Reduction of acetaldehyde to ethanol by some micro-organisms and its stereospecificity

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The stereochemical course of the reduction of acetaldehyde to ethanol was investigated by evaluating, with the enzymic system yeast alcohol dehydrogenase/diaphorase and g.c.–m.s., the configuration of [1-²H]-ethanol obtained from [1-²H]acetaldehyde with different micro-organisms. Although only *S*-[1-²H]ethanol was formed, all the micro-organisms showed evidence of the existence of alcohol dehydrogenases with opposite stereospecificity.

INTRODUCTION

Enzyme stereospecificity is not limited to the reaction of chiral molecules for which the steric course of the transformation is evident from the configuration of the substrate or product. Some biochemical reactions take place at centres that are not chiral but are, however, still stereospecific although their steric course is not evident to the observer; for this kind of reaction the name 'stereochemically cryptic' has been proposed (Hanson & Rose, 1974). The stereospecificity of these reactions can be deduced by isotope labelling, which converts the reaction centre into a chiral centre.

The ethanol–acetaldehyde oxidation–reduction is a stereochemically cryptic reaction, and studies of alcohol dehydrogenase (ADH, EC 1.1.1.1) from *Saccharomyces cerevisiae* have shown that this enzyme introduces and removes stereospecifically the pro-*R*-hydrogen atom (Loewus *et al.*, 1953; Lemieux & Howard, 1962):

The same stereospecificity was also observed for ADH from horse liver [cited in Arigoni & Eliel (1969) as unpublished work by A. Umani-Ronchi, H. Webber & D. Arigoni] and for whole cells of *Candida utilis* (Günther *et al.*, 1973a).

Because the stereochemical course of the acetaldehyde reduction has no influence on the physiological–metabolic activity of the produced achiral ethanol, the existence of ADH with different stereospecificity could be expected in other organisms and could possess some phylogenetic and taxonomic significance. Consequently the steric course of the reduction of acetaldehyde to ethanol was studied in different micro-organisms; the configuration of [1-²H]ethanol formed from [1-²H]-acetaldehyde reduction with cells or cell extracts was determined by an enzymic system with yeast ADH/diaphorase (EC 1.6.4.3) and g.c.–m.s. Preliminary work was performed to screen micro-organisms able to reduce acetaldehyde as resting cells, because this ability cannot

be presumed for every fermenting micro-organism and can even be found in non-fermenting species (van Dijken & Scheffers, 1984).

EXPERIMENTAL

Micro-organisms and growth conditions

A preliminary screening was performed with 70 strains of yeasts, moulds, higher fungi and bacteria from the genera *Ashbya*, *Aureobasidium*, *Brettanomyces*, *Candida*, *Dekkera*, *Endomycopsis*, *Geotrichum*, *Hansenula*, *Kloeckera*, *Cluyveromyces*, *Pachisolen*, *Pichia*, *Rhodotorula*, *Saccharomyces*, *Saccharomycodes*, *Saccharomycopsis*, *Schizosaccharomyces*, *Sporobolomyces*, *Torulopsis*, *Zygosaccharomyces*, *Allescheria*, *Aspergillus*, *Dactilomyces*, *Fusarium*, *Humicola*, *Malbranchea*, *Myriococcum*, *Mucor*, *Nectria*, *Neurospora*, *Penicillium*, *Rhizopus*, *Bolletus*, *Lepiota*, *Morchella*, *Pholiota*, *Polyporus*, *Bacillus*, *Clostridium*, *Escherichia*, *Lactobacillus*, *Leuconostoc*, *Micrococcus*, *Proteus*, *Pseudomonas* and *Zymomonas*. Yeasts and mesophilic moulds were cultured in flasks with a growth medium containing 2% glucose, 0.5% (NH₄)₂SO₄, 0.1% K₂HPO₄, 0.1% Difco yeast extract (Difco, Detroit, MI, U.S.A.) and 0.02% MgSO₄, pH 5.8. The cultures were incubated for 24–48 h on a reciprocating shaker (100 strokes/min) at 26–28 °C. Thermophilic moulds and higher fungi were cultured in potato/glucose/yeast extract medium (Craveri *et al.*, 1964) and incubated respectively at 45 °C and 26 °C for 48–72 h on a reciprocating shaker (100 strokes/min).

Bacteria of the genera *Bacillus*, *Escherichia*, *Micrococcus*, *Proteus* and *Pseudomonas* were cultured in Difco Nutrient Broth, and *Zymomonas mobilis* was cultured in yeast extract/glucose medium (Swings & DeLey, 1977); all the flasks were incubated at 30 °C on a reciprocating shaker (100 strokes/min).

Bacillus thermoruber and *Bacillus stearothermophilus* were cultured in a medium containing 2% crude maltose, 0.8% soya-bean meal and 0.3% Difco yeast extract, pH 7, and the flasks were shaken respectively at 45 °C and 55 °C for 18 h.

Lactic bacteria were grown in Man–Rogosa–Sharpe medium (deMan *et al.*, 1960) for 24 h at 26 °C, 37 °C or

Abbreviation used: ADH, alcohol dehydrogenase.

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45 °C according to the species; *Lactobacillus sanfrancisco* was cultured in a medium containing 2% crude maltose, 1.8% Difco yeast extract, 0.6% Difco Bacto Tryptone and 0.5% Tween 80, pH 5.6, and incubated at 30 °C for 24 h.

Clostridium bifermentans and *Clostridium sordellii* were grown in Todd-Hewitt Broth (Biolife, Milan, Italy) and incubated at 37 °C in an anaerobe glove-cabinet (Forma Scientific, Marietta, OH, U.S.A.) in which the air was replaced by an atmosphere of N₂/H₂/CO₂ (17:2:1). The cells were harvested after 13–15 h, before sporulation.

[1-²H]Acetaldehyde

[1-²H]Acetaldehyde was prepared by the reduction of butane-2,3-dione with NaB²H₄ and by H₅IO₆ oxidation of the butane-2,3-[²H₂]diol obtained.

A 300 mg portion of butane-2,3-dione was dissolved in 6 ml of distilled water, and then 30 mg of NaB²H₄ was added. After 5 min at room temperature with magnetic stirring, the reaction mixture was acidified to pH 3 with 1 M-H₂SO₄, and 880 mg of H₅IO₆ was added. After 10 min the [1-²H]acetaldehyde was distilled off under N₂ stream and collected in 3 ml of cold water until we obtained 40 mg of [1-²H]acetaldehyde/ml, which corresponds to a 55% total yield. The deuterium percentage, measured by g.c.-m.s., was found to be higher than 95%.

Transformation of [1-²H]acetaldehyde by resting cells

Transformation of [1-²H]acetaldehyde was carried out with resting cells. Fresh cells from submerged or stationary cultures were collected by centrifuging and washed twice with 0.1 M-sodium phosphate buffer, pH 5.6 in experiments with eumycetes and lactic bacteria and pH 7.0 in experiments with the other schizomycetes. The cells were suspended in the same buffer (15 mg dry wt. of cells/ml for eumycetes and 1.5 mg dry wt. of cells/ml for schizomycetes), and 2 mg of [1-²H]acetaldehyde/ml was added (5 ml total volume).

To assess the ability of the tested strains to produce endogenous ethanol or to ferment glucose, parallel experiments were carried out by incubating respectively the cell suspension alone and the cell suspension in the presence of glucose (10 mg/ml). Both suspensions were incubated for 48 h under limited O₂ supply in carefully closed vials and at the growth temperature of each strain, then they were assayed for ethanol production (by g.c.) and, after addition of 100 mg of NaHSO₃, they were distilled to isolate it.

Transformation of [1-²H]acetaldehyde by cell extracts

Transformation of [1-²H]acetaldehyde was performed by *Bacillus stearothermophilus*, *Lactobacillus sanfrancisco* and *Zymomonas mobilis* with use of the cell extracts. Fresh cells from cultures were harvested, washed twice with 0.1 M-sodium phosphate buffer, pH 7 (pH 5.6 for *L. sanfrancisco*), and resuspended in the same buffer (600 mg dry wt. of cells in 3 ml of buffer), and then they were broken up by a double passage at 83 MPa (12000 lbf/in²) through a French press (Wabash Hydraulic Press; Metal Products Co., Wabash, IN, U.S.A.) at 4 °C.

To the cell extract, maintained at 4 °C, 1000 units of deoxyribonuclease (2000 units/mg, from bovine pancreas; Boehringer, Mannheim, West Germany) were added; after 10 min the extract was diluted 1:10 with the buffer and centrifuged at 18000 g for 30 min at 4 °C, and

the supernatant was again diluted to a protein concentration of 0.8 mg/ml determined by the Lowry method. The solution was then dialysed at 4 °C for 24 h against 0.1 M-sodium phosphate buffer, pH 7 (pH 5.6 for *L. sanfrancisco*). Transformations of [1-²H]acetaldehyde were performed by cell extracts in the conditions described above and in the presence of NADH (1.1 mol/mol of substrate; Boehringer).

Determination of the configuration of [1-²H]ethanol

The configuration of [1-²H]ethanol formed from microbial reduction of [1-²H]acetaldehyde was determined by removing the hydrogen (deuterium) atom from the pro-*R* position at C-1 of ethanol with yeast ADH/NAD diaphorase (Günther *et al.*, 1973a,b) and evaluating the residual deuterium percentage by g.c.-m.s. To 0.5 ml of the aqueous solution containing 2–5 mg of [1-²H]ethanol from distillation of the reaction mixture were added 12 units of yeast ADH (400 units/mg of enzyme protein; Boehringer), 4 units of diaphorase (80 units/mg of enzyme protein; Boehringer) and 3.2 mg of NAD⁺ (Boehringer). After incubation at 35 °C for 72 h the deuterium percentage in ethanol was determined by g.c.-m.s. by the ratio:

$$\frac{I_{32}}{I_{31} + I_{32}}$$

where *I*₃₁ and *I*₃₂ are the intensity of the fragmentation peaks 31 *m/z* (CH₂=OH) and 32 *m/z* (CH²H=OH) respectively. With this method the minimal detectable amount of 1-²H was 5%.

Gas chromatography-mass spectrometry

G.c.-m.s. analyses were performed on a Finnigan 4021 instrument with the SuperINCOS data system. Conditions were as follows: 2000 mm × 2 mm (internal diam.) Porapak Q (80–100 mesh) directly coupled to the ion source via a micro capillary shut-off valve; helium flow 30 ml/min; oven temperature 200 °C; injector temperature 250 °C; ionizer temperature 250 °C; electron energy 70 eV; emission current 0.25 mA; electron multiplier 1500 V; pre-amplifier sensitivity 10⁻⁷ A/V (10⁻⁶ A/V for control ethanol). Spectra were recorded over the range 20–200 atomic mass units in 0.55 s ± 0.05 s.

Analytical methods

Butane-2,3-dione, butane-2,3-diol, acetaldehyde and ethanol, during the preparation of [1-²H]acetaldehyde and its transformation into ethanol, were checked by g.c. with a Carlo Erba Fractovap G1 chromatograph fitted with a flame ionization detector and a 1500 mm × 4 mm (internal diam.) glass column of Porapak Q (80–100 mesh). For ethanol and acetaldehyde the column temperature was 170 °C, the injection temperature 230 °C and the N₂ flow 50 ml/min. For the determination of butane-2,3-dione and butane-2,3-diol the column temperature was 230 °C, the injection temperature 270 °C and the N₂ flow 75 ml/min.

Chemicals

All reagents were of analytical grade. NaB²H₄ (99 atoms % ²H) was from Fluka, Buchs, Switzerland.

RESULTS AND DISCUSSION

The ability to reduce acetaldehyde was evaluated for 70 strains of schizomycetes and eumycetes, including

Table 1. Reduction of [1-²H]acetaldehyde by several micro-organisms, and deuterium percentage of the ethanol obtained after isolation (a) and after enzymic removal of the pro-*R*-hydrogen atom (b)

Resting cells of the micro-organisms were incubated (15 mg dry wt. of cells/ml for eumycetes and 1.5 mg dry wt. of cells/ml for bacteria) in the presence of [1-²H]acetaldehyde (2 mg/ml) for 48 h at the growth temperature of each. The production of ethanol was evaluated by g.c. and is expressed as a percentage of the stoichiometric transformation. For some strains, indicated with an asterisk, the [1-²H]acetaldehyde reduction was performed with the cell extract. Deuterium percentage was evaluated by g.c.-m.s. on ethanol after distillation and after the enzymic treatment. Key to culture collections: A.T.C.C., American Type Culture Collection, Rockville, MD, U.S.A.; C.B.S., Centraalbureau voor Schimmelcultures, Baarn, The Netherlands; C.C.Y., Czechoslovak Collection of Yeasts and Yeast-Like Organisms, Bratislava, Czechoslovakia; M.I.M., Microbiologia Industriale, Milano, Italy; N.C.I.B., National Collection of Industrial Bacteria, Aberdeen, U.K.; N.C.T.C., National Collection of Type Cultures, London, U.K.; W.F.P.L., Western Forest Products Laboratories, Vancouver, B.C., Canada.

Micro-organism	Ethanol from acetaldehyde	² H (%) (a)	² H (%) (b)
<i>Ashbya gossipii</i> M.I.M.	15 %	71	70
<i>Candida boidinii</i> C.B.S. 2428	40 %	86	88
<i>Candida utilis</i> C.B.S. 621	50 %	88	89
<i>Dekkera intermedia</i> C.B.S. 2796	75 %	85	83
<i>Endomycopsis fibuligera</i> C.C.Y. 42.3.1	30 %	69	69
<i>Hansenula glucozyma</i> C.B.S. 5766	35 %	82	83
<i>Pachisolen tannophilus</i> C.B.S. 4044	70 %	76	77
<i>Saccharomyces cerevisiae</i> C.B.S. 1171	40 %	70	71
<i>Sporobolomyces albo-rubescens</i> C.B.S. 482	15 %	76	74
<i>Sporobolomyces singularis</i> C.B.S. 5109	100 %	86	85
<i>Aspergillus niger</i> C.B.S. 626.26	30 %	72	71
<i>Fusarium lini</i> M.I.M.	60 %	86	85
<i>Fusarium semitectum</i> C.B.S. 190.60	20 %	74	76
<i>Nectria gliocladioides</i> M.I.M.	95 %	70	68
<i>Neurospora crassa</i> C.B.S. 239.37	20 %	69	67
<i>Polyporus tulipiferus</i> W.F.P.L. 103A	70 %	76	76
<i>Bacillus stearothermophilus</i> * N.C.T.C. 10003	40 %	86	83
<i>Clostridium sordellii</i> M.I.M.	15 %	85	86
<i>Lactobacillus sanfrancisco</i> * M.I.M.	95 %	84	83
<i>Pseudomonas putida</i> A.T.C.C. 4359	25 %	89	88
<i>Zymomonas mobilis</i> * N.C.I.B. 8938	80 %	83	82

species representative of physiological groups whose ability to produce ethanol from glucose has been recognized, excluded or is unknown (Doelle, 1975; Cochrane, 1976; Corry, 1978; Holt & Krieg, 1984; Kreger-van Rij, 1984; Holt & Sneath, 1986).

For many micro-organisms a behaviour consistent with their described fermenting or non-fermenting character was noticed. However, anomalous situations were also observed. The non-fermenting yeast *Sp. singularis* transformed acetaldehyde with a 100 % yield and was the fastest reducer of all the tested micro-organisms and the only one to give a quantitative reduction. On the other hand *Bac. stearothermophilus*, *Lb. sanfrancisco* and *Zm. mobilis*, of known fermentation ability, were unable to transform acetaldehyde to ethanol as resting cells; this reduction was carried out with cell extracts, giving yields of 40 % for *Bac. stearothermophilus*, 80 % for *Zm. mobilis* and 95 % for *Lb. sanfrancisco*.

Endogenous ethanol formation was generally not observed, except for *Endomycopsis fibuligera* and *Neurospora crassa*, which required a starvation period of 2 h at 28 °C in 0.1 M-sodium phosphate buffer, pH 5.8, before acetaldehyde reduction could be carried out without interference from the endogenous fermentation.

Subsequently, micro-organisms representative of different physiological groups and able to reduce the acetaldehyde in a good yield (Table 1) were used to convert [1-²H]acetaldehyde into [1-²H]ethanol, which was

then isolated by distillation. The deuterium percentage of ethanol evaluated by g.c.-m.s. ranged from 69 to 89 %, showing the presence of some unlabelled ethanol (Table 1).

The configuration of [1-²H]ethanol was determined by exchanging the hydrogen (deuterium) atom at the C-1 pro-*R* position with water hydrogen atoms by using yeast ADH/NAD diaphorase under equilibrating conditions. The concentrations of both enzymes and the reaction time were preliminarily established by using *RS*-[1-²H]ethanol (obtained by reducing acetaldehyde with NaB²H₄) as substrate. After the enzyme reaction, the deuterium present was 52 %, giving evidence of a 96 % exchange of ²H/¹H atoms at the pro-*R* position. When this enzymic exchange reaction was performed on [1-²H]-ethanol formed from microbial reduction of [1-²H]-acetaldehyde, a parallel determination on *RS*-[1-²H]-ethanol was always carried out as a control assay of the reaction efficiency and always showed 95–98 % exchange.

For all the tested micro-organisms the g.c.-m.s. of [1-²H]ethanol after pro-*R*-hydrogen (-deuterium) exchange treatment showed a retention of the deuterium atom at C-1, giving evidence of *S*-[1-²H]ethanol formation by the microbial reduction of [1-²H]acetaldehyde.

On the basis of this result the existence of different microbial enzymes showing an identical steric course in the reduction of acetaldehyde could be claimed, but this

possibility is invalidated by the presence of undeuterated ethanol found in amounts of up to 30%. It could derive from the fermentation of endogenous substrates, and this would be consistent with the hypothesis that only pro-*R* enzyme exists, or from the recycle of deuterated ethanol by ADHs with opposite stereospecificity. In fact, when *S*-[1-²H]ethanol from pro-*R* enzyme is dehydrogenated by pro-*S* enzyme (or *R*-[1-²H]ethanol is dehydrogenated by pro-*R* enzyme), undeuterated ethanol could result. An endogenous origin of undeuterated ethanol was ruled out because endogenous ethanol production in control experiments with cells suspended in buffer was not observed or occurred in amounts less than 0.1 mg/ml. Consequently the occurrence of undeuterated ethanol after microbial reduction of [1-²H]acetaldehyde provides indirect evidence of the existence, in the same micro-organism, of ADHs with opposite stereospecificity.

Considering the large range of microbial species tested and the consequent probable existence of different ADH isoenzyme systems, it is interesting to observe that the presence of pro-*S* enzymes never caused the formation of detectable amounts of *R*-[1-²H]ethanol. The absence of this enantiomeric form of [1-²H]ethanol could be explained by the prevalent activity of a 'fermentative' ADH and/or by different K_m values for ethanol of pro-*S* and pro-*R* ADH isoenzymes. In this regard, in microbial species such as *Sacch. cerevisiae* and *Zm. mobilis*, in which at least two ADHs with different K_m values and with opposite metabolic roles are known (Wills, 1976; Wills *et al.*, 1981), a different stereospecificity for the reductive and the oxidative enzymes could be hypothesized.

When this work started we hypothesized that (1) ADH with pro-*S* stereospecificity would be found in some microbial species or, more probably, (2) a stereochemical conservatism of ADH pro-*R* stereospecificity would occur, as is commonly accepted (Rose, 1972). Therefore the results obtained, suggesting the generalized presence of ADHs with opposite stereospecificity in every tested microbial species and showing an unequivocal kinetic course leading to the formation of only *S*-[1-²H]ethanol despite the existence of both enzymes, seem rather surprising, but further clarity would require a different experimental approach.

Obtaining more detailed information on stereoselectivity by isolating ADH isoenzymes in different microbial species needs hard and time-consuming work, but could be rewarding from the biochemical evolution point of view.

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