A study of the maloalcoholic fermentation pathway in *Schizosaccharomyces pombe*

Elisabetta MACONI,* Pier Luigi MANACHINI,* Fabrizio ARAGOZZINI,* Cesare GENNARI†
 and Giuliana Severini RICCA†

*Istituto di Microbiologia Agraria e Tecnica, Facoltà di Agraria, and †Istituto di Chimica Organica, Facoltà di Scienze, Università degli Studi di Milano, Milano, Italy

(Received 31 October 1983/Accepted 15 November 1983)

The pathway of the maloalcoholic fermentation in *Schizosaccharomyces pombe* was investigated by a ^1H -, ^2H - and ^{13}C -n.m.r.-spectroscopic study of hydrogen and deuterium distribution on the ethanol produced by *S. pombe* from L-malic acid in $^2\text{H}_2\text{O}$ and from L-[2- ^2H]malic acid. Our findings rule out a double-decarboxylation mechanism and agree with a pathway that involves acetaldehyde as intermediate.

Maloalcoholic fermentation, which mainly occurs in some yeasts of the genus *Schizosaccharomyces*, has been suggested as a means of removal of malic acid from musts and wines. This new interest has stimulated several studies on ecological, physiological and technological aspects of these yeasts (Riberau-Gayon & Peynaud, 1962; Mayer & Temperli, 1963; Peynaud *et al.*, 1964; Florenzano *et al.*, 1977). Nevertheless, experimental evidence for the mechanism of the degradation of L-malic acid to CO_2 and ethanol is still lacking. Mayer & Temperli (1963), by feeding oxaloacetic acid and pyruvic acid (the possible metabolic intermediates) to resting cells of *Schizosaccharomyces pombe*, obtained a significant amount of acetic acid and acetoin, not formed by feeding malic acid, and a lower production of ethanol. On the basis of this result, they formulated the hypothesis that malic acid is converted into ethanol by a direct 1,4-decarboxylation (Scheme 1, pathway *a*). Later, Temperli *et al.* (1965) isolated a decarboxylating L-malate dehydrogenase (malic enzyme, EC 1.1.1.38) from *S. pombe*, and Heer *et al.* (1976) demonstrated the existence of four different isoenzymes of malate dehydrogenase (EC 1.1.1.37) in the same yeast. Therefore the alternative pathway proceeding through pyruvate and acetaldehyde (Scheme 1, pathways *b* and *c*) cannot be excluded.

The present paper is concerned with a ^1H -, ^2H - and ^{13}C -n.m.r.-spectroscopic study of hydrogen distribution in the ethanol produced by resting cells of *S. pombe* from L-malic acid in $^2\text{H}_2\text{O}$, or from L-[2- ^2H]malic acid, in order to obtain some information about the mechanism of the maloalcoholic fermentation pathway.

Materials and methods

Organism and growth conditions

All experiments were performed with the strain *S. pombe* 4Z (Florenzano *et al.*, 1977). The organism was cultured on a medium containing 5% (w/v) malt extract and 0.1% (w/v) yeast extract. The flasks were incubated on a reciprocating shaker (100 strokes/min) at 28°C for 48 h.

Experiments with resting cells

In a standard procedure, fresh cells, harvested by centrifugation and washed twice with distilled water, were suspended (1.5 g dry wt. of cells) in 100 ml of 0.1 M-sodium citrate/0.2 M-sodium phos-

Scheme 1. Alternative pathways for maloalcoholic fermentation

phate buffer, pH 3.2, with 3.2 g of DL-malic acid. The cell suspension was incubated anaerobically at 25°C for 72 h. The transformation gave ethanol in 40% yield. For the transformation in $^2\text{H}_2\text{O}$, the cells were previously suspended in $^2\text{H}_2\text{O}$ at 5°C for 20 h, then collected and resuspended in a buffer prepared with $^2\text{H}_2\text{O}$.

Isolation of ethanol

After centrifugal separation of cells, ethanol was distilled on a Vigreux column as a binary azeotropic mixture. A 5 ml fraction (b.p. 78–100°C) was collected. This fraction was distilled again on a Fisher–Spaltrohr column, with collection of the fraction boiling at 79°C (0.2 ml), which contained 80% of the total amount of ethanol, as determined by g.l.c. analysis. The hydroalcoholic mixture was diluted with 20 ml of anhydrous dioxan and dried over K_2CO_3 overnight.

Ethyl 3,5-dinitrobenzoate

Ethanol was esterified with 3,5-dinitrobenzoyl chloride. 3,5-Dinitrobenzoyl chloride (1.2 g), and then 0.4 ml of anhydrous pyridine, were added to the previously described ethanol/dioxan mixture containing 160 mg of ethanol. The reaction mixture was stirred and heated at 90°C for 2 h, then quenched by adding 15 ml of aq. 5% (w/v) NaHCO_3 solution. The mixture was then extracted with chloroform, and the organic extracts were washed with acetic acid (5%, v/v) and water and evaporated under vacuum. The residue (725 mg) was purified by flash chromatography (Still *et al.*, 1978), by elution with chloroform/light petroleum (b.p. 40–70°C) (3:1, v/v). The ethyl 3,5-dinitrobenzoate obtained (507 mg) was crystallized from anhydrous acetone/light petroleum (m.p. 93°C).

[2- ^2H]Malic acid

DL-[2- ^2H]Malic acid was prepared by reduction of oxaloacetic acid with NaB^2H_4 . Oxaloacetic acid (7.23 g) was suspended in water (30 ml), the pH was adjusted to 8.5 with 1 M-NaOH at 5°C, and then NaB^2H_4 (572 mg) was added. After 24 h at 25°C, the reaction mixture was acidified to pH 2 with 2 M-HCl, and DL-[2- ^2H]malic acid was continuously extracted with diethyl ether for 5 days. The extracts were evaporated under vacuum, and the residue was dissolved in methanol and evaporated under vacuum several times to eliminate boric acid. Crude DL-[2- ^2H]malic acid was purified by ion-exchange chromatography on Dowex 1 (formate form) by elution with an aqueous solution of 0–6 M-formic acid (Bhattacharjee *et al.*, 1968), and crystallized from acetone/chloroform.

Resolution of DL-[2- ^2H]malic acid

Racemic [2- ^2H]malic acid was resolved by

salification with *R*(+)-1-phenylethylamine, as reported by Ingersoll (1943). The molar ratio between the reagents was modified by using 1.5 mol of *R*(+)-1-phenylethylamine per mol of DL-malic acid. L-Malic acid was obtained in 42% yield with an optical purity of 95.5%.

Recovery of untransformed [2- ^2H]malic acid

Untransformed [2- ^2H]malic acid was recovered from the reaction mixture by extraction with diethyl ether as described above. It was separated from the citric acid (from the buffer) by flash chromatography by elution with methanol/25% (w/v) NH_3 /water (44:3:3, by vol.). Ammonium malate was converted into malic acid by treatment with Amberlite IR-120 (H^+ form) resin.

Spectral analyses

^1H -, ^2H - and ^{13}C -n.m.r. spectra were recorded on a Varian XL-200 system operating in the Fourier transform mode, at 200.000, 30.701 and 50.288 MHz respectively. Chemical shifts are in p.p.m. (δ) from internal tetramethylsilane for C^2HCl_3 solutions for both carbon atoms and protons. Chemical shifts for ^2H -n.m.r. spectra for H_2O and dimethyl sulphoxide solutions were obtained by using C^2HCl_3 (in dilute CHCl_3 solution in a coaxial tube) as external standard (7.25 p.p.m.).

Direct ^2H -n.m.r.-spectroscopic studies

Transformation of L-[2- ^2H]malic acid was also monitored directly by ^2H -n.m.r. spectroscopy. In this experiment only the transformable L-isomer was used, to decrease the signal from untransformed malic acid. The resting-cell suspension (2 ml), prepared as described above, and L-[2- ^2H]malic acid (32 mg) were incubated at 25°C in an n.m.r. tube (10 mm diameter). ^2H -n.m.r. spectra were recorded every 2 h for a 72 h period.

Another experiment with unlabelled DL-malic acid was carried out by adding 5 μl of [$^2\text{H}_6$]ethanol to 2 ml of the reaction mixture in the n.m.r. tube after 24 h of transformation. ^2H -n.m.r. spectra were recorded every 24 h for 3 days.

Analytical methods

Ethanol production and isolation were checked by g.l.c. with a Carlo Erba Fractovap 2150 chromatograph fitted with a flame ionization detector and a 4 mm $\times$ 1500 mm glass column of Porapak Q (80–100 mesh). The column temperature was 200°C, the injector temperature 230°C, and the N_2 flow 50 ml/min. Acetone was used as internal standard. Optical rotations were measured with a Perkin–Elmer 141 polarimeter. All t.l.c. was performed on silica-gel plates (Kieselgel 60 F₂₅₄; Merck). Purification of ethyl 3,5-dinitrobenzoate

was monitored by t.l.c. with chloroform/light petroleum (b.p. 40–70°C) (3:1, v/v) as eluent and u.v. light as detector. Preparation and purification of DL-[2-²H]malic acid were followed by t.l.c. with methanol/25% NH₃/water (44:3:3, by vol.) as eluent and 0.1 M-KMnO₄ solution as detector.

Chemicals

All reagents were analytical grade. ²H₂O (99.75 atom% ²H) was from Merck, Darmstadt, Germany. [²H₆]Ethanol (99 atom% ²H) was from Merck, Sharp and Dohme, Montreal, Que., Canada. NaB²H₄ (99 atom% ²H) and *R*(+)-1-phenylethylamine were from Fluka, Buchs, Switzerland.

Results and discussion

Transformation of L-malic acid in ²H₂O

Ethanol obtained in the transformation of L-malic acid with resting cells of *S. pombe* in ²H₂O buffer was isolated and esterified as described above. The ¹H-n.m.r. spectrum of ethyl 3,5-dinitrobenzoate in C²HCl₃ solution shows, beyond signals at 9.18 and 9.26 p.p.m. (*J* = 1.5 Hz) due to three protons in the aromatic ring, two broadened signals at 4.4 p.p.m. and 1.42 p.p.m. in a 1:2 ratio. This is consistent with the presence of one deuterium atom on C-1 and one deuterium atom on C-2 of ethanol. This conclusion is confirmed by the proton-carbon-undecoupled ¹³C-n.m.r. spectrum showing a doublet of triplets at 62.63 p.p.m. (*J*_{C-H} = 150 Hz; *J*_{C-²H} = 22.7 Hz) attributed to C-1, coupled both with one proton and with one deuterium atom, and a triplet of triplets at 13.8 p.p.m. (*J*_{C-H} = 140 Hz; *J*_{C-²H} = 19.4 Hz) assigned to C-2, coupled with two protons and one deuterium atom.

These results give evidence for 100% 1,2-[²H₂]ethanol production. The ethyl 3,5-dinitrobenzoate obtained had $[\alpha]_D^{25} = -0.58$ (*c* 5.17 in CHCl₃). This result shows that the C-1 of ethanol, which is chiral as a result of isotopic substitution, was formed with some degree of enantioselectivity. [1,2-²H₂]Ethanol formation is consistent with the double-decarboxylation mechanism in which two carboxylic groups are removed with introduction of two protons (deuterons) from H₂O (²H₂O). This result is also compatible with the pathway involving acetaldehyde, assuming that deuterated NADH is not present in a significant amount to reduce acetaldehyde. Therefore this first experiment does not allow discrimination between the two possible mechanisms of the transformation.

Transformation of L-[2-²H]malic acid

DL-[2-²H]Malic acid was fed to resting cells of *S. pombe*. The ¹H-n.m.r. spectrum of ethyl 3,5-

dinitrobenzoate in C²HCl₃ solution obtained in this experiment shows (beyond signals at 9.20 p.p.m. corresponding to three protons in the aromatic ring) the following signals: a quartet at 4.4 p.p.m. (*J*_{C-H} = 6.5 Hz), a triplet at 1.46 p.p.m. (*J*_{C-H} = 6.5 Hz) and a doublet at 1.44 p.p.m. (*J*_{C-H} = 6.5 Hz). The multiplicity of the signals points out very clearly both partial substitution of a proton with a deuterium atom on C-1 (about 50%) and the absence of deuterium atoms on C-2 of ethanol. These attributions are confirmed by the proton-carbon-undecoupled ¹³C-n.m.r. spectrum, which shows two quartets at 14.29 p.p.m. and 14.20 p.p.m. (*J*_{C-H} = 125 Hz), due to the presence of two methyl groups, one triplet at 63.1 p.p.m. (*J*_{C-H} = 150 Hz), attributed to C-1 coupled with two protons, and one doublet of triplets at 62.72 p.p.m. (*J*_{C-H} = 150 Hz; *J*_{C-²H} = 22.6 Hz) assigned to C-1, coupled in this case with one proton and one deuterium atom.

This result shows that only 50% of ethanol from L-[2-²H]malic acid has one deuterium atom on C-1. To exclude the possibility that a partial loss of deuterium had occurred from [2-²H]malic acid before the transformation, untransformed malic acid was re-isolated from the reaction mixture. Its ¹H-n.m.r. spectrum showed the total retention of ²H at C-2. To exclude the possibility that the deuterium loss had occurred from ethanol after its formation, a direct ²H-n.m.r.-spectroscopic study of the transformation of L-malic acid was carried out by adding [²H₆]ethanol to the reaction mixture after 24 h. The ²H-n.m.r. spectra recorded every 24 h for 3 days showed that there was no ²H/¹H exchange at C-1 of the labelled ethanol. This finding rules out the possibility of a double decarboxylation of malic acid, because, according to this mechanism, 100% retention of ²H would occur at C-1 of ethanol. On the contrary, the simultaneous formation of deuterated and undeuterated ethanol from L-[2-²H]malic acid is consistent with pathway *b* or *c* (Scheme 1), assuming that acetaldehyde reduction can be performed by deuterated and undeuterated NADH respectively. A substantial enrichment in deuterated NADH, with a consequent high degree of deuteration of ethanol, should be expected in view of the initial oxidation of the large amount of L-[2-²H]malic acid (16 g/l). The observed ratio between deuterated and undeuterated ethanol (about 1:1) could be explained in terms of an isotopic effect and/or by competitive amounts of hydrogenated reducing substrates from the cells. Accordingly, in a further experiment carried out with a greater concentration of cells (30 mg dry wt. of cells/ml), only 35% of deuterated ethanol was found.

In order to assess the fate of deuterium removed from L-[2-²H]malic acid and not found in ethanol,

a direct ^2H -n.m.r.-spectroscopic study was performed, with recording every 2 h for 72 h of the ^2H -n.m.r. spectra of the whole reaction mixture containing L-[2- ^2H]malic acid. After 4 h, the signal from deuterium on C-2 of malic acid ($\delta = 4.46$ p.p.m.) began to decrease, and only the signal from deuterium on C-1 ($\delta = 3.56$ p.p.m.) of ethanol appeared. No other signal was observed between 0 and 30 p.p.m. Since signals from deuterated compounds present within the cells were not recorded because of cell sedimentation, a spectrum of the cells harvested after 48 h and suspended in dimethyl sulphoxide was recorded. This new spectrum showed three broad signals at 4.09, 4.77 and 8.83 p.p.m., giving evidence for the presence of unidentified endocellular hydrogen acceptors.

These results all strongly suggest that *S. pombe* produces ethanol by oxidation and decarboxylation of malic acid to pyruvic acid, successive decarboxylation and then reduction of acetaldehyde. Since the enzymes operating in this pathway are also commonly found in several yeasts not fermenting or weakly fermenting malic acid, the rapid metabolism of malic acid by *S. pombe* could be explained by the high substrate affinity of its malic enzyme (Temperli *et al.*, 1965) and by the possible existence of a carrier protein for malate

similar to the one that has been described for *Saccharomyces bailii* (Kuczynski & Radler, 1982).

We thank Professor G. Florenzano (Istituto di Microbiologia Agraria e Tecnica, Università degli Studi di Firenze, Firenze, Italy) for kindly providing *S. pombe* 4Z.

References

- Bhattacharjee, J. K., Maragoudakis, M. E. & Strassman, M. (1968) *J. Bacteriol.* **95**, 495–497
- Florenzano, G., Balloni, W. & Materassi, R. (1977) *Vitis* **16**, 38–44
- Heer, B., Brändi, E. & Fiechter, A. (1976) *Proc. Int. Ferment. Symp.* **5th** 167
- Ingersoll, A. W. (1943) *Org. Synth. Collect. Vol.* **2**, 506–509
- Kuczynski, J. T. & Radler, F. (1982) *Arch. Microbiol.* **131**, 266–270
- Mayer, K. & Temperli, A. (1963) *Arch. Mikrobiol.* **46**, 321–328
- Peynaud, E., Domerq, S., Boidron, A.-M., Lafon-Lafourcade, S. & Guimbertau, G. (1964) *Arch. Mikrobiol.* **48**, 150–165
- Riberau-Gayon, J. & Peynaud, E. (1962) *C. R. Séances Acad. Agric. Fr.* **48**, 558–563
- Still, W. C., Kahn, M. & Mitra, A. (1978) *J. Org. Chem.* **43**, 2923–2925
- Temperli, A., Künsch, U., Mayer, K. & Busch, I. (1965) *Biochim. Biophys. Acta* **110**, 630–632