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Effect of a Nonmetabolizable Analog of Fructose-1,6-bisphosphate on Glycolysis and Ethanol Production in Strains of *Saccharomyces cerevisiae* and *Escherichia coli*

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Abstract

Fructose-1,6-bisphosphate (F-1,6-P2) is an allosteric activator of two key enzymes of glycolysis: phosphofructokinase and pyruvate kinase. Regulation of glycolysis in a wild-type *Saccharomyces cerevisiae* and a recombinant *Escherichia coli* by a dead-end structural analog of F-1,6-P2 was studied. 2,5-Anhydromannitol (2,5-AM), a structural analog of β-D-fructose, was used. On being taken up by the cells, 2,5-AM was converted into its monophosphate and diphosphate by the enzymes of the glycolytic pathway. The final product, 2,5-anhydromannitol-1,6-bisphosphate, could not be metabolized further and, therefore, accumulated inside the cells. Glucose and fructose were used as substrates. It was found that 2,5-AM at concentrations of 1 m*M* or less did not have any effect on either substrate consumption or ethanol production. At concentrations of 2,5-AM of 2.5 m*M* or greater, significant inhibition of both glucose and fructose was observed, with fructose inhibition much more severe. We discuss the possible mechanisms of glycolysis inhibition by 2,5-AM at high concentrations and the regulation of glycolysis by this compound.

Index Entries: Glycolysis regulation; *Saccharomyces cerevisiae*; *Escherichia coli*; β-D-fructose structural analog; 2,5-anhydromannitol; fructose-1, 6-bisphosphate.

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Introduction

Glycolysis is probably the most important biochemical pathway in industrial microbiology. It is the starting point of glucose metabolism. Several industrially important chemicals are derived directly from it, such as succinic acid, ethanol, and lactic acid. There has been a strong belief that phosphofructokinase 1 (PFK1) is the key enzyme controlling flux in glycolysis *(1)*. The product of PFK1, fructose-1,6-bisphosphate (F-1,6-P2), has been identified as one of the several allosteric activators of this enzyme *(2,3)*. F-1,6-P2 also is an allosteric activator of another key regulatory enzyme of glycolysis, pyruvate kinase (PK), which catalyzes the final step of the pathway *(4,5)*. With its important role in the regulation of the two key enzymes of glycolysis, it is of great interest to know the influences of F-1,6-P2 at various levels on the activity of this pathway. Overexpression of PFK1 was observed to have no effects on the glycolytic flux in *Saccharomyces cerevisiae (6)*. The intracellular concentrations of the product of this enzyme, F-1,6-P2, also stayed at the same levels as those in the controls. Thus, controlling the expression of PFK1 did not seem to be a suitable method to increase the intracellular levels of F-1,6-P2. We therefore chose a different approach and used a structural analog of fructose, 2-5-anhydromannitol (2,5-AM). Figure 1 illustrates the structural similarity of 2,5-AM and β-D-fructose. 2,5-AM, as an analog of β-D-fructose locked in the furan ring structure, can be taken up by the cells and converted into 2,5-anhydromannitol-1-monophosphate by hexokinase and subsequently into 2,5-anhydromannitol-1,6-bisphosphate (2,5-AM-1,6-P2) by PFK1 *(7–9)*. 2,5-AM-1,6-P2 is an analog of β-fructose-1,6-P2 rather than α-fructose-1,6-P2 and, therefore, is not readily hydrolyzed by fructose-1,6-bisphosphatase, which has preference for the α anomer *(10)*. 2,5-AM-1,6-P2 subsequently will accumulate in the cells *(11)*. The potential of 2,5-AM-1,6-P2 to act as a regulator of glycolysis was thus investigated.

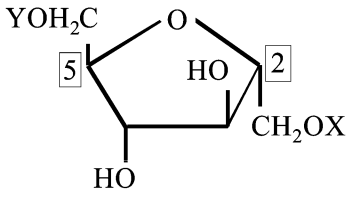
Materials and Methods

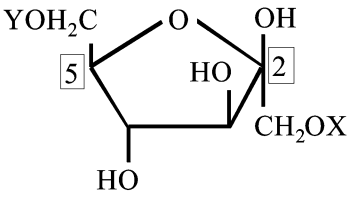
Materials

S. cerevisiae ATCC 24855 was obtained from the American Type Culture Collection (ATCC) (Manassas, VA). *Escherichia coli* KO11, a recombinant strain capable of producing ethanol from glucose and xylose at high rates and yields, was kindly provided by Professor Lonnie Ingram at the University of Florida (Gainesville). The stock cultures were maintained in 25% glycerol at –70°C. 2,5-AM was purchased from Sigma (St. Louis, MO).

Experimental Procedures

Cultures were grown in Luria medium *(12)* supplemented with 20 g/L of glucose. The medium was sterilized by autoclaving at 121°C for 20 min. One frozen vial containing 1.25 mL of stock culture was thawed and used to inoculate 500 mL of medium in a Fernbach flask. The flask was incubated





	X	Y
2,5-Anhydro-D-mannitol	H	H
2,5-AM-1-P	PO_3^{2-}	H
2,5-AM-1,6-P2	PO_3^{2-}	PO_3^{2-}

	X	Y
β-D-Fructose	H	H
β-D-Fructose-1-P	PO_3^{2-}	H
β-D-Fructose-1,6-P 2	PO_3^{2-}	PO_3^{2-}

Fig. 1. Structural similarity between 2,5-AM and β-D-fructose.

overnight at 30°C and shaken at 150 rpm. The cells used in each experiment were recovered from 20 mL of overnight culture by centrifugation. The cell pellet was washed twice with deionized water and resuspended in 20 mL of 50 m*M* Tris buffer. In the case of *S. cerevisiae* ATCC 24855, the pH of the buffer was adjusted to 5.5 with concentrated HCl, and for *E. coli* KO11, it was adjusted to 6.5, the optimum pH observed for ethanol production by this strain *(13)*. Substrates of glycolysis included glucose, fructose, and glycerol. Stock solutions of 2,5-AM, glucose, fructose, and glycerol were prepared fresh in suitable buffer solutions. Prior to starting an experiment, these solutions were preincubated in a water bath at 30°C. After the recovered and washed cells were resuspended in buffer, the test tube was placed in the same water bath at 30°C and allowed to reach thermal equilibrium, which normally took about 5 min. Then 2,5-AM was added first, followed by either glucose, fructose, or glycerol. In all experiments, the initial substrate concentration was fixed at 5 g/L unless otherwise stated. 2,5-AM concentrations were varied from 10 μ*M* to 10 m*M*. Several batches of cells were used for the experiments. A control experiment was performed in duplicates for each batch. The test tube containing the mixture was thoroughly mixed by vortexing and incubated without shaking at 30°C. Samples of 0.5 mL were taken at t = 0+ and then every 30 min. The test tube was thoroughly mixed by vortexing before a sample was taken. Metabolic activities were stopped by immediate addition of 0.5 mL of 1 *M* H_2SO_4. The samples were centrifuged on a microcentrifuge and the supernatants were stored in a –20°C freezer for analysis. Concentrations of substrates (glucose, fructose, glycerol) and product (ethanol) were plotted against time, and the initial rates were calculated. Experiments were performed in duplicates, and the reported results are averages of the two measurements.

Analytical Methods

Glucose, fructose, glycerol, and ethanol were analyzed using a high-performance liquid chromatography (HPLC) system consisting of a Waters 410 RI detector, a Waters 717 Plus Autosampler, and an Alltech 425 HPLC pump.

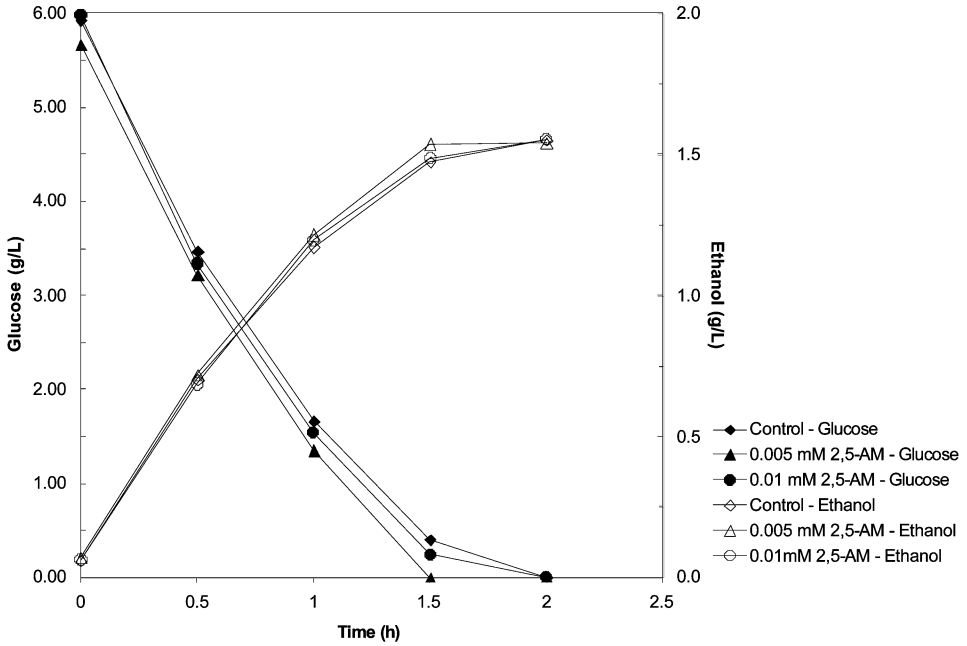


Fig. 2. Glucose consumption and ethanol production in experiments using *S. cerevisiae* ATCC 24855 plus 2,5-AM added at 0.005 and 0.01 m*M*.

The column was Aminex HPX-87H (Bio-Rad, Hercules, CA). The mobile phase was 5 m*M* H_2SO_4 pumped at a flow rate of 0.6 mL/min. Data acquisition and analysis were performed using Waters Millenium software.

Results and Discussion

S. cerevisiae *ATCC 24855*

Figures 2–4 show the concentration profiles of glucose and ethanol obtained with *S. cerevisiae* ATCC 24855. Table 1 summarizes the effects of 2,5-AM concentrations on glucose consumption and ethanol production. It can be seen that at concentrations of 1 m*M* or less 2,5-AM did not show any effect on both glucose consumption and ethanol production. At concentrations of 2.5 m*M* or greater, 2,5-AM inhibited both glucose consumption and ethanol production. The inhibition increased with increasing 2,5-AM concentration. Since β-D-F-1,6-P2 is an allosteric activator of both PFK and PK *(2–5)*, the fact that its dead-end structural analog, 2,5-AM-1,6-P2, which was produced from 2,5-AM, did not show any activation of either glucose consumption or ethanol production at concentrations of 1 m*M* or less may indicate that PFK and PK probably are not the rate-limiting steps of glycolysis in *S. cerevisiae*.

In an attempt to determine the location of inhibition by 2,5-AM at high concentrations (2.5 m*M* or greater), an experiment was performed in which

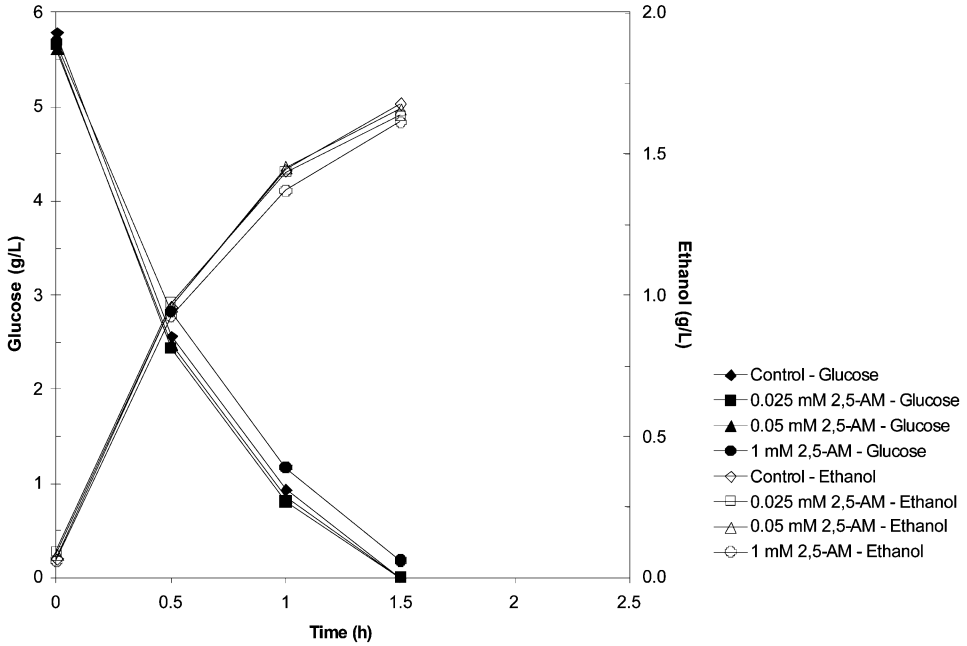


Fig. 3. Glucose consumption and ethanol production in experiments using *S. cerevisiae* ATCC 24855 plus 2,5-AM added at 0.025, 0.05, and 1 m*M*.

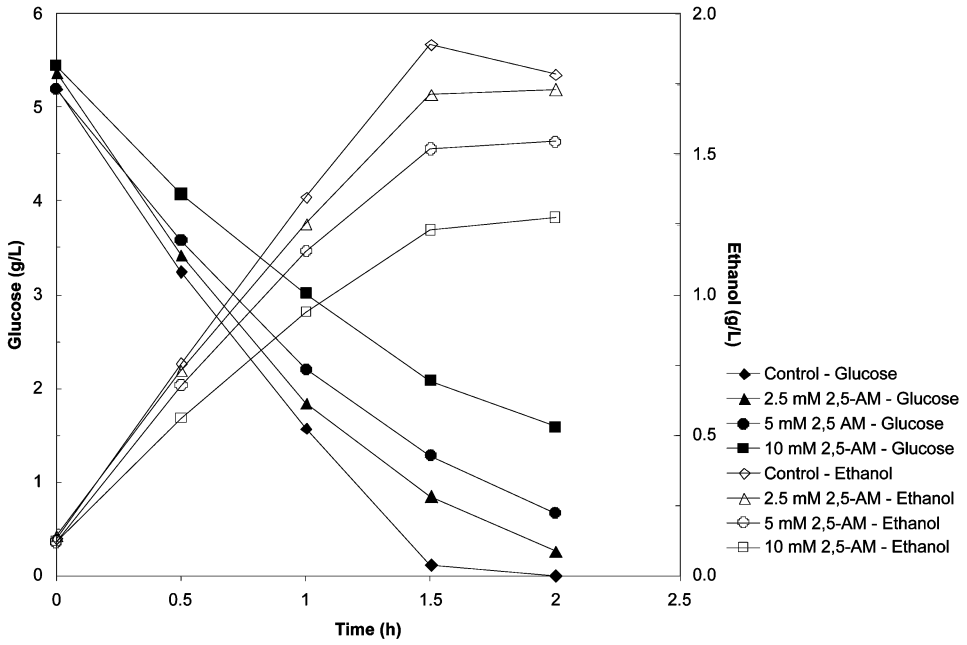


Fig. 4. Glucose consumption and ethanol production in experiments using *S. cerevisiae* ATCC 24855 plus 2,5-AM added at 2.5, 5, and 10 m*M*.

Table 1
Effects of 2,5-AM on Glucose Consumption and Ethanol Production by *S. cerevisiae* ATCC 24855[a]

2,5-AM concentration (m*M*)	Glucose consumption rate (g/[L·h])	Ethanol production rate (g/ [L·h])	Effect of 2,5-AM on glucose consumption rate (%)	Effect of 2,5-AM on ethanol production rate (%)	Ethanol yield (g ethanol/ g glucose consumed)
0.005	4.32 (4.26)	1.15 (1.11)	+1.4	+3.6	0.27 (0.26)
0.01	4.44 (4.26)	1.13 (1.11)	+4.2	+1.8	0.26 (0.26)
0.025	4.89 (4.89)	1.35 (1.38)	0	–2.2	0.29 (0.29)
0.05	4.80 (4.89)	1.38 (1.38)	–1.8	0	0.29 (0.29)
1	4.54 (4.89)	1.32 (1.38)	–7.2	–4.3	0.28 (0.29)
2.5	3.03 (3.39)	1.05 (1.18)	–10.6	–11	0.32 (0.34)
5	2.63 (3.39)	0.94 (1.18)	–22.4	–20.3	0.30 (0.34)
10	2.43 (3.39)	0.74 (1.18)	–28.3	–37.3	0.23 (0.34)

[a]The results obtained in control experiments are given in parentheses.

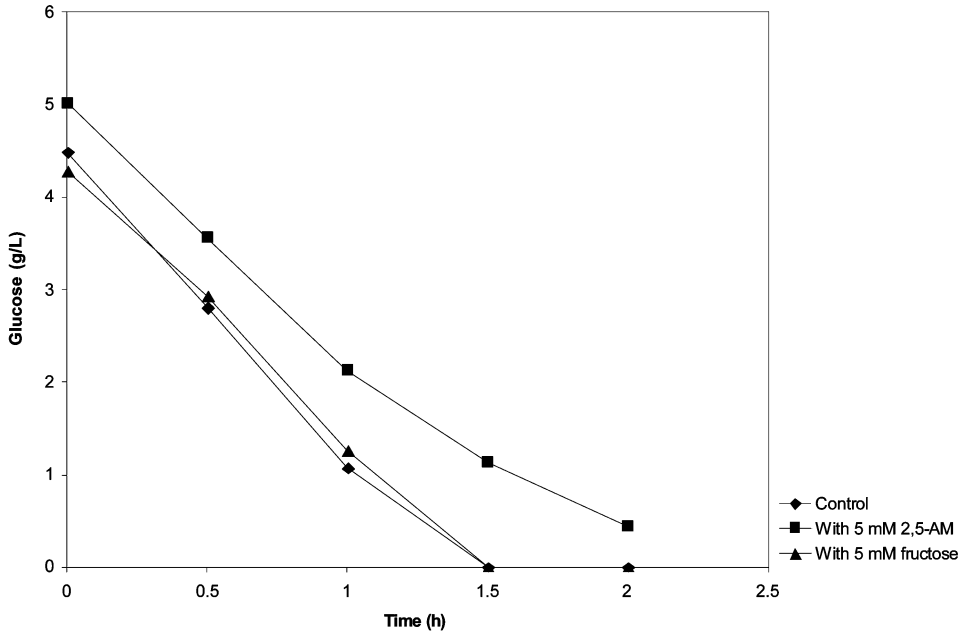


Fig. 5. Effects of 5 m*M* fructose and 5 m*M* 2,5-AM on glucose consumption by *S. cerevisiae* ATCC 24855.

5 m*M* fructose was added to the reaction mixture and the results were compared with those obtained with 5 m*M* 2,5-AM; the results are plotted in Fig. 5. Again, 5 m*M* 2,5-AM showed inhibition of both glucose consumption and ethanol production whereas 5 m*M* fructose had no effect. The initial glucose consumption rates obtained in the control, control plus 5 m*M* fructose, and control plus 5 m*M* 2,5-AM were 3.42, 3.39, and 2.90 g/(L·h), respectively. Therefore, the inhibition must be located after the hexokinase phosphorylation step. The observed inhibition could be the result of either competitive binding of 2,5-AM-1-P (which is structurally equivalent to 2,5-AM-6-P) to PFK against fructose-6-phosphate (F-6-P) or competitive binding of 2,5-AM-1,6-P2 to fructose bisphosphate aldolase against F-1,6-P2. Koerner et al. *(14)* have demonstrated that PFK preferred the β form of F-6-P, which is the structural analog of 2,5-AM-1-P (or 2,5-AM-6-P). In addition, 2,5-AM-1,6-P2 has been shown to be a competitive inhibitor of fructose bisphosphate aldolase against F-1,6-P2 *(9)*.

From Table 1 it also can be seen that 2,5-AM did not have any effect on ethanol yield (grams of ethanol produced per gram of glucose consumed) at all tested concentrations <10 m*M*. In other words, inhibition of glucose consumption by 2,5-AM at these concentrations was translated directly to inhibition of ethanol production. In fact, the percentages of inhibition of glucose consumption and ethanol production compared to the controls shown in Table 1 are approximately equal at each tested 2,5-AM concentration <10 m*M*. This resulted because the two steps that possibly were the

location of inhibition preceded the steps that led to ethanol production. When 10 m*M* 2,5-AM was used, significantly higher inhibition of ethanol production compared to inhibition of glucose consumption was observed. The rate of inhibition of ethanol production dropped by 24% compared with the rate of inhibition of glucose consumption, and ethanol yield was 32% lower than the yield obtained in the control experiment. This indicated that at the highest 2,5-AM concentration tested the inhibition of ethanol production was located at a step after the fructose bisphosphate aldolase. The most likely step probably was the one catalyzed by PK, and the effector probably was 2,5-AM-1,6-P2. It is possible that at high concentrations (10 m*M* or greater) of 2,5-AM its product, 2,5-AM-1,6-P2, flooded all the binding sites of the natural allosteric activator F1,6-P2 on the enzyme and, hence, reverted it to its original conformation or even changed it to a lower activity conformation.

Table 2 summarizes the effects of 2,5-AM on fructose consumption and ethanol production. Significantly higher inhibition of substrate consumption and ethanol production was observed compared to glucose. At 1 m*M* 2,5-AM, inhibition of fructose consumption and ethanol production was already >10% compared to the control experiment. When glucose was used as substrate, the inhibition of substrate consumption and ethanol production was about 10% at a 2,5-AM concentration of 2.5 m*M*. At the highest concentration of 2,5-AM tested (10 m*M*), more than 60% inhibition of both fructose consumption and ethanol production was observed. Inhibition of fructose consumption by 2,5-AM at concentrations of 1 m*M* and higher probably was exerted at the two locations where inhibition of glucose consumption occurred as previously discussed plus the fructose phosphorylation step. At these concentrations 2,5-AM seemed to compete successfully with fructose for fructokinase. Similar to the case of glucose, ethanol yield was not affected until 2,5-AM was added at 10 m*M*. The decrease in ethanol yield compared with the yield obtained in the control experiment was 33%, which is the same as the relative decrease in ethanol yield obtained with glucose as substrate and 2,5-AM at 10 m*M* (32%). The fact that the same degrees of reduction in ethanol yield were obtained at 10 m*M* 2,5-AM when glucose and fructose were used as substrates seems to indicate that the inhibition of ethanol production in addition to the inhibition translated from the inhibition of substrate consumption was located at the same place in both cases, which is suggested to be the PK, as we have already discussed.

E. coli *KO11*

Tables 3 and 4 summarize the results obtained with *E. coli* KO11 when glucose and fructose were used as substrates, respectively. The effects of 2,5-AM on the metabolism of glucose and fructose by *E. coli* KO11 were similar to those observed for *S. cerevisiae* ATCC 24855. When glucose was used, 2,5-AM did not seem to have any effect on either glucose consumption or ethanol production at concentrations at 1 m*M* or less. At 5 m*M*, 2,5-AM inhibited glucose consumption and ethanol production by 16 and 23%,

Table 2
Effects of 2,5-AM on Fructose Consumption and Ethanol Production by *S. cerevisiae* ATCC 24855[a]

2.5-AM concentration m*M*)	Fructose consumption rate (g/L·h])	Ethanol production rate (g/L·h])	Effect of 2,5-AM on fructose consumption rate (%)	Effect of 2,5-AM on ethanol production rate (%)	Ethanol yield (g ethanol/ g fructose consumed)
0.005	2.85 (2.94)	0.85 (0.89)	–3.1	–4.5	0.29 (0.30)
0.01	2.89 (2.94)	0.87 (0.89)	–1.7	–2.2	0.31 (0.30)
0.025	2.89 (2.86)	0.82 (0.82)	+1.0	0	0.29 (0.29)
0.05	2.82 (2.86)	0.81 (0.82)	–1.4	–1.2	0.29 (0.29)
1	2.47 (2.88)	0.8 (0.91)	–14.2	–12.1	0.33 (0.30)
2.5	2.1 (2.96)	0.64 (0.91)	–29.1	–29.7	0.29 (0.30)
5	1.58 (2.96)	0.44 (0.91)	–46.8	–51.6	0.28 (0.30)
10	1.12 (2.858)	0.3 (0.91)	–61.1	–67.0	0.20 (0.30)

[a]The results obtained in control experiments are given in parentheses.

Table 3
Effects of 2,5-AM on Glucose Consumption and Ethanol Production by *E. coli* KO11[a]

2,5-AM concentration (m*M*)	Glucose consumption rate (g/[L·h])	Ethanol production rate (g/[L·h])	Effect of 2,5-AM on fructose consumption rate (%)	Effect of 2,5-AM on ethanol production rate (%)	Ethanol yield (g ethanol/ g fructose consumed)
0.01	3.10 (2.90)	0.99 (1.05)	+6.9	–5.7	0.32 (0.36)
1	2.70 (2.93)	1.0 (1.06)	–7.9	–5.7	0.34 (0.35)
5	2.47 (2.93)	0.82 (1.06)	–15.7	–22.6	0.34 (0.35)

[a]The results obtained in control experiments are eiven in parentheses.

Table 4
Effects of 2,5-AM on Fructose Consumption and Ethanol Production by *E. coli* KO11[a]

2,5-AM concentration (m*M*)	Fructose consumption rate (g/L·h])	Ethanol production rate (g/L·h])	Effect of 2,5-AM on fructose consumption rate (%)	Effect of 2,5-AM on ethanol production rate (%)	Ethanol yield (g ethanol/ g fructose consumed)
0.005	2.91 (2.71)	0.94 (1.13)	+7.4	–16.8	0.33 (0.40)
0.01	2.69 (2.71)	1.03 (1.13)	–0.7	–8.9	0.39 (0.40)
1	2.43 (2.71)	0.97 (1.14)	–10.3	–14.9	0.50 (0.43)
5	1.21 (2.71)	0.51 (1.14)	–55.4	–55.3	0.40 (0.43)

[a]The results obtained in control experiments are given in parentheses.

respectively. 2,5-AM also showed much stronger effects when fructose was used compared to glucose. At 5 m*M* 2,5-AM, both fructose consumption and ethanol production were inhibited by 55%. At all 2,5-AM concentrations tested, with the highest being 5 m*M*, no effects were observed for ethanol yield. 2,5-AM at concentrations <5 m*M* also showed no effect on ethanol yield with *S. cerevisiae* ATCC 24855. We also tested the effect of 2,5-AM at 5 m*M* on the consumption of glycerol by *E. coli* KO11. At this concentration complete inhibition of both glycerol consumption and ethanol production by *E. coli* KO11 was observed. In *E. coli* the first step in glycerol metabolism is catalyzed by glycerol kinase, which is inhibited by F-1,6-P2 *(15)*. The results obtained here indicated that 2,5-AM indeed was converted into 2,5-AM-1,6-P2, the dead-end structural analog of F-1,6-P2.

Conclusion

The effects of the β-D-fructose analog 2,5-AM on the metabolism of glucose and fructose by a wild-type *S. cerevisiae* and a recombinant *E. coli* were investigated. In both cases the dead-end product of 2,5-AM conversion, 2,5-AM-1,6-P2, which is the structural analog of F-1,6-P2, the allosteric activator of two key regulatory enzymes of glycolysis, did not increase the rates of either substrate consumption or ethanol production when the parent compound, 2,5-AM, was used at low concentrations (1 mM or less). At concentrations of 2,5-AM at 2.5 mM or higher, significant inhibition of both glucose and fructose was observed, with fructose inhibition much more severe. Perhaps the most significant finding of this investigation was the possibility that PFK and PK are not the rate-controlling steps of glycolysis in the organisms tested.

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