Propionate metabolism in *Saccharomyces cerevisiae*: implications for the metabolon hypothesis

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Aerobic, glucose-limited chemostat of *Saccharomyces cerevisiae* CBS 8066 co-metabolized propionate when this compound was added to the reservoir medium. Co-metabolism of propionate led to an increase of the biomass and protein yields. Attempts to grow *S. cerevisiae* on propionate as a sole source of carbon and energy were not successful. Activities of propionyl-CoA synthetase in cell-free extracts were sufficient to account for the rates of propionate consumption observed in the chemostat cultures. Activities of propionyl-CoA carboxylase, a key enzyme of the methylmalonyl-CoA pathway of propionate metabolism, were negligible. In contrast, activities of 2-methylcitrate synthase, a key enzyme activity of the 2-methylcitrate pathway of propionate metabolism, increased substantially with increasing propionate-to-glucose ratios in the reservoir media, and were sufficient to account for the propionate consumption rates observed in the chemostat cultures. This suggested that the 2-methylcitrate pathway is the major pathway of propionate metabolism in *S. cerevisiae*. In the literature, labelling patterns observed after incubation of this yeast with [3-¹³C]propionate have been interpreted as evidence for channelling of tricarboxylic acid (TCA) cycle intermediates, possibly as a consequence of the organization of TCA cycle enzymes in a metabolon. However, this interpretation of ¹³C-labelling patterns rested on the assumption that propionate metabolism in *S. cerevisiae* occurs via the methylmalonyl-CoA pathway. Since the distribution of ¹³C in alanine reported in the literature is fully compatible with a major role of the 2-methylcitrate pathway in propionate metabolism, it cannot be interpreted as evidence for the existence of a TCA cycle metabolon in *S. cerevisiae*.

Keywords: *Saccharomyces cerevisiae*, propionate metabolism, 2-methylcitrate pathway, metabolic channelling

INTRODUCTION

Non-metabolizable weak acids such as benzoate, butyrate and, under anaerobic conditions, acetate, have a profound effect on the physiology of yeasts. When these acids are added to the medium feed of sugar-limited chemostat cultures, the biomass yield decreases and the rate of sugar dissimilation increases (Verduyn *et al.*, 1990a, b; 1992). This negative effect on growth efficiency can be attributed to an increased energy requirement for cytosolic pH

homeostasis by the proton-pumping plasma membrane ATPase.

As anticipated, the addition of propionate to anaerobic, glucose-limited chemostat cultures of *S. cerevisiae* also caused a decreased biomass yield and an increased rate of alcoholic fermentation (Verduyn *et al.*, 1990a, b). However, when low concentrations (5–10 mM) of propionate were added to aerobic glucose-limited chemostat cultures of *S. cerevisiae* growing at low dilution rates, virtually all propionate disappeared from the cultures (C. Verduyn, unpublished observations).

In a variety of prokaryotic organisms, propionate is metabolized by the methylmalonyl-CoA pathway of

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Fig. 1. Pathways of propionate metabolism. (a) Methylmalonyl-CoA pathway; (b) 2-methylcitrate pathway. Black dots indicate the fate of the C-3 atom of propionate when propionate is converted to pyruvate (or alanine), assuming that all intermediates are in equilibrium with the bulk phase water and that pyruvate does not re-enter the TCA cycle. Numbered reactions are catalysed by the following enzymes: 1, propionyl-CoA carboxylase; 2, methylmalonyl-CoA mutase; 3, succinyl thiokinase; 4, TCA cycle enzymes; 5, malate dehydrogenase, phosphoenolpyruvate carboxykinase and pyruvate kinase; 6, 2-methylcitrate synthase; 7, 2-methylcitrate dehydratase and 2-methylisocitrate dehydratase; 8, 2-methylisocitrate lyase.

propionate metabolism (Fig. 1a; Wegener *et al.*, 1968). However, we have found no evidence in the literature to indicate the involvement of this route in propionate metabolism in yeasts. One of the key enzymes of this pathway, methylmalonyl-CoA mutase, requires vitamin B12 as a cofactor (Wegener *et al.*, 1968), and *S. cerevisiae* is not known to be able to synthesize this vitamin (Schneider & Stroinski, 1987). An alternative pathway of propionate metabolism has been demonstrated in the lipid-degrading yeast *Yarrowia lipolytica* (Tabuchi & Uchiyama, 1975; Uchiyama *et al.*, 1982). This pathway is initiated by the synthesis of 2-methylcitrate from propionyl-CoA and oxaloacetate (Fig. 1b). Via a metabolic sequence similar to the 'normal' TCA cycle reactions, 2-methylcitrate is converted to 2-methylisocitrate, which is then cleaved into pyruvate and succinate (Fig. 1b).

Metabolism of [3-¹³C]propionate by *S. cerevisiae* has been investigated to study the occurrence of metabolic channeling in *S. cerevisiae* (Sumegi *et al.*, 1990). Since knowledge of the actual pathway of propionate metabolism by *S. cerevisiae* is essential for a correct interpretation of their results, we studied the activity of a number of key enzymes of propionate-degrading pathways in cell-free extracts.

METHODS

Micro-organisms and maintenance. *Saccharomyces cerevisiae* CBS 8066 was obtained from the Centraal Bureau voor Schimmelcultures, Delft, the Netherlands, and was maintained by monthly subculture on malt agar slopes. Stock cultures were stored at 4 °C. *Paracoccus denitrificans* LMD 22.21 was obtained from the Culture Collection of the Laboratory of Microbiology and Enzymology, Delft, the Netherlands, and maintained on peptone agar slants.

Chemostat cultivation. Aerobic chemostat cultivation of *S. cerevisiae* was performed at 30 °C in 1 litre Applikon laboratory fermenters at a stirring speed of 750 r.p.m. and at a dilution rate of 0.05 h⁻¹. The cultures were flushed with air (0.5 l min⁻¹). The dissolved-oxygen concentration in the cultures was above 80 % of air saturation. The culture pH was controlled at 5.0 by automatic addition of 2 M KOH. The condenser was connected to a cryostat and cooled at 2 °C. The working volume of the culture was kept at 1.0 litre by a peristaltic effluent pump coupled to an Applikon level controller. Biomass concentrations in samples taken directly from the culture differed by less than 1 % from biomass concentrations in the culture effluent. The mineral medium, supplemented with vitamins, was prepared according to Verduyn *et al.* (1992). Vitamins were filter-sterilized and added after autoclaving the mineral medium. Glucose was sterilized separately at 110 °C. Pure propionic acid was added to sterile reservoir media without prior sterilization.

Cultures were checked for the occurrence of oscillations (Sonnleitner, 1991) by continuous registration of the dissolved-oxygen concentration. All data presented were derived from steady-state cultures without detectable oscillations. The purity of chemostat cultures was routinely checked by phase contrast microscopy at 1000 $\times$ magnification and by plating on complex media.

Cultivation of *Paracoccus denitrificans*. *P. denitrificans* was grown at 30 °C in shake flask cultures on the mineral medium described by Van Schie *et al.* (1984), supplemented with 20 mM sodium propionate.

Determination of culture dry weight. Dry weights of culture samples were determined using a microwave oven and 0.45 μ m membrane filters as described by Postma *et al.* (1989). Parallel samples varied by less than 1%.

Analysis of substrates and metabolites. Glucose was assayed with the Boehringer GOD-PAP glucose kit (no. 676543). Propionate was determined by HPLC on an HPX-87H column (300 $\times$ 7.8 mm, Bio-Rad) at room temperature. Detection was by means of a Waters 441 UV detector at 214 nm, coupled to a Waters 741 data module. Peak areas were linearly proportional to concentrations. The detection limit for propionate was approximately 50 μ M. The same HPLC set-up was used for detection of organic acids in culture supernatants and for the detection of glycerol and ethanol. In the latter case, the same column was used, but detection was by means of an Erma 7515 A refractive index detector.

Protein determination. Protein concentrations in cell-free extracts were estimated by the Lowry method. The protein content of culture samples was estimated by a modified biuret method (Verduyn *et al.*, 1992). In both assays, bovine serum albumin (fatty-acid-free, Sigma) was used as a standard.

Preparation of cell-free extracts. Samples of steady-state chemostat cultures were harvested by centrifugation, washed twice with 10 mM potassium phosphate buffer (pH 7.5) containing 2 mM EDTA, concentrated to a final concentration of 10–15 mg dry wt ml⁻¹ and stored at -40 °C in 5 ml aliquots. Before preparation of extracts, the samples were thawed at room temperature, washed twice and resuspended in ice-cold 0.1 M potassium phosphate buffer (pH 7.5), containing 1 mM dithiothreitol and 2 mM MgCl₂. Extracts were prepared by sonication with 0.1 mm diameter glass beads at 0 °C for 2 min at 0.5 min intervals with an MSE sonicator (150 W output, 8 μ m peak-to-peak amplitude). Unbroken cells and debris were removed by centrifugation at 4 °C (20 min at 75000 g). The clear supernatant, typically containing 1–3 mg protein ml⁻¹, was used as the cell-free extract.

Enzyme assays. Enzyme activities were assayed immediately after preparation of the cell-free extracts. All enzyme assays were carried out at 30 °C. In all activity measurements, the reaction rates were linearly proportional to the amount of cell-free extract added.

Citrate synthase (EC 4.1.3.7). The reaction mixture (1 ml) contained: Tris/HCl buffer (pH 8.0), 100 μ mol; acetyl-CoA, 0.05 μ mol; 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), 0.1 μ mol; and cell-free extract. The reaction was started by the addition of 0.2 μ mol oxaloacetate. Release of CoA was monitored at 412 nm (Srere, 1969) and activities were calculated using an extinction coefficient of 13.6 mM⁻¹ cm⁻¹.

2-Methylcitrate synthase. The reaction mixture (1 ml) contained: Tris/HCl buffer (pH 8.0), 100 μ mol; propionyl-CoA,

0.1 μ mol; DTNB, 0.1 μ mol; and cell-free extract. The reaction was started by the addition of 0.2 μ mol oxaloacetate and monitored as described for citrate synthase.

Acetyl-CoA synthetase (EC 6.2.1.1) and propionyl-CoA synthetase. Enzyme activity was determined by means of a discontinuous assay, measuring the consumption of CoA (Webster, 1969). The reaction mixture (1 ml) contained: Tris/HCl buffer (pH 8.0), 100 μ mol; MgCl₂, 5 μ mol; NiCl₂, 0.02 μ mol; ATP, 2.5 μ mol; CoA, 1 μ mol; and cell-free extract. The reaction was started by the addition of either 2 μ mol sodium acetate or 20 μ mol sodium propionate. At appropriate time intervals, 50 μ l samples were withdrawn from the reaction mixture and added to 950 μ l of a solution containing 100 μ mol Tris/HCl buffer (pH 8.0), and 0.2 μ mol DTNB. Decrease of the CoA concentration was linear with time and proportional to the amount of cell-free extract added.

Propionyl-CoA carboxylase (EC 6.4.3.1). Enzyme activity was measured as incorporation of [¹⁴C]bicarbonate (Kaziro, 1969). The reaction mixture (1 ml) contained: Tris/HCl buffer (pH 8.0), 100 μ mol; MgCl₂, 5 μ mol; reduced glutathione, 2 μ mol; ATP, 2.5 μ mol; NaH¹⁴CO₃ (37 GBq mol⁻¹), 2.5 μ mol; and cell-free extract. The reaction was started by the addition of 0.1 μ mol propionyl-CoA. At appropriate time intervals, 50 μ l samples were withdrawn from the mixture and added to 200 μ l acetic acid, to expel non-bound CO₂. After evaporation of acetic acid at 60 °C, the pellet was dissolved in 0.5 ml water. Beckman Ready-Safe scintillation cocktail (5 ml) was added and radioactivity was measured in a Beckman LS 3801 scintillation counter. Cell-free extracts of propionate-grown *Paracoccus denitrificans* LMD 22.21 were included as a positive control, since this organism is known to metabolize propionate via methylmalonyl-CoA (Smith & Kornberg, 1967).

Chemicals. Sodium [¹⁴C]bicarbonate (2.11 TBq mol⁻¹) was obtained from Amersham. All other chemicals were reagent grade and obtained from commercial sources.

RESULTS AND DISCUSSION

Utilization of propionate by *S. cerevisiae*

When 10 mM propionic acid was added to the reservoir media of glucose-limited chemostat cultures of *S. cerevisiae*, the residual concentration of propionate in the culture remained below 100 μ M. At this low residual concentration, propionate does not have a significant uncoupling effect on the energy metabolism of *S. cerevisiae* (Verduyn *et al.*, 1990a). Instead, the consumption of propionic acid by the cultures coincided with an increase of the biomass concentration (Fig. 2). A further increase of the reservoir concentration of propionic acid led to a linear increase of the steady-state biomass concentration (Fig. 2), with the residual propionate concentration remaining below 100 μ M. Also, the protein content of the cultures increased linearly over the range of propionate concentrations tested (Fig. 2).

Only small increases of the propionic acid concentration in the reservoir media could be made in the mixed-substrate cultures: increases of over 10 mM propionic acid at a time resulted in wash-out. Attempts to switch cultures growing on 14 mM (2.5 g l⁻¹) glucose and 50 mM propionic acid to growth on 40 mM propionic acid as the sole source of carbon and energy were

Fig. 2. Growth of *S. cerevisiae* CBS 8066 on glucose (2.5 g l⁻¹) with increasing concentrations of propionic acid in the reservoir media. Growth conditions: $D = 0.05 \text{ h}^{-1}$, pH 5.0, $T = 30^\circ \text{C}$, d.o.t. > 80% air saturation. $\circ$, Culture dry weight; $\bullet$, protein concentration. Data are presented as means $\pm$ SD of two independent chemostat runs.

unsuccessful: the cultures washed out. These experiments do not necessarily imply that *S. cerevisiae* is unable to grow on propionate as a sole carbon source. Propionate is toxic to *S. cerevisiae*, and it may well be that propionate-limited steady-state cultures can be attained by a more careful manipulation of the composition of the reservoir medium or at a lower dilution rate.

In addition to the effects on biomass yield, addition of propionic acid to the reservoir media also affected the morphology of the yeast cells. Glucose-limited cultures mainly contained free cells and cells with one bud. In mixed-substrate cultures, a substantial fraction of the buds did not dissociate from the mother cells, leading to the formation of small aggregates (typically 3–8 cells). The occurrence of these aggregates did not influence the removal of cells with the culture effluent: biomass concentrations in samples taken directly from the culture and in samples taken from the effluent line differed by less than 1%. Electron microscopy of thin sections revealed an increase of the mitochondrial volume with increasing propionate flux. Proliferation of peroxisomes, as reported for propionate-grown *Candida tropicalis* (Ueda *et al.*, 1983, 1989), was not observed (data not shown).

Enzymology of propionate metabolism

To investigate which pathway of propionate metabolism is operating in *S. cerevisiae*, the activity of three potential key enzymes was assayed in cell-free extracts. Propionyl-CoA synthetase catalyses the activation of propionate (Fig. 1). Propionyl-CoA carboxylase and 2-methylcitrate

synthase are key enzymes of the methylmalonyl-CoA pathway and the 2-methylcitric acid cycle, respectively (Fig. 1).

Acetyl-CoA synthetase (EC 6.2.1.1) from *S. cerevisiae* can also catalyse the formation of propionyl-CoA from propionate and coenzyme A (Jones & Lipmann, 1955). In cell-free extracts, activities with both substrates could be detected at the various ratios of propionic acid to glucose in the reservoir media (Fig. 3). The propionyl-CoA synthetase activities measured in cell-free extracts were sufficient to account for the flux of propionate in the chemostat cultures (Fig. 3). The ratio between acetyl-CoA synthetase activity and propionyl-CoA synthetase activity (3.6 ± 1.2) did not exhibit a clear correlation with the composition of the reservoir medium. A similar ratio (3.5) was observed in ethanol-limited chemostat cultures. These observations are consistent with catalysis of the initial step of propionate metabolism in *S. cerevisiae* by acetyl-CoA synthetase. Acetyl-CoA synthetase levels in aerobic, glucose-limited *S. cerevisiae* cultures decrease with increasing growth rate (Postma *et al.*, 1989). This may explain the observation that, at a dilution rate of 0.30 h^{-1} , propionate was not consumed by aerobic, glucose-limited chemostat cultures of *S. cerevisiae* CBS 8066 (Postma *et al.*, 1989).

The initial step in propionyl-CoA metabolism via the methylmalonyl-CoA pathway is the carboxylation of propionyl-CoA, yielding the key intermediate of this pathway, methylmalonyl-CoA (Fig. 1a). This reaction is catalysed by the enzyme propionyl-CoA carboxylase (EC 6.4.1.3). Activities of this enzyme in cell-free extracts of all chemostat cultures were below $0.5 \text{ nmol min}^{-1} \text{ mg}^{-1}$. This is over 100-fold lower than the maximum observed propionate consumption rates in the chemostat cultures, which makes it unlikely that the enzyme plays a significant role in propionate metabolism in the cultures. In control experiments, high activities of propionyl-CoA carboxylase ($30\text{--}50 \text{ nmol min}^{-1} \text{ mg}^{-1}$) were observed in cell-free extracts of propionate-grown *Paracoccus denitrificans*, indicating that the low activities observed in the *S. cerevisiae* extracts were not due to errors in the assay procedure.

The 2-methylcitrate pathway of propionate metabolism is initiated by the condensation of propionyl-CoA and oxaloacetate, yielding 2-methylcitrate (Tabuchi & Uchiyama, 1975). The activity of the enzyme responsible, 2-methylcitrate synthase, increased with increasing propionate-to-glucose ratios in the reservoir media of the chemostat cultures and was paralleled by an increase of the citrate synthase activity (Fig. 4). When suboptimal concentrations of propionyl-CoA were added to the enzyme assays, the amount of CoA released was equal to the amount of propionyl-CoA added (data not shown), indicating that the observed activities were not due to contamination of the propionyl-CoA with acetyl-CoA. Apparently, the 2-methylcitrate synthase reaction is catalysed by the *S. cerevisiae* citrate synthase. Likewise, the *Yarrowia lipolytica* 2-methylcitrate synthase exhibits this dual substrate specificity (Uchiyama & Tabuchi, 1976).

Fig. 3

Fig. 4

Fig. 3. Activities of acetyl-CoA synthetase (●) and propionyl-CoA synthetase (○) in cell-free extracts of *S. cerevisiae* CBS 8066 pregrown in glucose-limited chemostat cultures supplemented with increasing concentrations of propionic acid in the reservoir medium (growth conditions as in legends to Fig. 2). Data are presented as means $\pm$ SD of two independent chemostat runs. The dashed line indicates estimated *in vivo* rates of propionate consumption in the chemostat cultures, calculated on the basis of a soluble-protein content of yeast biomass of 33 % (Postma *et al.*, 1989).

Fig. 4. Activities of citrate synthase (●) and 2-methylcitrate synthase (○) in cell-free extracts of *S. cerevisiae* pregrown in glucose-limited chemostat cultures supplemented with increasing concentrations of propionic acid in the reservoir medium (growth conditions as in legends to Fig. 2). Data are presented as means $\pm$ SD of two independent chemostat runs. The dashed line (plotted against the right ordinate) indicates the estimated *in vivo* rates of propionate consumption in the chemostat cultures (see legend to Fig. 3).

The 2-methylcitrate synthase activity was sufficient to account for the fluxes of propionate observed in the chemostat cultures of *S. cerevisiae* growing on mixtures of glucose and propionate (Fig. 4).

The above data suggest that instead of the methylmalonyl-CoA pathway, the 2-methylcitrate pathway is the major route of propionate metabolism in *S. cerevisiae*. The net result of propionate conversion via this pathway is its oxidation to pyruvate. Although toxic at high concentrations, propionate is not an unnatural product in the intermediary metabolism of yeasts. For example, propionyl-CoA is an intermediate in the degradation of isoleucine. Recently, it has been suggested that the 2-methylcitrate pathway may be responsible for the formation of succinate from isoleucine by *S. cerevisiae* during beer fermentation (Shindo *et al.*, 1993).

Metabolic channelling

It has been suggested in the literature that the enzymes of the TCA cycle should not be considered to be soluble in the mitochondrial matrix but, instead, form a supra-molecular complex bound to the inner surface of the mitochondrial inner membrane, called a metabolon (Srere, 1985, 1990). Organization of enzymes in metabolons could lead to a phenomenon called 'channelling', i.e. the transfer of intermediates between the enzymes of a

metabolic pathway occurs without equilibration with the bulk water phase.

The distribution of ^{13}C in alanine after incubation of *S. cerevisiae* cells with $[3-^{13}\text{C}]$ -propionate has been used to study channelling of TCA cycle intermediates in yeast mitochondria (Sumegi *et al.*, 1990). For interpretation of their experimental data, Sumegi *et al.* (1990) assumed that metabolism of propionate by *S. cerevisiae* occurs via the methylmalonyl-CoA pathway (Fig. 1a). Starting with $[3-^{13}\text{C}]$ propionate, this pathway results in the formation of $[2-^{13}\text{C}]$ succinyl-CoA. Upon removal of the CoA moiety, succinate is formed. Since this is a symmetrical molecule, no enrichment of ^{13}C in either C-2 or C-3 of alanine is expected when transfer of TCA cycle intermediates between the enzymes of the cycle occurs by free diffusion through the bulk liquid phase of the mitochondrial matrix. However, Sumegi *et al.* (1990) did observe a significant enrichment of ^{13}C label in C-3 of alanine. This was interpreted as evidence for channelling of TCA cycle intermediates in *S. cerevisiae* mitochondria (Sumegi *et al.*, 1990; Srere, 1990; Evans *et al.*, 1993).

The data presented in this paper suggest that propionate metabolism in *S. cerevisiae* occurs predominantly via the 2-methylcitrate pathway. Conversion of $[3-^{13}\text{C}]$ propionate into pyruvate via this linear pathway should yield exclusively $[3-^{13}\text{C}]$ pyruvate (Fig. 1b). Only when pyruvate re-enters the citric acid cycle may some label be scrambled

and appear at C-2 of alanine as well. Accordingly, a major involvement of the 2-methylcitrate pathway is fully compatible with the labelling pattern of alanine formed from [3-¹³C]propionate as observed by Sumegi *et al.* (1990). Consequently, their data cannot be interpreted as proof for the occurrence of metabolic channelling of TCA cycle intermediates in *S. cerevisiae* or the existence of a TCA cycle metabolon in this yeast.

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