Identification of rate-limiting steps in yeast heme biosynthesis

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Abstract

The heme biosynthesis pathway in the yeast Saccharomyces cerevisiae is a highly regulated system, but the mechanisms accounting for this regulation remain unknown. In an attempt to identify rate-limiting steps in heme synthesis, which may constitute potential regulatory points, we constructed yeast strains overproducing two enzymes of the pathway: the porphobilinogen synthase (PBG-S) and deaminase (PBG-D). Biochemical analysis of the enzyme-overproducing strains revealed intracellular porphobilinogen and porphyrin accumulation. These results indicate that both enzymes play a rate-limiting role in yeast heme biosynthesis.

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Heme is an important molecule synthesized practically in all cells of most organisms. The heme biosynthesis pathway is well characterized and appears to be evolutionarily conserved in cells ranging from bacteria to humans (for reviews, see [1,2]). It consists of eight reactions (Fig. 1), the first of which is the synthesis of 5-aminolevulinic acid (in animals, fungi, and some bacteria from glycine and succinyl-CoA), and the last the synthesis of heme. In eukaryotes the pathway is located partly in the mitochondria and partly in the cytosol. In the yeast Saccharomyces cerevisiae the enzymes catalyzing the subsequent reactions have been identified [4–11] and their genes have been cloned [12–18]. The enzymes have been analyzed biochemically and the kinetic parameters of most of them have been determined (see Table 5). Well-described yeast heme mutants, partially or totally blocked in different steps of heme biosynthesis, are available and often they have been extensively characterized biochemically [19–24]. Therefore, Saccharomyces cerevisiae constitute a very convenient system for studying heme biosynthesis.

The production of heme in yeast cells does not remain constant under all conditions. It depends strongly on the carbon source and the availability of oxygen in the medium. Normal biosynthesis of heme requires oxygen (the substrate of two heme enzymes: the copro- and protoporphyrinogen oxidases), but anaerobically cultured yeast cells do produce small amounts of heme. This is usually assumed to be due to traces of oxygen remaining in the media, although recently it has been shown that under stress conditions (during treatment with high concentrations of salt or with heat) oxygen is not an obligatory electron acceptor for heme synthesis in yeast [25]. However, under anaerobic conditions heme accounts only for 3–7% of the pathways overall productivity and the main product is ALA (5-aminolevulinic acid), the product of the first reaction, which constitutes about 50–70% of the total sum of tetrapyrrole equivalents synthesized. The remaining 30–40% are accumulated in the form of various porphyrins—products of the spontaneous oxidation of the pathways’ porphyrinogen intermediates [3]. Under aerobic conditions heme biosynthesis is strongly regulated by the carbon source. Fermentable carbon sources, such as glucose or—to a somewhat lesser extent—galactose, cause repression of the pathway. Growth on non-fermentable carbon sources results in induction of heme biosynthesis; during growth on ethanol the total sum of tetrapyrroles produced increases about 2- to 3-fold in comparison with glucose-grown cells [3]. The only doc-
In this work we have made an approach to identify the rate-limiting steps in yeast heme biosynthesis by examining the effects of overproducing the PBG-S in yeast cultured aerobically in the presence of fermentable carbon sources, and here we present our results, which confirm the rate-limiting character of this enzyme. We also overproduced two more enzymes of the pathway, the porphobilinogen deaminase (PBG-D) and the uroporphyrinogen III decarboxylase (URO-D). Our results demonstrate that PBG-D, which follows PBG-S in the pathway and catalyzes the reaction of PBG polymerization, constitutes a further block in heme formation. The effects of URO-D overproduction suggest that this enzyme might be the next rate-limiting step, but this conclusion remains speculative and still requires further analysis and confirmation.

**Materials and methods**

**Yeast strains and growth conditions.** The *Saccharomyces cerevisiae* strains used in this study are listed in Table 1. Standard complete (YPD) and minimal (SD) media and standard methods were used for yeast cultivation [28]. For heme-deficient mutants media were supplemented with hemine (15 mg/liter). For biochemical analysis and immunoassays the strains were cultivated in standard liquid YPD medium (1% yeast extract, 1% bacto-peptone) containing 2% glucose (YPD), 1% galactose, and 1% glucose (YPGal), or 2% ethanol and 0.2% glucose (YPE) as the carbon source. The media were supplemented with hemine and with various concentrations of 5-aminolevulinic acid (ALA), as indicated, and the cells were harvested at the onset of the stationary phase of growth. Yeast transformation was performed by the lithium acetate method [29].

**DNA manipulations and plasmid construction.** Standard protocols were used for all DNA manipulations [30]. *Escherichia coli* XL1-Blue MRF' was used for cloning and propagation of plasmids. PCR amplification of DNA [31] was carried out with the high-fidelity Expand DNA polymerase (Boehringer–Mannheim).

The plasmid pPBGS1, carrying the His-tagged *S. cerevisiae* HEM2 gene under the control of the GAL1 promoter, was constructed by insertion of the PCR-amplified yeast HEM2 gene (coding for porphobilinogen synthase) into the episomal high-copy-number pYES2 vector (Invitrogen). The HEM2 gene was amplified from total yeast genomic DNA by PCR with the following primers: 5'-CCC AAG CTT ATG CAT ACA GCT TTA TTT GAAA TAC ACA CGT ACT ATG and 5'-CCG GAA TTC AAA TAC ACA CGT ACT ATG GGC CCT G3 and 5'-CC GAA TTC TCA TTA AGC GTA ATC AGG AAC ATC GTA TGG GTA TTT GAT TCT GTT TAA ATT.
AAT TTC-3'. The obtained DNA fragment was digested with the EcoRI and BamHI enzymes and ligated into the multiple cloning site of the prepared vector. The plasmid fully complemented the growth defect of the hem3-1 yeast mutant.

The plasmid YEp351–HEM12 was described previously [27]. It carries the wild-type S. cerevisiae HEM12 gene, coding for URO-D, under the control of its own promoter, inserted into the multicopy vector YEp351 [32].

**Immunodetection of the His-tagged PBG synthase and HA-tagged PBG deaminase.** Total proteins (ca. 100 μg) extracted from yeast cells [33] were resolved by SDS–PAGE and transferred electrophoretically to nylon membranes. The membranes were probed with mouse antibodies against either the histidine (Qiagen) or hemagglutinin (Babco) epitopes and secondary anti-mouse-IgG antibodies conjugated with alkaline phosphatase (Promega). Alkaline phosphatase activity was detected directly on the membranes with the Western Blue reagent (Promega).

**Biochemical analysis.** Published methods were used to determine intracellular 5-aminolevulinic acid (ALA) and porphobilinogen (PBG) content [21]. Cells were cultured in liquid YPGal medium, collected, washed twice with water, and extracted twice with 5% TCA. PBG content in the extracts was measured colorimetrically at 555 nm after 15 min of reaction with an equal volume of the modified Ehrlich reagent. For ALA measurements 0.5 ml of 2.6 M sodium acetate and 40 μl acetylacetone were added to 1 ml of extract and the samples were boiled for 10 min. After cooling on ice an equal volume of the modified Ehrlich reagent was added for 15 min and ALA was measured colorimetrically at 555 nm. Porphyrins accumulating in the cells were measured as described in [23]. The cells were cultured in liquid YPGal medium supplemented with hemine, collected, washed three times with water, and extracted three times with 3 N HCl in the dark. The amount of porphyrins in the extracts was assayed spectrophotometrically at 404 nm. The values given for the porphyrin measurements are the results of single, representative experiments (the results differed between individual measurements by no more than ±20%); for the highly porphyrin-accumulating strains the results varied strongly, so the range of values occurring is given.

**Results**

**Intracellular PBG levels in yeast cells overproducing PBG-S and PBG-D**

We transformed the wild-type S150-2B yeast strain with the pPBGS1 and pPBGD1 plasmids (coding for the PBG-S and PBG-D enzymes, respectively), both separately and simultaneously. We could regulate the level of enzyme overproduction in the cells by culturing them in media containing various carbon sources, since the GAL1 promoter becomes induced in the presence of galactose, is repressed by glucose, and remains in a derepressed state in media containing ethanol. Proper regulation of enzyme synthesis from both plasmid-encoded genes was confirmed by immunooassays (Fig. 2).

Intracellular PBG measurements revealed that cells overproducing solely the PBG synthase accumulate significant amounts of porphobilinogen (Table 2). In cells cultured in YPGal medium (induction of the GAL1 promoter), the intracellular PBG level has increased almost 4-fold compared to wild-type yeast. The addition of exogenous ALA caused further accumulation of PBG (Table 2), in correlation with the rise in intracellular ALA availability (Table 3). Eventually, for an ALA concentration of 1 mg/ml, PBG reached a level about 40 times higher than wild-type values. At the same time
control cells lacking the plasmid did not accumulate PBG when cultured in medium supplied with additional ALA. These results indicate that PBG formation is one of the rate-limiting steps of heme biosynthesis.

Overproduction of the PBG deaminase resulted in a decrease of the PBG content (Table 2). Cells carrying only the pPBGD1 plasmid and grown in the presence of galactose had half as much PBG as wild-type yeast. Cells harboring both plasmids and therefore overproducing both enzymes showed a 2- to 4-fold decrease in PBG accumulation in comparison to cells overproducing only PBG-S and grown under the same conditions.

Intracellular porphyrin levels in cells overproducing PBG-S, PBG-D, and URO-D

We also measured the amounts of porphyrins accumulated in the cells of the S150-2B strain carrying various combinations of the pPBGS1, pPBGD1, and Yep351–HEM12 plasmids, and in a mutant strain blocked in heme biosynthesis and harboring the pPBGS1 and pPBGD1 plasmids. This mutant, lacking the URO-S enzyme (hem4Δ), accumulates uroporphyrins (Table 4). If overproduction of PBG-S and/or PBG-D resulted in increased accumulation of any pathway intermediate ranging from hydroxymethylbilane (the product of PBG-D) to protoporphyrin IX (the substrate of ferrochelatase), this should be visible in the plasmid-carrying strains, both mutant and wild-type, as an increase in porphyrin accumulation. An increase in heme production would be indistinguishable in the heme mutant, but porphyrin accumulation in the wild-type background should then be less pronounced than in the mutant. Overproduction of the PBG-S and PBG-D enzymes in yeast cells cultured under inducing conditions (in YPGal medium) was confirmed by immunoaassays (data not shown). The Yep351–HEM12 plasmid has been previously shown to cause overexpression of the HEM12 gene in transformed yeast cells [27].

Under the conditions tested none of the strains overproducing solely PBG-S showed any increase in porphyrin accumulation. Cultivating the cells in medium supplemented with exogenous ALA, the substrate of the PBG synthase, did not increase porphyrin accumulation either (Table 4). These results together with the PBG measurements suggest that the PBG deaminase plays a strongly rate-limiting role in the yeast heme biosynthesis pathway.

Yeast cells overproducing only PBG-D accumulated 1.5-3 times more porphyrins than the corresponding parental strains (Table 4), which remains consistent with the 2-fold decrease in the intracellular PBG content in the S150-2B [pPBGD1] cells. These differences did not exceed the accuracy of the method, so their significance remains uncertain, but the tendencies seem stable.

Strains overproducing both PBG-S and PBG-D could accumulate significant amounts of porphyrins (Table 4). This effect was absolutely clear when ALA in

Table 2
Intracellular PBG levels in S150-2B yeast cells overproducing the PBG synthase and/or PBG deaminase

<table>
<thead>
<tr>
<th>Strain</th>
<th>Intracellular PBG (nmol/g of dry weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td>~ALA</td>
<td>+250 μg/ml ALA</td>
</tr>
<tr>
<td>S150-2B</td>
<td>50 ± 14</td>
</tr>
<tr>
<td>S150-2B [pPBGD1]</td>
<td>24 ± 7</td>
</tr>
<tr>
<td>S150-2B [pPBGS1] [pPBGD1]</td>
<td>41 ± 5</td>
</tr>
</tbody>
</table>

Table 3
ALA uptake by S150-2B cells cultivated with exogenous ALA supplemented in the medium

<table>
<thead>
<tr>
<th>ALA concentration in the medium</th>
<th>Intracellular ALA (nmol/g of dry weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1153 ± 153</td>
</tr>
<tr>
<td>250 μg/ml</td>
<td>6230 ± 4</td>
</tr>
<tr>
<td>1 mg/ml</td>
<td>13257 ± 1877</td>
</tr>
</tbody>
</table>

Table 4
Intracellular porphyrin concentrations in wild-type and hem4Δ cells overproducing one, two or three of the enzymes PBG-S, PBG-D, and URO-D, compared to parental-type cells

<table>
<thead>
<tr>
<th>Strain</th>
<th>Intracellular porphyrins (nmol/g of dry weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td>~ALA</td>
<td>+ALA (250 μg/ml)</td>
</tr>
<tr>
<td>S150-2B</td>
<td>5</td>
</tr>
<tr>
<td>S150-2B [pPBGS1]</td>
<td>5</td>
</tr>
<tr>
<td>S150-2B [pPBGD1]</td>
<td>13</td>
</tr>
<tr>
<td>S150-2B [pPBGS1] [pPBGD1]</td>
<td>19</td>
</tr>
<tr>
<td>S150-2B [pPBGS1] [Yep351–HEM12]</td>
<td>7</td>
</tr>
<tr>
<td>S150-2B [pPBGD1] [Yep351–HEM12]</td>
<td>4</td>
</tr>
<tr>
<td>S150-2B [pPBGS1] [pPBGD1] [Yep351–HEM12]</td>
<td>6</td>
</tr>
<tr>
<td>S150-2B hem4Δ</td>
<td>25</td>
</tr>
<tr>
<td>S150-2B hem4Δ [pPBGS1]</td>
<td>44</td>
</tr>
<tr>
<td>S150-2B hem4Δ [pPBGD1]</td>
<td>73</td>
</tr>
<tr>
<td>S150-2B hem4Δ [pPBGS1] [pPBGD1]</td>
<td>107</td>
</tr>
</tbody>
</table>
Discussion

Previous attempts to identify the rate-controlling steps of yeast heme biosynthesis were based mainly on the analysis of the biochemical properties of the enzymes (Table 5) and on the comparison of their steady-state activities, measured both in vitro and in situ, with estimated in vivo activities. The in vivo values were calculated from experimental concentrations of accumulated and excreted pathway intermediates and intracellular heme content, combined with growth rates of yeast cells. This approach led to the conclusion that the enzymes PBG-S, PBG-D, and URO-D function in vivo at rates close to their maximal activity in vitro, whereas ALA-S and COPROX operate significantly below their theoretical maximal activity, and the two final enzymes, PROTOX and FERROC, function in the cells at only a small fraction of their maximum velocity ([1], and ref. within). It is therefore unlikely that the two final steps of the pathway should limit the rate of heme formation. The enzyme URO-S is the least characterized enzyme of the pathway and the kinetic properties of the yeast protein have not been determined yet, but uroporphyrinogen III synthases from other organisms have already been purified and they display very high $V_{\text{max}}$ and fairly low $K_M$ values (Table 5). The substrate of URO-S does not accumulate in wild-type yeast cells. These data suggest that this enzyme does not play a rate-limiting role in the pathway. Also the initial enzyme, ALA-S, which is the rate-controlling step of heme biosynthesis in mammalian cells, does not seem to play such a role in yeast, where its product, ALA, is normally present in excess. Thus, it seems that the best candidates for rate-limiting steps of heme biosynthesis in yeast are the PBG synthase, PBG deaminase, and uroporphyrinogen III decarboxylase.

In this work we applied a different approach to the problem and investigated heme biosynthesis in yeast cells overproducing some of the enzymes considered potentially rate-limiting. The results we obtained remain consistent with the predictions discussed above.

Analysis of the effects of porphobilinogen synthase overproduction in yeast cells confirmed its rate-limiting role in heme biosynthesis under the conditions investigated. An increase in intracellular PBG-S content resulted in an increase in PBG availability. The addition of exogenous ALA enhanced this effect. The second enzyme studied, the porphobilinogen deaminase, also proved rate-limiting. Its overproduction caused a decrease in PBG content and—if enough PBG was available, that is in PBG-S-overproducing cells—enhanced porphyrin accumulation, indicating an increase in the rate of the reaction it catalyzes.

Enhanced porphyrin accumulation in the S150-2B and hem4Δ strains overproducing both the PBG synthase and deaminase suggests that further blocks exist in the pathway, downstream of these enzymes. Our results do not allow their precise localization, since the method applied for the measurements of intracellular porphyrin content does not discriminate between various porphyrins (which are the products of spontaneous oxidation of porphyrinogens and as such accumulate upon porphyrinogen accumulation). The observed increase in porphyrin content in cells overproducing PBG-S and

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>$K_M$ [M]</th>
<th>$V_{\text{max}}$ (mol of product/sec/mol of enzyme active form)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALA-S</td>
<td>$3.0 \times 10^{-3}$ (glycine); $2 \times 10^{-6}$ (succinyl-CoA)</td>
<td>1.15</td>
<td>[4]</td>
</tr>
<tr>
<td>PBG-S</td>
<td>$3.6 \times 10^{-4}$</td>
<td>1.2</td>
<td>[5]</td>
</tr>
<tr>
<td>PBG-D</td>
<td>$1.9 \times 10^{-4}$</td>
<td>$0.5^a$</td>
<td>[6,34]</td>
</tr>
<tr>
<td>URO-S</td>
<td>$5.0 \times 10^{-1} - 2.6 \times 10^{-5b}$</td>
<td>$250^a$</td>
<td>[34–37]</td>
</tr>
<tr>
<td>URO-D</td>
<td>$6.0 \times 10^{-9}$</td>
<td>0.021</td>
<td>[7]</td>
</tr>
<tr>
<td>COPROX</td>
<td>$5.0 \times 10^{-3}$</td>
<td>0.10</td>
<td>[9]</td>
</tr>
<tr>
<td>PROTOX</td>
<td>$1.0 \times 10^{-7}$</td>
<td>0.62</td>
<td>[10]</td>
</tr>
<tr>
<td>FERROC</td>
<td>$9.0 \times 10^{-4}$</td>
<td>0.39</td>
<td>[11]</td>
</tr>
</tbody>
</table>

*No data on S. cerevisiae enzymes available; mean values of parameters for enzymes from other organisms are given.

*b No data on S. cerevisiae enzymes available; range of values occurring in other organisms is given.
PBG-D may therefore indicate the accumulation of any pathway intermediate between hydroxymethylbilane (which undergoes spontaneous, non-enzymatic cyclization to uroporphyrinogen I) and protoporphyrin IX. Some indications concerning the nature of these downstream blocks arise though from the analysis of the URO-D-overproducing strains.

The overproduction of this enzyme caused a significant decrease in the level of porphyrin accumulation in cells in which the flux through the initial fragment of the pathway was increased, that is in cells overproducing both PBG-S and PBG-D and supplied with additional ALA. It seems possible that the overproduction of the URO-D enzyme resulted in an increase in the amount of heme produced in these cells. It is unlikely that any significant increase in heme production occurs in the S150-2B [pPBGS1] [pPBGD1] strain, since the amount of porphyrins it accumulates is roughly the same as the amount accumulated by the hem4a mutant strain carrying these plasmids. This indicates that in this case the extra hydroxymethylbilane (the product of PBG-D) is arrested somewhere along the pathway, prior to heme formation. The decrease of intracellular porphyrins in the strain overproducing additionally the URO-D enzyme suggests that in these cells this extra flux from PBG-D might accumulate in the form of uroporphyrinogen III. Overexpression of the HEM12 gene would then cause a reversion of this block. The reversion was only partial: porphyrin accumulation was decreased 4-fold in comparison to the same strain without the YEp351–cumulation was decreased 4-fold in comparison to the

Acknowledgments

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References


