this temperature.

Substrate Specificity of the QH-EDH Electrode

Soluble QH-EDH has been shown to have affinity for a large range of alcohols and secondary alcohols *(8,10,17)*. This specificity is also found in the enzyme electrodes described here. The kinetic data for the enzymatic conversion are derived by the method of Lineweaver and Burk from the plots of substrate conversion vs current density; examples are shown in Fig. 4. It can be noticed that at higher concentrations substrate inhibition is observed for some of the substrates. Data on the conversion of a large number of alcohols are given in Table 1. A relation was found between the V_{max} and K_m of the enzyme and the chain length of the alcohols together with the position of the hydroxyl function in the chain (Table 1). For primary alcohols, increasing oxidation rates and decreasing K_m values are observed in the range of ethanol up to 1-pentanol *(8,10,17,19)*. For the series of 2-alcohols, a relatively low activity was observed with 2-propanol (*see* Table 1).

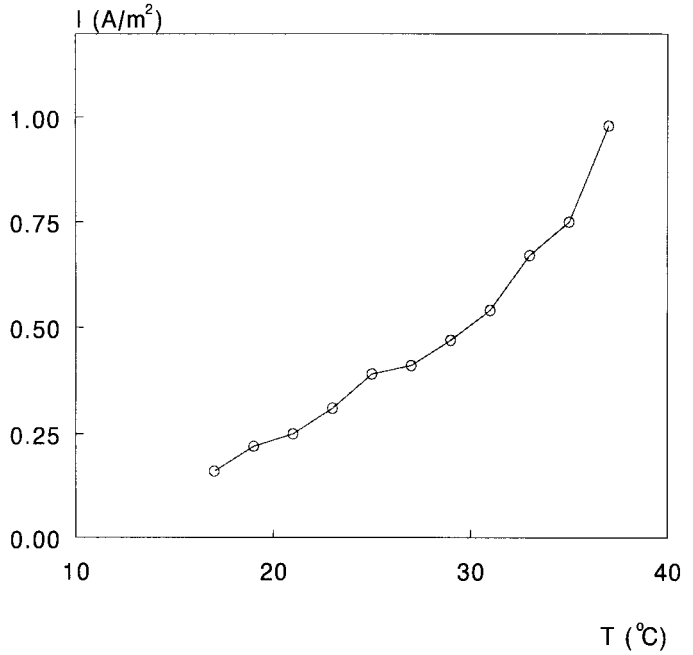

Fig. 3. Temperature-dependent activity of QH-EDH electrodes. Substrate, 1 m*M* 2-(R,S)-octanol; pH 7.7; other conditions, *see* "Materials and Methods."

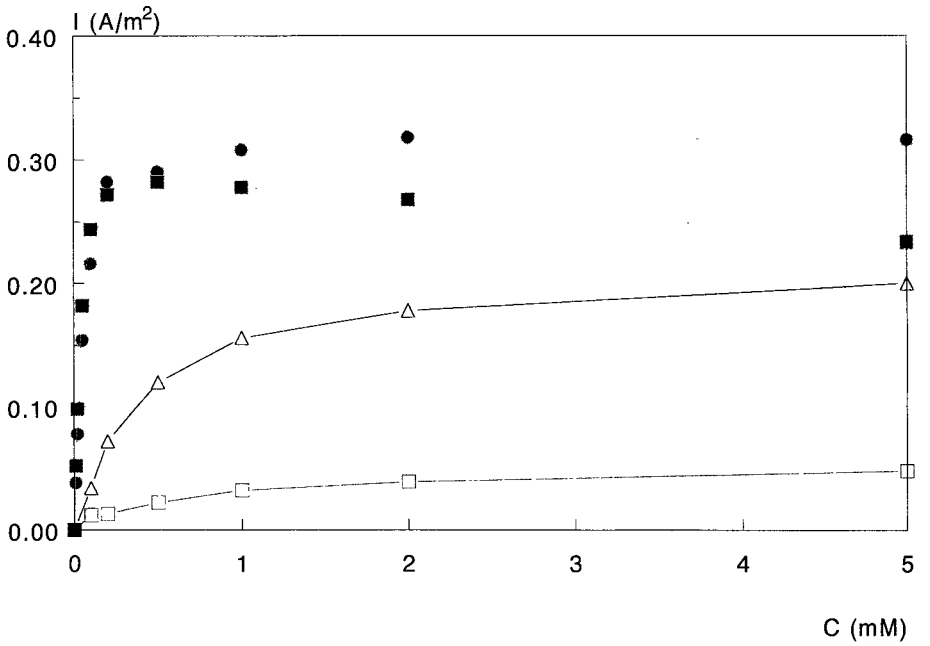

Fig. 4. Conversion of secondary alcohols at a QH-EDH electrode. Conditions as described in "Material and Methods." △, (R,S)-2-pentanol; ○, R-2-hexanol; ●, S-2-hexanol; □, R-2-heptanol; ■, S-2-heptanol.

Table 1
Oxidation of Alcohols at QH-EDH Electrodes

	Enzyme electrode[a]		Soluble enzyme *(8,10)*	
Substrate	K_m (m*M*)	V_{max} (%)[b]	K_m (m*M*)	V_{max} (%)[b]
Ethanol	2.2	86	2.2	75
1-Propanol	0.06	93	0.06	90
1-Butanol	0.005	100	0.006	100
1-Pentanol	0.006	93	0.005	100
1-Octanol	0.006	95	0.005	95
2-Propanol	22	9	30	6
2-Butanol	12	17	8	10
2-Pentanol	0.4	40	0.6	34
2-Octanol	0.05	84	0.05	80
3-Pentanol	28	1	30	1.4
3-Hexanol	6.3	6.5	5.3	9
3-Heptanol	4.3	3.7	2.9	1
3-Octanol	0.9	10	0.9	10
3-Nonanol	0.8	4.7	0.8	4.5
4-Heptanol	0.7	9.3	0.7	10
4-Decanol	0.4	3.7	0.4	3.6
1,3-Butanediol	0.3	91		
4-Methyl-2-pentanol	0.75	5.6	n.d.[c]	n.d.
3-Methyl-2-pentanol	0.9	9.3	n.d.	n.d.
5-Methyl-2-hexanol	0.24	3.7	n.d.	n.d.
Cyclohexanol	0.8	11	0.83	9

[a] No response for the following sugars: D-glucose, D-galactose, D-fructose, α-D-methylglucoside, mannitol.
[b] V_{max} (100%) = 0.43 A m^{-2} (electrode) and 6.6 U mg^{-1} (Soluble Enzyme)
[c] n.d., not determined

Increasing velocity and affinity was found with an increasing chain length of the alcohol with a V_{max} and K_m of 0.39 A m^{-2} and 0.05 m*M*, respectively, for 2-octanol. The affinity of the enzyme for 2-octanol is a factor 10 lower than, for example, for 1-butanol and 1-pentanol. The same phenomenon was observed in the series of 3-alcohols where K_m decreases with increasing chain length (Table 1), 3-octanol and 3-nonanol (K_m = 0.8 m*M*) being the preferred substrates. V_{max} values varied between 0.004 A m^{-2} and 0.04 A m^{-2} for the oxidation of 3-pentanol and 3-octanol, respectively.

The results show that the optimal substrate—that is the substrate with the highest affinity and reaction rate—should contain an 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,

Table 2
Enantioselective Oxidation of Alcohols at QH-EDH Electrodes

	Enzyme electrode			Soluble enzyme *(8–10,24)*		
Substrate	K_m (m*M*)	V_{max} (%)[a]	E[b]	K_m (m*M*)	V_{max} (%)[a]	E[b]
S-2-butanol	5	14	13	4.5	13	13.5
R-2-butanol	30	6.5		28	6	
S-2-hexanol	0.09	74	35	0.09	71	105
R-2-hexanol	0.6	14		1.2	9	
S-2-heptanol	0.06	70	82	0.04	63	315
R-2-heptanol	0.7	10		1.1	5.5	
S-2-octanol	0.05	82	82	0.05	80	>800
R-2-octanol	0.4	8		ND[d]	nihil	
S-solketal	1.0	9	39	0.99	0.7	117
R-solketal	0.1	35		0.11	10	
S-glycidol	4.5	30	0.9	ND[d]	1.6[c]	1
R-glycidol	4.8	37		ND[d]	1.6[c]	
1-butanol	0.005	100		0.006	100	

[a] V_{max} (100%) = 0.43 A m^{-2} (electrode) and 6.6 U mg^{-1} (soluble enzyme).
[b] 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.
[c] Concentration of glycidol, 27 m*M*.
[d] ND, not determined.

and so on. An increase in the short alkyl chain of the secondary alcohol then results in a less optimal fit of the substrate in the enzymes active site. Methyl-substituted aliphatic alcohols appear to be substrates and the affinity of the enzyme is comparable with the affinities for the alcohols (*see* f.e. 3-methyl-2-pentanol and 4-methyl-2-pentanol). The rates of conversion however, are considerably lower. A number of sugars (*see* Table 1) are not a substrate for the enzyme.

Enantioselective Oxidation of Secondary Alcohols with the QH-EDH Electrode

From Table 2, it can be concluded 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 enantiomeric ratio [E-value], *see* Table 2) increases a factor 6 when the conversion of 2-butanol and 2-octanol are compared.

The properties of the enzyme can be compared with the behavior observed for the alcohol dehydrogenases from *Thermoanaerobium* species *(20,21)*. The reaction mechanism behind the selectivity can then 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 1]), but the fit becomes less optimal with increasing chain length, thus forcing the longer alkyl chains in the "larger alkyl pocket" and in this way promoting the selectivity. The fact that for QH-EDH from *C. 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. Owing to the orientation of the small alkyl pocket, the fit of R-alcohols with increasing chain length in the enzymes active site becomes less favorable, resulting in less favorable kinetic parameters.

A number of differences is observed when the enzyme electrode is compared with the behavior of the soluble enzyme. First of all, the enantiomeric ratios of the electrode are generally lower than the value found for the soluble enzyme (Table 2). The S-enantiomers are oxidized with the same efficiency by both the immobilized enzyme and the soluble enzyme. Evaluation of the electrode data for the R-enantiomers (with respect to K_m[app] and V_{max}[app]) shows that, through the series of R-2-hexanol to R-2-octanol, a lower K_m and higher V_{max} is found when compared with the soluble enzyme. With R-2-octanol, no activity was observed with the soluble enzyme. The enzyme electrode is characterized by a V_{max} of 0.035 A m^{-2}, being 8% of the maximum observed current density. R-hexanol and R-heptanol are also better substrates at the electrode (lower K_m and higher V_{max}). Although these characteristics cannot be readily understood, a possible explanation might be the different electron acceptor than the one used in the studies on the soluble enzyme (i.e., potassium ferricyanide). Therefore, it cannot be excluded that the osmium bipyridyl functionality in the redox polymer plays a role in the specificity of the enzyme for the different alcohols. Other QH-EDH with activity towards secondary alcohols were reported by Toyama et al. *(22)*. The kinetic parameters of this enzyme from *Pseudomonas putida* (denominated ADH IIB), are comparable with the values obtained for QH-EDH from *C. testosteroni*. For both QH-EDH and ADH IIB, long-chain primary and secondary alcohols are the preferred substrates. No data, however, were reported on the enantioselectivity of ADH IIB for secondary alcohols.

Oxidation of C_3-synthons can also be accomplished with the enzyme electrode. Table 2 shows that the enzyme has the expected specificity with regard to the oxidation of glycidol and solketal. High enantioselectivity

was obtained in the conversion of solketal, R-solketal being the preferred substrate. No enantioselectivity was observed with R- and S-glycidol, in agreement with the results previously found for the soluble enzyme *(9,23)*. Again however, an increased activity in terms of higher V_{max} values was found with these synthons when the results of the enzyme electrode were compared with literature data *(9,23)*. It is known that the R-solketal aldehyde is a better substrate than R-solketal (K_m = 0.5 m*M* and 0.1 m*M*, V_{max} = 8.29 μmol min^{-1} mg^{-1} and 2.12 μmol min^{-1} mg^{-1}, respectively), and therefore the conversion of the aldehyde might interfere with the V_{max} observed for R-solketal on the electrode. However, this explanation does not seem to be plausible, because the enzyme electrode is operated under flow through conditions and accumulation of the aldehyde is not expected to occur. Glycidol is converted with an approximately 20-fold increase in V_{max}. The results indicate that the enzyme electrode is an attractive tool for an efficient conversion of these substrates.

CONCLUSIONS

QH-EDH electrodes are an effective tool for the enantioselective oxidation of secondary alcohols. The maximum current density obtained at room temperature is 0.43 A m^{-2} in the conversion of primary alcohols. Secondary alcohols (i.e., 2-heptanol and 2-octanol) are converted with a current density of 0.35 A m^{-2} and an enantiomeric ratio (E) of approximately 80. This means that the substrate is converted with a rate of 8×10^{-3} moles m^{-2} h^{-1}, which can be considered as a very interesting starting point for preparative use, especially when the production of high added value products is considered.

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