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RESOLUTION OF THE COENZYME B-12-DEPENDENT DEHYDRATASES OF *KLEBSIELLA* SP. AND *CITROBACTER FREUNDII*

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Summary

Diol dehydratase (1,2-propanediol hydro-lyase, EC 4.2.1.28) and glycerol dehydratase (glycerol hydro-lyase, EC 4.2.1.30) are shown to be distinct, separable enzymes that occur individually or together in different strains of *Klebsiella* sp. Anaerobic growth with propan-1,2-diol induces diol dehydratase alone, whereas glycerol fermentation induces both enzymes in *K. pneumoniae* ATCC 25955 and in *Citrobacter freundii* NCIB 3735. The dehydratases can be resolved by polyacrylamide-gel electrophoresis or separated by anion-exchange chromatography alone. Sucrose density gradient centrifugation failed to distinguish the enzymes and indicated a molecular weight of $1.9 \cdot 10^5$ for both. The enzymes can be assayed individually, even when present in the same crude extract, using the 67-fold difference in their K_m values for coenzyme B-12. For both enzymes inactivation kinetics are observed with glycerol as substrate, and monovalent cations influence both the inactivation rate and catalytic rate of the reaction.

Introduction

Diol dehydratase (1,2-propanediol hydro-lyase, EC 4.2.1.28) was originally discovered [1] and characterized [2,3,4] in extracts of *Klebsiella pneumoniae* ATCC 8724 grown anaerobically with propan-1,2-diol; the enzyme requires coenzyme B-12 (Coα-[α-(5,6-dimethylbenzimidazolyl)]-Coβ-adenosylcobamide). A glycerol dehydratase (glycerol hydro-lyase, EC 4.2.1.30) was found in [5] and purified from [6] *K. pneumoniae* ATCC 25955 (formerly *Aerobacter aerogenes* PZH 572, Warsaw) grown anaerobically in glycerol media. Both enzymes catalyse the conversion of ethan-1,2-diol, propan-1,2-diol or glycerol to acetal-

dehyde, propionaldehyde or 3-hydroxypropionaldehyde, respectively.

Although these two dehydratases have other features in common (e.g. irreversible inactivation of the holoenzyme by O_2 [7,8] or during the reaction with glycerol [4,8]), Toraya and Fukui [9] have more recently reported that the dehydratases are indeed different enzymes. They detected the diol enzyme immunochemically in *K. pneumoniae* ATCC 25955 after growth with propan-1,2-diol but not after growth with glycerol, and concluded that each substrate induces specifically its own dehydratase.

The resolution of the two dehydratases is described here, with a convenient method for their individual assay even when present together. In our experiments, glycerol induces both enzymes in strain 25955.

Methods

Organisms

Klebsiella pneumoniae ATCC 8724 and ATCC 25955, *Klebsiella aerogenes* NCIB 418 and *Citrobacter freundii* NCIB 3735 were grown in media containing per l of demineralized water: K_2HPO_4, 7 g; KH_2PO_4, 3 g; $(NH_4)_2SO_4$, 1.4 g; and $MgSO_4 \cdot 7H_2O$, 0.1 g. The indicated supplements were: Difco yeast extract, 10 g; glucose, 3 g; glycerol, 15 ml; (+)-L-arabinose, 3 g; propan-1,2-diol, 4 ml; and ethan-1,2-diol, 10 ml.

Growth was in full 1 l flasks or 10 l carboys, the medium being initially de-aerated by vigorous bubbling with N_2. The inoculum was 6 mg dry wt. per l from a culture growing logarithmically in the same medium; the culture was harvested after 24 h at 30° C.

Preparation of extracts

Cells were washed with 10 mM Tris, suspended in 10 mM potassium phosphate buffer (pH 8.2) containing 50 mM $(NH_4)_2SO_4$ and propan-1,2-diol (2% v/v), and subjected to ultrasound (approx. 25 kHz; 600 W) for 12 min in an ice-cooled stainless steel flask. The preparation was kept at pH 8.2 by adding 1 N KOH, then treated with active charcoal (Darco G60; 0.1 g/g dry wt. of organisms) for 15 min before centrifuging (31 000 × *g* for 45 min). The supernatant extract retained activity for several months at −15° C. Manipulations with extracts were at 0—4° C.

Dehydratase assays

Two methods were used to measure both enzymes using propan-1,2-diol as substrate. Incubations were at 37° C in dim red light using stoppered tubes.

In the less sensitive method using 2,4-dinitrophenylhydrazine, the enzyme was incubated in 0.4 ml of 25 mM potassium (or ammonium) phosphate buffer (pH 7.85) with 10 mM propan-1,2-diol and 12 μM coenzyme B-12. After 10 min, 0.44 ml of 2,4-dinitrophenylhydrazine (0.1% w/v) in 2 N HCl was added, then 0.4 ml of 5 N KOH 30 min later. After a further 30 min, the samples were centrifuged (10 000 × *g* for 4.5 min) and their absorbance read at 520 nm.

The more sensitive method was based on the reaction of aldehydes with 3-methylbenzo-2-thiazolone hydrazone [10]. The reaction mixtures (0.2 ml) contained the test sample in 40 mM ammonium *N*-2-hydroxyethylpiper-

azine-*N'*-2-ethane sulphonate buffer (pH 8.2) with 10 mM propan-1,2-diol and 12 μM coenzyme B-12. After 10 min the reaction was stopped with 0.1 ml of 375 mM glycine/HCl buffer (pH 2.7) containing 0.6 mg of the hydrazone. The tubes were heated for 3 min in a boiling water bath, cooled rapidly to room temperature then treated with 1 ml of 0.33% (w/v) $FeCl_3 \cdot 6H_2O$. After 30 min the absorbance at 670 nm was measured.

One unit of enzyme activity is defined as the amount that catalyses the formation of 1 μmol of propionaldehyde per min. The protein content of extracts was measured spectrophotometrically [11].

To follow the reaction with glycerol, the product was converted to acrolein and reacted with tryptophan [12]. The enzyme was incubated in 25 mM potassium (or ammonium) phosphate buffer (pH 7.85) containing 100 mM glycerol and 12 μM coenzyme B-12. At intervals, aliquots (0.2 ml) were removed into ice-cold ethanol (0.2 ml) to which was then added conc. HCl (0.8 ml), and DL-tryptophan (0.15 ml of 0.3% w/v solution in 2 N HCl). The mixture was heated at 60°C for 5 min and cooled to room temperature before reading its absorbance at 550 nm.

Polyacrylamide gel electrophoresis

The Tris/glycine system of Jovin [13] was used. The gels were made according to Chrambach et al. [14] so that the running gels contained 8% (w/v) acrylamide (2% crosslinked) and the two stacking gels 5% (w/v) acrylamide (20% crosslinked). The catalyst of polymerization was both ammonium persulphate (150 μg/ml) and riboflavin (5 μg/ml). Gels were prepared in 5 mm diameter tubes, using 1 ml of running gel and 0.2—0.25 ml of stacking gel, and all solutions used contained propan-1,2-diol (1% v/v).

Samples were diluted with the electrode buffer and sucrose added (5% w/v), so that the volume (10—50 μl) layered on each gel contained 0.03—0.07 units of activity. A trace of bromophenol blue was the tracking dye. The electrophoresis was at 0—4°C, using 2.5 mA per gel, until the dye had moved 42 mm into the running gel. Gels were rimmed with distilled water, frozen in liquid N_2 and cut into 0.5 mm slices with a Mickle gel slicer.

Each slice was shaken for 5 min at 0°C in 1 ml of 10 mM Tris buffer (pH 8.5) then its enzyme content assayed by the more sensitive method above. The total enzyme activity measured in the slices was at least 90% of that applied to the gel.

Results are plotted as activity per slice (in terms of absorbance at 670 nm) against mobility relative to bromophenol blue (R_B).

Sucrose density gradient centrifugation

This was according to Martin and Ames [15] using a linear 5—20% (w/v) sucrose gradient in 100 mM Tricine (*N*-tris(hydroxymethyl)methylglycine) adjusted to pH 8.2 with KOH, and containing 7 mM 2-mercaptoethanol. Tubes containing 5.1 ml total volume were centrifuged at 114 000 × *g* for 16 h in the SW50.1 rotor of a Beckman Spinco model L2-65B centrifuge. Fractions (0.15 ml) were taken from the top using an ISCO model 185/568 fraction collector fitted with the UA5 absorbance monitor.

Results

Polyacrylamide gel electrophoresis

A crude extract of *K. pneumoniae* ATCC 8724 grown in glycerol medium was used as an unambiguous source of diol dehydratase [3] for gel electrophoresis; it gave a single band of activity with mobility $R_B = 0.18$ (Fig. 1). Glycerol dehydratase was provided by a crude extract of *K. pneumoniae* ATCC 25955 grown and subsequently induced in a complex glycerol-containing medium [16]; this gave a major band of activity with $R_B = 0.30$ and only a trace of activity at the position of diol dehydratase.

In contrast an extract of strain 25955 grown in simple glycerol medium showed two comparable bands of activity corresponding with the two dehydratases (Fig. 1). Both enzymes were found, in approximately constant proportions, for cultures in middle or late logarithmic phases of growth; the total specific activity rose to a maximum in the stationary phase. A similar electrophoretic pattern was observed when the growth was in medium containing yeast extract and glycerol. Since glycerol induces both enzymes in this strain (unlike strain 8724 which forms only diol dehydratase), it was then of interest to test propan-1,2-diol as an inducer. However, this organism (and the others used here) did not grow with propan-1,2-diol as sole C source, either anaerobically or aerobically. Therefore it was cultured on yeast extract with propan-1,2-diol; the extract now showed only diol dehydratase on electrophoresis. This was also the only enzyme found when the organism was grown in fully defined medium with arabinose as C source and propan-1,2-diol or ethan-1,2-diol as inducer; no dehydratase could be detected after growth with glucose, yeast extract or arabinose without either propan-1,2-diol or glycerol.

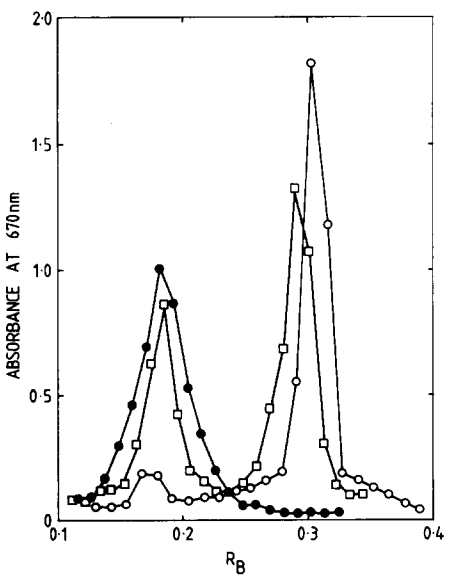

Fig. 1. Polyacrylamide gel electrophoresis of dehydratase extracts. Strain 8724 grown on glycerol medium (●); strain 25955 grown according to [16] (○) or on defined glycerol medium (□). In no case was activity detected at $R_B \leq 0.1$ or ≥ 0.4.

TABLE I

DEHYDRATASE ACTIVITIES OF *KLEBSIELLA* STRAINS GROWN WITH DIFFERENT CARBON SOURCES

Values are the specific activities (units/mg of protein) of extracts prepared as described under Methods and assayed with propan-1,2-diol using the 2,4-dinitrophenylhydrazine method. Symbols in parentheses refer to the forms of dehydratase found by gel electrophoresis: D = diol dehydratase (R_B = 0.18 ± 0.01, 10 determinations); G = glycerol dehydratase (R_B = 0.29 ± 0.01, 8 determinations).

Strain number	Carbon source			
	Glycerol alone	Yeast extract + glycerol	Yeast extract + propan-1,2-diol	Arabinose + propan-1,2-diol
ATCC 8724	0.21 (D)	0.05 (D)	0.26 (D)	
ATCC 25955	1.45 (G + D)	0.06 (G + D)	0.45 (D)	0.47 (D) *
NCIB 418	0.63 (G)			<0.02 **

* Identical results were obtained when ethan-1,2-diol replaced the propan-1,2-diol in the growth medium.

** Insufficient activity for gel electrophoresis.

Electrophoresis showed that *K. aerogenes* NCIB 418 growing in glycerol medium formed only glycerol dehydratase; growth on arabinose plus propan-1,2-diol gave insignificant activity. An extract of *Citrobacter freundii* grown with glycerol had both dehydratases, in similar proportion to that found for the corresponding extract of *K. pneumoniae* ATCC 25955.

Table I summarizes the form(s) and total specific activity of dehydratase found in extracts of the different organisms after growth in the various media. The overall ability of the extracts to dehydrate propan-1,2-diol varies markedly. In particular, when the medium contains yeast extract, glycerol behaves as only a weak inducer of the enzyme(s) compared with propan-1,2-diol.

Ion-exchange chromatography

Since the dehydratases resolve readily on electrophoresis they could be sufficiently dissimilar in charge to give a simple separation on ion-exchange chromatography.

K. pneumoniae ATCC 25955 grown in glycerol medium provided a convenient source of both dehydratases. The extract (12 mg of protein/ml) was first partially purified by treatment with protamine sulphate (20 mg/ml in 10 mM potassium phosphate buffer, pH 8.2, containing 50 mM ammonium sulphate and 2% (v/v) propan-1,2-diol), using 0.6 ml solution/ml of extract. The suspension was stirred for 30 min, and centrifuged at 3500 × *g* for 40 min; to the supernatant solution, ammonium sulphate was added (0.3 g/ml) while the pH was maintained at pH 8.2 by adding 2 N KOH. The pellet obtained by centrifuging at 25 000 × *g* for 60 min was then triturated with 100 mM Tricine buffer (pH 8.2) containing 2% (v/v) propan-1,2-diol (buffer 1), using 2 ml per 100 ml of initial crude extract. After 1 h, this ammonium sulphate fraction was again centrifuged (25 000 × *g* for 20 min). After dialysis against three 600-ml lots of buffer 1, to remove ammonium sulphate, the supernatant solution usually contained only 20% of the protein of the initial crude extracts but at

least 60% of the total dehydratase activity. The proportion of the two enyzmes, estimated by electrophoresis, was unchanged.

In a trial experiment, a sample (2 ml) of the ammonium sulphate fraction was brought to 60 mM phosphate, by adding 0.5 ml of buffer 1 containing 300 mM potassium phosphate, then fractionated on a column of DEAE-cellulose (Fig. 2). Gel electrophoresis of the two most active fractions representing the two peaks of enzymic activity in the eluant, showed that the material passing directly through the column had only diol dehydratase; the glycerol dehydratase was held by the column until eluted free from diol dehydratase by the buffer with 150 mM potassium phosphate.

In subsequent separations on a larger scale, buffer 1 containing 0.5 M KCl was used to elute glycerol dehydratase in a smaller volume.

The two enzymes were further purified by pooling the appropriate fractions and chromatographing them separately on a column (54 cm × 14.5 cm^2) of Sephadex G-200 (Pharmacia, U.K., Ltd.) using buffer 1. In each case, the more active fractions were combined. At this stage the diol dehydratase had 8 units/mg of protein, and the glycerol dehydratase 13 units/mg of protein. The enzymes were concentrated by precipitation with 2 vols. of saturated

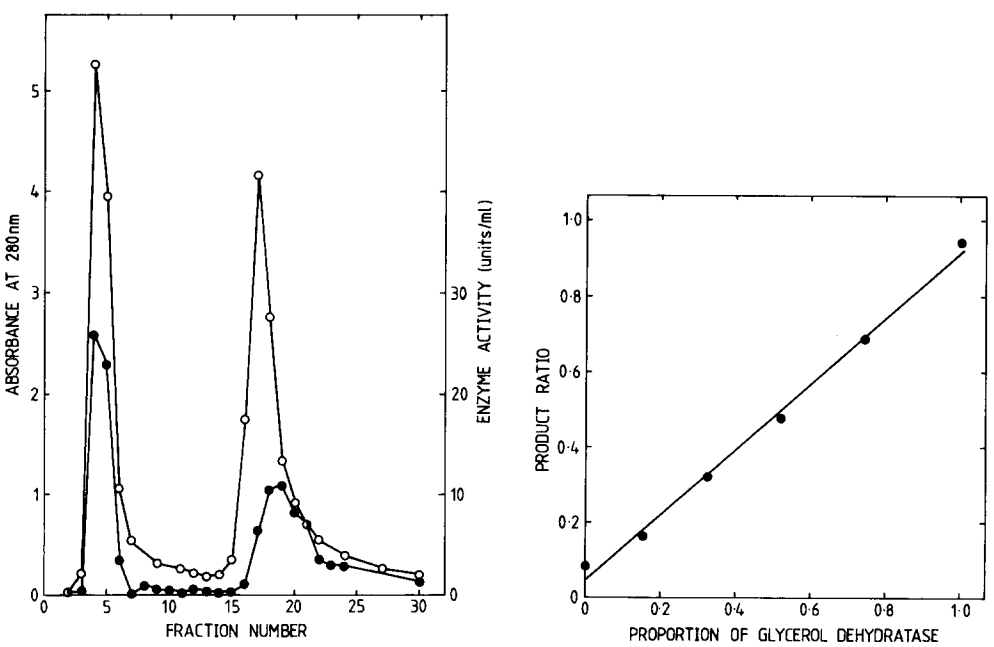

Fig. 2. Separation of dehydratases with DEAE-cellulose, A sample of partially purified extract of strain 25955 (grown on glycerol medium) as described in the text was applied to a column (7 cm × 1.8 cm^2) of Whatman DE52-cellulose equilibrated with buffer 1 containing 60 mM potassium phosphate, and washed through with 25 ml of the same buffer. The column was then eluted with buffer 1 containing 150 mM potassium phosphate. Dehydratase in each fraction (2.2 ml) was assayed using the 2,4-dinitrophenylhydrazine method (•). Absorbance at 280 nm (○).

Fig. 3. Differential assay of mixtures of diol and glycerol dehydratases. Admixtures of the two purified dehydratases were assayed in parallel by the 2,4-dinitrophenylhydrazine method using 12 μM and 0.12 μM coenzyme B-12. Results are plotted as the ratio of the product obtained with 0.12 μM coenzyme B-12 to that with 12 μM coenzyme, against the known proportion of glycerol dehydratase in the mixture.

ammonium sulphate solution. After 60 min, the suspensions were centrifuged (30 000 × g for 60 min) and the pellets dissolved in buffer 1 before dialysing against two 500 ml lots of the same buffer.

Differential assay of dehydratases

Although the complete electrophoretic resolution of the enzymes affords a means of estimating them, the technique is relatively tedious and subject to the cumulative errors of assaying of many slices. Therefore we sought a more convenient and accurate method for separate assay of the enzymes.

The apparent K_m for coenzyme B-12 of purified diol dehydratase of *K. pneumoniae* ATCC 8724 has been reported as $6 \cdot 10^{-7}$ M when propan-1,2-diol was used as substrate [17]. But the K_m for coenzyme B-12 of purified glycerol dehydratase of strain 25955 has been measured as only $2 \cdot 10^{-8}$ M [18].

We have measured the apparent K_m values for the two separated dehydratases of strain 25955 using propan-1,2-diol as substrate and K^+ as cofactor. Double reciprocal plots gave K_m values of $1.4 \cdot 10^{-6}$ M and $2.1 \cdot 10^{-8}$ M for the diol dehydratase and glycerol dehydratase respectively.

Therefore if total dehydratase activity with propan-1,2-diol is assayed using 12 μM coenzyme B-12, then both enzymes act maximally. However, in parallel assays using only 0.12 μM coenzyme B-12 the diol and glycerol dehydratases operate at about 4% and 95% of their respective maximum values. Hence by difference the individual activities can be derived. Fig. 3 shows the results obtained by this procedure using known admixtures of the separated and purified enzymes.

This method applied to a crude extract of *K. pneumoniae* ATCC 25955 (grown in defined glycerol medium), indicated 64% glycerol dehydratase, which agrees with the 59% obtained for the same extract by summing the activities of slices in an electrophoretic analysis. Assay of the slices from a duplicate gel using only 0.12 μM coenzyme B-12 confirmed that the band with greater mobility is glycerol dehydratase.

As an example of its value, the differential assay was used to compare the sedimentation of the two purified enzymes in a sucrose gradient. The enzymes could not be resolved when centrifuged together. Comparison separately or together with yeast alcohol dehydrogenase as a marker of molecular weight $1.5 \cdot 10^5$ [19] showed that both dehydratases have a molecular weight of $1.9 \cdot 10^5$.

Activity of the enzymes with glycerol

The dehydratases progressively become inactivated during the reaction with glycerol. The inactivation follows first order kinetics, the rate constants for inactivation (k_{in}) at 37° C with K^+ being reported as 0.35 min^{-1} for strain 25955 glycerol dehydratase [20], and k_{in} = 1.30 min^{-1} for strain 8724 diol dehydratase [21].

We have compared the kinetics of the glycerol reaction for the two enzymes under the same conditions and prepared from the same strain, ATCC 25955. When the data for diol dehydratase (Fig. 4a) are plotted according to Poznanskaya et al. [20], i.e. assuming first order inactivation, a k_{in} of 0.63 min^{-1} at 37° C is found irrespective of whether the cofactor is K^+ or NH_4^+. How-

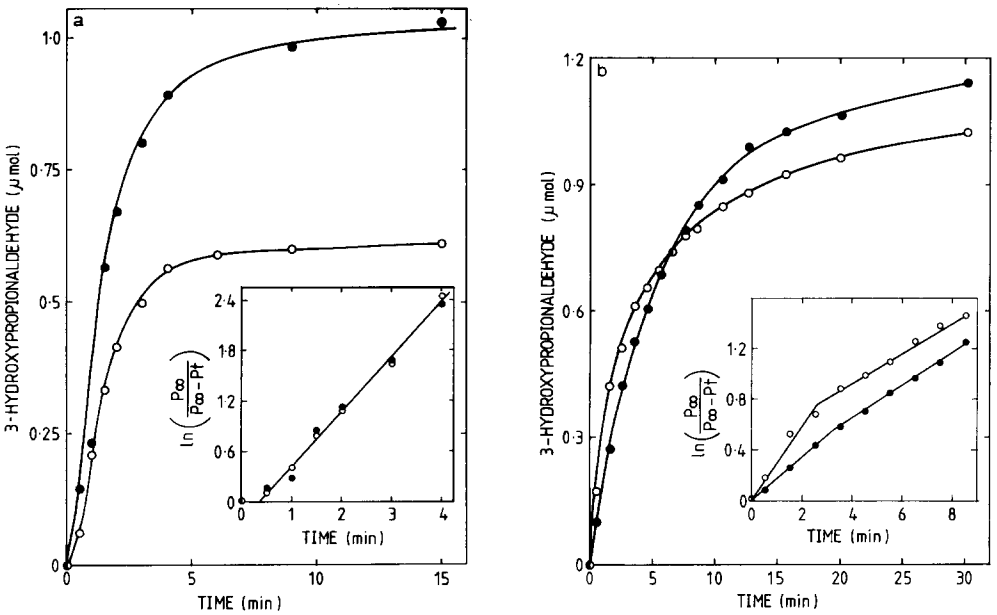

Fig. 4. Progress of the reaction with glycerol using (a) purified diol dehydratase, 0.54 units and (b) purified glycerol dehydratase, 0.09 units, after dialysis against 100 mM triethanolamine-HCl buffer, pH 8.2. The reactions (see Methods) were in either K^+ buffer (○) or NH_4^+ buffer (●). Insets show the data plotted according to [20], where P_∞ is the final amount of product and P_t is the amount after t min.

ever, the calculated initial reaction velocity, v_0, is 1.6-fold higher with NH_4^+ than with K^+. With either ion, the glycerol reaction shows a lag period of about 20 s.

In contrast, using glycerol dehydratase, no lag is seen and the monovalent cation affects both v_0 and k_{in} (Fig. 4b). With NH_4^+, v_0 is now 1.7-fold less than with K^+, but during the first 2 min of reaction k_{in} is 0.17 min^{-1} with NH_4^+ and 0.35 min^{-1} with K^+, i.e. not only different but also significantly less than k_{in} for diol dehydratase. At later times, the inactivation rates decrease with either K^+ (to k_{in} = 0.11 min^{-1}) or NH_4^+ (to k_{in} = 0.13 min^{-1}).

Discussion

During glycerol fermentation, strain 8724 forms only diol dehydratase, strain 418 only glycerol dehydratase but strain 25955 forms both simultaneously. It is not clear why diol dehydratase was not previously detected by immunoprecipitation in this last strain (grown in a complex glycerol medium) using antibody against purified diol dehydratase of strain 8724 [19]; it may relate to the very small amount of this enzyme found in extracts of cultures in similarly complex media (Fig. 1, Table I). The diol enzyme was precipitated this way from extracts of a culture grown with propan-1,2-diol; our results confirm that the activity here is indeed diol dehydratase, and that propan-1,2-diol is not an inducer for glycerol dehydratase in either strain 25955 or strain 418.

The identical behaviour of the two enzymes during sucrose gradient centrifugation reinforces their many similarities noted previously [21]. The apparent molecular weight of $1.9 \cdot 10^5$ agrees well with the values of $1.88 \cdot 10^5$

reported for the aquocobalamin-apoenzyme complex of glycerol dehydratase [16], and $2.1 \cdot 10^5$ calculated for the native apoenzyme [22]. On the other hand a significantly higher molecular weight of $2.6 \cdot 10^5$ has been claimed for diol dehydratase from strain 8724 [23].

Despite the similarity in size, the good electrophoretic resolution and separation of the two enzymes by anion exchange chromatography shows that there are important structural differences between them. It is not surprising, therefore, that their interaction with small ligands should vary, e.g. the 67-fold difference in K_m for coenzyme. Although the K_m values for glycerol are similar, 1.6 mM for the diol enzyme [21] and 1.5 mM for the glycerol enzyme [24], the kinetics with this substrate are very different and depend on the monovalent cation used. For strain 25955, the inactivation of the diol enzyme is significantly faster than that of the glycerol enzyme. However, the difference between these two is actually smaller than that between the inactivation rate ($k_{in} = 0.63\ min^{-1}$) found here for the diol enzyme of strain 25955 and that reported [21] for the same enzyme of strain 8724 ($k_{in} = 1.30\ min^{-1}$). This suggests that there are subtle strain differences within at least diol dehydratase.

The inactivation by glycerol of the glycerol dehydratase seems especially complex, although mechanistically it may well occur predominantly by reaction of the holoenzyme with (*S*)-glycerol as shown for diol dehydratase [21]. The cation affects both product formation and inactivation, i.e. K^+ favours both processes compared with NH_4^+. In practice this means that the apparent relative activities of cations in product formation will vary with the reaction time. Furthermore, inactivation of glycerol dehydratase appears to proceed by more than one mechanism since a decreased inactivation rate obtains at later reaction times.

The physiological role of glycerol dehydratase may be to produce a preferred electron acceptor during glycerol fermentation [28]. But there is no obvious distinctive role for the isozymic diol dehydratase, when both enzymes are present in the same strain; the puzzling relationship between the two is only emphasized by the apparent ability of strains 8724 and 418 each to produce only one dehydratase. The specific induction of diol dehydratase by propan-1,2-diol or ethan-1,2-diol is also remarkable since these alcohols are not fermented by strain 25955, nor does there appear to be any advantage in their formation from other fermentable compounds.

The differences between propan-1,2-diol and glycerol as inducers strongly suggest that the synthesis of the two enzymes are under separate control; the ability to assay the enzyme individually within a mixture will facilitate investigation of this. Furthermore, we have been able to isolate mutants of strain 25955 producing either enzyme constitutively (unpublished data).

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