

Metabolic Responses of Pyruvate Decarboxylase-Negative *Saccharomyces cerevisiae* to Glucose Excess

MARCEL T. FLIKWEERT, JOHANNES P. VAN DIJKEN, AND JACK T. PRONK*

Department of Microbiology and Enzymology, Kluyver Laboratory of Biotechnology,
Delft University of Technology, 2628 BC Delft, The Netherlands

Received 28 January 1997/Accepted 17 June 1997

In *Saccharomyces cerevisiae*, oxidation of pyruvate to acetyl coenzyme A can occur via two routes. In pyruvate decarboxylase-negative (Pdc^-) mutants, the pyruvate dehydrogenase complex is the sole functional link between glycolysis and the tricarboxylic acid (TCA) cycle. Such mutants therefore provide a useful experimental system with which to study regulation of the pyruvate dehydrogenase complex. In this study, a possible in vivo inactivation of the pyruvate dehydrogenase complex was investigated. When respiring, carbon-limited chemostat cultures of wild-type *S. cerevisiae* were pulsed with excess glucose, an immediate onset of respiro-fermentative metabolism occurred, accompanied by a strong increase of the glycolytic flux. When the same experiment was performed with an isogenic Pdc^- mutant, only a small increase of the glycolytic flux was observed and pyruvate was the only major metabolite excreted. This finding supports the hypothesis that reoxidation of cytosolic NADH via pyruvate decarboxylase and alcohol dehydrogenase is a prerequisite for high glycolytic fluxes in *S. cerevisiae*. In Pdc^- cultures, the specific rate of oxygen consumption increased by ca. 40% after a glucose pulse. Calculations showed that pyruvate excretion by the mutant was not due to a decrease of the pyruvate flux into the TCA cycle. We therefore conclude that rapid inactivation of the pyruvate dehydrogenase complex (e.g., by phosphorylation of its E1 α subunit, a mechanism demonstrated in many higher organisms) is not a relevant mechanism in the response of respiring *S. cerevisiae* cells to excess glucose. Consistently, pyruvate dehydrogenase activities in cell extracts did not exhibit a strong decrease after a glucose pulse.

In comparison with many other yeasts, *Saccharomyces cerevisiae* (baker's yeast) has a strong tendency towards alcoholic fermentation. Even under fully aerobic conditions, a mixed respiro-fermentative metabolism is observed when the sugar concentration in the growth medium exceeds a threshold value (typically ca. 1 mM [25]) or when the growth rate is higher than the so-called critical growth rate (usually ca. two-thirds of the maximum specific growth rate on glucose [13, 14]). In view of the low ATP yield from alcoholic fermentation and the detrimental effects of fermentation products on biomass formation, alcoholic fermentation should be prevented during industrial production of *S. cerevisiae* biomass or (heterologous) proteins (5, 24). In industrial practice, a low sugar concentration is maintained by sugar-limited fed-batch cultivation at growth rates below the critical growth rate. Nevertheless, local sugar concentrations above the respiro-fermentative threshold are difficult to avoid due to imperfect mixing in large reactors, augmented by the use of high sugar concentrations in the feeds (20). Therefore, studies of the mechanisms involved in the switch to respiro-fermentative metabolism in *S. cerevisiae* are of industrial relevance. In this study, the regulation of pyruvate metabolism after transition of *S. cerevisiae* from sugar limitation to sugar excess was examined.

Pyruvate, the product of glycolysis, is located at an important branching point in the metabolism of carbohydrates by *S. cerevisiae* (for a review, see reference 16). During fermentative sugar metabolism, pyruvate is decarboxylated to acetaldehyde by pyruvate decarboxylase and subsequently reduced to ethanol. Respiratory dissimilation of pyruvate requires its conver-

sion to acetyl coenzyme A (acetyl-CoA), the fuel of the tricarboxylic acid (TCA) cycle. In *S. cerevisiae*, this conversion can occur in two ways. A direct oxidative decarboxylation of pyruvate to acetyl-CoA is catalyzed by the mitochondrial pyruvate dehydrogenase complex. Alternatively, this conversion can be accomplished by the concerted action of pyruvate decarboxylase, acetaldehyde dehydrogenase, and acetyl-CoA synthetase (6, 15). This indirect pathway is frequently referred to as the pyruvate dehydrogenase bypass.

During glucose-limited respiratory growth, the pyruvate dehydrogenase complex is primarily responsible for the conversion of pyruvate into acetyl-CoA (15). However, a small flux through the pyruvate dehydrogenase bypass is essential during growth on sugars, probably to provide cytosolic acetyl-CoA for lipid synthesis (3). In pyruvate decarboxylase-negative (Pdc^-) mutants (in which the bypass cannot operate), this requirement can be met by adding an exogenous source of acetyl-CoA (e.g., acetate or ethanol) to the feed of sugar-limited chemostat cultures. Sugar-limited cultivation is required for prolonged sugar consumption by Pdc^- mutants of *S. cerevisiae* (albeit only in the presence of ethanol or acetate [3]). In the presence of excess sugar, synthesis of respiratory enzymes is repressed, whereas alcoholic fermentation cannot provide ATP.

In mammalian cells, activity of the pyruvate dehydrogenase complex is regulated by phosphorylation of three serine residues on its E1 α subunit, leading to a rapid and complete inactivation of the complex. Phosphorylation and dephosphorylation of the complex are catalyzed by a specific kinase and phosphatase, respectively (17, 29). Sequencing of the *PDA1* gene, encoding the E1 α subunit of the *S. cerevisiae* pyruvate dehydrogenase complex, has revealed a single putative phosphorylation site (19), which can be phosphorylated in vitro by kinases isolated from mammalian cells (11, 21). Recently, it has been reported that phosphorylation of the E1 α subunit occurs in *S. cerevisiae* mitochondria and leads to inactivation of

* Corresponding author. Mailing address: Department of Microbiology and Enzymology, Kluyver Laboratory of Biotechnology, Delft University of Technology, Julianalaan 67, 2628 BC Delft, The Netherlands. Phone: 31 15 278 3214. Fax: 31 15 278 2355. E-mail: j.t.pronk@stm.tudelft.nl.

TABLE 1. Properties of wild-type *S. cerevisiae* T2-3D and the isogenic, pyruvate decarboxylase-negative strain GG570 in aerobic, steady-state chemostat cultures ($D = 0.10 \text{ h}^{-1}$) grown on a mixture of glucose and acetate^a

Strain	Pyruvate decarboxylase (U · mg of protein ⁻¹)	Biomass yield (g · mol of C ⁻¹)	Protein content (g · g [dry wt] ⁻¹)	$q_{\text{O}_2}^b$ (mmol of O ₂ · g [dry wt] ⁻¹ · h ⁻¹)
T2-3D	0.9 ± 0.1	14.8 ± 0.5	0.41 ± 0.02	2.3 ± 0.1
GG570	<0.001	15.1 ± 0.4	0.42 ± 0.02	2.3 ± 0.1

^a Mixture of 90% glucose and 10% acetate on a carbon basis. Data are the averages ± standard deviations of two independent cultures.

^b q_{O_2} , specific rate of O₂ consumption.

the complex (7). However, the physiological significance of this mechanism is unclear at present.

A rapid inactivation of the pyruvate dehydrogenase complex could, at least in theory, be an important mechanism in the switch to respiro-fermentative metabolism observed after exposure of respiring sugar-limited *S. cerevisiae* cultures to excess glucose. The ensuing redirection of pyruvate metabolism via pyruvate decarboxylase would be consistent with the accumulation of ethanol and acetate observed under such conditions. At the same time, respiratory metabolism could continue via the pyruvate dehydrogenase bypass.

The aim of the present study was to investigate whether inactivation of the pyruvate dehydrogenase complex occurs *in vivo* during exposure of respiring *S. cerevisiae* cells to excess glucose. To this end, the metabolic response of wild-type *S. cerevisiae* after exposure of respiring cells to glucose excess was compared with that of an isogenic, pyruvate decarboxylase-negative strain. The latter was used since it can use only the pyruvate dehydrogenase complex to link glycolysis and the TCA cycle.

MATERIALS AND METHODS

Strains and maintenance. *S. cerevisiae* T2-3D (*HO/HO*) is a homozygous diploid strain derived from the heterozygous strain *S. cerevisiae* CBS 8066 (27). *S. cerevisiae* GG570 (*HO/HO pdc1::Tn5ble/pdc1::Tn5ble pdc5::Tn5ble/pdc5::Tn5ble pdc6::APT1/pdc6::APT1*) is an isogenic mutant in which all three structural genes encoding pyruvate decarboxylase have been inactivated by using dominant antibiotic resistance marker genes (3). Both strains were grown to stationary phase in shake flask cultures on YPE medium (yeast extract [Difco], 10 g · liter⁻¹; peptone from casein (Merck), 20 g · liter⁻¹; and ethanol, 2% [vol/vol]). After addition of glycerol (15% [vol/vol]), 2-ml aliquots were transferred to sterile vials and stored at -70°C. YPE plates streaked with samples from these frozen stock cultures were used to inoculate precultures for growth experiments.

Chemostat cultivation. Aerobic chemostat cultivation was performed at 20°C in laboratory fermentors (Applikon, Schiedam, The Netherlands), at a stirrer speed of 800 rpm and a dilution rate of 0.10 h⁻¹. The working volume of the cultures was kept at 1.0 liter by a peristaltic effluent pump coupled to an electrical level sensor. This setup ensured that under all growth conditions, the biomass concentrations in samples taken directly from the cultures differed by <1% from those in samples taken from the effluent line. The culture pH was kept at 5.0 by automatic addition of 2 mol of KOH liter⁻¹. The fermentor was flushed with air at a flow rate of 0.5 liter · min⁻¹ by using a Brooks 5876 mass flow controller. The dissolved-oxygen concentration was monitored with an oxygen electrode (model 34 100 3002; Ingold) and remained above 50% of air saturation. Steady-state data refer to cultures without detectable metabolic oscillations. Chemostat cultures were checked for purity by using phase-contrast microscopy and plating on complex media. Mineral medium, supplemented with vitamins and trace elements, was prepared as described previously (3). Glucose and vitamins were added to the autoclaved mineral medium after separate sterilization. Growth of *Pdc⁻ S. cerevisiae* in glucose-limited chemostat cultures requires the addition of ethanol or acetate to reservoir media (3). Steady-state, carbon-limited cultures in which all substrate carbon was consumed could readily be obtained on mixtures of 6.75 g of glucose liter⁻¹ and 0.75 g of acetate liter⁻¹ (10% acetate on a carbon basis). For the sake of comparison, cultures of the isogenic wild-type strain T2-3D were grown on the same medium. The biomass yields and protein contents of wild-type and *Pdc⁻* strains pregrown under these conditions were not significantly different (Table 1).

Glucose pulse experiments. At zero time, glucose was added to steady-state chemostat cultures to give an initial concentration of 50 mM. To prevent the C₂ requirement of the *Pdc⁻* mutant from interfering with the pulse experiments, the influent and effluent pumps were run continuously. At appropriate intervals, samples were taken from the effluent line and analyzed for biomass and metabolite concentrations.

Determination of culture dry weight. The dry weight of washed culture samples was determined by using 0.45-μm-pore-size membrane filters and a microwave oven (14). Parallel samples varied by <1%. During glucose pulse experiments, only small samples could be taken. Therefore, culture dry weights were calculated from the optical density at 660 nm (OD₆₆₀) of culture samples, measured in a VitaLab 20 spectrophotometer (Vita Scientific, Dieren, The Netherlands). To obtain a linear relationship between OD₆₆₀ and culture dry weight, samples were diluted with demineralized water to give an OD₆₆₀ between 0.1 and 0.3. Control experiments confirmed that the biomass-dry weight relationship did not change significantly during the pulse experiments.

Metabolite analysis. Organic acids, glycerol, and ethanol in culture supernatants were determined by high-pressure liquid chromatography analysis using a Phenomenex column (Rezex ROA organic acid 00H-0138-KO) at 60°C. The column was eluted with 0.5 g of sulfuric acid liter⁻¹ at a flow rate of 0.5 ml · min⁻¹. Organic acids were detected by use of a Waters 441 UV meter at 214 nm coupled to a Waters 741 data module. Ethanol and glycerol were detected with an Erma ERC 7515 refractive-index detector coupled to a Hewlett-Packard 3390A RI integrator. Twenty-microliter samples were injected with a Hamilton syringe. Glucose in reservoir media and supernatants was determined enzymically by the glucose oxidase (GOD-PAP) method (Merck Systems kit 14144; detection limit, ca. 5 μM). Ethanol was also assayed enzymically, using alcohol oxidase from *Hansenula polymorpha* (Bird Engineering, Schiedam, The Netherlands).

Preparation of cell extracts. Culture samples were harvested by centrifugation, washed twice with 10 mM potassium phosphate buffer (pH 7.5) containing 2 mM EDTA, concentrated fourfold, and stored at -20°C. Before disruption, the samples were thawed on ice, washed, and resuspended in 100 mM potassium phosphate buffer (pH 7.5) containing 2 mM MgCl₂ and 1 mM dithiothreitol. Cell disruption was performed either by sonication or by use of a French press. Sonication was carried out at 0°C for 2 min at 0.5-min intervals with a Measuring & Scientific Equipment Ltd. (MSE) sonicator (150-W output, 7-μm peak-to-peak amplitude). French press disruption was performed by two passages at 4°C and 1,000 lb/in² through an American Instrument Company FA-073 French pressure cell. After cell disruption, unbroken cells and debris were removed by centrifugation at 4°C (20 min at 36,000 × g).

Enzyme assays. Pyruvate decarboxylase activity was routinely determined in extracts from cultures of the pyruvate decarboxylase-negative strain to confirm culture purity and stability. These assays were performed as described previously (3), using cell extracts prepared by sonication. Activity of the pyruvate dehydrogenase complex was assayed in cell extracts prepared with the French pressure cell. Enzyme activity was assayed immediately after preparation of the extracts, at 30°C with a Hitachi 100-60 spectrophotometer set at 340 nm. The assay mixture, a modified version of that used by Kresze and Ronft (10), contained the following: potassium phosphate buffer (pH 8.0), 100 mM; MgCl₂, 1 mM; thiamine pyrophosphate, 0.2 mM; NAD⁺, 2.5 mM; cysteine-HCl, 2 mM; pyruvate, 5 mM; Triton X-100, 0.05%; and cell extract. The reaction was started by the addition of 0.15 mM coenzyme A. Reaction rates were linearly proportional to the amount of cell extract added.

Protein determination. Protein concentrations in cell extracts were estimated by the Lowry method. Bovine serum albumin (fatty acid free; Sigma Chemical Co.) was used as a standard. The protein content of whole cells was determined by a modified biuret method (26).

Calculation of metabolic fluxes. Specific rates of metabolite conversions (q) were estimated from plots of metabolite concentration (C_s) and biomass concentration (C_x) versus time, according to equation 1:

$$q_s = \frac{\frac{dC_s}{dt} + D(C_s - C_{s,i})}{C_x} \quad (1)$$

In equation 1, D is the dilution rate and $C_{s,i}$ is the metabolite concentration in the reservoir medium. dC_s/dt was estimated by differentiating spline functions fitted to plots of C_s versus time, fitted with the software package Fig.P (Fig.P Software Corporation). We stress that this approach is sensitive to small experimental variation in the determination of C_s . Therefore, the calculated fluxes give only an indication of the true metabolic activity. According to equation 1, metabolite production is indicated by a positive value of q_s , whereas consumption of a metabolite is indicated by a negative value.

Gas analysis. The exhaust gas of the fermentor was cooled in a condenser (2°C) and dried with a Perma Pure dryer (PD-625-12P). O₂ and CO₂ concentrations were determined with a Servomex 1100A analyzer and a Beckman model 864 infrared detector, respectively. The exhaust gas flow rate was measured as described previously (28). Specific rates of CO₂ production and O₂ consumption were calculated as described by van Urk et al. (23). In cultures of wild-type *S. cerevisiae*, it was not possible to accurately monitor the rapid changes in O₂ consumption and CO₂ production after a glucose pulse due to delays in the

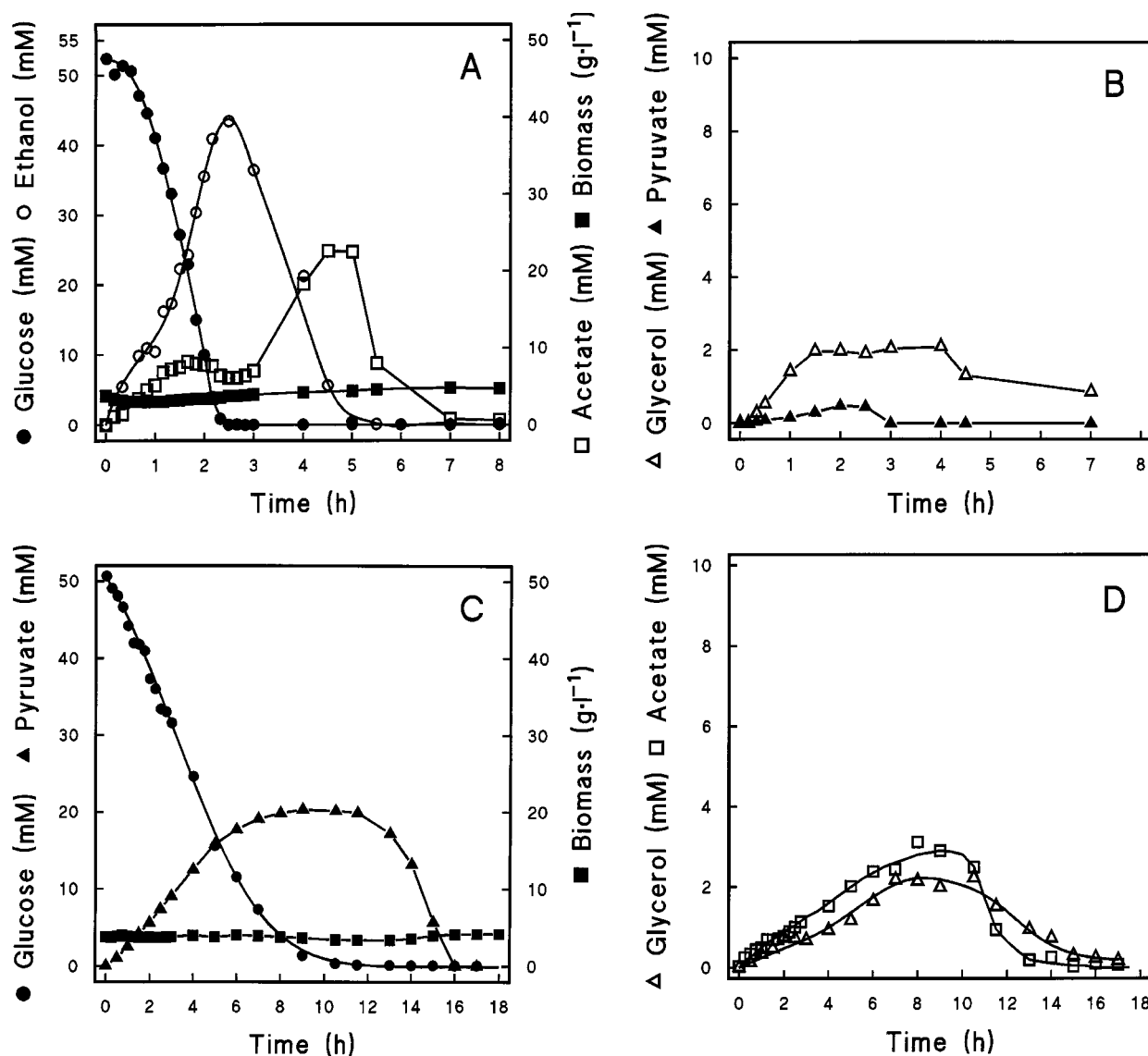


FIG. 1. Concentrations of biomass and metabolites after addition of a glucose pulse (50 mmol) to steady-state chemostat cultures ($D = 0.10 \text{ h}^{-1}$, 1-liter working volume) of wild-type *S. cerevisiae* T2-3D (A and B) and the pyruvate decarboxylase-negative strain GG570 (C and D). Metabolite concentrations in independent duplicate experiments differed by less than 10% from those shown.

experimental setup (caused by gas transfer, headspace mixing in the fermentor, and length of the tubing from fermentor to analyzers).

RESULTS

Metabolic responses of wild-type *S. cerevisiae* to glucose excess. To provide a reference with which to compare the Pdc⁻ strain *S. cerevisiae* GG570, glucose pulse experiments were carried out with the isogenic wild-type strain T2-3D, pregrown on a mixture of glucose and acetate. After injection of glucose to an initial concentration of $50 \text{ mmol} \cdot \text{liter}^{-1}$, the cultures exhibited the typical response of respiring *S. cerevisiae* cells to glucose excess, i.e., the glucose was rapidly consumed, and ethanol and acetate accumulated in the culture (Fig. 1A). In addition, small amounts of glycerol and pyruvate were produced (Fig. 1B). The observed pattern of metabolite formation was essentially the same as that observed with cultures pregrown on glucose as the sole carbon source (1).

The specific rate of glucose consumption, which was 1

$\text{mmol} \cdot \text{g}^{-1} \cdot \text{h}^{-1}$ during the carbon-limited steady state, increased after the glucose pulse, reaching a maximum of ca. $10 \text{ mmol} \cdot \text{g}^{-1} \cdot \text{h}^{-1}$ (Fig. 2A). This increase was not instantaneous but occurred over ca. 1.5 h (Fig. 2A). This suggests that the increased rate of glucose metabolism involved de novo synthesis of key enzymes.

Peculiarly, the specific rate of glucose consumption hardly changed during the first 30 min after the glucose pulse (note the constant concentration of glucose in Fig. 1A). Nevertheless, ethanol and acetate production accelerated during this period (Fig. 1A and 2A). This phenomenon was also observed with chemostat cultures of *S. cerevisiae* T2-3D grown on glucose as the sole carbon source (1) and may indicate the mobilization of storage carbohydrates after exposure to glucose excess. Indeed, it has been reported that the glycogen content of *S. cerevisiae* CBS 8066 decreased from 6%, of the culture dry weight to 2% during the first 30 min after a glucose pulse (23).

When, after just over 2 h, residual glucose was no longer

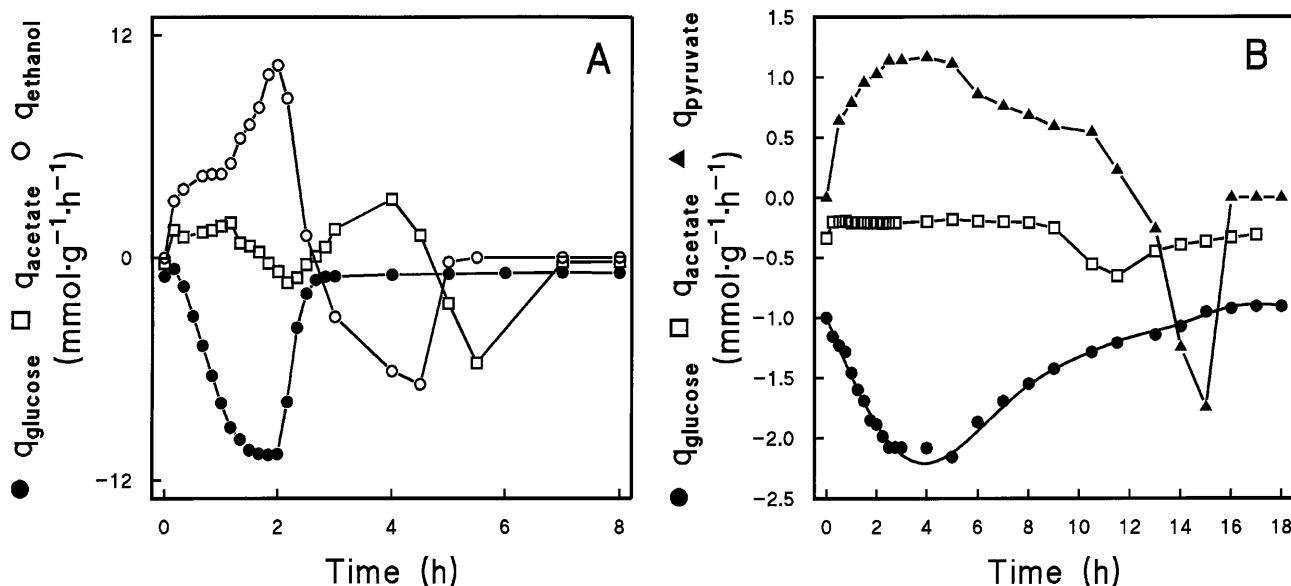


FIG. 2. Specific rates of glucose consumption and metabolite production (q) after addition of a glucose pulse (50 mmol) to steady-state chemostat cultures ($D = 0.10 \text{ h}^{-1}$, 1-liter working volume) of wild-type *S. cerevisiae* T2-3D (A) and the pyruvate decarboxylase-negative strain GG570 (B).

detectable in the feed, the ethanol that had been accumulated was consumed, leading to a further accumulation of acetate in the culture. Finally, the acetate was also consumed (Fig. 1A and 2A).

Metabolic responses of Pdc^- *S. cerevisiae* to glucose excess.

After addition of 50 mmol of glucose to a steady-state, respiring culture of the Pdc^- strain *S. cerevisiae* GG570, the pattern of metabolite formation was completely different from that observed with wild-type cells (Fig. 1, compare panels A and B with C and D). The most notable difference was the complete absence of ethanol, the major metabolite produced by wild-type *S. cerevisiae* under these conditions. Some acetate accumulated after the glucose pulse (Fig. 1D), but this could be explained by the incomplete utilization of the acetate present in the medium feed. In fact, although in the first hours after glucose addition the rate of acetate consumption was lower than in the preceding carbon-limited steady state, a net consumption of acetate continued throughout the pulse experiment (Fig. 2B).

In the Pdc^- strain, the rate of glucose consumption after a pulse was much lower than in wild-type cultures (compare Fig. 2A and B). The maximum rate of glucose consumption in Pdc^- cultures (ca. $2 \text{ mmol} \cdot \text{g}^{-1} \cdot \text{h}^{-1}$) was ca. fivefold lower than the maximum rate of glucose consumption observed in the wild-type cultures. Consequently, it took 11 h before all glucose was consumed, compared to 2.5 h in the wild-type cultures. As observed in the wild-type cultures, the specific rate of glucose consumption gradually increased during the initial phase after the pulse, suggesting that synthesis of glucose-metabolizing enzymes occurred.

In vitro determination of pyruvate dehydrogenase activity.

It is difficult to assay activity of the pyruvate dehydrogenase complex in crude cell extracts of wild-type *S. cerevisiae* due to interference by pyruvate decarboxylase. No such interference was expected in extracts of a Pdc^- strain. Indeed, it was possible to measure pyruvate dehydrogenase activity in crude extracts as pyruvate- and coenzyme A-dependent reduction of NAD^+ . The measured activity was linearly proportional to the amount of cell extract added to the reaction mixture. The

activity of the pyruvate dehydrogenase complex measured in cell extracts of chemostat cultures of the Pdc^- strain (grown at a dilution rate of 0.10 h^{-1}) was $25 \text{ nmol of pyruvate} \cdot \text{min}^{-1} \cdot \text{mg of protein}^{-1}$.

In aerobic, glucose-limited chemostat cultures of wild-type *S. cerevisiae*, the acetyl-CoA requirement for biosynthesis has been estimated at $3.4 \text{ mmol} \cdot \text{g of biomass}^{-1}$ (15). Furthermore, for each g of biomass synthesized, 3.0 mmol of glucose (6.0 mmol of pyruvate) is completely catabolized to carbon dioxide and water (15). At a dilution rate of 0.10 h^{-1} , this results in a total flux from pyruvate to acetyl-CoA of $0.94 \text{ mmol} \cdot \text{g}^{-1} \cdot \text{h}^{-1}$. A rough estimate of the in vivo flux through pyruvate dehydrogenase in the carbon-limited chemostat cultures of the Pdc^- strain can be made by assuming that the acetate present in the reservoir medium stoichiometrically replaces the acetyl-CoA that in wild-type cultures is produced via pyruvate dehydrogenase. In the steady-state cultures, the specific rate of acetate consumption was $0.33 \text{ mmol} \cdot \text{g}^{-1} \cdot \text{h}^{-1}$. This leaves a net flux through the pyruvate dehydrogenase complex of $0.94 - 0.33 = 0.61 \text{ mmol} \cdot \text{g}^{-1} \cdot \text{h}^{-1}$. With an estimated soluble-protein content of the biomass of 30% (14), this corresponds to a pyruvate dehydrogenase activity of $30 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{mg of protein}^{-1}$, which is close to the activity measured in cell extracts.

Does inactivation of the pyruvate dehydrogenase complex occur after a glucose pulse?

As calculated above, the dissipatory flux from pyruvate into the TCA cycle in aerobic, glucose-limited chemostat cultures of the Pdc^- mutant equals ca. $0.6 \text{ mmol of pyruvate} \cdot \text{g of biomass}^{-1} \cdot \text{h}^{-1}$. Complete oxidation to CO_2 requires a specific oxygen uptake rate of $2.5 \times 0.6 = 1.5 \text{ mmol of O}_2 \cdot \text{g}^{-1} \cdot \text{h}^{-1}$, which is 65% of the total oxygen uptake rate in the steady-state cultures ($2.3 \text{ mmol} \cdot \text{g}^{-1} \cdot \text{h}^{-1}$ [Table 1]). The remaining oxygen consumption (i.e., 35%) must be ascribed to oxidation of the cytosolic NADH formed in glycolysis and in biosynthetic reactions.

If after a glucose pulse the pyruvate dehydrogenase complex were completely inactivated, this would be expected to cause a 60% decrease in the rate of oxygen consumption; such a decrease was not observed (Fig. 3). Instead, immediately after a

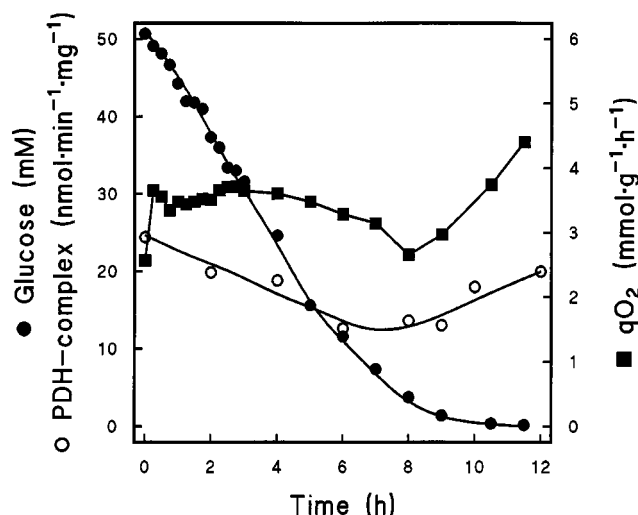


FIG. 3. Regulation of the pyruvate dehydrogenase complex after addition of a glucose pulse to a steady-state chemostat culture ($D = 0.10 \text{ h}^{-1}$) of the Pdc⁻ *S. cerevisiae* strain GG570. Symbols: ●, glucose concentration in the culture; ■, specific rate of oxygen consumption by the culture; ○, specific activity of the pyruvate dehydrogenase complex in cell extracts.

glucose pulse, the specific rate of oxygen uptake increased from 2.3 to 3.5 mmol of O₂ · g⁻¹ · h⁻¹. About half of this increase can be attributed to the incomplete oxidation of glucose to pyruvate (the maximum observed rate of pyruvate production was ca. 1 mmol · g⁻¹ · h⁻¹ [Fig. 2B]). This suggests that in the steady-state chemostat cultures, respiratory pyruvate metabolism operates close to saturation.

After the initial increase of the oxygen uptake rate after a pulse, the respiration rate remained constant for about 4 h (Fig. 3). Apparently, a rapid inactivation of the pyruvate dehydrogenase complex did not occur. After 4 h, a slow decrease of the oxygen uptake rate was observed. This may be indicative of glucose repression of the synthesis of respiratory enzymes, a well-known phenomenon in *S. cerevisiae* (4). During the pulse, the specific rate of carbon dioxide production, which is caused primarily by the pyruvate dehydrogenase reaction and the TCA cycle, followed the rate of oxygen consumption (data not shown). When the residual-glucose concentration in the culture decreased below 5 mM, pyruvate consumption started (Fig. 1C and 2B). This was accompanied by a steady increase of the respiration rate (Fig. 3), probably reflecting a relief of glucose repression of respiratory enzymes.

DISCUSSION

The switch to respiro-fermentative metabolism observed after exposure of respiring *S. cerevisiae* cultures to excess glucose (13, 23) is relevant for industrial applications that require a high biomass yield (5, 24). In the literature, this phenomenon has been explained from a limited capacity of respiratory sugar metabolism (8, 13). Theoretically, such a limited respiratory capacity can reside in three metabolic processes: (i) the reoxidation of NADH that is generated in the cytosol during glycolysis and biosynthetic processes, (ii) any of the reactions leading from pyruvate to carbon dioxide and reduced cofactors (transport of pyruvate from cytosol to mitochondrial matrix, pyruvate dehydrogenase complex, pyruvate dehydrogenase bypass, and TCA cycle), and (iii) the reoxidation of the reduced cofactors generated inside the mitochondrion during respiratory dissimilation of pyruvate. In *S. cerevisiae*, respiratory oxida-

tion of NADH in the cytosol and in the mitochondrial matrix is catalyzed by two separate NADH-dehydrogenase activities. These are both associated with the mitochondrial inner membrane and coupled to the mitochondrial respiratory chain but face different subcellular compartments (2).

If in a Pdc⁻ mutant the capacity for respiratory oxidation of cytosolic NADH were not limiting, glycolysis could proceed at the same rate as in a wild-type strain. Depending on the capacity for dissimilatory pyruvate metabolism, pyruvate might then either be excreted or oxidized to CO₂ in the TCA cycle. Instead, the glycolytic flux observed in glucose pulse experiments with a Pdc⁻ strain was much lower than the flux observed in experiments with the isogenic wild type (Fig. 2). A similar reduction of the specific rate of glucose consumption in strains with a reduced level of pyruvate decarboxylase has been found in shake flask cultures (18). Our observations strongly suggest that also in *S. cerevisiae* pregrown under aerobic, glucose-limited conditions, the capacity for reoxidizing cytosolic NADH by respiration is limited to such an extent that alcoholic fermentation is required to sustain high glycolytic fluxes. This hypothesis can be verified by the simultaneous addition of excess glucose and an electron acceptor to chemostat cultures of Pdc⁻ *S. cerevisiae*. If the presence of an external NADH acceptor enhances the glycolytic flux, this would confirm that the cytosolic NADH/NAD⁺ ratio plays a pivotal role in regulation of the glycolytic flux.

The normal physiological response of *S. cerevisiae* to NADH excess is the production of glycerol (22). Indeed, it has been reported that glycerol production is enhanced in strains with a low pyruvate decarboxylase activity (12). However, increased production of glycerol was not observed either in aerobic batch cultures of the Pdc⁻ strain used in this study (3) or during glucose pulse experiments with chemostat cultures (Fig. 1B and D). The main difference from the work of Nevoigt and Stahl (12) is that those authors measured glycerol production under anaerobic conditions. It is quite possible that oxygen limitation is needed for full induction of the key enzymes of glycerol formation and excretion: under aerobic conditions, wild-type cells can probably rely on respiration to reoxidize any excess NADH formed in biosynthesis and thus do not require glycerol formation (in such cultures, NADH formed in glycolysis can be reoxidized either by fermentation or by respiration). Due to their apparent inability to efficiently reoxidize the NADH formed in glycolysis, Pdc⁻ strains may offer an interesting system for cofactor regeneration in NADH-coupled biological reductions. Experiments to investigate the viability of such a system are in progress in our laboratory.

Only a relatively small increase in the specific rate of glucose consumption was observed after glucose pulses to the Pdc⁻ strain (Fig. 2B). Nevertheless, some accumulation of pyruvate occurred (Fig. 1C). This indicates that in the aerobic, glucose-limited steady-state situation preceding the pulse, respiratory pyruvate metabolism in the Pdc⁻ mutant was operating fairly close to saturation. This is consistent with activities of the pyruvate dehydrogenase complex in cell extracts, which were close to the calculated *in vivo* flux through the complex.

Immediately after the glucose pulse to the Pdc⁻ cultures, the rate of acetate consumption by the cultures decreased (Fig. 2B). This is indicative of a decrease of the *in vivo* activity of acetyl-CoA synthetase, a key enzyme in the pyruvate dehydrogenase bypass (16). If the same phenomenon occurs in wild-type cells, this may be an important factor in the formation of acetate, a metabolite that is invariably formed during exposure of respiring *S. cerevisiae* cells to excess glucose (23). Several mechanisms might explain the reduction of *in vivo* acetyl-CoA synthetase activity after a glucose pulse, including short-term

regulation of acetyl-CoA synthetase activity by covalent modification or allosteric regulation. An alternative explanation is that this enzyme (which in *S. cerevisiae* is at least partially located in the cytosol [1, 9]) has to compete with the hexose kinases for ATP.

It has recently been reported that, in contrast to earlier findings (10), *S. cerevisiae* is able to phosphorylate the E1 α subunit of its pyruvate dehydrogenase complex, leading to loss of enzyme activity (7). Remarkably, phosphorylation could be demonstrated in exponentially growing batch cultures only when they were grown on galactose. The presence in the *S. cerevisiae* genome of an open reading frame that exhibits homology with mammalian pyruvate dehydrogenase kinases (B. Barrel and M. A. Rajandream, gene Y1L042C; GenBank accession no. Z46861) provides a further indication that phosphorylation of the pyruvate dehydrogenase complex may be a relevant regulatory mechanism in this yeast under some growth conditions. However, the lack of inhibition of respiration by a Pdc⁻ mutant after a glucose pulse to a carbon-limited chemostat culture (Fig. 3) suggests that this mechanism is not relevant during the transient exposure of *S. cerevisiae* cells to excess glucose in large-scale industrial bioreactors.

ACKNOWLEDGMENTS

We thank our colleagues of the Delft-Leiden Yeast Group for many stimulating discussions.

This work is supported by a grant from the Dutch Ministry of Economic Affairs (via the ABON program "Metabolic Fluxes in Yeasts and Fungi") and by the European Community (via the research project "From Gene to Product in Yeast: a Quantitative Approach," which is part of the EC Framework IV Cell Factory Program).

REFERENCES

- de Jong-Gubbels, P., J. P. van Dijken, and J. T. Pronk. Unpublished data.
- de Vries, S., and C. A. M. Marres. 1987. The mitochondrial respiratory chain of yeast. Structure and biosynthesis and the role in cellular metabolism. *Biochim. Biophys. Acta* **895**:205-239.
- Flikweert, M. T., L. van der Zanden, W. M. T. M. Janssen, H. Y. Steensma, J. P. van Dijken, and J. T. Pronk. 1996. Pyruvate decarboxylase: an indispensable enzyme for growth of *Saccharomyces cerevisiae* on glucose. *Yeast* **12**:247-257.
- Gancedo, J. M. 1992. Carbon catabolite repression in yeast. *Eur. J. Biochem.* **206**:297-313.
- Hensing, M. C. M., R. J. Rouwenhorst, J. J. Heijnen, J. P. van Dijken, and J. T. Pronk. 1995. Physiological and technological aspects of large-scale heterologous-protein production with yeasts. *Antonie Leeuwenhoek* **67**:261-279.
- Holzer, H., and W. H. Goedde. 1957. Zwei Wege von Pyruvate zu Acetyl-Coenzym A in Hefe. *Biochem. Z.* **329**:175-191.
- James, A. G., R. M. Cook, S. M. West, and J. G. Lindsay. 1995. The pyruvate dehydrogenase complex of *Saccharomyces cerevisiae* is regulated by phosphorylation. *FEBS Lett.* **373**:111-114.
- Käppeli, O. 1986. Regulation of carbon metabolism in *Saccharomyces cerevisiae* and related yeasts. *Adv. Microb. Physiol.* **28**:181-209.
- Kisfal, G., J. Cseko, I. Alkonyi, and A. Sandor. 1991. Isolation and characterization of carnitine acetyltransferase from *Saccharomyces cerevisiae*. *Biochim. Biophys. Acta* **1085**:217-222.
- Kresze, G. B., and H. Ronft. 1981. Pyruvate dehydrogenase complex from bakers' yeast. I. Properties and some kinetic and regulatory properties. *Eur. J. Biochem.* **119**:573-579.
- Mistry, S. C., D. A. Priestman, A. L. Kerbey, and P. J. Randle. 1991. Evidence that rat liver pyruvate dehydrogenase kinase activator protein is a pyruvate dehydrogenase kinase. *Biochem. J.* **275**:775-779.
- Nevoigt, E., and U. Stahl. 1996. Reduced pyruvate decarboxylase and increased glycerol-3-phosphate dehydrogenase [NAD⁺] levels enhance glycerol production in *Saccharomyces cerevisiae*. *Yeast* **12**:1331-1337.
- Petrik, M., O. Käppeli, and A. Fiechter. 1983. An expanded concept for the glucose effect in the yeast *Saccharomyces uvarum*: involvement of short-term and long-term regulation. *J. Gen. Microbiol.* **129**:43-49.
- Postma, E., C. Verduyn, W. A. Scheffers, and J. P. van Dijken. 1989. Enzymic analysis of the Crabtree effect in glucose-limited chemostat cultures of *Saccharomyces cerevisiae*. *Appl. Environ. Microbiol.* **55**:468-477.
- Pronk, J. T., T. J. Wenzel, M. A. H. Luttik, C. C. M. Klaassen, W. A. Scheffers, H. Y. Steensma, and J. P. van Dijken. 1994. Energetic aspects of glucose metabolism in a pyruvate-dehydrogenase-negative mutant of *Saccharomyces cerevisiae*. *Microbiology* **140**:601-610.
- Pronk, J. T., H. Y. Steensma, and J. P. van Dijken. 1996. Pyruvate metabolism in *Saccharomyces cerevisiae*. *Yeast* **12**:1607-1633.
- Reed, L. J., and S. J. Yeaman. 1987. Pyruvate dehydrogenase. *The Enzymes* **28**:77-95.
- Schmitt, H. D., and F. K. Zimmermann. 1982. Genetic analysis of the pyruvate decarboxylase reaction in yeast glycolysis. *J. Bacteriol.* **151**:1146-1152.
- Steensma, H. Y., L. Holterman, I. Dekker, C. A. van Sluis, and T. J. Wenzel. 1990. Molecular cloning of the gene for the E1 α subunit of the pyruvate dehydrogenase complex from *Saccharomyces cerevisiae*. *Eur. J. Biochem.* **191**:769-774.
- Sweere, A. P. J., J. Giesselbach, R. Barendse, R. de Krieger, G. Honderd, and K. C. A. M. Luyben. 1988. Modelling the dynamic behaviour of *Saccharomyces cerevisiae* and its application in control experiments. *Appl. Microbiol. Biotechnol.* **28**:116-127.
- Uhlinger, D. J., C. Y. Yang, and L. J. Reed. 1986. Phosphorylation-dephosphorylation of pyruvate dehydrogenase from yeast. *Biochemistry* **25**:5673-5677.
- van Dijken, J. P., and W. A. Scheffers. 1986. Redox balances in the metabolism of sugars by yeasts. *FEMS Microbiol. Rev.* **32**:199-224.
- van Urk, H., P. R. Mak, W. A. Scheffers, and J. P. van Dijken. 1988. Metabolic responses of *Saccharomyces cerevisiae* CBS 8066 and *Candida utilis* CBS 621 upon transition from glucose limitation to glucose excess. *Yeast* **4**:283-291.
- Verduyn, C. 1991. Physiology of yeasts in relation to growth yields. *Antonie Leeuwenhoek* **60**:325-353.
- Verduyn, C., T. P. L. Zomerdiijk, J. P. van Dijken, and W. A. Scheffers. 1984. Continuous measurement of ethanol production by aerobic yeast suspensions with an enzyme electrode. *Appl. Microbiol. Biotechnol.* **19**:181-185.
- Verduyn, C., E. Postma, W. A. Scheffers, and J. P. van Dijken. 1990. Physiology of *Saccharomyces cerevisiae* in anaerobic glucose-limited chemostat cultures. *J. Gen. Microbiol.* **136**:395-403.
- Wenzel, T. J., M. A. van den Berg, W. Visser, J. A. van den Berg, and H. Y. Steensma. 1992. Characterization of *Saccharomyces cerevisiae* mutants lacking the E1 α subunit of the pyruvate dehydrogenase complex. *Eur. J. Biochem.* **209**:697-705.
- Weusthuis, R. A., W. Visser, J. T. Pronk, W. A. Scheffers, and J. P. van Dijken. 1994. Effects of oxygen limitation on sugar metabolism in yeasts: a continuous-culture study of the Kluyver effect. *Microbiology* **140**:703-715.
- Yeaman, S. J. 1989. The 2-oxo acid dehydrogenase complexes: recent advances. *Biochem. J.* **257**:625-632.