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Regulation of Glycolytic Flux and Ethanol Production in *Saccharomyces cerevisiae*: Effects of Intracellular Adenine Nucleotide Concentrations on the In Vitro Activities of Hexokinase, Phosphofructokinase, Phosphoglycerate Kinase, and Pyruvate Kinase†

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The progressive decline in the glycolytic activity of *Saccharomyces cerevisiae* during batch fermentation is accompanied by changes in adenine nucleotide pools. The relative activities of four glycolytic enzymes were examined in vitro in the presence of nucleotide concentrations equivalent to intracellular pools. Phosphofructokinase and pyruvate kinase were not inhibited. Phosphoglycerate kinase was inhibited by AMP but was judged unlikely to be of physiological consequence owing to enzyme abundance. Both isoenzymes of hexokinase were strongly inhibited by AMP. The degree of hexokinase inhibition was sufficient to account for the observed decline in glycolytic activity during batch fermentation.

The commercial production of fuel ethanol is based upon the fermentation of hexoses by *Saccharomyces cerevisiae* (19, 21, 23). Ethanol productivity per milligram of cell protein is greatest during the early hours of fermentation and progressively declines as ethanol accumulates (8, 9). This decline begins when low concentrations of ethanol accumulate (<2% by volume), which coincides with the shift in metabolism from balanced growth to stationary phase (8). Eliminating the decline in glycolytic flux and ethanol production would decrease the time required to complete fermentations, increasing production capacity in commercial plants.

The biochemical basis for the decline in ethanol productivity has been investigated by many laboratories (5, 15, 18, 24). This decline is exacerbated by nutritional limitations for compounds such as magnesium (8, 9) and unsaturated membrane lipids (3, 5) and by environmental stress, such as elevated temperatures (3, 18, 24). The decline appears to result from a combination of the inhibitory effects of accumulated ethanol and from changes in cellular physiology (9, 11). Possible causes for this decline, such as inactivation of glycolytic enzymes (10), inadequate levels of coenzymes (11), inhibition of glucose uptake (11), and changes in internal pH (10), have been eliminated. Phosphorylated intermediates of glycolysis did not accumulate during the decline, indicating that no single enzyme in the pathway between glucose 6-phosphate and ethanol represented a major restriction of glycolysis (11).

Intracellular AMP concentration increased dramatically during batch fermentation and was accompanied by other changes in adenine nucleotide pools (11). Hexokinase is competitively inhibited by AMP (13, 22), and other glycolytic enzymes, such as phosphoglycerate kinase (1), may also be inhibited by changes in nucleotide levels. In this study, we have examined the relative activities of four glycolytic enzymes involved in sugar phosphorylation and ATP synthesis in the presence of adenine nucleotide concentrations equivalent to those found within cells at different times during fermentation (11).

Enzymes were prepared from *S. cerevisiae* KD2 (7). Phosphofructokinase was purified as a 35 to 55% ammonium sulfate fraction (20) and was also used as a source of pyruvate kinase. Hexokinase was also purified by ammonium sulfate fractionation (2) and was used as a source of the hexokinase isoenzymes (PI and PII) and phosphoglycerokinase. Preparations were dialyzed immediately before use. Hexokinase PII was irreversibly inactivated by a 6-h incubation with xylose and MgATP (12) to allow investigation of PI alone (55% of total hexokinase activity). Activities were determined in the forward direction for glycolysis as follows: hexokinase and pyruvate kinase (6, 17), phosphoglycerate kinase (4), and phosphofructokinase (20). Coenzyme concentrations for these reactions were 1 mM ADP or 1 mM ATP (as appropriate), 0.2 mM NADH (phosphoglycerate kinase, pyruvate kinase, and phosphofructokinase), and 0.3 mM NADP (hexokinase). Fructose 1,6-bisphosphate (1 mM) was included as an allosteric activator with pyruvate kinase. Buffers, reducing agents, and substrates were as indicated in the original references. Further increases in the concentrations of glycolytic intermediates as substrates did not increase catalytic activity, and thus the concentrations were presumed to be saturating.

The effects of AMP on phosphofructokinase, phosphoglycerate kinase, pyruvate kinase, and hexokinase activities are shown in Fig. 1. Both the mixture of the two hexokinase isoenzymes and hexokinase PI were very similar in the extent to which AMP inhibited glucose phosphorylation. Phosphoglycerate kinase was inhibited to a lesser extent. The concentrations of AMP required to cause a 50% inhibition of hexokinase and phosphoglycerate kinase were approximately 5.5 and 15 mM, respectively. Pyruvate kinase

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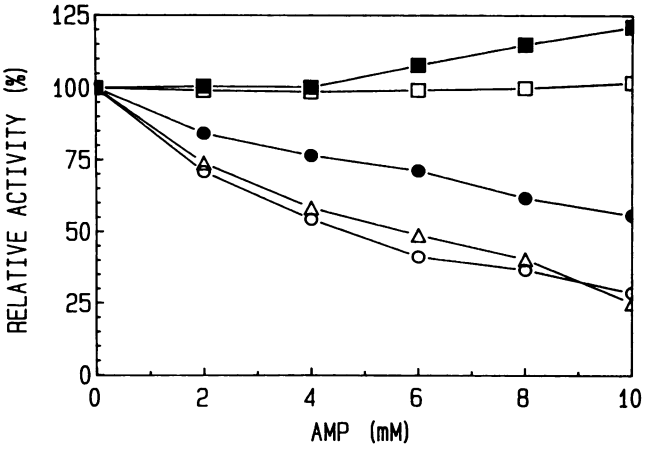

FIG. 1. Effects of AMP on enzyme activity. Symbols: ○, mixed hexokinase isoenzymes; △, hexokinase PI; ●, phosphoglycerate kinase; □, pyruvate kinase; ■, phosphofructokinase.

was not affected by AMP, and phosphofructokinase was stimulated by AMP addition.

The effects of nucleotide pools on enzymatic activity are summarized in Fig. 2. Adenine nucleotide concentrations are summarized in Table 1. Glycolytic activity of washed cells is included for comparison (10). Phosphofructokinase was not significantly affected by the changes in AMP and ATP concentrations. The changes in pyruvate kinase activity primarily reflect variations of ADP levels around the K_m, 4 mM (14). Since both the mixture of hexokinase isoenzymes and hexokinase PI were similar with respect to AMP inhibition, only total hexokinase activity was examined. Hexokinase and phosphoglycerate kinase were maximal at nucleotide concentrations measured in cells after 12 h, declining in the presence of nucleotide concentrations equivalent to those in cells during the latter stages of fermentation. The decline in hexokinase activity coincided with that of glycolytic activity.

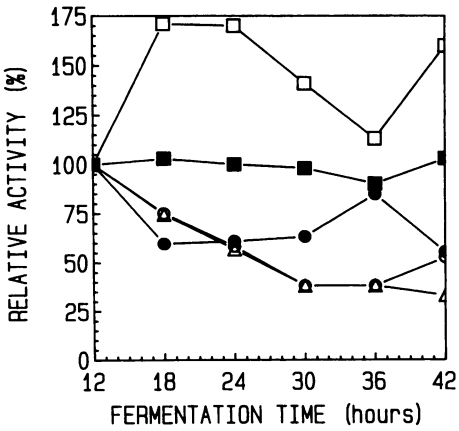

FIG. 2. Effects of intracellular coenzyme levels present at various times during fermentation on the activity of selected enzymes in vitro. Enzymes were assayed in the presence of AMP and ADP or of AMP and ATP concentrations equivalent to those measured within cells at different times during fermentation (15). Data are presented relative to the rates of 12 h, the peak of glycolytic activity and ethanol production per unit of cell protein. Symbols: ○, mixed hexokinase isoenzymes (AMP + ATP); ●, phosphoglycerate kinase (AMP + ADP); □, pyruvate kinase (AMP + ADP); ■, phosphofructokinase (AMP + ATP); △, relative glycolytic activity (10).

TABLE 1. Intracellular concentrations of adenine nucleotides at various times during batch fermentation[a]

Time (h)	Ethanol concn (%, vol/vol)	Nucleotide concn (mM)		
		AMP	ADP	ATP
12	1.0	0.7	1.3	1.7
18	3.7	3.3	3.4	2.1
24	6.6	6.9	2.5	0.7
30	8.5	6.9	1.6	0.2
36	10.4	5.4	1.1	0.2
42	11.5	8.6	2.2	0.6

[a] Data presented are from a standardized fermentation with *S. cerevisiae* KD2 that contained 200 g of glucose per liter and were previously presented as a graph in reference 14.

In evaluating the role of AMP or ADP in the decline of ethanologenic activity during batch fermentation, glycolytic flux and the abundance of individual glycolytic enzymes must also be considered. Glycolytic flux was estimated to be 0.5 μmol of hexose per min and 1.0 μM triose per min per mg of cell protein in fermenting cells after 12 h (9). This activity fell by 60% after 30 h and by 67% after 42 h. Phosphoglycerate kinase and pyruvate kinase activities were 10- to 50-fold higher than those required to support glycolytic flux (10) and do not appear to contribute to the decline. However, pyruvate kinase is strongly dependent upon fructose 1,6-bisphosphate, an allosteric activator (14) which may serve to regulate activity in vivo. Phosphofructokinase responds to a number of allosteric effectors, including inhibition by ATP and activation by fructose 2,6-bisphosphate (16, 20). This enzyme was stimulated by AMP and appears to also remain in excess (10).

Hexokinase activity in crude extracts from cells during fermentation was 0.8- to 4-fold higher than glycolytic flux under substrate-saturating conditions (10). In vivo catalysis by hexokinase can be estimated from the product of the fraction of activity observed in vitro in the presence of nucleotide concentrations equivalent to those observed in vivo and the measurements of activity in cell extracts at various times during fermentation (10). The predicted in vivo activities of hexokinase after 30 and 42 h were 0.42 and 0.39 IU, respectively, sufficient to cause the observed decline in glycolytic flux and ethanol production during batch fermentation.

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