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Characterization of the Enantioselective Properties of the Quinohemoprotein Alcohol Dehydrogenase of *Acetobacter pasteurianus* LMG 1635. 1. Different Enantiomeric Ratios of Whole Cells and Purified Enzyme in the Kinetic Resolution of Racemic Glycidol

Sonia Salgueiro MACHADO, Ute WANDEL, Jaap A. JONGEJAN,† Adrie J. J. STRAATHOF, and Johannis A. DUINE

Department of Microbiology and Enzymology, Kluyver Institute for Biotechnology, Delft University of Technology, Julianalaan 67, 2628 BC Delft, The Netherlands

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Resting cells of *Acetobacter pasteurianus* LMG 1635 (ATCC 12874) show appreciable enantioselectivity ($E=16$–18) in the oxidative kinetic resolution of racemic 2,3-epoxy-1-propanol, glycidol. Distinctly lower values ($E=7$–9) are observed for the ferricyanide-coupled oxidation of glycidol by the isolated quinohemoprotein alcohol dehydrogenase, QH-ADH, which is responsible for the enantiospecific oxidation step in whole cells. The accuracy of *E*-values from conversion experiments could be verified using complementary methods for the measurement of enantiomeric ratios. Effects of pH, detergent, the use of artificial electron acceptors, and the presence of intermediate aldehydes, could be accounted for. Measurements of *E*-values at successive stages of the purification showed that the drop in enantioselectivity correlates with the separation of QH-ADH from the cytoplasmic membrane. It is argued that the native arrangement of QH-ADH in the membrane-associated complex favors the higher *E*-values. The consequences of these findings for the use of whole cells versus purified enzymes in biocatalytic kinetic resolutions of chiral alcohols are discussed.

Key words: *Acetobacter pasteurianus*; quinohemoprotein alcohol dehydrogenase; oxidation; enantioselectivity; kinetic resolution

Enantiomerically pure glycidol provides an attractive building block for the production of various fine chemicals, including β-blockers and antivirals.[1,2] While both (*R*)- and (*S*)-glycidol (*e.e.* ≈ 0.90) can be synthesized by the Sharpless asymmetric epoxidation of allyl alcohol,[3] and (*S*)-glycidol (*e.e.* = 0.96) has been prepared in single-run lipase-catalysed hydrolyses of racemic glycidol esters,[4,5] enantiopure (*R*)-glycidol (*e.e.* > 0.99) can be readily obtained by *Acetobacter pasteurianus*-catalyzed kinetic resolution of a racemic substrate.[6] The organism catalyzes the enantiospecific oxidation of glycidol to glycidic acid with $E=16$–18, well above the selectivity ($E=10$) that is commonly accepted as a lower limit for industrial applications. Optimal rates of oxidation are of the order of 25 mmol/h per gram dry weight, however, serious inhibition occurs at glycidol concentrations in excess of 1 M of glycidol.[6]

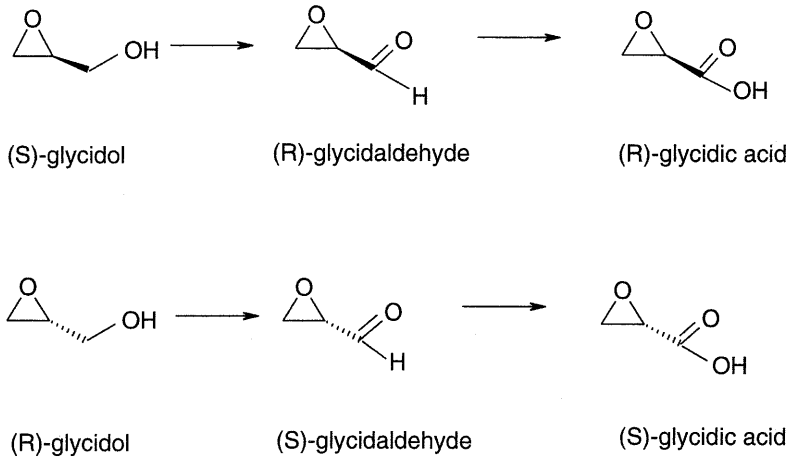
Bacteria of the genera *Acetobacter* and *Gluconobacter* have long been known for their capacity to catalyze the oxidation of various alcohols to the corresponding aldehydes and acids.[7–9] Comprehensive reviews of the distribution and characterization of the enzymes involved in the alcohol and sugar oxidizing systems have appeared.[10,11] Studies of *A. pasteurianus* in chemostat cultures showed that both NAD(P)-dependent and dye-linked alcohol dehydrogenase are expressed during growth on ethanol, acetate, and lactate. The dye-linked quinohemoprotein alcohol dehydrogenase, QH-ADH Type II, which is expressed most efficiently in ethanol-grown cells, has been found to be strictly responsible for the oxidation of glycidol.[12,13]

Quinoprotein alcohol dehydrogenases containing pyrroloquinoline quinone, 2,7,9-tricarboxy-1*H*-pyrrolo[2,3-*f*]quinoline-4,5-dione, PQQ, as a cofactor have been isolated from various Gram-negative bacteria.[14–18] Methanol dehydrogenases, MDHs, from methylotrophic bacteria,[19–22] and ethanol dehydrogenases, EDHs, occurring in Pseudomondas[23] are prominent members of this class. Quinohemoprotein alcohol dehydrogenases, QH-ADHs, form a second class containing heme *c* as an additional organic cofactor. Type I quinohemoprotein alcohol dehydrogenases are soluble, monomeric proteins containing one molecule of PQQ and a single *c*-type heme.[24–30] Type II QH-ADHs have been isolated from various acetic acid bacteria. They are membrane-associated enzymes composed of different subunits.[11,31–35] The two types of quinohemoprotein alcohol dehydrogenase show different substrate specificity and enantioselectivity for chiral C_3-alcohols.[12] The pH optima differ by 2–3 units (7–8 for Type I and 4–6 for Type II). No activator is required for catalysis.

Considering the potential applicability of *A. pasteurianus* LMG 1635 for the kinetic resolution of racemic glycidol (Scheme 1), identification of the factors involved in the inhibition that is observed when glycidol concentrations in excess of 1 M are used[6] is of interest. To find whether the toxicity of glycidol for whole cells involves (ir)reversible inhibition of QH-ADH we set out

† To whom correspondence should be addressed. Phone: +31152782371; Fax: +31152782355; E-mail: jaap@dutsh15.tudelft.nl

Abbreviations: *E*, enantiomeric ratio; ee_S, enantiomeric excess of substrate; QH-ADH, quinohemoprotein alcohol dehydrogenase



Scheme 1. Oxidation of (*R*, *S*)-Glycidol by *A. pasteurianus* LMG 1635.

to investigate the effects of glycidol on the isolated and purified enzyme. To our surprise, kinetic resolutions of racemic glycidol with isolated enzyme showed QH-ADH to be substantially less enantioselective than the cells from which it was isolated. Although it is not uncommon for whole cells to show better catalytic performance than the isolated enzymes in terms of rate and stability, the overall enantioselectivity is not normally expected to exceed that of the key-selective enzyme. This prompted us to investigate possible causes for this intriguing observation.

Materials and Methods

Chemicals. (*R*, *S*)-Glycidol of 96% chemical purity was obtained from Sigma-Aldrich Chemie BV and distilled before use to remove small amounts of polymeric material. (*R*)- and (*S*)-glycidol, of 96% enantiomeric purity, were obtained from Aldrich. (*R*, *S*)- and (*R*)-glycidaldehyde were synthesized by methods in the literature.[36] (*R*)- and (*S*)-2-butanol, of enantiomeric purity of 98% and 99%, respectively, were obtained from Fluka Chemie, Buchs. β-Dodecylmaltoside, 99% HPLC grade, was obtained from Anatrace. Decylubiquinone, approx. 98% chemical purity (TLC) was from Sigma/Aldrich. Other chemicals were of analytical grade and obtained from commercial suppliers (Merck, Aldrich).

Microorganism. Acetobacter pasteurianus LMG 1635 (ATCC 12874) was grown in chemostat cultures at a dilution rate of 0.05 h^{-1}, under ethanol-limited conditions in a mineral medium without vitamins, pH 6.0, 30°C, and dissolved-oxygen concentrations above 25% of air saturation as described previously.[13] The effluent from the chemostat cultures under steady-state conditions was concentrated by ultrafiltration/microfiltration (Hollow fiber filter, model CFP-1-E-9A, 0.1 μm, A/G Technology Corporation, connected to a Watson Marlow model 601 pump). The cell paste was stored at −80°C.

Enzyme purification. A. pasteurianus quinoprotein alcohol dehydrogenase was purified essentially as described by Tayama and coworkers[31] with minor modifications:

a. Chemostat-grown cells were suspended (1 gram cell paste: 1 ml buffer) in potassium phosphate buffer, 10 mM, pH 6.0, followed by disruption using a French Pressure cell at 110 MPa. Intact cells were removed by centrifugation at 10,000 × *g* for 15 min. The cell-free extract was stirred in the presence of detergent (1% Triton X-100, final concentration) at ambient temperature for three hours and centrifuged at 50,000 × *g* for three hours. The clear supernatant containing the solubilized QH-ADH was purified further. The pellet consisting of membrane particles with approx. 2% of the total glycidol dehydrogenase activity originally present was stored at −80°C.

b. Ion-exchange chromatography was done on DEAE Sepharose (Fast Flow, 1.5 × 28.0 cm) equilibrated with potassium phosphate buffer, 10 mM, pH 7.0, containing 0.1% Triton. Following application of the solution containing solubilized QH-ADH and extensive washing (3 column volumes) with the same buffer, a linear gradient of 10 to 50 mM potassium phosphate buffer containing 0.1% Triton at pH 7.0 was put in for 15 h at a flow rate of 0.5 ml/min. Eluted fractions were assayed for dye-linked ethanol and glycidol oxidizing activity. Active fractions were pooled and concentrated (Amicon Centriprep, 4,000 × *g* for 30 min, three times).

c. Concentrated material was put onto a hydroxyapatite Bio-Gel HT column (1.5 × 13.5 cm) equilibrated with 10 mM potassium phosphate buffer, pH 7.0, and eluted with a linear gradient of 10–100 mM potassium phosphate buffer pH 7.0 for 7 h at a flow rate of 2 ml/min. Active fractions were assayed, pooled, concentrated (Amicon Centriprep), and stored at −40°C until further use.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was done on 7.5% cross-linked polyacrylamide gels in Tris-glycine buffer, pH 8.8. Protein fractions were denatured by heat treatment for 5 min at 100°C in the presence of SDS (2.5%) and 2-mercaptoethanol (5%). Coomassie Brilliant Blue was used for staining. Reference proteins were from Pharmacia/Biotech (LMWCK). Native polyacrylamide gel electrophoresis (native-

PAGE) was done by the method of Toyama[30] on 7.5% polyacrylamide gels in Tris-glycine buffer, pH 8.3. QH-ADH activity staining was done by soaking the gels in an ethanol-PMS-DCIP-containing assay mixture.[13]

Enzyme activity measurements. **a. Dye-linked activity.** The assay mixture contained 20 mM ethanol, 1.0 mM potassium ferricyanide, 0.5 ml of McIlvaine buffer, pH 4.0, and enzyme sample in a total volume of 1.0 ml. The ferricyanide consumption rate was monitored spectrophotometrically at $\lambda = 420$ nm ($\varepsilon_{420} = 1020\ M^{-1} \cdot cm^{-1}$) and 25°C.[37] One unit of dye-linked activity is defined as the amount of enzyme catalyzing the conversion of 1 μmole of alcohol (2 μmoles of ferricyanide) per min under these conditions. **b. Quinone reductase assay.** Reduction of decylubiquinone was measured spectrophotometrically by following the decrease in absorbance at $\lambda = 275$ nm ($\varepsilon_{275} = 12.5\ M^{-1} \cdot cm^{-1}$) in time. The reaction mixture contained 20 mM ethanol, 100 μM decylubiquinone, 0.5 ml McIlvaine buffer, pH 4.0, and enzyme sample in a total volume of 1.0 ml containing 0.25%(w/w) of β-dodecylmaltoside.[38] One unit of quinone-reductase activity is defined as the amount of enzyme catalyzing the conversion of 1 μmole of alcohol (1 μmole of decylubiquinone) per min under these conditions. Protein was measured by the method of Bradford[39] with desalted bovine serum albumin as a standard.

Calculation of the enantiomeric ratio, E. The enantioselective properties of enzymes and microorganisms are conveniently expressed by the enantiomeric ratio (*E*-value).[40,41] Different methods were used for the calculation of *E*-values.

Method A. Evaluation of the ratio of the specificity constants for the pure enantiomers by the defining expression for the enantiomeric ratio,[40,42] Eq. (1):

$$E = \frac{(k_{cat}/K_M)^S}{(k_{cat}/K_M)^R} \cdot \qquad (1)$$

Kinetic constants k_{cat} and K_M for (*S*)- and (*R*)-substrate were obtained by non-linear regression of initial reaction rates as a function of the enantiomer concentration. For numerical convenience (*E*-values > 1) *E* is chosen to represent *S*-over *R*-specificity.

Method B. Evaluation of initial rate measurements for samples with identical concentrations but varying molar fractions of the enantiomers,[43] Eq. (2):

$$E = \frac{r_S(r_x - r_R)}{r_R(r_S - r_x)} \cdot \frac{(1-x)}{x} \qquad (2)$$

r_S, r_R and r_x represent the initial reaction rates on equal total concentrations of pure (*S*)-enantiomer, pure (*R*)-enantiomer, and (*R*, *S*)-mixtures of mole fraction *x* of (*S*)-enantiomer, respectively.

Note: a misprint of Eq. (2) in Geerlof *et al.*[12] as compared to the correct expression.[43]

Method C. Evaluation of the enantiomeric excess-value of the remaining substrate, ee_S as a function of the extent of conversion, ξ, in batch kinetic resolutions of racemic substrate, Eq. (3):[40]

$$E = \frac{\ln\{(1-\xi)(1-ee_S)\}}{\ln\{(1-\xi)(1+ee_S)\}} \qquad ee_S = \frac{C_S^R - C_S^S}{C_S^R + C_S^S} \qquad \xi = 1 - \frac{C_S^R + C_S^S}{C_{S,0}^R + C_{S,0}^S} \qquad (3)$$

with $C_{S,0}^R$, $C_{S,0}^S$ and C_S^R, C_S^S, the initial and actual concentrations of (*R*)- and (*S*)-substrate, respectively.

For all three methods, the *E*-value is calculated using the non-linear regression algorithm incorporated in the program SIMFIT developed by van Tol and coworkers.[44] Standard deviations represent lower estimates on the assumption of (normally distributed) errors of the dependent variables, ee_S, *r*, only.

Measurement of the ee_S-values. Extraction solvent (diethyl ether: 2-butanone, 1:3 v/v, 1 ml) was added to samples (0.25 ml) of the bioconversion reaction mixture and mixed thoroughly in a 2-ml vial. The organic phase was removed and dried over anhydrous $MgSO_4$ (2 h). The volume was reduced to about 100 μl by evaporation of the solvent in a gentle stream of dry air. Samples (1–5 μl) were injected on an HP 5890 serie II GC with a chiral column (2,3,6-tri-*O*-trifluoroacetyl-g-cyclodextrin as a stationary phase) at a carrier gas flow rate of 0.6 ml/min, a column head pressure of 30 kPa, splitter ratio of 1:150, and an oven temperature of 65°C. Under these conditions (*R*)-glycidol elutes at 10.7 min, (*S*)-glycidol at 11.2 min, (*R*)-glycidaldehyde at 5.3 min, and (*S*)-glycidaldehyde at 5.4 min. The enantiomeric excess-value was measured from the ratio of the areas of the base-line separated peaks.

Measurement of the extent of conversion, ξ. The extent of conversion was measured from the concentration of unreacted glycidol and produced glycidaldehyde and glycidic acid in the reaction mixture using HPLC. Aqueous samples (50 μl) were injected onto a Bio-Rad Aminex column HPX-87H, coupled to an HPLC system consisting of a Waters pump (Model 510), an automatic injector (Waters, WISP 710B), and a differential refractometer (Waters, Model 410) connected to a chromatography data system from Chrompack. Samples were eluted with 5 mM H_2SO_4 at a flow rate of 0.5 ml/min at 30°C. Under these conditions glycidol and glycidic acid are base-line separated with retention times of 16.2 and 19.5 min, respectively. Two peaks are observed in the elution profiles of authentic glycidaldehyde (>98% pure, as judged from ^{1}H NMR measurements and GC analysis). A major peak, accounting for 83% of the combined peak area elutes at 14 min; a minor peak (17%) co-elutes with glycidol at 16.5 min. Upon chromatographic analysis at 54°C the minor peak is absent. ^{1}H NMR measurements at various temperatures support the conclusion that the minor peak represents the (hydrated) glycidaldehyde dimer. Since the glycidol and glycidic acid peaks tend to overlap at the higher elution temperatures, analysis was routinely done at 30°C. The glycidol, glycidaldehyde, and glycidic acid concentrations in the samples were calculated from the peak areas at 14 min (glycidaldehyde monomer, 83% of total

glycidaldehyde), 16 min (glycidol), 16 min (glycidaldehyde dimer, 17% of total glycidaldehyde), and 19.5 min (glycidic acid), respectively, as compared to calibration curves for the separate components.

Conversion experiments with A. pasteurianus whole cells. *A. pasteurianus* LMG 1635 cell paste (0.7 g) was suspended in 50 ml of 10 mM potassium phosphate buffer, pH 6.0. Racemic glycidol (0.37 g, 5 mmol) was added at $t=0$. The conversion assays were run at 30°C while the pH was kept constant by addition of 1.0 M NaOH using a pH-Stat system consisting of a Metrohm Dosimat (Model 655), an Impulsomat (Model 614), and a pH-meter (Model 691). One-ml samples were taken at regular intervals and the conversion was stopped by removing the cells by centrifugation (5 min at $48{,}000 \times g$).The supernatant was used to measure the degree of conversion and the *e.e.*-value of the remaining glycidol as described above. The extent of conversion of glycidol was calculated after correction for the amount of reaction mixture removed with each sample.

Conversion experiments with purified QH-ADH. Fifteen U of purified QH-ADH from *A. pasteurianus* were added to 25 ml of 4 mM potassium phosphate buffer, pH 6.0, containing 25 mM (0.63 mmol) of racemic glycidol and 160 mM (4.0 mmol) of potassium ferricyanide as an electron acceptor. The conversion was monitored as described for the whole cells. Samples (1.0 ml) were taken at regular intervals. Part of the sample (0.50 ml) was acidified immediately by the addition of concentrated hydrochloric acid (3.0 µl) to stop the reaction. Following centrifugation, the amounts of glycidol, glycidaldehyde, and glycidic acid were measured by HPLC. The remaining part of the sample was used to calculate the *e.e.*-value.

Enantioselective conversion of (R, S)-2-butanol. Calculation of the *E*-values of QH-ADH in the ferricyanide-coupled oxidation of (*R*, *S*)-2-butanol was done by Method B. Reaction conditions were as described for (*R*, *S*)-glycidol.

Results and Discussion

Characterization of QH-ADH

QH-ADH Type II, the "relevant enzyme" in kinetic resolutions of racemic glycidol using *A. pasteurianus* whole cells and cell-free extracts of low specific activity[12] was isolated and purified. On DEAE-Sepharose chromatography two fractions were obtained. A major fraction of homogeneous enzyme (3260 U on ethanol) and a minor fraction (50 U on ethanol) eluting separately from the main peak. The results reported here have been obtained with the enzyme from the major fraction only. Purification steps are summarized in Table 1.

Specific activities of 139 and 67 U/mg protein were determined for ethanol and (*rac*)-glycidol, respectively. The specific activity for ethanol is of the same order of magnitude as the values of 189 U/mg for *A. aceti* QH-ADH and 125 U/mg protein reported for *A. methanolicus* QH-ADH.[33,35,37] The ratio of specific activities for ethanol and glycidol as substrates remains virtually unchanged during the purification steps (Table 1). The absorption spectrum of the enzyme (reduced form) shows maxima at $\lambda=418$, 524, and 555 nm, respectively (Fig. 1a). The ratio of absorbance $\varepsilon_{280}/\varepsilon_{418}=1.75$ is close to the value of 1.80 estimated from spectra of Type II QH-ADHs of other acetic acid bacteria (*e.g. A. polyoxygenes* QH-ADH).[31]

On SDS-PAGE, purified QH-ADH showed bands around of 75, 45, and 19 kDa, after protein staining (Fig. 1b). The molecular masses of the three subunits are consistent with those reported for *A. pasteurianus* NCI1452 QH-ADH by Kondo *et al.*[34,45] On native PAGE (pH 8.3) and protein staining of the purified preparation again 3 bands were observed with mobilities that are virtually identical to those observed on SDS-PAGE. Dissociation of QH-ADH into the subunits under these conditions has also been observed by Adachi and co-workers.[32] Activity staining of native gels using ethanol/DCIP-PMS was inconclusive. It appears that at least one of the subunits, most probably 75 kDa subunit, has low activity, however, due to diffusion during the large exposure times discoloration covers the area of all 3 bands.

Whole cells versus purified QH-ADH in the enantioselective oxidation of glycidol

Enantiomeric excess-values of remaining glycidol as a function of the extent of conversion in the kinetic resolution of racemic glycidol with whole cells of *A. pasteurianus* LMG 1635 grown in chemostat cultures and stored

Table 1. Purification of QH-ADH from *Acetobacter pasteurianus* LMG 1635

Step	Total protein (mg)	Total activity[c] (U)		Specific activity (U/mg protein)		Recovery[b] (%)	Ratio (EtOH/Glycidol)
		EtOH	(*R*, *S*)-Glycidol	EtOH	(*R*, *S*)-Glycidol		
Cell homogenate[a]	3999	4142	2001	1.0	0.5	100	2.0:1
Solubilized fraction	586	3485	1802	6.0	3.7	84	2.0:1
DEAE-Sepharose	44.80	3258	1920	72.7	42.9	78	1.7:1
Hydroxyapatite	16.65	2301	1222	139.0	67.4	56	2.0:1

[a] Cell homogenate treated with Triton X-100.
[b] Recovery calculation based on the activities for ethanol.
[c] Activities measured using potassium ferricyanide as an electron acceptor.

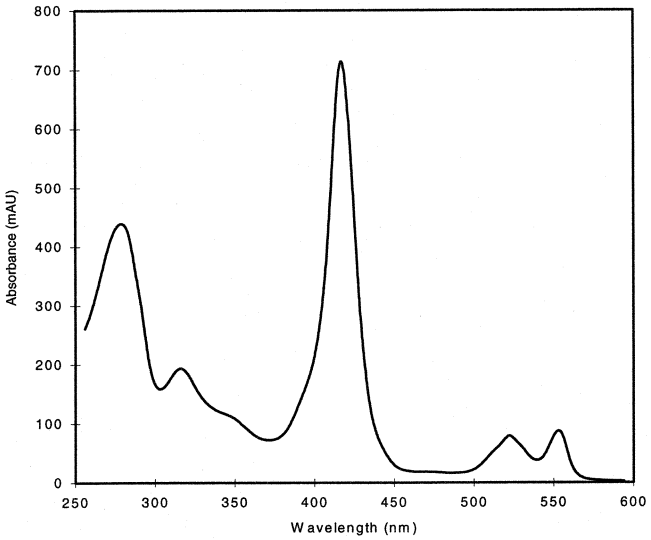


Fig. 1a. Ultraviolet/Visible Absorption Spectrum of Purified QH-ADH from *A. pasteurianus* LMG 1635.

The absorption spectrum of purified *A. pasteurianus* QH-ADH was taken in 10 mM potassium phosphate buffer, pH 7.0, at a concentration of 175 μg/ml in the presence of 0.1% Triton X-100 using an SLM-Aminco spectrophotometer.

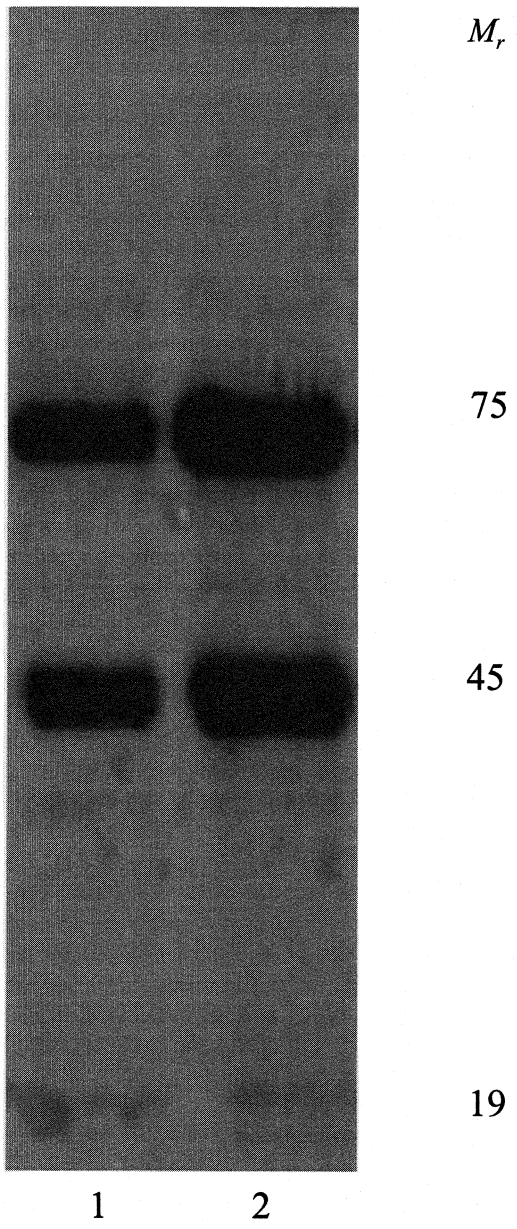


Fig. 1b. SDS-PAGE of QH-ADH from *Acetobacter pasteurianus* LMG 1635.

SDS-polyacrylamide gel electrophoresis (7.5%) of the purified QH-ADH (17 and 38 μg of protein were put on lanes 1 and 2 respectively) in Tris-glycine buffer pH 8.8 and staining with Coomassie Brilliant Blue (0.125%). The molecular mass (M_r) of each band is indicated on the side in kDa.

at -80°C, show a convincing fit for $E=15.5$, using Method C (Fig. 2, Curve A). In similar experiments, data obtained using purified QH-ADH of specific activity 67 U/mg on glycidol, lead to $E=8$ (Fig. 2, Curve B). In addition, the latter reaction is observed to become exceedingly sluggish after $\xi>0.6$, when most of the enzyme appears to be inhibited. In contrast to samples from whole cell conversions, samples drawn from conversions with purified enzyme contain appreciable amounts of (enantiomerically enriched) glycidaldehyde (Figs. 3, 4).

It has been established that E-value calculations based on Method C may be seriously biased by (systematic) errors of the presumingly independent variable, ξ.[42,44] In this case, direct evaluation of the extent of conversion from the consumption of base and/or ferricyanide is not feasible due to the iterative procedures required to account for the variable overall stoichiometry in mixed product formation. Alternative methods to measure the enantiomeric ratio were done as well.

Evaluation of the enantioselectivity of purified QH-ADH

In order to validate the results obtained for the QH-ADH enantioselectivity using Method C, E-value measurements using Methods A and B were done using potassium ferricyanide (Table 2). The drop in the enantioselectivity in going from whole cells to purified QH-ADH appears to be consistently reproduced independent of the method used, also at different pHs. When ubiquinone was used as an electron acceptor to check whether the redox potential of the electron acceptor might have an effect (Table 3), the E-value was found to be low as well ($E=5.1$). In previous investigations[12,46] the drop in E-value was not observed, probably because of a limited extent of purification. This prompted us to investigate the enantioselective properties of homogenates, cell-free extracts, and chromatographic fractions at every step of the purification protocol.

Enantioselectivity at each step of the purification

A slight decrease of the E-value is observed following addition of Triton X-100 to the cell homogenate (Table 4). A statistically relevant lowering of the enantioselectivity, however, becomes manifest only after ultracentrifugation, when the solubilized QH-ADH is separated from the membrane fraction. The QH-ADH activity

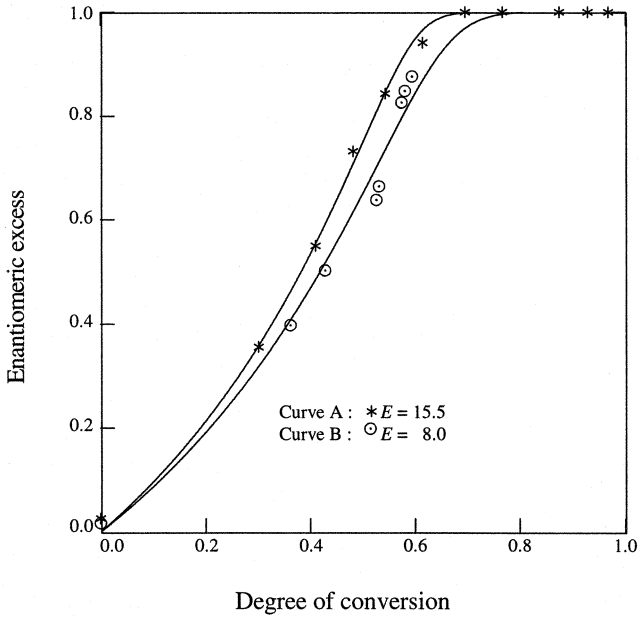


Fig. 2. Selective Oxidation of Glycidol by *A. pasteurianus* LMG 1635 Whole Cells and Purified QH-ADH.

Experiments were done in a pH-stat at 30°C with: Curve A (*) cells; 0.7 g cells, 0.37 g of (*R*, *S*)-glycidol were added to 50 ml of 10 mM potassium phosphate buffer, pH 6.0; Curve B (◉) purified QH-ADH (15 U), 0.62 mmol of (*R*, *S*)-glycidol, 3.9 mmol of potassium ferricyanide were added to 25 ml of potassium phosphate buffer, pH 6.0. The lines represent the best fits, according to method C.

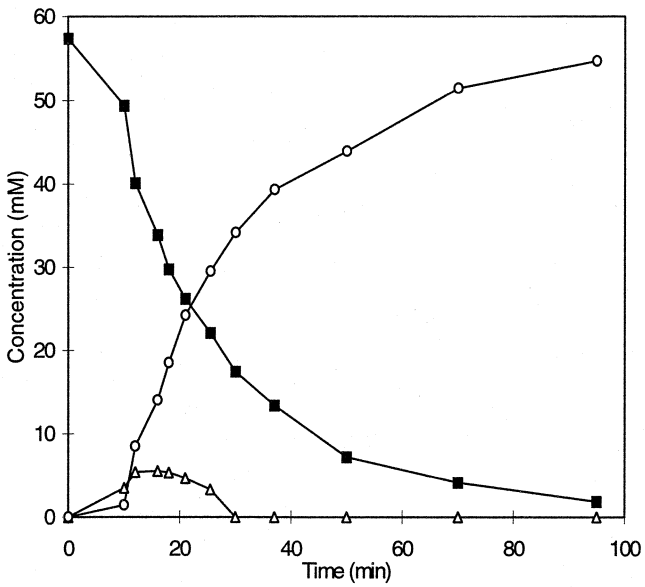


Fig. 3. Glycidol Conversion by *A. pasteurianus* LMG 1635.

Concentrations of glycidol (—■—), glycidaldehyde (—△—), glycidic acid (—○—) as a function of the time. The same experimental conditions as described in Fig. 2, curve A.

(2% of the total activity) retained in the membrane fraction has $E=13.4\pm1.9$, close to the value observed for the cell homogenate (14.3 ± 1.8) and for the bioconversion with whole cells at pH 4.0 (Fig. 2 curve A). Different *E*-values for the dye-linked alcohol oxidizing activity retained in the membrane fraction and the solubilized

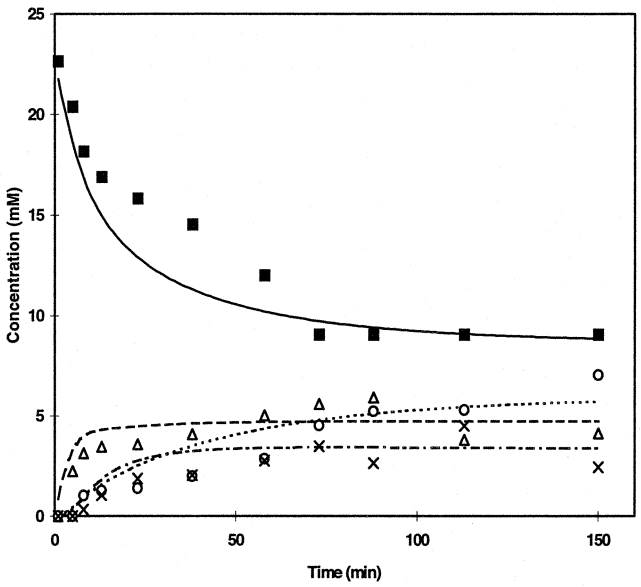


Fig. 4. Glycidol Bioconversion by Purified QH-ADH from *A. pasteurianus* LMG 1635.

Concentrations of glycidol (—■—), glycidaldehyde (—△—), glycidic acid (—○—) and the dimer (—×—) as a function of the time, obtained under same experimental conditions as described in Fig. 2 curve B. The lines represent simulations based on the equations 5–10, glycidol (——), glycidaldehyde (– – – –), glycidic acid (· · · ·) and the dimer (–·–·–).

Table 2. Calculation of the Enantioselectivity of Purified QH-ADH for the Oxidation of (*R*, *S*)-Glycidol

Method[a]	pH	Enantiomeric ratio $E(\sigma)$[b]
A	4.0	7.6(0.9)
B	4.0	7.9(0.7)
C	6.0	8.0(0.6)

[a] See Materials and Methods.
[b] σ, the standard deviation of 15 data points in triplicate for each method.

particulate enzyme are also observed in the kinetic resolution of (*R*, *S*)-2-butanol (Table 5).

Possible causes for the observed change of E-value

1. *The accuracy of E-value measurements.*

Two sources of error must be indicated that can readily lead to variations as large as those observed here for whole cell and purified enzyme catalyzed resolutions. First, since the *E*-value is expressed as a *ratio* of kinetic constants, minor absolute errors of the individual constants will generate large statistical uncertainties of the lumped parameter. Secondly, when certain kinetic and mechanistic features of the catalytic reaction are not properly identified the 'apparent' nature of the resulting *E*-values may not be recognized. In this work, care was taken to reduce the statistical uncertainty resulting from random measurement errors by increasing the number of experimental data points. When systematic errors were suspected, *e.g.* overlap of glycidol and glycidaldehyde-derived peaks in the measurement of the extent of

Table 3. Kinetic Parameters of *A. pasteurianus* QH-ADH for Different Alcohols Calculated from Initial Rate Measurements, Using Potassium Ferricyanide or Decylubiquinone as Electron Acceptors

Substrate	Ferricyanide			Ubiquinone		
	K_M^{app} (mM)	k_{cat}^{app} (μmol·min^{-1}·mg^{-1})	E^{app} [a]	K_M^{app} (mM)	k_{cat}^{app} (μmol·min^{-1}·mg^{-1})	E^{app} [a]
ethanol	0.17±0.02	17.8±0.3		0.13±0.01	17.2±0.3	
(*S*)-glycidol	1.77±0.12	20.2±0.5	7.65	2.95±0.42	13.9±0.5	5.10
(*R*)-glycidol	10.03±0.88	14.9±0.8		14.90±2.3	13.8±0.8	

[a] Enantiomeric ratio for (*S*) and (*R*)-glycidol is given as E^{app} since the K_M and k_{cat} values do not apply to enantiomerically pure compounds.

Table 4. Enantioselectivity of *A. pasteurianus* Cell Fractions in the Kinetic Resolution of (*R*, *S*)-Glycidol

Cell fraction	Enantiomeric ratio $E(\sigma)$[d]
CH[a]	14.3(1.8)
CH+Triton X-100 (t=0 h)	12.1(1.7)
CH+Triton X-100 (t=3 h)	11.2(1.4)
Supernatant[b]	8.8(0.9)
Membrane fraction[b]	13.4(1.9)
QH-ADH[c]	7.4(0.7)

[a] CH=cell homogenate.
[b] After ultracentrifugation.
[c] Purified QH-ADH.
[d] E and σ as in the legend of Table 2.

Table 5. Enantioselectivity of the Mixtures of Purified QH-ADH and Membrane Fraction Obtained After Ultracentrifugation

Preparations	Incubation time (min)	*Enantiomeric ratio* $E(\sigma)$	
		(*R*, *S*)-Glycidol	(*R*, *S*)-2-butanol
QH-ADH (25 U)	0	7.4(0.7)	9.7(1.3)
MF (5.0 U)	n.d.	13.4(1.9)	n.d.
QH-ADH (25 U) +MF (5.0 U)	<5	10.5(1.5)	n.d.
QH-ADH (25 U) +MF (5.0 U)	30	21.8(3.4)	18.1(3.4)

MF=Membrane fraction after removal of the bulk QH-ADH activity.
Incubation time=time during which purified QH-ADH and membrane fractions are in contact.
U=total activity on ethanol.
n.d.=not determined.

conversion by HPLC (Method C), alternative methods were used. The sensitivity of methods A through C for random and systematic errors in general applications has been reviewed.[42-44] In this case evaluation of E from the ratio of specificity constants according to the defining equation, Method A, appears to be of limited value since the results are affected by minor contamination of the commercial alcohols with the enantiomer of opposite chirality.[42] Sufficient quantities of both chemically and optically pure enantiomers of glycidol were not available to us. Application of Method B obviates this problem. Effects of products, notably the presence of glycidaldehyde of varying enantiomeric composition in conversion experiments, can also be excluded by using this method.[43] Method B is inheritantly less accurate than Method C, in particular since along with E, two more parameters, r_R and r_S, are evaluated from the same data set. Standard deviations of reasonable magnitude could, however, still be obtained by raising the number of trials, *e.g.* triplicate data points (Table 2). Since application of Method B does not allow calculation of the individual kinetic constants, we used Method A to evaluate the enantiomeric ratio for purified QH-ADH using decylubiquinone as an electron acceptor. The values of the kinetic constants collected in Table 3 are apparent values, restricted by the limited solubility of the quinone. The standard deviation addresses the goodness of fit to the available data points. Considering the different sources and magnitudes of possible systematic errors in the methods used, the fact that consistent values for the enantiomeric ratio of isolated QH-ADH were obtained from all three methods strongly supports the authenticity of the observed effect: E-values calculated for *A. pasteurianus* QH-ADH (E=7–8) and whole cell preparations (E=16–18) under the conditions stated differ by a statistically relevant factor.

2. *pH-effects*

Effects of pH on the enantioselectivity of alcohol dehydrogenases have been reported by Secundo and Phillips for the oxidation of 2-butanol by secondary alcohol dehydrogenase from *Thermoanaerobacter ethanolicus* when lowering the pH from 9 (E=2.5) to 5.5 (E=4.2). No effect was observed for the reaction catalyzed by horse liver ADH.[47] In this case, oxidations with whole cells at pHs substantially different from 6 were not feasible. Despite the low activity of isolated QH-ADH at pH 6, conversion experiments (Method C) could be done, affording E-values identical to those obtained at pH 4 (Methods A, B) (Table 2). Although pH effects on E in the QH-ADH-catalyzed kinetic resolution of glycidol cannot be ruled out on the basis of these observations, a major contribution to the observed difference appears to be unlikely.

3. *Effects of detergent*

The effects of the presence of Triton X-100, the detergent used throughout this investigation, on the E-value appear to be equally limited. As is shown in Table 4, a time-dependent effect of low statistical relevance is observed on exposure of cell-free extracts to Triton X-100. Whether a direct effect of the detergent on the enantioselectivity of isolated QH-ADH occurs could not be unequivocally established since complete removal of Triton X-100 from the purified enzyme solutions leads to inactivation. Circumstantial evidence suggesting a limited effect of the presence of the detergent will be discussed below in relation with the enantioselectivity of mem-

brane preparations retaining what is most probably the membrane-bound form of QH-ADH.

4. *Low-molecular weight compounds*

The conspicuous presence of (chiral) glycidaldehyde as an intermediate in the reaction is evident. While whole cells catalyze the conversion of glycidol to glycidic acid with concomitant release of only minimum amounts of intermediate glycidaldehyde into the medium, conversions with isolated QH-ADH give rise to substantial accumulation of glycidaldehyde, eventually bringing the reaction to a complete halt (Fig. 4). In analogy with the kinetic bi-bi ping-pong scheme including uncompetitive substrate inhibition reported for the mechanistically similar reaction catalyzed by the QH-ADH Type I of *Comamonas testosteroni*,[46] the oxidation rate of a single enantiomer of the alcohol in the presence of aldehyde by the Type II enzyme can be expressed as:

$$r_{alc}=\frac{(k_{cat(alc)}/K_{M(alc)})[alc][E]}{D_{Fe,alc}}$$

$$D_{Fe,alc,ald}=1+\frac{[alc]}{K_{M(alc)}}+\frac{[alc]/K_{M(alc)}}{[Fe]/K_{M(Fe,alc)}}+\frac{[alc]^2}{K_{M(alc)}K_{i(alc)}}+\frac{[ald]}{K_{M(ald)}}+\frac{[ald]/K_{M(ald)}}{[Fe]/K_{M(Fe,ald)}}+\frac{[ald]^2}{K_{M(ald)}K_{i(ald)}}. \quad (4)$$

With $[alc]$, $[ald]$, $[E]$, and $[Fe]$, the concentration of the alcohol-, aldehyde, enantiomer, active enzyme, and ferricyanide, respectively. It can be readily shown that when both enantiomers of the alcohol and the aldehyde are present, analogous expressions 5 and 6 will hold:

$$r_{alcR}=\frac{(k_{cat(alcR)}/K_{M(alcR)})[alcR][E]}{D_{Fe,alcS,alcR,aldS,aldR}} \quad (5)$$

$$r_{alcS}=\frac{(k_{cat(alcS)}/K_{M(alcS)})[alcS][E]}{D_{Fe,alcS,alcR,aldS,aldR}}. \quad (6)$$

Since the denominators of 5 and 6 are identical, the ratio of rates for the conversion of (*R*)- and (*S*)-alcohol will still be governed by the simple relationship serving to define the enantiomeric ratio. Thus, within the framework of this analysis, the enantioselective performance is not expected to be affected by the presence of intermediate aldehydes. A possible exception has been pointed out by van Tol and coworkers for the case of lipase enantioselectivity, where reversible 'internal' equilibria between the thermodynamically stable enzyme species, *e.g.* free enzyme and acyl-enzyme can play a role.[5] Although the kinetic schemes are equivalent, it is highly unlikely that such a situation will occur for the enzymatic reaction considered here: the redox potentials for the species involved are so far apart that complete irreversibility, also for 'internal' steps, is expected. This conclusion is supported by the results of the experiments in which decylubiquinone is used as an electron acceptor. Given the lower value for the midpoint potential of this compound, an even more drastic effect on *E* would have been predicted. Yet, the decrease in *E* is quite similar to that observed with ferricyanide as the electron acceptor. It should be noted in this respect that the slightly lower *E*-value calculated from Method A may be biased by the relatively low solubility of the quinone, possibly leading to an underestimate of the k_{cat} on (*S*)-glycidol, relative to its (*R*)-enantiomer. There is no indication that the use of an electron acceptor with redox potential similar to that of the probable physiological acceptor restores the higher *E*-values observed for whole cells.

The consistency of this analysis is supported by the course of the reaction of racemic glycidol catalyzed by purified QH-ADH. The concentrations of all relevant reactants and products were measured as a function of time. Eq(s). (5)–(10) were used for the simulation of the traces (Fig. 4). Kinetic parameters of the isolated enzyme for glycidol were taken from Table 3. Kinetic parameters for the aldehyde were obtained from Wandel *et al.* (in preparation).

$$r_{aldR}=\frac{(k_{cat(aldR)}/K_{M(aldR)})[aldR][E]}{D_{Fe,alcR,alcS,aldR,aldS}} \quad (7)$$

$$r_{aldS}=\frac{(k_{cat(aldS)}/K_{M(aldS)})[aldS][E]}{D_{Fe,alcR,alcS,aldR,aldS}} \quad (8)$$

$$r_{Enzyme}=-k_{inact}([aldS]+[aldR])[E] \quad (9)$$

$$r_{dimer}=k_{dimer}(([aldS]+[aldR])^2-[dimer]/K_{dimer}) \quad (10)$$

$$D_{Fe,alcS,alcR,aldS,aldR}=1+\frac{[dimer]}{K_{i(dimer)}}+\frac{[alcR]}{K_{M(alcR)}}+\frac{[alcS]}{K_{M(alcS)}}+\frac{[aldR]}{K_{M(aldR)}}+\frac{[aldS]}{K_{M(aldS)}}.$$

Dimerization of the aldehyde (Eq. (10)) has been included in the model as a reversible reaction ($k_{dimer}=0.01$ $l\cdot min^{-1}\cdot mmol^{-1}$, $K_{dimer}=0.1$ $l\cdot mmol^{-1}$). Inactivation of the enzyme by the aldehyde (Eq. (9), $k_{inact}=0.0045$ $l\cdot min^{-1}\cdot mmol^{-1}$) and inhibition by the dimer ($K_{i(dimer)}=3$ mM) account for the abortion of the catalytic reaction that is observed to take place before glycidol is fully converted. All constants are apparent values with respect to the ferricyanide concentration. A detailed analysis of the kinetic model will be given elsewhere. Of particular importance for this evaluation of possible effects of glycidaldehyde on the value of the apparent enantioselectivity is the observation that its inhibitory action can be, convincingly, albeit semi-quantitatively, accounted for by Eq(s). (5) through (10). Thus, the model contains contributions of the aldehyde enantiomers to the conversion rates of the alcohol enantiomers only in the denominator, leading to a complete separation of the effects of glycidaldehyde on absolute (overall inhibition of catalytic reaction) and relative (preferential conversion of one of the two enantiomeric alcohols) rates. Observation of a similar decrease of *E*-value both for the kinetic resolution of (*rac*)-glycidol and for (*rac*)-2-butanol excludes chemical modification of the catalyst by glycidol or glycidaldehyde as a primary cause since in the latter case methylethylketone is produced as a potentially harmless product.

5. *Multiple enzymatic activities*

Dye-linked alcohol dehydrogenase activity has been identified as the sole enzymatic activity involved in the oxidation of glycidol in cell-free extracts of *A. pasteurianus*.[13] Evidence for the participation of QH-ADH Type

II as a single enzyme species may be obtained from the data presented in Table 1, showing the ratio of specific activities for ethanol and glycidol to remain fairly constant during the purification protocol. The overall recovery of 56% of glycidol oxidizing activity, not including losses when collecting fractions of substantial specific activity only, supports the conclusion that additional dye-linked dehydrogenases in whole cells and cell-free extracts are not present or do not contribute appreciably to the observed change of *E*-value. No indications for the presence of genes in *A. pasteurianus* encoding other dye-linked alcohol dehydrogenases or iso-enzymes in addition to QH-ADH have appeared so far.

6. *Subunit composition and conformational changes*

Macromolecular effects on the alcohol oxidizing activity of acetic acid bacteria have been reported for a number of situations, including culture conditions, carbon source, pH, aeration conditions, and subunit association of the alcohol dehydrogenase.[11] Although the ability to catalyze the oxidation of ethanol and glycidol appears to be expressed constitutively,[13] Takemura *et al.*[48] report the QH-ADH activity to increase more than 10-fold when *A. pasteurianus* is cultivated with ethanol as a carbon source. Recent studies[49] showed that the higher activity is attributable to the larger amount of the large subunit (subunit I) of the QH-ADH in *A. pasteurianus* and *A. methanolicus* cells grown in the presence and the absence of ethanol, respectively. Equal amounts of QH-ADH protein are produced under both growth conditions. Matsushita *et al.*[50] report the production of an "inactive" form of QH-ADH in *Gluconobacter suboxydans* when this organism is cultivated under acidic or high aeration growth conditions. The inactive form has a 'loose' conformation due to some defect in the interaction between subunits. Inactivation of the QH-ADH is attributed to a change detected in one of the heme *c* sites leading to impaired subunit interaction.

Interaction and localization of QH-ADH subunits has been studied extensively. Of particular interest for this investigation is the observation by Kondo *et al.*[34] that the medium subunit (subunit II) has so far only been detected in the membrane fractions. They conclude that this subunit appears to be embedded in the membrane and may be active as a component of the respiratory chain linking the terminal oxidase to the other dehydrogenases while the large and small subunits (subunits I and III) can be found in the cytoplasmic membrane as a complex. Matsushita *et al.*[33] report that subunit II binds to the subunit I/III complex with an equimolar ratio to form the fully active QH-ADH complex. Studies by Frébortová *et al.*[51] show separation, albeit inefficiently, of the subunits during ultracentrifugation of *A. methanolicus* QH-ADH preparations.

Although SDS-PAGE of the isolated QH-ADH preparation used in this work shows bands of all three subunits, the implications for the enantioselective properties of active QH-ADH in the possible involvement of inactive forms present in the cell-free preparations drew our attention. In a preliminary investigation, isolated and purified QH-ADH was mixed in various proportions with membrane fractions containing low amounts of dye-linked alcohol dehydrogenase activity. No effects on the *E*-value (glycidol) were observed unless substantial quantities of membrane fraction were added. Under the latter conditions *E*-values of a magnitude similar to that observed for whole cells were again observed. It must be considered highly unlikely that the observed raise of *E* can be attributed to the actual reconstitution of QH-ADH with the membrane. More likely, the active QH-ADH still present in the membrane preparation overrides the lower *E*-value of the soluble QH-ADH present. This could be confirmed by kinetic resolution experiments using large amounts of membrane particles: high *E*-values were indeed observed (Table 5). Similarly high values were also observed for the kinetic resolution of 2-butanol using membrane particles.

Extensive extraction of membrane fractions containing residual alcohol dehydrogenase activity using Triton X-100 as before, afforded membrane-free preparations containing dye-linked alcohol dehydrogenase activity indistinguishable with respect to activity and enantioselectivity from the isolated QH-ADH prepared earlier. SDS-PAGE and protein staining of the solubilized preparation again showed the three bands characteristic of QH-ADH. From these results, we tentatively conclude that the change of *E*-value upon detergent-assisted release of QH-ADH from the membrane is most probably related to small conformational differences between the membrane-associated and solubilized forms of the enzyme. The effects of small structural perturbations on enzyme activity have been documented.[52] Effects on enantioselective properties may be expected as well. In the latter case, it needs to be emphasized that the observed change of the enantiomeric ratio by a factor of 2 relates to a change of the Gibbs free energy difference in the selectivity-determining transition states for reaction of (*R*)- and (*S*)-enantiomer of less than 2 kJ/mol at room temperature.

Concluding remarks

The finding of different *E*-values in the enantioselective conversion of chiral alcohols by whole cells of *A. pasteurianus* and the quinohemoprotein alcohol dehydrogenase responsible for the key selective step in the reaction addresses an important issue regarding the application of either whole cells or purified enzymes in biocatalysis. It would appear that in addition to such factors as handling, contamination, stability, and cofactor regeneration, enantioselective performance must be taken into consideration as well. A major implication of the current findings is the likely importance of conformational changes when membrane (associated) enzymes are involved in enantioselective conversions. So far, effects of conformational changes on the enantiomeric ratio have been suggested to play a role in the interfacial activation of certain lipases.[5] Similar behavior can, however, also be anticipated for other membrane-bound enzymes of potential biotechnological applicability, notably P450 oxidoreductases. The use of complementary methods, as reported in this paper, in order to deal with the experimental problems involved in the accurate measurement of *E*-values for such systems is

strongly recommended.

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