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# Isolation of a Regulatory Mutant of Fructose-1,6-diphosphatase in *Saccharomyces carlsbergensis*

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By selecting for growth on glycerol, but absence of growth on glucose, a mutant of *Saccharomyces carlsbergensis* was isolated which does not grow on glucose, fructose, mannose, or sucrose, which shows long-term adaptation to maltose, but which can grow normally on galactose, ethanol, or glycerol. In the mutant, fructose diphosphatase is not inactivated after the addition of glucose, fructose or mannose to the medium, resulting in the simultaneous presence of fructose diphosphatase and phosphofructokinase activity. Under these conditions, a cycle is probably catalyzed between fructose-6-phosphate and fructose-1,6-diphosphate, resulting in the net consumption of adenosine triphosphate and an immediate stop of protein synthesis.

In prokaryotes and in simple eukaryotes, the synthesis of a number of enzymes is sensitive to catabolite repression (for reviews see 11 and 23). It is generally assumed that in *Escherichia coli* the degree of catabolite repression is mediated by changes in the intracellular level of cyclic 3',5'-adenosine monophosphate (cAMP). In yeast, the same correlation was observed between the degree of repression and the level of cAMP (25, 22). Moreover, cAMP stimulates the respiratory adaptation in protoplasts of *Saccharomyces cerevisiae* in the presence of 10% glucose (5). Subsequently the existence in yeast of phosphodiesterase (20), cAMP binding protein (21), and adenyl cyclase (13, 22) was shown. On the other hand, both in wild-type cells of *S. cerevisiae* and in a mutant which is insensitive to catabolite repression, no correlation could be found between the degree of catabolite repression and the intracellular level of cAMP (16).

Apart from this, in yeast cells the synthesis of some key enzymes of carbon metabolism, such as fructose diphosphatase (D-fructose-1,6-diphosphate 1-phosphohydrolase; EC 3.1.3.11) (7), the cytoplasmic malate dehydrogenase (6, 26, 19), and the maltose permease (10, 19), is not only repressed by glucose, but the preexisting amount of enzyme is irreversibly inactivated in the presence of this compound. The mechanism of inactivation repression of these enzymes is not known. On the other hand, in yeast ornithine transaminase (1) and tryptophan synthase (15) can be inactivated, depending on the culture conditions, by specific inactivating enzymes (1, 12).

By selecting for growth on glycerol but absence of growth on glucose, a mutant of *Saccharomyces carlsbergensis* was isolated which does not grow on glucose, fructose, mannose, or sucrose, which shows long-term adaptation to maltose, but which can grow normally on galactose, glycerol, or ethanol. The characterization of this mutant is the subject of this paper. It appears that, in the mutant, derepressed fructose diphosphatase is not inactivated in the presence of glucose, fructose, or mannose.

## MATERIALS AND METHODS

**Strains.** The *fdp* mutant was isolated in a haploid derivative from *S. carlsbergensis* with the genotype $\alpha$, $lys_2$, *FDP*. This strain was used as the wild-type strain throughout this study.

**Growth conditions and determinations of enzymes and intermediates.** Cells were grown in rich medium containing 2% peptone (Difco Laboratories, Detroit, Mich.), 1% yeast extract (Difco), and various carbon sources, or in minimal medium containing 0.7% yeast nitrogen base (without amino acids; Difco), 30 mg of lysine per liter, and various carbon sources. For the determination of fructose diphosphatase, 50 ml of cells were centrifuged at 3,000 × *g*, suspended in 10 ml of water, centrifuged again, and suspended in 1 ml of water. Subsequently, crude extracts were prepared by ultrasonification. Cell debris was removed by centrifugation at 3,000 × *g*. Fructose diphosphatase in these extracts was determined as described by Gancedo and Gancedo (9). Maltose permease was determined in intact cells as described before (3). The intracellular levels of adenosine triphosphate (ATP) and glucose-6-phosphate (G-6-P) were determined as described by Polakis and Bartley (17). Overall protein synthesis was deter-

mined as described before (18). Overall protein was determined by the method of Lowry et al. (14).

## RESULTS

**Isolation of the fdp mutant.** After mutagenesis with 1-nitroso-imidazolidone-2 (2) of a strain with the genotype $\alpha$, $lys_2$, *FDP*, the *fdp* mutant was isolated by selecting for growth on glycerol but absence of growth on glucose. The diploid, obtained by crossing of the *fdp* mutant with a strain of the genotype $a$, $ade_1$, *FDP*, showed normal growth on glucose, indicating that the *fdp* mutation is recessive. After sporulation of this diploid strain, 20 complete tetrads were obtained, all showing a two-plus, two-minus segregation for growth on glucose, indicating that the genotype of the mutant is caused by a single mutation.

**Growth characteristics of the fdp mutant.** The results of a number of growth experiments are presented in Table 1. It can be seen from this table that the *fdp* mutant did not grow on glucose, fructose, mannose, sucrose, or maltose within 2 days, but grew normally on galactose, ethanol, and glycerol. After further incubation, growth was observed on all sugars after a variable number of days. However, this growth was due to revertants, except in the case of maltose, indicating that the initial absence of growth on this carbon source was due to long-term adaptation.

The mutant did not grow on glucose, sucrose, fructose, or mannose in combination with glycerol, but grew on maltose in combination with this compound. Under the latter conditions, both in the wild type and in the mutant, maltose stimulated growth, and maltase and the maltose permease were induced (not shown).

**Localization of the mutation.** Since growth was normal on glycerol, it was thought that the mutation is possibly localized in that part of glycolysis before its metabolites enter this pathway. Moreover, the mutation must be localized in carbon metabolism common to all sugars except galactose. Consequently hexokinase, phosphofructokinase, aldolase, and fructose diphosphatase might be possible candidates. However, the absence of growth of the mutant must be caused by a regulatory disorganization and not by the complete absence of one of these enzymes, since growing on galactose a normal glycolysis, and growing on glycerol or ethanol a normal gluconeogenesis, must occur. This latter conclusion was verified by measuring the intracellular levels of these four enzymes. From these experiments, it appeared that hexokinase, phosphofructokinase, and aldolase (after growth on maltose + glycerol) and fructose diphosphatase (after growth on glycerol) were present in normal amounts in the wild type and in the mutant (not shown).

TABLE 1. *Growth of the fdp mutant on different media*[a]

| Carbon source (2%) | Growth |
|---|---|
| Glucose | − |
| Fructose | − |
| Mannose | − |
| Sucrose | − |
| Maltose | − |
| Galactose | + |
| Glycerol | + |
| Ethanol | + |
| Glucose + glycerol | − |
| Fructose + glycerol | − |
| Mannose + glycerol | − |
| Sucrose + glycerol | − |
| Maltose + glycerol | + |

[a] Rich medium containing carbon sources as indicated above was inoculated, and growth was determined after 48 h of incubation at 28 C. Under these conditions, wild-type cells were grown until the stationary phase on all media. Cells of the *fdp* mutant either did the same or showed a complete absence of growth within the time of the experiment. Growth was determined as the increase in optical density at 590 nm.

**Inactivation repression in the fdp mutant.** Because of the probability of a regulatory disorganization, the inactivation repression of fructose diphosphatase was tested. After growth on glycerol, about the same level of this enzyme was found both in the wild type and in the mutant. However, after addition of glucose, the fructose diphosphatase was inactivated in the wild type but not in the mutant (Table 2).

Inactivation repression was observed in the presence of hexoses which can be used constitutively, such as glucose, mannose, and fructose. Fructose diphosphatase of the mutant was also insensitive to the presence of fructose and mannose in the medium (Table 3). Although inactivation repression could play a role in the metabolism of other sugars, testing for the presence of this regulatory mechanism is much more complicated, since cells first must adapt to these carbon compounds.

It seemed of interest to know whether the absence of inactivation repression was specific for fructose diphosphatase. Consequently, the effect of glucose was studied on the induced

TABLE 2. *Inactivation repression of glucose in the wild type and the fdp mutant*

| Culture fraction | Fructose diphosphatase[b] | | Maltose transport[c] | |
|---|---|---|---|---|
| | 0 min | 60 min | 0 min | 60 min |
| Wild type | 30 | 32 | 9.1 | 8.4 |
| Wild type + glucose | 30 | 2 | 9.1 | 1.8 |
| *fdp* mutant | 32 | 32 | 13.5 | 12.4 |
| *fdp* mutant + glucose | 32 | 25 | 13.5 | 1.2 |

[a] Cells were grown until the early log phase on rich medium containing 2% glycerol for the determination of fructose diphosphatase and on rich medium containing 2% glycerol and 2% maltose for the determination of maltose transport. At zero time, glucose was added to part of the cultures to a final concentration of 2%.

[b] Expressed as nanomoles of fructose-1,6-diphosphate split per minute per milligram of protein.

[c] Expressed as picomoles of [$^{14}$C]maltose taken up per minute per milligram (dry weight) of cells.

TABLE 3. *Inactivation repression of fructose and mannose in the wild type and the fdp mutant*[a]

| Culture fraction | Fructose diphosphatase[b] | | |
|---|---|---|---|
| | 0 min | 60 min | 120 min |
| Wild type + fructose | 38 | 11 | 3 |
| *fdp* mutant + fructose | 26 | 34 | 37 |
| Wild type + mannose | 27 | 6 | 2 |
| *fdp* mutant + mannose | 16 | 15 | 15 |

[a] Cells were grown until the early log phase on rich medium containing 2% glycerol. At zero time, fructose or mannose was added to the cultures to a final concentration of 2%.

[b] Expressed as in Table 2.

maltose permease, which is also known to be sensitive to inactivation repression (10) (Table 2). From this table, it is clear that the maltose permease is inactivated by glucose both in the wild type and in the mutant, indicating that the effect on the inactivation of fructose diphosphatase is probably specific for this enzyme.

**Effect of glucose on the intracellular level of ATP and G-6-P.** If fructose diphosphatase is not inactivated after the addition of one of the hexoses, a serious disorganization of carbon metabolism is likely to occur. This is verified by measuring the levels of G-6-P and ATP (Table 4). These results prove that, in cells of the *fdp* mutant pregrown on glycerol, the level of ATP is lowered after addition of glucose, whereas the intracellular concentration of G-6-P is hardly affected. As for ATP, comparable results were obtained in mutant cells pregrown on galactose. However, under these conditions the intracellular level of G-6-P showed an increased after the addition of glucose.

TABLE 4. *Effect of glucose on the intracellular level of ATP and G-6-P*[a]

| Culture fraction | Wild type | | *fdp* mutant | |
|---|---|---|---|---|
| | G-6-P | ATP | G-6-P | ATP |
| Growth medium + glycerol | 0.1 | 1.4 | 0.1 | 1.1 |
| Growth medium + glycerol + 2% glucose | 0.7 | 1.1 | 0.7 | 0.1 |
| Growth medium + galactose | 1.5 | 1.3 | 1.3 | 0.9 |
| Growth medium + galactose + 2% glucose | 1.9 | 1.1 | 4.3 | 0.0 |

[a] Cells were grown until the early log phase on minimal medium containing 2% Casamino Acids and 2% glycerol or 2% galactose. At zero time, glucose was added to part of the cultures to a final concentration of 2%. After 30 min of incubation in this medium, ATP and G-6-P were determined. For the determination of these compounds, cells were harvested by centrifugation and immediately resuspended in 5% perchloric acid in the cold. After three times freezing and thawing, the extracts were neutralized and centrifuged, and G-6-P and ATP were determined. The intracellular levels of both compounds are expressed as micromoles per gram (dry weight) of cells.

**Effect of glucose on the rate of protein synthesis.** The effect of glucose on the rate of protein synthesis in wild-type and in mutant cells pregrown on glycerol was studied (Fig. 1). From this figure, it is clear that glucose stimulates protein synthesis in the wild type, but in the *fdp* mutant, immediately inhibits protein synthesis completely. Comparable results were obtained in cells pregrown on galactose.

**Levels of fructose diphosphatase after growth on different carbon sources.** Because of the absence of inactivation repression of fructose diphosphatase in the *fdp* mutant, it seemed of interest to measure the levels of this enzyme after growth on different carbon sources. The results are presented in Table 5. From this table, it can be seen that a normal derepressed level of fructose diphosphatase is observed after growth on glycerol, but low levels are found after growth on galactose or maltose together with glycerol.

**AMP inhibition of fructose diphosphatase.** Fructose diphosphatase activity is allosterically inhibited by AMP (9). This was verified both in the wild type and in the *fdp* mutant. It ap-

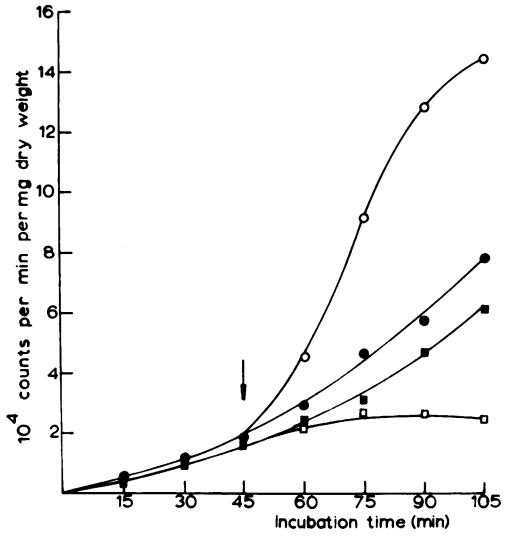


FIG. 1. *Protein synthesis in the wild type and in the fdp mutant in the presence of glucose. Cells of the wild type and of the fdp mutant were grown on minimal medium containing 2% glycerol and 2% Casamino Acids to the same optical density in the early logarithmic phase. Cells were centrifuged and resuspended in a similar medium containing 0.5% Casamino Acids and 10 μCi of $^{14}$C-labeled Casamino Acids. At the time indicated by the arrow, glucose was added to part of the cultures to a final concentration of 2%. Symbols: ●, wild-type cells; ○, wild-type cells in the presence of glucose; ■, cells of the fdp mutant; □, cells of the fdp mutant in the presence of glucose.*

TABLE 5. *Levels of fructose diphosphatase after growth in the presence of different carbon sources*[a]

| Carbon source | Sp act of fructose diphosphatase[b] | |
|---|---|---|
| | Wild type | *fdp* mutant |
| 2% Glucose | <1 | |
| 2% Galactose | <1 | <1 |
| 2% Maltose + 2% glycerol | <1 | <1 |
| 2% Glycerol | 23 | 22 |

[a] For the determination of fructose diphosphatase, cells were grown until the early log phase on rich medium with a carbon source as indicated above.

[b] Expressed as nanomoles of fructose-1,6-diphosphate split per minute per milligram of protein.

peared that, in both strains, fructose diphosphatase activity is almost completely inhibited in the presence of AMP (1 mM) (not shown).

## DISCUSSION

The observation that fructose diphosphatase is not inactivated in the mutant after addition of glucose, fructose, or mannose proves that somehow the regulation of this enzyme is affected. The absence of inactivation repression is probably specific for fructose diphosphatase since the maltose permease is still sensitive to this type of regulation. The presence of glucose in the medium results in the loss of most of the intracellular ATP and inhibits protein synthesis completely in the mutant. Although an effect of glucose on the amino acid pool, and consequently on the rate of incorporation of $^{14}$C amino acids, cannot be excluded, the complete absence of protein synthesis in the *fdp* mutant in the presence of glucose suggests that the process of inactivation repression of the maltose permease is independent of continuous protein synthesis. This result would be in good agreement with the observation that inactivation repression of fructose diphosphatase (7) and of the maltose permease (10) is not, or only slightly, affected in the presence of cycloheximide. Moreover, the inactivation process of the maltose permease appears to be energy independent, as was observed for fructose diphosphatase (7).

After addition of one of the hexoses, no inactivation repression of fructose diphosphatase was observed. Two hypotheses can explain this result: either the metabolism of glucose is changed in such a way that inactivation repression of fructose diphosphatase is impossible, or we are dealing with a regulatory mutant of fructose diphosphatase, resulting in a disorganization of carbon metabolism under certain circumstances. At present we favor the latter hypothesis, since the results discussed above indicate that the inactivation process is relatively insensitive to metabolic alterations and since the absence of inactivation repression is specific for fructose diphosphatase. Moreover, a regulatory disorganization of this enzyme can satisfactorily explain the observations without additional assumptions: the disappearance of ATP in the presence of glucose was probably the direct result of the simultaneous presence of phosphofructokinase and fructose diphosphatase, catalyzing an energetically wasteful cycle between fructose-1,6-diphosphate and fructose-6-phosphate (4). The intensity of cycling is not directly related to the levels of both enzymes, since cycling tends to be decreased by the fact that phosphofructokinase is activated by AMP, whereas fructose diphosphatase is inhibited by this compound (4). However, on additon of glucose in the mutant, energy production is impaired, and consequently protein synthesis and growth stop immediately because of lack of ATP. Complete absence of growth is observed on sugars, such as glucose, fructose, mannose,

and sucrose, which leads to a high degree of catabolite repression (24). Under these conditions, the level of fructose diphosphatase is low (8) and should be low since energy is derived mainly from glycolysis, and cycling between fructose-6-phosphate and fructose-1,6-diphosphate could easily reduce net ATP production.

As can be expected, a normal rate of growth of the mutant is observed under conditions of complete derepression of fructose diphosphatase for gluconeogenesis, i.e., after growth on glycerol or ethanol.

Since normal growth on galactose is observed, it must be assumed that, for growth on this derepressing sugar, inactivation is less important. In these experiments, no significant difference was found between the levels of fructose diphosphatase after growth on glucose or galactose. However Gancedo et al. (8) observed a certain degree of derepression of fructose diphosphatase under the latter conditions.

Apparently, cycling in the presence of maltose is too high for this sugar to be metabolized immediately, but long-term adaptation is possible. Growth on maltose leads to partly derepressed levels of the enzymes sensitive to catabolite repression (24). Therefore, glycerol can be metabolized under these conditions. It is thought that the less efficient ATP production delays adaptation to maltose, but the ATP production from glycerol compensates for this effect.

It is interesting to note that, in the mutant after growth on galactose or on maltose with glycerol, the level of fructose diphosphatase is low. Consequently, it must be assumed that catabolite repression still can exert its effect on the synthesis of this enzyme. However, it is not clear why, although the level of fructose diphosphatase is already low, cells once adapted to galactose or maltose do not grow in the presence of glucose. The most likely explanation of this observation is that catabolite repression and inactivation are two separate phenomena. Catabolite repression determines the level of fructose diphosphatase, and inactivation, which occurs in the presence of glucose, fructose, mannose, sucrose, and maltose, would reduce any activity present and is a prerequisite for the (immediate) utilization of these carbon compounds.

It is thought that a more detailed study of this mutant will give more information on the mechanism of inactivation repression. At least it is clear that AMP inhibition and inactivation repression of fructose diphosphatase are two independent phenomena. If this inactivation is exerted by a specific inactivating enzyme, as is observed for tryptophan synthase in yeast (1, 12), this would be in good agreement with the specificity and with the recessive character of the *fdp* mutation.

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