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Glycolytic Flux Is Conditionally Correlated with ATP Concentration in *Saccharomyces cerevisiae*: a Chemostat Study under Carbon- or Nitrogen-Limiting Conditions

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Anaerobic and aerobic chemostat cultures of *Saccharomyces cerevisiae* were performed at a constant dilution rate of 0.10 h^{-1}. The glucose concentration was kept constant, whereas the nitrogen concentration was gradually decreasing; i.e., the conditions were changed from glucose and energy limitation to nitrogen limitation and energy excess. This experimental setup enabled the glycolytic rate to be separated from the growth rate. There was an extensive uncoupling between anabolic energy requirements and catabolic energy production when the energy source was present in excess both aerobically and anaerobically. To increase the catabolic activity even further, experiments were carried out in the presence of 5 mM acetic acid or benzoic acid. However, there was almost no effect with acetate addition, whereas both respiratory (aerobically) and fermentative activities were elevated in the presence of benzoic acid. There was a strong negative correlation between glycolytic flux and intracellular ATP content; i.e., the higher the ATP content, the lower the rate of glycolysis. No correlation could be found with the other nucleotides tested (ADP, GTP, and UTP) or with the ATP/ADP ratio. Furthermore, a higher rate of glycolysis was not accompanied by an increasing level of glycolytic enzymes. On the contrary, the glycolytic enzymes decreased with increasing flux. The most pronounced reduction was obtained for HXK2 and ENO1. There was also a correlation between the extent of carbohydrate accumulation and glycolytic flux. A high accumulation was obtained at low glycolytic rates under glucose limitation, whereas nitrogen limitation during conditions of excess carbon and energy resulted in more or less complete depletion of intracellular storage carbohydrates irrespective of anaerobic or aerobic conditions. However, there was one difference in that glycogen dominated anaerobically whereas under aerobic conditions, trehalose was the major carbohydrate accumulated. Possible mechanisms which may explain the strong correlation between glycolytic flux, storage carbohydrate accumulation, and ATP concentrations are discussed.

The yeast *Saccharomyces cerevisiae* is an organism with a remarkable metabolic flexibility. It is one of the few yeasts which are able to grow fermentatively under strictly anaerobic conditions (40). It is also capable of using an entirely respiratory metabolism when sufficient oxygen is available. These two types of catabolism are, however, often used simultaneously. Respiration is the more efficient pathway in terms of ATP production per substrate utilized, but apparently with a limited capacity for high rates compared with fermentation. For instance, by gradually increasing the dilution rate in chemostat cultures, it has been demonstrated that *S. cerevisiae* uses a strictly respiratory metabolism at low dilution rates whereas at high dilution rates, fermentation is used in addition to respiration (19, 29, 41).

The rates of energy production in these catabolic pathways are not always correlated with the anabolic energy requirements; i.e., more ATP is produced than needed by biosynthetic and maintenance demands (19, 20, 36). This phenomenon of metabolic uncoupling has also been reported for bacteria (14, 24, 25, 35). During growth of *S. cerevisiae* in a glucose-limited chemostat, the cells face an energy rather than a carbon limitation (30, 39). Consequently, under such conditions, a low extent of uncoupling is to be expected. A nitrogen limitation, on the other hand, may provoke metabolic uncoupling, since this represents an energy-excess situation. Indeed, this has been reported for aerobic chemostat cultures of *S. cerevisiae* where a gradual change from glucose (energy) limitation to nitrogen limitation and energy excess was studied (19). The primary response during energy excess was an increased respiratory activity; however, when the maximum respiratory activity was reached, also the rate of fermentation increased (19). An increased respiratory activity does not necessarily mean a higher ATP production rate, since the P/O ratio may be adjusted to compensate for the increased flux and avoid excessive ATP production. A higher rate of ethanol production, however, implies that more ATP is being produced unless futile cycles, such as between fructose 1,6-phosphate and fructose 6-phosphate, exist in glycolysis. Other ways of minimizing ATP production under fermentative conditions would include increased glycerol production, which is an ATP-consuming reaction. There may also be different types of futile cycles operating. For instance, the synthesis and subsequent degradation of the storage carbohydrates glycogen and trehalose could function as potential futile cycles. There does seem to be a connection between the rate of glycolysis and storage carbohydrates, since accumulation of these compounds is considered to be associated with periods of reduced growth rate (21); i.e., it might be that at low fluxes storage carbohydrates accumulate, whereas at high fluxes these pathways are either not in use or involved in futile cycling.

The rate of glycolysis can potentially be regulated both by allosteric control of existing enzymes and by changes in the amount of glycolytic enzymes. However, attempts to improve

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flux by increasing the amount of certain key enzymes have in general proved unsuccessful (9). The glycolytic flux in *S. cerevisiae* is controlled at the level of phosphofructokinase (PFK) and pyruvate kinase, but also glucose transport or phosphorylation is thought to play an important role (4, 5). The regulation of glycolytic rate as well as rate of respiration involves nucleotides and especially adenine nucleotides (3–5, 11). The adenine nucleotides act as allosteric effectors, but they are also involved as substrates and products of glycolysis. Therefore, both their absolute concentrations and their mass action ratios may be of importance for the regulation of glycolytic and respiratory flux. Beauvoit et al. (3) found that different respiratory and glycolytic rates were induced in transfer experiments using different carbon sources and conditions, i.e., aerobic or anaerobic, and a correlation was found between ATP/ADP ratio and glycolytic rate. The respiratory rate was also influenced by the ATP/ADP ratio, but in this case it was also dependent on the redox potential. Several studies on glycolytic control have focused on one particular aspect of yeast metabolism, the Pasteur effect (i.e., the decreased glycolytic rate obtained when respiratory-competent cells are transferred from anaerobic to aerobic conditions) (5, 7, 8, 18, 28).

In this study, many different glycolytic and respiratory rates (at constant growth rate) were obtained under steady-state situations in chemostat cultures anaerobically as well as aerobically. The changes in glycolytic flux were brought about by imposing on the cells a gradually increasing nitrogen limitation, and hence increasing surplus of energy, i.e., by inducing different degrees of metabolic uncoupling. To reach even higher fluxes of both glycolysis and respiration, we also took advantage of the uncoupling effect of weak organic acids, e.g., acetic acid and benzoic acid. The aims of this study were to (i) investigate the levels of intracellular nucleotides and glycolytic enzymes at different glycolytic fluxes and (ii) determine whether there is a correlation between any of these concentrations and rate of glycolysis. Furthermore, we examined how changes in catabolic flux, at constant growth rate, affected the accumulation of the storage carbohydrates glycogen and trehalose.

MATERIALS AND METHODS

Yeast strains and media. The baker's yeast strain, supplied by a Swedish baker's yeast manufacturer (Jästbolaget AB, Rotebro, Sweden), was used. In the anaerobic experiments, also strain W303-1A (*ade2 his3 leu2 trp1 ura3 can1-100*) was used. Qualitatively identical results were obtained with this strain; i.e., metabolic uncoupling was observed, and the pattern of storage carbohydrate accumulation was the same even though the levels were lower (the correlation between ATP concentration and glycolytic flux of W303-1A is included in Fig. 2). However, apart from this result, only the experiments with the baker's yeast strain are shown.

Cells were cultured in a defined medium described by Albers et al. (1), with $(NH_4)_2SO_4$ as the nitrogen source. Ergosterol and Tween 80 were omitted in the aerobic experiments. In experiments with the auxotrophic mutant, the necessary amino acids and bases were added to the medium at a concentration of 120 mg/liter or, for leucine, 240 mg/liter. The glucose concentration was kept constant at 20 (anaerobic) or 5 (aerobic) g/liter, whereas the ammonium sulfate concentration was varied in order to obtain medium C/N ratios ranging from 10 to 640 (g/g) (anaerobic) or 2.5 to 160 (aerobic). In the experiments with acetic acid and benzoic acid addition, a feed medium concentration of 5 mM was used.

Growth conditions. The chemostat cultures were conducted at a constant dilution rate of 0.10 h^{-1} in a 2-liter CMF minifermentor (Chemap AG, Volketswil, Switzerland). The working volume was 1.5 liters, the temperature was 30°C, the stirring rate was 500 (anaerobic) or 600 (aerobic) rpm, and the pH was controlled at 5.0 by addition of 1 M NaOH. Anaerobic conditions were obtained by flushing the fermentor with N_2 at a rate of 0.2 vol/vol/min by using a mass flow controller (Bronkhorst High-Tech B.V., Ruurlo, The Netherlands). Aerobic conditions were established by flushing air at a rate of 0.3 vol/vol/min. This ensured a dissolved oxygen concentration above 65% air saturation.

The activity of the culture was monitored continuously by measuring the CO_2 production or, aerobically, also the O_2 consumption with an acoustic carbon dioxide and oxygen monitor (type 1308; Brüel and Kjaer, Naerum, Denmark). Alternatively, the total activity (rate of heat production) of the culture was measured with a microcalorimeter (Thermal Activity Monitor 2277; Thermometric AB, Järfälla, Sweden) operating in the flowthrough mode.

After inoculation, the substrate feed for continuous operation was initiated once the cells had reached the late exponential or stationary phase of batch growth. The experiments were performed by starting at the lowest C/N ratio without any addition. After attainment of steady state and sampling, 5 mM acetic acid was added to the medium reservoir (at the same C/N ratio); thereafter, acetic acid was replaced by 5 mM benzoic acid. Subsequently, this was repeated at the next-higher medium C/N ratio (no addition and addition of 5 mM acetic acid and 5 mM benzoic acid). Samples were taken, once a steady state had been established, for determination of biomass concentration, extracellular concentrations of glucose, ethanol, glycerol, acetate, and ammonium, intracellular concentrations of nucleotides, content of proteins and the storage carbohydrates glycogen and trehalose, and protein composition by two-dimensional (2D) polyacrylamide gel electrophoresis (PAGE).

Test for steady-state conditions. A constant heat production rate or a constant CO_2 production rate was used as the criterion for the establishment of steady-state conditions.

Dry weight determinations. Samples (two of 5 ml each) were centrifuged for 5 min at 5,000 × *g*, washed twice with 0.9% (wt/vol) NaCl, dried for 24 h at 105°C, and stored in a desiccator before being weighed.

Determination of extracellular glucose, ethanol, acetate, glycerol, and ammonium concentrations. Samples (two of 1.5 ml each) were centrifuged for 2 min at 15,000 × *g*, and the supernatants were stored in the freezer (−20°C). The concentrations were determined with enzyme combination kits (Biochemica Test Combination; Boehringer Mannheim GmbH, Mannheim, Germany).

Determination of glycogen and trehalose content of the cells. Samples (two of 10 ml each) were mechanically extracted as described previously (32) except that 0.2 M citrate buffer (pH 4.8) was used. Glycogen was enzymatically hydrolyzed by 1.4 U of amyloglucosidase (Boehringer Mannheim) per ml in 0.2 M citrate buffer (pH 4.8). Trehalose was hydrolyzed in a separate assay by 0.2 U of trehalase (Sigma Chemical Company, St. Louis, Mo.) per ml in 0.2 M citrate buffer (pH 5.7). The enzymatic digestion was incubated overnight, and the released glucose was analyzed with an enzymatic kit (Boehringer Mannheim).

Determination of total protein content of the cells. Samples (two of 10 ml each) were centrifuged at 5,000 × *g* for 5 min and washed twice with 0.9% (wt/vol) NaCl. The pellet was resuspended in 3 ml of NaOH, and total protein was determined by a modified biuret method (39), using bovine serum albumin as a standard.

Calculation of Y_{ATP}. ATP yield (Y_{ATP}; grams of biomass formed per mole of ATP consumed) was calculated by assuming that each ethanol and acetate formed was accompanied by formation of 1 mol of ATP. Glycerol production, on the other hand, results in the consumption of 1 mol of ATP.

Determination of intracellular nucleotides. Samples (two of 1 ml each) were extracted by a trichloroacetic acid method previously described (13). Analysis was performed by ion-pair reversed-phase high-pressure liquid chromatography. The column was Hypersil BDS C18 (Hewlett-Packard Company, Camas, Wash.), 250 by 4.6 mm and a particle size of 5 μm. The mobile phase consisted of 60 mM $NH_4H_2PO_4$ plus 5 mM tetrabutylammonium dihydrogen phosphate (pH 6.50). The acetonitrile concentration was linearly increased from 0 to 8% during the first 15 min, and this concentration was maintained until the end of the run. The temperature was 42°C, and the flow rate was 1 ml/min. Detection was performed by measuring the absorbance at 254 nm.

2D PAGE analysis. The 2D-PAGE was performed as described previously (26), with first-dimensional separation in an immobilized pH 3 to 10 gradient subsequently resolved on sodium dodecyl sulfate-containing 10% acrylamide gels (26). Resolved proteins have previously been identified by microsequencing (26), and image data can be found on our World Wide Web server (http://yeast-2DPAGE.gmm.gu.se). The proteins were silver stained by using a silver nitrate-based protocol, with prior treatment of the gels with dithiothreitol (23). To properly quantitate the stained 2D PAGE-resolved proteins, a calibration strip with stepwise increased concentrations of bovine serum albumin was simultaneously stained and used in the computer-aided image analysis. By applying this quantitation procedure, the silver staining technology showed a protein-invariant linear response (25a). 2D PAGE-resolved proteins were automatically quantified by image analysis using the PDQUEST software (version 5.1), which is a commercial variant of the QUEST system (12).

RESULTS

All experiments were carried out in chemostat cultures at a constant dilution rate of 0.10 h^{-1}. Energy excess was imposed by a stepwise decrease in the ammonium concentration of the feed medium while keeping the glucose level constant, with C/N ratios ranging from 10 to 640 (g/g). Sampling was done when steady state was established. In some experiments, 5 mM acetic acid or 5 mM benzoic acid was added to the feed medium to obtain even higher glycolytic rates (37) (at a constant growth rate).

TABLE 1. Changes in measured variables during anaerobic chemostat cultures of *S. cerevisiae* at various feed medium C/N ratios[a]

C/N (g/g) ratio	Weak acid	Biomass (g/liter)	Glucose (mM)	NH_4 (mM)	Ethanol (mM)	Glycerol (mM)	Acetate (mM)	Glucose (mmol/g/h)	Ethanol (mmol/g/h)	Glycerol (mmol/g/h)	Y_{ATP} (g/mol)	Protein (%)	Glycogen (%)	Trehalose (%)	ATP (μmol/g)	ADP (μmol/g)
10	None	2.08	0.4	47.0	153.5	14.2	0.1	5.6	7.4	0.7	14.9	52.0	7.1	1.0	8.6	1.5
40	None	1.83	0.8	5.1	159.4	11.9	0.1	6.4	8.7	0.7	12.4	53.4	7.9	1.7	9.1	2.3
80	None	1.74	0.6	0.1	160.4	11.0	0.4	6.9	9.2	0.6	11.6	42.2	7.4	2.7	6.2	1.7
160	None	1.14	15.4	<0.1	145.4	12.1	1.3	9.1	12.7	1.1	8.5	34.8	3.6	1.3	4.7	0.8
320	None	0.58	56.2	<0.1	88.4	7.7	1.2	10.9	15.3	1.3	7.1	32.9	1.0	0.2	3.6	0.9
640	None	0.42	85.2	<0.1	56.2	5.2	1.0	7.4	13.3	1.2	8.1	25.4	0.2	0.9	3.5	0.7
10	Benzoic	0.87	2.2	50.2	185.5	6.7	0.2	13.0	21.3	0.8	4.9	55.7	2.5	0.5	8.2	2.9
40	Benzoic	0.99	1.4	7.4	182.5	6.9	0.2	12.0	18.5	0.7	5.6	55.9	3.7	0.8	7.7	2.6
80	Benzoic	0.96	0.8	2.1	194.8	7.1	0.2	12.2	20.4	0.7	5.1	55.9	2.4	0.7	6.6	1.8
160	Benzoic	0.67	2.0	0.1	190.4	6.3	0.2	17.7	28.3	0.9	3.6	49.9	1.3	0.0	4.8	1.9
320	Benzoic	0.41	33.7	<0.1	136.7	4.7	0.4	20.6	33.3	1.1	3.1	43.3	0.2	0.0	3.7	1.5
640	Benzoic	0.25	73.0	<0.1	85.9	3.1	0.6	16.4	34.8	1.3	3.0	39.3	0.0	0.3	2.8	1.1

[a] Performed at a constant dilution rate of 0.10 h^{-1} and with or without addition of 5 mM benzoic acid.

Anaerobic growth characteristics. At the lowest C/N ratios, the cells were energy limited, with a high residual ammonium concentration (Table 1). When the nitrogen content of the feed decreased, glucose accumulated in the chemostat and the cells faced energy excess. The growth yields could be calculated to 0.10 g of biomass/g of glucose during energy limitation, whereas energy excess resulted in an approximately 50% reduction in yield. However, the reduction in biomass concentration was not proportional to the decrease in the nitrogen content of the feed, implying that the nitrogen content of the cells was lower under nitrogen limitation than under glucose limitation. When 5 mM benzoic acid was added to the feed medium, the biomass concentration decreased (Table 1) and the growth yield never exceeded 0.05 g/g. It should be noted, however, that energy excess also in this case caused a reduction in yield compared to energy limitation. There was also a slightly higher residual glucose concentration under glucose limitation in the presence of benzoic acid, with values in the range of 1.4 to 2.2 mM. Without addition of benzoic acid, the residual glucose concentration was always well below 1 mM. The highest ethanol concentrations were obtained in the presence of benzoic acid, with an increase of 21% compared to no addition (Table 1). For glycerol, the result was the opposite; i.e., benzoic acid addition yielded a significant decrease in the concentration of this product (Table 1). The presence of acetate produced results more or less identical to those without weak acid addition. In fact, with the exception of storage carbohydrate accumulation (see below), acetate addition did not change any of the variables measured; hence, these data are not shown.

Anaerobic substrate consumption and product formation rates. Changing the conditions from energy limitation to energy excess had a strong effect on the specific glucose consumption and ethanol production rates (Table 1). During these conditions, the specific ethanol production rate rose approximately twofold. Benzoic acid addition yielded even higher ethanol production rates (Table 1), especially when the presence of benzoic acid was combined with energy excess, where values well above 30 mmol/g h were recorded. The specific glycerol production rates were approximately doubled when the conditions changed from energy limitation to energy excess. Except perhaps for a slightly higher glycerol production under glucose limitation and benzoic acid addition, the effect of weak acid on the productivity of glycerol seemed limited (Table 1).

Anaerobic protein content and Y_{ATP}. The protein content of the cells decreased from 52% during glucose limitation to only 25% under nitrogen limitation at the highest feed medium C/N ratio tested (Table 1). Benzoic acid addition provoked a higher protein content of the cells compared to no addition, especially at high C/N ratios under nitrogen limitation (Table 1). Y_{ATP} was 15 g/mol of ATP under energy-limiting conditions, whereas energy excess induced a more wasteful metabolism, resulting in values of 7 to 8 g/mol of ATP (Table 1). Benzoic acid had a strong negative effect on Y_{ATP}. The presence of this acid decreased Y_{ATP} to 5 to 6 g/mol of ATP under energy limitation, and energy excess caused a further reduction to only 3 g/mol of ATP (Table 1).

Anaerobic storage carbohydrate accumulation. Glycogen, and also to a certain extent trehalose, accumulated to a higher degree during energy-limiting conditions (Table 1). When the C/N ratio of the feed was changed and there was an excess of carbon and energy, the cells responded by decreasing the accumulation of storage carbohydrates. These experiments were repeated with the haploid strain W303-1A with similar results, i.e., high accumulation during energy limitation and a gradually decreasing content of storage carbohydrate as the nitrogen content of the feed was reduced and energy excess was imposed. Addition of benzoic acid lowered the accumulation of these compounds (Table 1). Also the presence of acetic acid reduced the accumulation of storage carbohydrates, although not to the same extent as found with benzoic acid (data not shown). The fact that the cells were almost completely depleted of storage carbohydrates and the drastic reduction in protein content during nitrogen limitation brings into question the composition of the dry biomass. In our opinion, the most likely explanation is an increasing contribution from the cell wall. Another possibility is an increased lipid accumulation, but if this were the case, also the energy content of the cells should increase. This has not been found under nitrogen-limiting conditions (18a) except for oleaginous yeast (2).

Anaerobic intracellular nucleotide content. The intracellular content of both ATP and ADP decreased as the conditions changed from energy limitation to energy excess (Table 1). Consequently, the ATP/ADP ratio was largely unaffected, with all values between 4 and 6. The ATP content decreased gradually from 8 to 9 μmol/g during glucose limitation to 3 to 4 μmol/g at the highest feed medium C/N ratio tested. Benzoic acid had no effect on intracellular ATP content, whereas the ADP levels were elevated (Table 1). The values were on average approximately two times higher, and as a result the ATP/ADP ratio in this case ranged from 2 to 3.

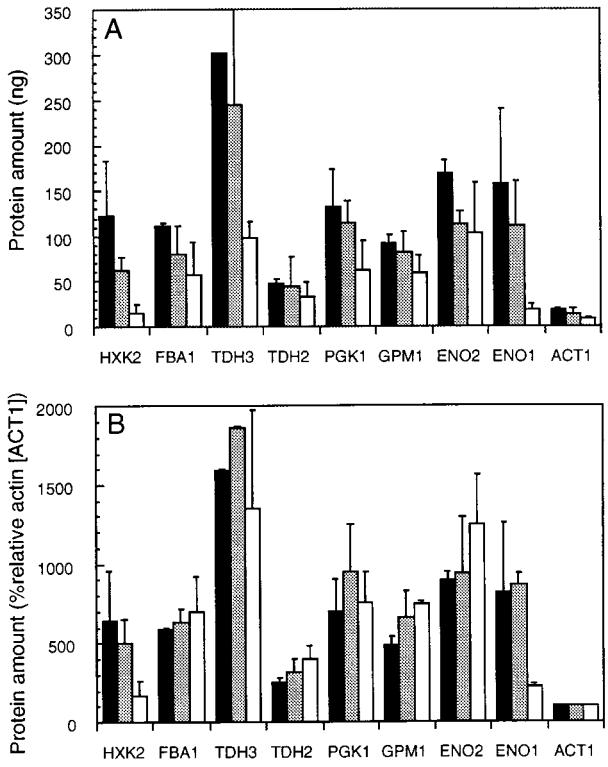

FIG. 1. Changes in the levels of glycolytic enzymes analyzed by 2D PAGE during anaerobic chemostat cultures of *S. cerevisiae* at a dilution rate of 0.10 h^{-1} and feed medium C/N ratios of 10 (black bars), 80 (grey bars) and 320 (open bars). (A) Protein amount per 20 μg of total protein; (B) amount of protein as percentage of the value for actin.

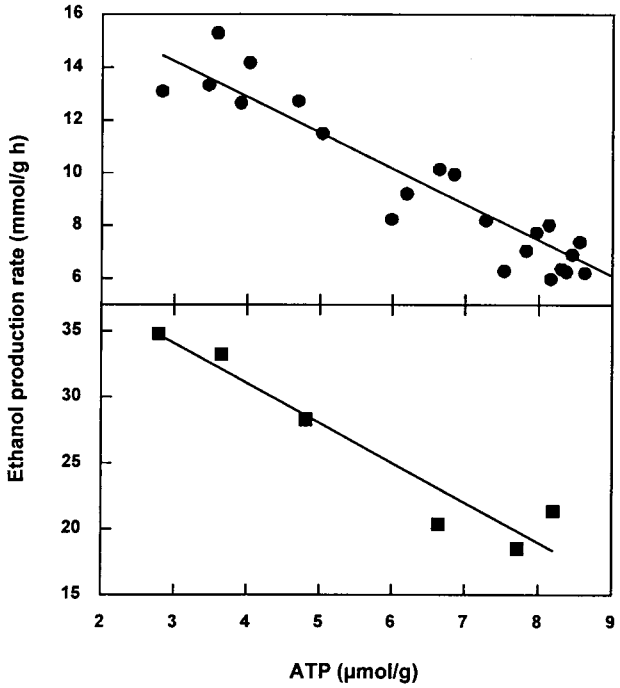

FIG. 2. Correlation between glycolytic rate (measured as ethanol production rate) and intracellular ATP content during anaerobic chemostat cultures of *S. cerevisiae* at a dilution rate of 0.10 h^{-1} with (■) and without (●) addition of 5 mM benzoic acid. The results without addition of weak acid are based on two entirely separate experiments using the baker's yeast strain and one experiment with strain W303-1A. r^2 was 0.85, and the t value was −10.9 (n = 22). Benzoic acid additions were performed with the baker's yeast strain. r^2 was 0.92, and the t value was −6.8 (n = 6).

Anaerobic protein pattern. We used 2D PAGE analysis to determine whether the changes in glycolytic flux were accompanied by changes in the levels of glycolytic enzymes. There seemed to be a general trend in that the relative amounts of glycolytic enzymes decreased when the conditions changed from energy limitation to energy excess (Fig. 1A). However, also the actin content decreased, and if the results were normalized to actin concentration, only the hexokinase HXK2 and ENO1 still showed a significant decrease in abundance during energy excess (Fig. 1B).

Correlation between rate of glycolysis and adenine nucleotide content under anaerobic conditions. For each series (e.g., no addition or addition of benzoic acid) of varying C/N ratio there was a strong negative correlation between glycolytic flux and intracellular ATP content; i.e., the higher the ATP content, the lower the rate of glycolysis (Fig. 2). No correlation was found with the other nucleotides tested (ADP, GTP, and UTP) or with the ATP/ADP ratio (data not shown). However, the same intracellular ATP content yielded a much higher glycolytic flux in the presence of benzoic acid than without addition (Fig. 2).

Correlation between storage carbohydrate accumulation and rate of glycolysis under anaerobic conditions. There was also a correlation between the extent of storage carbohydrate accumulation and rate of glycolysis; i.e., higher rate was accompanied by lower accumulation (Fig. 3). Even though there was still a linear correlation between flux and accumulation when benzoic acid was added, the relationship between rate of glycolysis and storage carbohydrate content was changed (Fig. 3).

To determine whether the correlation between glycolytic flux, storage carbohydrate accumulation, and ATP content was restricted to anaerobic conditions, the experiments were repeated under aerobic conditions. However, due to the higher yield (biomass per glucose) under aerobic conditions, the range of C/N ratios was changed to 2.5 to 160 (g/g).

Aerobic growth characteristics. At the two lowest feed medium C/N ratios, ammonium could be detected in the chemo-

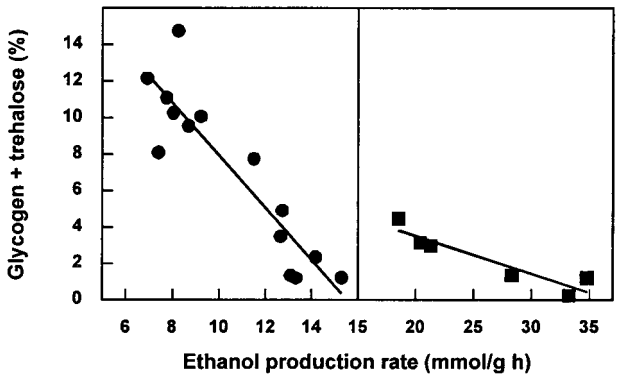

FIG. 3. Correlation between storage carbohydrate accumulation and glycolytic rate (measured as ethanol production rate) during anaerobic chemostat cultures of *S. cerevisiae* at a dilution rate of 0.10 h^{-1} with (■) and without (●) addition of 5 mM benzoic acid. The results without addition of weak acid are based on two entirely separate experiments using the baker's yeast strain. r^2 was 0.82, and the t value was −7.5 (n = 14). Benzoic acid addition yielded an r^2 of 0.87 and a t value of −5.1 (n = 6).

TABLE 2. Changes in measured variables during aerobic chemostat cultures of *S. cerevisiae* at various feed medium C/N ratios[a]

C/N (g/g) ratio	Weak acid	Biomass (g/liter)	Glucose (mM)	NH_4 (mM)	Glucose (mmol/g/h)	Oxygen (mmol/g/h)	Ethanol (mmol/g/h)	Y_{ATP} (g/mol)[b]	Protein (%)	Glycogen (%)	Trehalose (%)	ATP (μmol/g)	ADP (μmol/g)
2.5	None	2.70	0.2	41.3	1.0	2.3	0.0	16.0	44.9	1.5	4.5	7.4	0.8
10	None	2.30	0.0	4.9	1.2	3.0	0.0	12.5	46.8	1.2	3.5	7.2	1.2
20	None	2.04	0.1	<0.1	1.4	3.6	0.0	10.3	35.7	1.6	4.0	5.2	1.0
40	None	1.37	0.2	<0.1	2.0	3.0	1.2	10.7	26.9	1.1	2.1	4.2	0.7
80	None	0.77	0.9	<0.1	3.5	2.6	4.0	9.2	24.9	0.5	1.3	4.3	0.7
160	None	0.44	8.8	<0.1	4.4	2.8	5.4	7.8	24.3	0.0	1.1	3.5	0.6
2.5	Benzoic	1.53	0.1	43.2	1.8	7.2	0.0	5.2	49.7	0.6	1.5	6.9	2.7
10	Benzoic	1.72	0.0	4.2	1.6	6.0	0.0	6.3	47.0	0.5	1.4	6.3	2.2
20	Benzoic	1.53	0.1	<0.1	1.8	7.0	0.0	5.3	40.7	0.3	1.0	5.8	1.8
40	Benzoic	0.86	0.2	<0.1	3.3	7.1	2.4	4.7	39.2	0.4	0.9	5.9	2.0
80	Benzoic	0.53	0.4	<0.1	5.2	5.5	6.2	4.8	32.0	0	0.8	3.9	1.2
160	Benzoic	0.39	1.2	<0.1	7.0	5.2	9.7	4.2	27.2	0	0.8	2.2	0.7

[a] Performed at a constant dilution rate of 0.10 h^{-1} and with or without addition of 5 mM benzoic acid.
[b] Calculated by assuming a P/O ratio of 1.0.

stat and the cells were glucose or energy limited (Table 2). When the C/N ratio was increased above 10, the residual ammonium concentration was below 0.1 mM and there was a progressive decrease in biomass concentration (Table 2). In other words, the conditions shifted to energy excess.

Aerobic substrate consumption and product formation rates. The change from energy limitation to energy excess resulted in an increase in specific glucose consumption rate from 1.0 up to a maximum of 4.4 mmol/g/h (Table 2). Benzoic acid addition resulted in an increased glucose consumption from 1.7 to a maximum of 7.0 mmol/g/h under the same conditions. The ethanol production rate was negligible up to a feed medium C/N ratio of 20. When the ratio was further increased, there was a sharp increase in ethanol production, with the highest values attained in the presence of benzoic acid (Table 2). The increase in ethanol production rate did not coincide with any decrease in respiratory activity. The specific oxygen consumption rate was more or less the same throughout the whole range of C/N ratios tested, with typical values at about 3 mmol/g/h (Table 2). However, in the presence of benzoic acid, the respiratory activity increased and the values centered around 7 mmol/g/h, with a slight reduction at the highest C/N ratios.

Aerobic protein content and ATP yield. The protein content of the cells was slightly lower aerobically compared to anaerobically, ranging from 45 to 47% during glucose limitation down to 24 to 25% under nitrogen limitation (Table 2). There was, as under anaerobic conditions, a higher protein content in the presence of benzoic acid, although the difference was smaller aerobically compared to anaerobically (Tables 1 and 2). By assuming a constant P/O ratio of 1.0, (39), Y_{ATP} could be calculated to be 15 to 16 g/mol of ATP under energy limitation (Table 2). When the cells were subjected to nitrogen limitation and energy excess, the values decreased down to a minimum of 8 g/mol of ATP whereas benzoic acid caused a further reduction in Y_{ATP} (Table 2), with a minimum value of 4.2 g/mol of ATP.

Aerobic storage carbohydrate accumulation. As under anaerobic conditions, storage carbohydrate accumulation was most pronounced during glucose and energy limitation (Table 2). The amount of storage carbohydrates gradually decreased as the nitrogen feed was reduced and the excess of carbon and energy source increased. However, one difference was that aerobically, trehalose was the dominating carbohydrate (Table 2). Addition of benzoic acid reduced the accumulation of these compounds.

Aerobic intracellular nucleotide content. Intracellular ATP and ADP levels were very similar to those under anaerobic conditions; i.e., change of the conditions from energy limitation to energy excess was accompanied by a decrease in both ATP and ADP (Table 2). Furthermore, the contents were similar to those under anaerobic conditions and with ATP levels largely unaffected by the addition of benzoic acid to the feed medium, whereas the ADP levels were much higher in the presence of benzoic acid (Table 2).

Correlation between rate of glycolysis and adenine nucleotide content under aerobic conditions. There was still a linear correlation between rate of glycolysis and the intracellular ATP content (Fig. 4). As under anaerobic conditions, benzoic acid addition resulted in a different response between glycolytic flux and ATP content (although there was also in this case a linear correlation). A different response was also found when we compared anaerobic and aerobic conditions; i.e., anaerobically, the glycolytic flux was higher (at the same ATP content).

Correlation between rate of glycolysis and storage carbohydrate accumulation under aerobic conditions. The correlation between rate of glycolysis and storage carbohydrate accumulation found under anaerobic conditions was also observed aerobically (Fig. 5). However, also in this case the change from anaerobic to aerobic conditions resulted in a different relationship between rate of glycolysis and storage carbohydrate accumulation, with the most pronounced accumulation obtained anaerobically (Fig. 3 and 5).

DISCUSSION

Metabolic uncoupling. It is clear from this and previous studies (19, 20, 36) that uncoupling between anabolic energy demands and catabolic energy production can occur in *S. cerevisiae*. Anaerobically, when the nitrogen feed was decreased and energy excess was introduced, the catabolic activity of the cells increased (at a constant growth rate) until a saturation value in ethanol production rate of about 15 mmol/g/h was reached (Table 1). However, the presence of benzoic acid somehow enabled the cells to increase the fermentation rate above what seemed to be a saturation level (Table 1). Similar effects have earlier been reported for respiratory activity under aerobic conditions, where oxygen consumption rates of 19 to

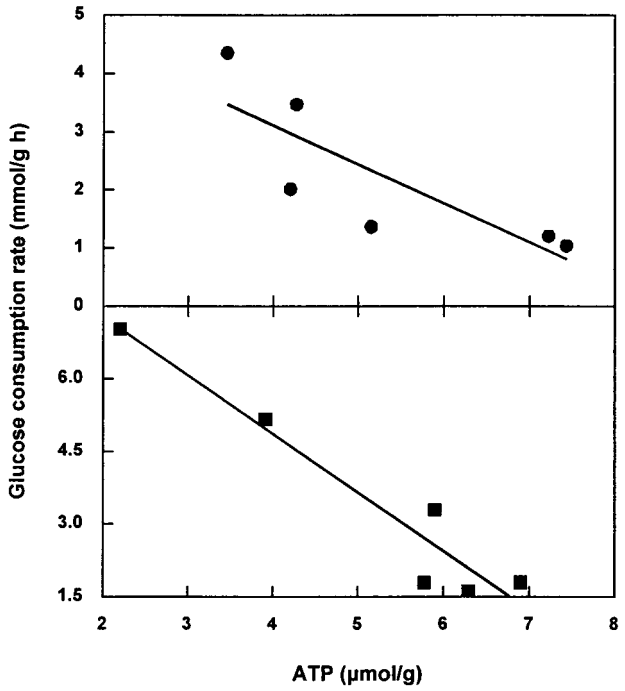

FIG. 4. Correlation between glycolytic rate (measured as glucose consumption rate) and intracellular ATP content during aerobic chemostat cultures of *S. cerevisiae* at a dilution rate of 0.10 h^{-1} with (■) and without (●) addition of 5 mM benzoic acid. r^2 without addition was 0.67, and the *t* value was −2.9 ($n = 6$); for benzoic acid, r^2 was 0.92 and the *t* value was −7.0 ($n = 6$).

21 mmol/g/h were recorded in the presence of benzoic acid (37). This value is much higher than the maximum respiratory rate reported by, e.g., Rieger et al. (29) for *S. cerevisiae*.

ATP production and consumption. Much interest has been devoted to the efficiency of energy generation in yeast (10, 19, 20, 31, 38, 39). Y_{ATP} values calculated under aerobic conditions by assuming a P/O ratio of 1.0 were similar to the values found during anaerobic conditions (Tables 1 and 2). This similarity was obtained both under energy limitation and energy excess, indicating that the P/O ratio is not regulated in order to minimize ATP production when the anabolic energy requirements

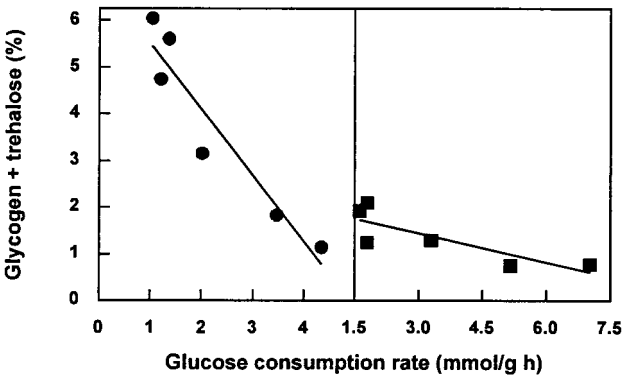

FIG. 5. Correlation between storage carbohydrate accumulation and glycolytic rate (measured as glucose consumption rate) during aerobic chemostat cultures of *S. cerevisiae* at a dilution rate of 0.10 h^{-1} with (■) and without (●) addition of 5 mM benzoic acid. r^2 without addition was 0.90, and the *t* value was −6.1 ($n = 6$), while benzoic acid addition yielded an r^2 of 0.68 and a *t* value of −2.9 ($n = 6$).

are low. Furthermore, the increased glycolytic and hence ATP production rate resulting from energy excess and/or benzoic acid addition under anaerobic conditions (Table 1) means that the cells must possess a mechanism(s) for disposal of this overproduction of ATP. The problem of how *S. cerevisiae* disposes of ATP, apparently not needed for known anabolic functions, was raised by Lagunas in 1976 (17). However, this is still an unanswered question. One such potential mechanism would be glycerol production, which is an ATP-consuming reaction. The rationale for glycerol production under anaerobic conditions is to maintain a cytoplasmic redox balance (27) by consuming the excess NADH formed during biosynthesis, mainly amino acid synthesis (1). Indeed, it did seem as if the cells used glycerol formation for consumption of unnecessary ATP under some but not all conditions. There was an increased specific glycerol production both anaerobically and aerobically, when nitrogen limitation was imposed and the ATP production was higher than what could be explained by anabolic requirements (Table 1). However, the ratio between ethanol production and glycerol production remained largely unaffected (Table 1); hence, increased glycerol production could not be the sole explanation for how the cells dispose of excess ATP. The increased glycerol production during energy excess could instead, at least under anaerobic conditions, also be a reflection of an increased protein turnover (33). Since protein synthesis is a very energy demanding process, an increased turnover of proteins might be an effective way of disposing of excess ATP.

Regulation of glycolytic flux. The strong negative correlation between intracellular ATP content and the rate of glycolysis suggests that ATP levels are involved in the regulation of this pathway. The other explanation would be that the rate of glycolysis in combination with the energy demand determines the ATP levels. However, if this was the case, there ought to be a positive correlation between ATP content and glycolytic flux. Instead, our study showed that the highest rates of glycolysis were obtained during energy excess and low ATP levels (Tables 1 and 2; Fig. 2 and 4). A correlation between dilution rate and intracellular ATP content can also be calculated from the data published by Sierkstra et al. (34). The authors did not mention this themselves, but by plotting the ATP contents versus dilution rate and by excluding the highest dilution rate (where the metabolism changed from respiratory to mixed respiratory-fermentative), a linear correlation could be obtained, with a correlation coefficient (r^2) of 0.83 and a *t* value of −4.4. In their study, the growth yield and RQ (respiratory quotient; moles of CO_2 produced per mole of O_2 consumed) were constant within this range of dilution rates; hence, changes in dilution rate were directly proportional to changes in glycolytic flux. The clear correlation between intracellular ATP content and glycolytic flux is probably a reflection of allosteric regulation of glycolysis. Another possibility might be that glycolytic flux is regulated by adjustment of the level of glycolytic enzymes. However, this seems not to be the case, since the enhanced glycolytic flux obtained under energy excess was in fact accompanied by a decrease in the amount of glycolytic enzymes, most notably HXK2 and ENO1 (Fig. 1). HXK and PFK are considered key enzymes in the control of glycolytic flux. PFK is subject to a number of allosteric regulators, including an inhibition by ATP (11, 28). Reibstein et al. (28) reported that PFK activity decreased when the ATP concentration was changed from 1.5 to 3.1 mM, and different ADP concentrations were without effect. However, PFK activity was found to be virtually independent of ATP concentrations ranging from 2 to 5 mM (28). Similarly, Caubet et al. (6) showed a very low effect on PFK activity when the ATP concentration was raised above 0.5 mM. By assuming an intracellular volume

of 2 μl/mg (22), the ATP concentrations in the present study ranged from approximately 1.1 to 4.6 mM. In other words, by extrapolating these in vitro data, the measured ATP concentrations seems to be higher than the range where it controls PFK activity. It has been reported that the activity of HXK2 is inhibited by ATP concentrations above 2 mM ATP (15). When the conditions change gradually from energy limitation to energy excess, the resulting increased glycolytic flux might be due to an increased HXK2 activity regulated by the ATP level. However, this proposition is based on in vitro data, and it may very well be that in vivo, ATP controls glycolytic flux by regulating the activity of HXK2 as well as that of PFK (or even more enzymes). Furthermore, regulation of the glycolytic rate involves more than just ATP. For instance, benzoic acid addition or transition from anaerobic to aerobic conditions changed the relationship between ATP content and rate of glycolysis, implying that more factors are involved in this regulation.

Regulation of storage carbohydrate accumulation. The increased catabolic rate had a large effect on the accumulation of storage carbohydrates (Fig. 3 and 5). It is well known that yeast cells sequentially accumulate glycogen and trehalose under nitrogen-limiting conditions in the presence of excess carbon and energy (21). In addition, the growth rate also appears to be an important parameter for determination of storage carbohydrate accumulation. Chemostat studies under glucose limitation showed that synthesis of reserve carbohydrates decreased with increasing growth rate (16). However, this study suggests that it is the rate of glycolysis rather than the growth rate which is the determinant for storage carbohydrate accumulation (Fig. 3 and 5). The magnitude of glycolytic flux seemed to be far more important than the type of limitation, i.e., nitrogen or glucose. In fact, a higher accumulation of reserve carbohydrates under glucose limitation than under nitrogen limitation at low dilution rates was also reported earlier (16). The ATP and ADP concentrations could also have a direct role in the regulation of storage carbohydrate synthesis due to their allosteric effects on the involved enzymes (11). However, the known regulatory effects of ATP and ADP cannot explain the results obtained in this study. The glycogen synthase is inhibited by ATP and ADP. The active dephosphorylated form is, however, less inhibited than the inactive phosphorylated form (11), and our results showed the highest accumulation of glycogen concomitant with the highest levels of ATP plus ADP (Table 1). It could be, though, that the inhibitory effect of ATP plus ADP was counteracted by an elevated concentration of the substrate of storage carbohydrate synthesis under these circumstances.

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