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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.

Keywords: *Saccharomyces cerevisiae*; Heme; Porphyrins

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-

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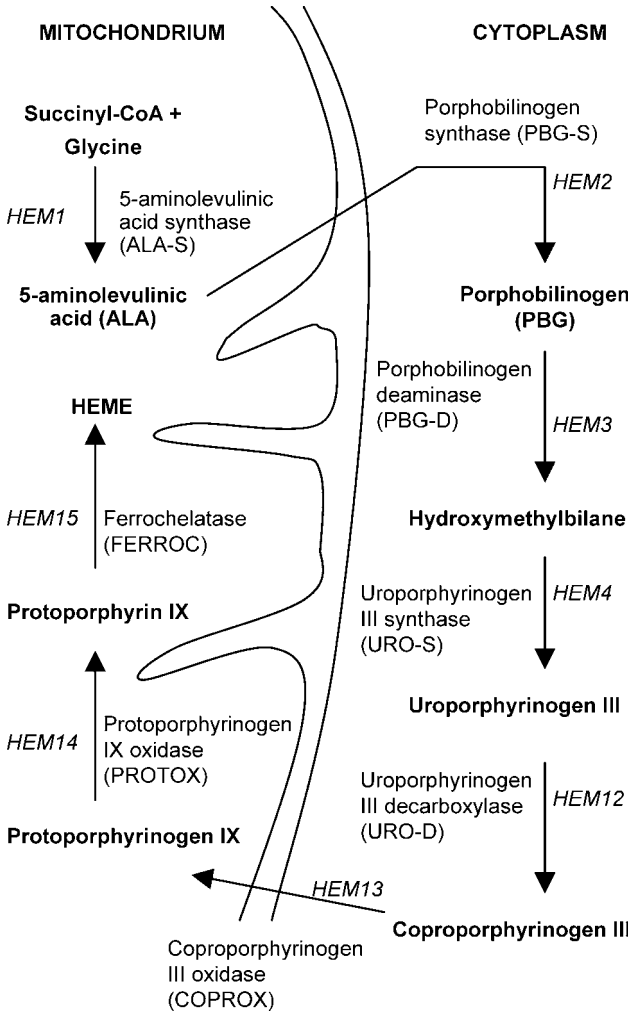


Fig. 1. The heme biosynthesis pathway of *Saccharomyces cerevisiae*. (Courtesy of Anna Chełstowska and Jonna Rytka [3]).

umented change in enzyme activity concerns the coproporphyrinogen oxidase (COPROX), which is transcriptionally activated in response to heme- or oxygen-deficiency.

The regulatory mechanisms underlying these events remain unknown. We considered the establishing of potential regulatory target points to be the first step for further studies. Enzymes that are rate-limiting under certain conditions—that is those for which the intracellular enzyme content limits the rate of the catalyzed reaction—are good candidates for regulatory points. It had been previously suggested that the porphobilinogen synthase (PBG-S) may play such a role, due to a relatively low specific activity and high K_M value, which result in intracellular accumulation of its substrate, ALA [1,3,26]. It has also been demonstrated that supplying yeast cells with additional ALA does not cause increased production of any of the further intermediates nor of heme [1], indicating that a block occurs in the pathway at the level of porphobilinogen (PBG) synthesis.

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 YP 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]. The *Escherichia coli* strain 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 GAA TTT TTG G-3′ and 5′-CCG **GAA TTC** TCA TTA GTG ATG GTG ATG GTG ATG GTT TTC TTC ATC TAA CCA GTC-3′. The obtained PCR product was digested with the *Eco*RI and *Hin*dIII enzymes and ligated into the pYES2 multiple cloning site, downstream of the regulatable yeast *GAL1* promoter. The plasmid fully complemented the *hem2*Δ phenotype of the ΔYGL040c-4A strain.

The pPBGD1 plasmid, carrying the hemagglutinin-tagged *S. cerevisiae HEM3* gene under the control of the *GAL1* promoter, was constructed by ligation of the yeast wild-type *HEM3* gene into a novel vector consisting of a 3956-bp *Pvu*I–*Pvu*I fragment of the pYES2 vector and a 3700-bp *Pvu*I–*Pvu*I fragment of the pRS424 vector (Stratagene). The resulting vector differed from the pYES2 vector only by the replacement of the yeast *URA3* marker gene with a *TRP1* gene from the pRS424 vector. The *HEM3* gene, coding for PBG deaminase, was amplified from total yeast genomic DNA by PCR with the following primers: 5′-CC **GGA TTC** AAA TAC ACA CGT ACT ATG GGC CCT G-3′ and 5′-CC **GAA TTC** TCA TTA AGC GTA ATC AGG AAC ATC GTA TGG GTA TTT GAT TCT GTC TAA ATT

Table 1
Saccharomyces cerevisiae strains used in this work

Name	Genotype	Source or reference
S150-2B	*MAT a his3Δ1 leu2-3,112 trp1-289 ura3-52*	[27]
ΔYGL040c-4A	*MATα his3Δ1 leu2Δ0 ura3Δ0 hem2Δ::KANMX4*	EUROSCARF
G210-1C	*MAT a his⁻ leu2-3,112 trp1-289 ura3-52 hem3-1*	[21]
S150-2B hem4Δ	*MAT a his3Δ1 leu2-3,112 trp1-289 ura3-52 hem4Δ*	Our collection

AAT TTC-3′. The obtained DNA fragment was digested with the *Eco*RI and *Bam*HI 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).

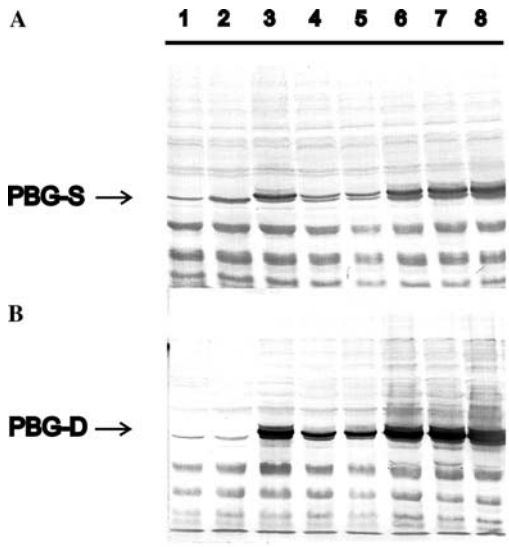
Biochemical analysis. Published methods were used to determine intracellular 5-aminolevulinic acid (ALA) and porphobilinogen (PBG) content [21]. Cells were cultured in liquid YPGal medium, collected, washed twice with water, and extracted twice with 5% TCA. PBG content in the extracts was measured colorimetrically 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 immunoassays (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



Lanes: 1 - WT cultured in YPGal, 2 - [pPBGS1] in YPGal, 3 - [pPBGD1] in YPGal, 4 - [pPBGS1 + pPBGD1] in YPD, 5 - [pPBGS1 + pPBGD1] in YPE, 6 - [pPBGS1 + pPBGD1] in YPGal, 7 - [pPBGS1 + pPBGD1] in YPGal + 250 μg/ml ALA, 8 - [pPBGS1 + pPBGD1] in YPGal + 1 mg/ml ALA.

Fig. 2. Western blots showing overproduction of PBG-S (A) and PBG-D (B). S150-2B cells carrying the indicated plasmids were grown in YPGal, YPE, and YPD media, resulting in induction, repression, and derepression of the *GAL1* promoter, respectively. Total yeast protein extracts (ca. 100 μg) were separated electrophoretically, transferred to nylon membranes, and probed with anti-His (A) or anti-HA (B) antibodies, followed by alkaline-phosphatase-based detection. The bands indicated correspond to proteins of approximately 38 kDa (PBG-S) and 37 kDa (PBG-D).

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

Strain	Intracellular PBG (nmol/g of dry weight)		
	−ALA	+250 μg/ml ALA	+1 mg/ml ALA
S150-2B	50 ± 14	59 ± 3	Not determined
S150-2B [pPBGS1]	198 ± 19	1201 ± 116	2025 ± 10
S150-2B [pPBGD1]	24 ± 7	Not determined	Not determined
S150-2B [pPBGS1] [pPBGD1]	41 ± 5	500 ± 38	1163 ± 69

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

ALA concentration in the medium	Intracellular ALA (nmol/g of dry weight)
0	1153 ± 153
250 μg/ml	6230 ± 4
1 mg/ml	13257 ± 1877

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 (*hem4*Δ), 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 immunoassays (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 4
Intracellular porphyrin concentrations in wild-type and *hem4*Δ cells overproducing one, two or three of the enzymes PBG-S, PBG-D, and URO-D, compared to parental-type cells

Strain	Intracellular porphyrins (nmol/g of dry weight)	
	−ALA	+ALA (250 μg/ml)
S150-2B	5	6
S150-2B [pPBGS1]	5	7
S150-2B [pPBGD1]	13	16
S150-2B [pPBGS1] [pPBGD1]	19	172–422
S150-2B [pPBGS1] [Yep351–HEM12]	7	4
S150-2B [pPBGD1] [Yep351–HEM12]	4	5
S150-2B [pPBGS1] [pPBGD1] [YEp351–HEM12]	6	54
S150-2B hem4Δ	25	25
S150-2B hem4Δ [pPBGS1]	44	66
S150-2B hem4Δ [pPBGD1]	73	41
S150-2B hem4Δ [pPBGS1] [pPBGD1]	107	433–622

a concentration of 250 μg/ml was added to the medium. The porphyrin content would then reach even 20 times the initial value, with no differences between the wild-type and mutant backgrounds.

The intracellular porphyrin content was measured also in S150-2B cells harboring the Yep351–*HEM12* plasmid along with one or both of the pPBGS1 and pPBGD1 plasmids. Overproduction of URO-D caused a significant decrease in porphyrin accumulation in the S150-2B [pPBGS1] [pPBGD1] [YEp351–*HEM12*] strain. The simultaneous overproduction of the three enzymes in cells cultured in the presence of exogenous ALA resulted in an approximately 4-fold decrease in porphyrin accumulation when compared with the S150-2B [pPBGS1] [pPBGD1] strain cultivated in the same medium (Table 4).

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 maximal 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_{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 *hem4*Δ 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 5
Kinetic parameters of the yeast heme biosynthesis enzymes

Enzyme	K_M [M]	V_{max} (mol of product/sec/mol of enzyme active form)	Reference
ALA-S	3.0×10^{-3} (glycine); 2×10^{-6} (succinyl-CoA)	1.15	[4]
PBG-S	3.6×10^{-4}	1.2	[5]
PBG-D	1.9×10^{-5}	0.5[a]	[6,34]
URO-S	5.0×10^{-6}–2.6×10^{-5}[b]	250[a]	[34–37]
URO-D	6.0×10^{-9}	0.021	[7]
COPROX	5.0×10^{-8}	0.10	[9]
PROTOX	1.0×10^{-7}	0.62	[10]
FERROC	9.0×10^{-8}	0.39	[11]

[a] 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 *hem4*Δ 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–*HEM12* plasmid, but it still exceeded wild-type levels. This may result from the fact that in our experiments the level of URO-D overproduction was lower than those of the PBG-S and PBG-D enzymes, or from the rate-limiting character of still another downstream enzyme, which under these conditions would most probably be COPROX. It may also be due to a combination of these factors.

The results we obtained suggest further that the PBG synthase and deaminase are not subjected to post-transcriptional regulation in yeast cells grown aerobically in the presence of glucose, or at least no mechanism functions under these conditions to prevent futile, unnecessary synthesis of PBG and hydroxymethylbilane. They might though be targets of regulatory mechanisms acting at the level of gene transcription, since the intracellular enzyme content of both PBG-S and PBG-D has proved to limit the rate of the reactions they catalyze. Any increase in the overall pathway productivity in response to environmental changes would require some kind of induction or activation of the reactions accounting for all of the pathway's blocks. Therefore some mechanism increasing the PBG-S and PBG-D content probably functions in vivo, relieving under certain conditions the blocks they constitute.

Acknowledgments

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