Expression of PEP carboxylase from *Escherichia coli* complements the phenotypic effects of pyruvate carboxylase mutations in *Saccharomyces cerevisiae*

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Received 27 June 1997

Abstract We investigated the effects of the expression of the *Escherichia coli* ppc gene encoding PEP carboxylase in *Saccharomyces cerevisiae* mutants devoid of pyruvate carboxylase. Functional expression of the ppc gene restored the ability of the yeast mutants to grow in glucose-ammonium medium. Growth yield in this medium was the same in the transformed yeast than in the wild type although the growth rate of the transformed yeast was slower. Growth in pyruvate was slowed down in the transformed strain, likely due to a futile cycle produced by the simultaneous action of PEP carboxykinase and PEP carboxylase.

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Key words: PEP carboxylase; Pyruvate carboxylase; TCA cycle; Glucose metabolism (yeast)

1. Introduction

Intermediates of the tricarboxylic acid (TCA) cycle are continuously withdrawn for synthesis of cell constituents. Therefore replenishment of the cycle is critical to maintain its functionality. Different reactions are used by diverse organisms to this end (for reviews see [1–3]). In yeast growing in a medium with glucose and ammonium the replenishment of the TCA cycle is carried out by pyruvate carboxylase that synthesizes oxalacetate from pyruvate. The important anaplerotic role of this enzyme in yeast is shown by the phenotype exhibited when the two genes *PYC1* and *PYC2* encoding two isoenzymes of pyruvate carboxylase are disrupted. These mutants are unable to grow on a glucose-ammonium medium while they grow on a medium with glucose-aspartate [4].

*Escherichia coli* synthesizes oxalacetate not from pyruvate, but from phosphoenolpyruvate (PEP) in a reaction catalyzed by PEP carboxylase [2,5]. Since it is not obvious why different organisms have evolved different starting points to replenish the TCA cycle we decided to investigate if the phenotype of a yeast devoid of pyruvate carboxylase could be complemented by expression of a foreign PEP carboxylase. Functional expression of *E. coli* PEP carboxylase will create a branch point in the metabolic pathway that could result in a rerouting of metabolism towards gluconeogenic substrates (Fig. 1). We document in this article the positive phenotypic complementation of the pyruvate carboxylase mutation and some differences observed between the wild-type yeast strain and the one expressing the *E. coli* protein.

2. Materials and methods

2.1. Yeast strains and culture conditions

*Saccharomyces cerevisiae* W303-1A Mat a ade2-1 his3-11,15 leu2-3,112 trpl-1 ura3-52 transformed with plasmid pAN10, a *yeast-E. coli* shuttle plasmid carrying the URA3 marker and a *HindIII* site between the yeast *ADH1* promoter and terminator [6] was used as wild-type control. *Saccharomyces cerevisiae* CJM 238 ade2-1 his3-11,15 leu2-3,112 trpl-1 ura3-52 pyc1::LEU2 pyc2::HIS3 carries disruptions in *PYC1* and *PYC2* genes as described in [4]. The strains were grown at 30°C in 0.17% yeast nitrogen base with 40 mM ammonium sulfate or aspartate as nitrogen source and the adequate auxotrophic requirements. Glucose 2% or pyruvate 2% were used as carbon sources.

2.2. Plasmid construction

Plasmid pAN10-ppc carrying the *E. coli* ppc gene under the control of the yeast *ADH1* promoter was constructed as follows: The ppc gene was isolated by PCR using plasmid pS2 [7] as template. The primers used were: upstream 5'-GGGAAAGCTTATATGAGAA-CATATTC-3' and downstream 5'-GGGAAAAACCTTAGGCGGTA-3'. *HindIII* sites (underlined) were introduced to facilitate cloning into pAN10. The 2.7-kbp fragment obtained was cloned into pGEMT (Promega) and the resulting plasmid digested with *HindIII*. The fragment containing the ppc gene was cloned into *HindIII* site of pAN10.

2.3. Enzyme assays

Cell extracts were obtained by shaking 100 mg of cells (wet weight) in 0.5 ml of 50 mM Tris hydrochloride pH 7.4 with 1 g of glass beads (0.5 mm diameter) in a vortex for five periods of 1 min each, with 1 min intervals on ice. PEP carboxylase was assayed as in [8]. The reaction mixture contained: 0.1 M Tris-HCl, 0.1 mM NADH, 10 mM KC0₃, 10 mM magnesium acetate, 5 mM PEP, 0.5 mM acetyl CoA, 1 unit malic dehydrogenase and the adequate amount of extract. When tested as an inhibitor, aspartate was routinely added at 0.8 mM. Pyruvate kinase was assayed as in [9]. Protein was determined with the Pierce reagent, using bovine serum albumin as standard.

2.4. Other methods

Measurements of respiration and fermentation were carried out at 30°C in a conventional Warburg respirometer. The vessels contained in a final volume of 1 ml: 1% glucose, 25 mM potassium phosphate pH 6 and an adequate amount of yeast (usually 10-20 mg wet weight). Growth was followed measuring the optical density of the culture at 600 nm. For growth yield determinations, samples of culture were filtered through glass fiber filters (Whatman GF/C) and dried until constant weight. Glucose was determined in parallel in the corresponding supernatants as in [10]. All determinations were performed in three independent cultures in different days.

3. Results and discussion

3.1. Expression of *E. coli* PEP carboxylase in *S. cerevisiae* mutants lacking pyruvate carboxylase

A multicopy plasmid carrying the coding region of *E. coli*
3.2. Effect of PEP carboxylase expression on different physiological parameters

Replacement of pyruvate carboxylase by E. coli PEP carboxylase might produce qualitative and quantitative differences with respect to a wild-type yeast. Qualitatively, the organism will have a new branching point at the level of PEP and pyruvate kinase and PEP carboxylase will compete for PEP (Fig. 1) and qualitatively the proportion of glycolytic inter-

mediate fanned into oxaloacetate could be different. It is difficult to predict the distribution of PEP between pyruvate kinase and PEP carboxylase because the actual activities of both enzymes depend on the concentration of their respective allosteric activators, fructose-1,6-bisphosphate and acetyl CoA [12,18]. While reliable data exist for the concentrations of fructose-1,6-bisphosphate in yeast [13], this is not the case for acetyl CoA. For pyruvate kinase we measured an activity in glucose of 7 units/mg protein in the presence of 1 mM fructose-1,6-bisphosphate and for PEP carboxylase activities varied between 40 mU/mg protein without addition of acetyl CoA and 2 mU/mg protein at 0.5 mM of the activator.

In a wild-type yeast pyruvate carboxylase, pyruvate dehydrogenase and pyruvate dehydrogenase compete for pyruvate. The capacity of pyruvate dehydrogenase in a yeast growing in glucose is low, so that at a semiquantitative level its contribution may be neglected. The $K_m$ values for pyruvate carboxylase and pyruvate dehydrogenase are respectively 0.4 mM [4] and 1 mM [14] and the activities 20-40 mU/mg protein pyruvate carboxylase (4, our own results) compared with 1.5 units/mg protein of pyruvate dehydrogenase [15,16]. Therefore it can be calculated that in the case of a wild-type yeast the deviation to oxaloacetate by pyruvate carboxylase will be about 1/50 of the incoming flux while in the case of the yeast expressing PEP carboxylase the proportion of PEP yielding oxaloacetate may range from 1/15 to 1/7000 of the flux depending on the actual concentration of acetyl CoA in the cytoplasm. Differences in the rate at which oxaloacetate is supplied to the TCA cycle may influence the fermentative behavior normally observed in glucose batch cultures [17,18]. To see if this was the case we measured the fermentation and respiration rates of a wild-type yeast and of a pyruvate carboxylase mutant expressing PEP carboxylase (Table 1).
In resting cells the amount of glucose fermented was the same in both strains but the rate of respiration although low was twofold higher in the PEP carboxylase expressing strain than in the wild type. This suggests that a higher amount of the incoming glucose is being fuelled to the TCA cycle. Both strains showed the same glucose consumption in resting cells. During growth, both strains consumed glucose at higher rate than in the resting state, a phenomenon well known in yeast but not yet satisfactorily explained [19], but the transformed yeast used glucose at a slower rate than the wild type. This could indicate that modifications in the systems that metabolize PEP and pyruvate are able to influence the glycolytic flux. One possibility is that the increased respiration inhibits glucose uptake. Although no mechanism relating transport and respiration has been identified yet, glucose consumption appears to be related with the respiratory capacity of the yeast [20,21]. Whereas growth rate was decreased in the yeast expressing the heterologous protein, growth yield was similar in both cases (Table 1) and the value was in the same range as that found by others for yeast growing in minimal media [22].

When pyruvate was the carbon source the growth rate of the PEP carboxylase expressing strain was also impaired (Table 1). This is likely due to the existence of a futile cycle between PEP carboxylase and the antagonistic gluconeogenic PEP carboxykinase, active under these conditions [23,24]. In yeast, operation of futile cycles between glycolytic and gluconeogenic enzymes produces a decrease in the fitness of the organism although less deleterious than could have been anticipated [25].

From our results it appears that at least in a laboratory setting, there is no basic incompatibility between fermentative yeast metabolism and use of the PEP carboxylase instead of pyruvate carboxylase to replenish the TCA cycle. The differences observed in the glucose grown cultures could be related more with an excess in the amount of PEP carboxylase expressed than with the nature of the reaction catalyzed itself. It is therefore possible that the different solutions adopted by

<table>
<thead>
<tr>
<th>Strain</th>
<th>Fermentation (µmol CO₂/g yeast/min)</th>
<th>Respiration (µmol O₂/g yeast/min)</th>
<th>Glucose consumption (µmol/g yeast/min)</th>
<th>Growth yield (g yeast/g glucose)</th>
<th>Generation time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Resting</td>
<td>Growing</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>W303-1A/pAN10</td>
<td>141 ± 35</td>
<td>18 ± 3</td>
<td>102</td>
<td>0.15</td>
<td>110</td>
</tr>
<tr>
<td>CJM238/pAN10-ppc</td>
<td>136 ± 26</td>
<td>36 ± 7</td>
<td>101</td>
<td>0.15</td>
<td>155</td>
</tr>
</tbody>
</table>

Yeasts were grown and parameters measured as described in Section 2. For fermentation and respiration measurements cells were grown on glucose and harvested in the exponential phase of growth. The rate of glucose consumption during growth was calculated using the formula (0.693/ generation time) × (g glucose/g yeast). Yeast weights are dry weights.
diverse organisms to replenish the TCA cycle are the result of aleatory events and not a specific adaptation to different metabolic styles.

Acknowledgements: We thank Prof. K. Izui (Kyoto, Japan) for sending plasmids carrying the E. coli ppc1 gene and information on the test of PEP carboxylase and Prof. Juana M. Gancedo (Madrid, Spain) for critical reading of the manuscript. This work is part of the project ‘From gene to products in yeast: a quantitative approach’ supported by the European Community (DGXII Framework IV Program Cell factories). It has benefitted from grant PB94-0091-CO2-01 of the Spanish DGICYT. C.-L. Flores was supported in the initial stages of the work by a Fellowship of the Spanish Instituto de Cooperacion Iberoamericana.

References