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Porphyrin Biosynthesis Intermediates Are Not Regulating δ-Aminolevulinic Acid Transport in *Saccharomyces cerevisiae*

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In *Saccharomyces cerevisiae*, as in all eukaryotic organisms, δ-aminolevulinic acid (ALA) is a precursor of porphyrin biosynthesis, a very finely regulated pathway. ALA enters yeast cells through the γ-aminobutyric acid (GABA) permease Uga4. The incorporation of a metabolite into the cells may be a limiting step for its intracellular metabolization. To determine the relationship between ALA transport and ALA metabolization, ALA incorporation was measured in yeast mutant strains deficient in the δ-aminolevulinic acid-synthase, uroporphyrinogen III decarboxylase, and ferrochelatase, three enzymes involved in porphyrin biosynthesis. Results presented here showed that neither intracellular ALA nor uroporphyrin or protoporphyrin regulates ALA incorporation, indicating that ALA uptake and its subsequent metabolization are not related to each other. Thus a key metabolite as it is, ALA does not have a transport system regulated according to its role.

Key Words: **δ-aminolevulinic acid; γ-aminobutyric acid; Uga4 permease; membrane transport; porphyrin biosynthesis; transport regulation; *Saccharomyces cerevisiae*.**

The biological importance of porphyrins and their metal complexes lies in their ability to act as mediators of biological oxidation reactions. These compounds carry out vital functions such as transport and storage of oxygen, generation of cellular energy, detoxification reactions, peroxidase-mediated reductions, biosynthesis of certain steroids and regulation of protein synthesis, and cell development (1).

For this reason, the biosynthesis of porphyrins has been extensively studied in a wide variety of organisms. The first committed step is the condensation of glycine with succinyl CoA, forming δ-aminolevulinic acid (ALA) catalysed by ALA-synthase. In most organisms studied, this is the rate limiting step in the porphyrin biosynthetic pathway, controlled by the intracellular free heme pool. ALA can also be formed via C5-pathway, using the intact C5-skeleton of glutamate or 2-oxoglutarate as the first substrate. The synthesis of ALA occurs within the mitochondria and then it moves to the cytoplasm, where two molecules are condensed and cyclized by the enzyme ALA-dehydratase to form the monopyrrole porphobilinogen (PBG). Four molecules of PBG are then condensed in a head to tail manner by the cytosolic PBG-deaminase, forming the linear tetrapyrrole hydroxymethylbilane, which is finally isomerized and cyclized to uroporphyrinogen III by the uroporphyrinogen III synthase. The last cytosolic enzyme uroporphyrinogen III decarboxylase leads to the formation of coproporphyrinogen III. The pathway now re-enters the mitochondria where the coproporphyrinogen oxidase forms protoporphyrinogen IX, which is converted to protoporphyrin IX by the protoporphyrinogen oxidase. The final step in the porphyrin pathway is the insertion of ferrous iron into protoporphyrin IX, catalysed by the enzyme ferrochelatase to form heme (1).

In *Saccharomyces cerevisiae* the heme biosynthetic pathway has also been exhaustively studied. Although this pathway is very similar in all organisms investigated so far, it should be noted that in yeast coproporphyrinogen oxidase is cytosolic (2) and the regulation of this via involves not only the enzyme ALA-synthase but also ALA-dehydratase (3–5).

It is known that ALA-synthase deficient strains of *S. cerevisiae* are able to grow using the heme precursor ALA, provided by the growth medium (6). On these grounds, ALA transport through plasma membrane was studied in yeast cells (7, 8). It was established (9) that the only permease involved in this process is the one known as the γ-aminobutyric acid (GABA) specific

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TABLE 1

Yeast Strains Used in This Study

Strain	Genotype	Reference
SP4	*leu1 arg4*	15
Sm2	*leu1 arg4 hem12-3*	16
DCT1-3D	*leu1 arg4 ctt1-1*	16
Sm12	*leu1 arg4 ctt1-1 hem15-3*	17
W303	*ade2 his3 leu2 trp1 ura3*	J. Verdière[a]
W303 *hem1::ADE2*	*his3 leu2 trp1 ura3 hem1*	J. Verdière[a]

[a] Personal communication.

permease encoded by *UGA4* gene. GABA can also be incorporated by the specific proline permease Put4 and the general amino acid permease Gap1 (10).

UGA4 expression is highly sensitive to nitrogen catabolite repression (NCR) (11) and is inducible by GABA (10). This regulation requires several specific and pleiotropic transcriptional factors (12–14).

The incorporation of a metabolite into the cells may be a limiting step for its intracellular metabolization. Because porphyrin biosynthesis is one of the most finely regulated pathways, we decided to determine the relationship between ALA transport and ALA metabolization.

For this purpose, we studied whether or not ALA incorporation is affected by the accumulation of different intermediates of porphyrin biosynthetic pathway.

MATERIALS AND METHODS

Chemicals. δ-[4-^{14}C]Aminolevulinic acid hydrochloride (^{14}C-ALA) was from New England Nuclear. Amino acids and other chemicals were of analytical grade and obtained from Sigma Chemical Co. (St. Louis, MO). Yeast extract and peptone were from Difco Laboratories (Detroit, MI).

Strains. The *Saccharomyces cerevisiae* strains used in this study are shown in Table 1. Mutant strains Sm2, Sm12, and W303 *hem1::ADE2* were derived from the wild-type strains SP4, DCT1-3D, and W303, respectively.

These strains were kindly supplied by J. Verdière from the Centre de Génétique Moleculaire du Centre National de Recherche Scientific, Université Pierre et Marie Curie, Gif-sur-Yvette, France and by M. Gora from the Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Warsaw, Poland.

Growth conditions. Minimal medium (MM1) used was described by Vavra and Johnson (18). The nitrogen source was either 10 mM proline (MPM1) or 10 mM $(NH_4)_2SO_4$ (MAM1).

When indicated, ALA was added to the medium at a final concentration of 0.3 mM. Cells were grown at 28°C with constant stirring (280 rpm) in darkness.

ALA uptake measurements. ALA uptake measurements were performed as already reported (10). Briefly, 0.1 mM ^{14}C-ALA was added ($t = 0$) to a culture at an absorbance of 0.300–0.900 at 570 nm. At different incubation times 1 ml samples were withdrawn and filtered through S&S 3362 filters and the cells were washed three times with 2 ml portions of ice-cold 20 mM potassium phthalate buffer. The dried filters were transferred to vials containing 5 ml of toluene scintillation cocktail and radioactivity was measured in a scintillation counter.

In each experiment all measurements were run in duplicates and the values presented are the average of three experiments. The deviation of these values from the mean was less than 15%.

Intracellular ALA, PBG, and porphyrins. Intracellular ALA was determined as reported by Malamud *et al.* (19) using the modified Ehrlich reaction developed by Mauzerall and Granick (20). PBG was measured using the Ehrlich reactive.

Porphyrins were measured spectrophotometrically (21).

Data represent the means of three independent experiments. Values varied ± 20% from experiment to experiment.

RESULTS

To elucidate the effect of different intermediates of porphyrin biosynthesis on ALA uptake we used mutant strains of *S. cerevisiae* partially defective in the functioning of ALA-synthase (encoded by *HEM1* gene), uroporphyrinogen III decarboxylase (encoded by *HEM12* gene), and ferrochelatase (encoded by *HEM15* gene).

To measure the activity of Uga4 permease, either ALA or GABA can be used as substrates. However, as ALA enters the yeast cells solely through this permease, while GABA can also be taken up by other two permeases (Put4p and Gap1p), we decided to determine ALA rather than GABA incorporation.

On the other hand, Uga4p was described by Grenson *et al.* (10) as a GABA inducible protein in yeast cells when grown on the minimal medium reported by Jacobs *et al.* (22). However, we have recently demonstrated that in cells grown on the minimal medium described by Vavra and Johnson (18) (MPM1) *UGA4* expression is constitutive and so not dependent on the presence of GABA (23). Therefore, Uga4p activity can be studied measuring ALA uptake in cells grown on MPM1 medium without previous induction with GABA.

Effect of Porphyrins

Mutants *hem12* and *hem15* are characterized by the accumulation within the cells of uroporphyrin and protoporphyrin, respectively. Intracellular ALA, PBG, and porphyrins were measured in these cells and results are shown in Table 2. As expected Sm2 and Sm12 strains accumulate great amounts of porphyrins while there is not overproduction of ALA and PBG.

ALA uptake in SP4 cells and its *hem12* mutant Sm2 are similar (Fig. 1) indicating that transport of ALA is not significantly affected by the presence of intracellular uroporphyrin (Table 2, lines 1 and 2).

ALA incorporation assays in DCT1-3D and its *hem15* mutant Sm12 suggest that protoporphyrin has no effect either on the ALA transport process (Fig. 2; Table 2, lines 3 and 4).

Effect of ALA

Mutants *hem1* are unable to synthesize ALA from glycine and succinyl CoA. So, W303 *hem1::ADE2* cells need the presence of ALA in the culture medium for

TABLE 2

Intracellular ALA, PBG, and Porphyrins

Strain	Relevant genotype	ALA	PBG	Porphyrins
1. SP4	*HEM12*	20	155	7
2. Sm2	*hem12*	27	228	1300
3. DCT1-3D	*HEM15*	53	175	95
4. Sm12	*hem15*	144	170	485
5. W303	*HEM1*	145	200	50
6. W303[a]	*HEM1*	3120	255	8
7. W303 *hem1::ADE2*[a]	*hem1*	2375	400	5
8. W303[b]	*HEM1*	3515	250	10
9. W303 *hem1::ADE2*[b]	*hem1*	3250	375	15
10. W303[c]	*HEM1*	385	180	7
11. W303 *hem1::ADE2*[c]	*hem1*	592	230	14
12. W303[d]	*HEM1*	1180	170	9
13. W303 *hem1::ADE2*[d]	*hem1*	1120	196	10

Note. ALA is expressed as nmol (g cell dry weight)$^{-1}$; PBG as nmol (g cell dry weight)$^{-1}$; porphyrins as pmol (g cell dry weight)$^{-1}$. Cells were grown until exponential growth phase on MPM1 medium. Then intracellular ALA, PBG, and porphyrins were measured.

[a] Cells at the exponential growth phase and grown on MPM1 supplemented with 0.3 mM ALA were transferred to fresh ALA-free medium where they were incubated for 2 h. Then intracellular ALA, PBG, and porphyrins were measured.

[b] Cells at the exponential growth phase and grown on MPM1 supplemented with 0.3 mM ALA were transferred to fresh ALA-free medium and immediately after intracellular ALA, PBG, and porphyrins were measured.

[c] Cells at the exponential growth phase and grown on MAM1 supplemented with 0.3 mM ALA were transferred to fresh ALA-free medium where they were incubated for 2 h. Then intracellular ALA, PBG, and porphyrins were measured.

[d] Cells at the exponential growth phase and grown on MAM1 supplemented with 0.3 mM ALA were transferred to fresh ALA-free medium and immediately after intracellular ALA, PBG, and porphyrins were measured.

their growth. Even though W303 wild-type strain does not need an external ALA supply for its growth, these cells were also grown in the presence of ALA, so that the growth conditions of this strain and its mutant were equal making the results comparable. These assays were carried out in W303 and W303 *hem1::ADE2* cells grown in the presence of 0.3 mM ALA for 24 h, then transferred to a fresh medium without ALA and incubated for 2 h. Intracellular ALA levels found in these strains are similar and very high (Table 2, lines 6 and 7), due to the incorporation of ALA from the medium and the fact that ALA-Dehydratase, the enzyme responsible for its metabolization, is a limiting rate enzyme of porphyrin pathway in yeast. The time course of ALA uptake is also similar in both strains (Fig. 3).

Moreover, there are no differences either between ALA incorporation in the wild-type cells grown with or without ALA in the growth medium (Fig. 3), although their intracellular ALA values differ significantly (Table 2, lines 5 and 6). In other words, intracellular ALA does not affect ALA uptake.

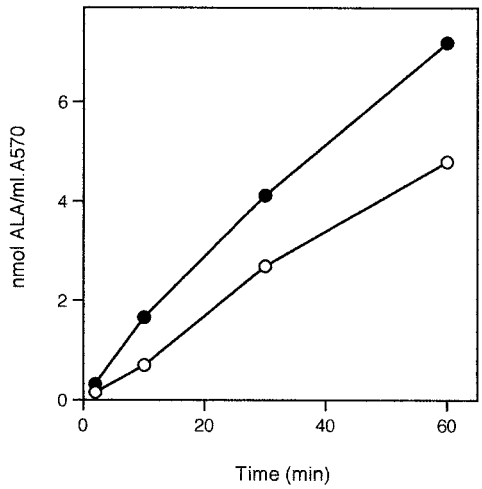

FIG. 1. Time course of ALA uptake. The assay was carried out in SP4 (○) and Sm2 (●) cells grown on MPM1 medium.

As expected, when W303 and W303 *hem1::ADE2* cells were grown for 24 h in the presence of 0.3 mM ALA, transferred to a fresh medium without ALA and immediately used for the assays, intracellular ALA values are very high (Table 2, lines 8 and 9). However ALA uptake is almost undetectable (Fig. 4). So, the low values of ALA uptake are probably due to the damage produced by extracellular ALA on the plasma membrane proteins rather than to any effect produced by intracellular ALA.

On the other hand, when *hem1* cells are grown on ammonium as the nitrogen source, the uptake of ALA dramatically decreases (Fig. 4). However the ALA incorporated is sufficient to allow the normal growth of these cells and after 24 h of growth there is an important pool of intracellular ALA (Table 2, lines 10 to 13) showing that the metabolization of ALA in yeast is strictly regulated.

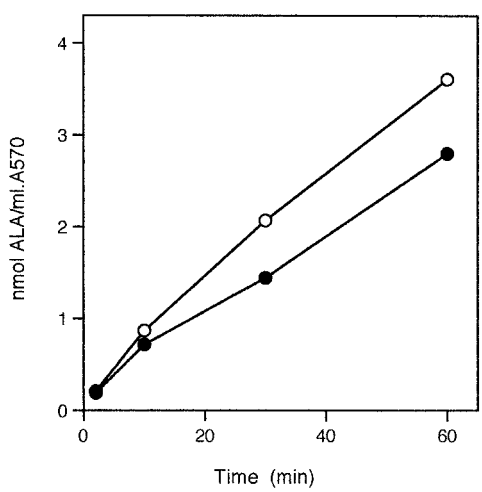

FIG. 2. Time course of ALA uptake. The assay was carried out in DCT1-3D (○) and Sm12 (●) cells grown on MPM1 medium.

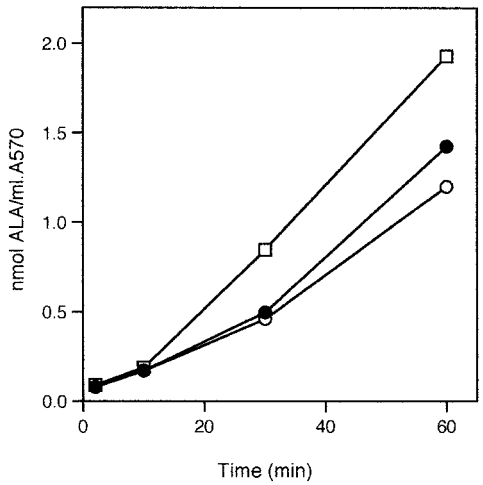

FIG. 3. Time course of ALA uptake. The assays were carried out in W303 cells grown on MPM1 medium (○) or on MPM1 supplemented with 0.3 mM ALA (●) and in W303 *hem1::ADE2* cells grown on MPM1 supplemented with 0.3 mM ALA (□). In all cases, cells were transferred to fresh ALA-free medium and incubated for 2 h before ALA uptake measurements.

DISCUSSION

The regulation of porphyrin biosynthesis is a very good example of how cells balance specific and global physiological needs. ALA is the first and unique precursor of this pathway and does not taking part in any other metabolic reactions. Since the incorporation of a metabolite into the cell may be a limiting step for its intracellular metabolization we decided to investigate whether or not there is a relationship between the regulation of the transport of ALA into yeast cells and its subsequent metabolization.

Using mutant strains deficient in the uroporphyrinogen III decarboxylase and ferrochelatase enzymes, we found that neither intracellular uroporphyrin nor protoporphyrin regulate ALA incorporation.

Results here presented also show that intracellular ALA does not produce any effect on its own incorporation. However, when cells were exposed to external ALA for 24 h, ALA uptake is strongly inhibited. This inhibition cannot be attributed to the presence of an important pool of intracellular ALA, because after ALA starvation for 2 h, intracellular ALA remained high while Uga4p activity is recovered. More likely, the observed decrease in ALA uptake could be due to the well-known oxidative stress produced by external ALA (24). Thus, when cells are exposed to ALA for 24 h, the subsequent reactive oxygen species that are generated, could cause a great deal of damage to the plasma membrane proteins and thereby reduce the activity of the permeases. Our results show that when cells are grown for 2 h in a fresh ALA-free medium, the activity of Uga4p is recovered. Instead intracellular ALA does not seem to produce any oxidative effect. This is probably due to highly developed stress defense mechanisms that act to protect yeast cells from oxidative threats (25).

In cells grown in the presence of ALA high intracellular levels are found and these pools do not diminish after a 2 h starvation. The reason for this may be that in yeast ALA metabolization is strictly regulated and the condensation of two molecules of ALA is a rate limiting step (3–5).

We conclude that regulation of ALA uptake is not related to regulation of ALA metabolization. From a physiological point of view, uptake systems make sense to a cell only when their substrates are both available in the medium and useful to the cell. As a matter of fact, the expression of the permease structural genes and the activity of the corresponding permeases are controlled and modulated by different types of regulatory systems.

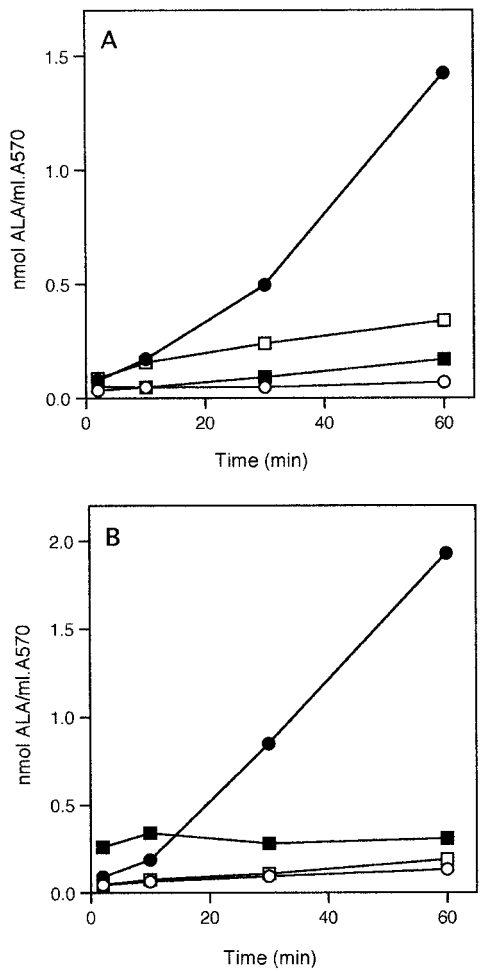

FIG. 4. Time course of ALA uptake. The assays were carried out in W303 (A) and W303 *hem1::ADE2* (B) strains. Cells were grown on MAM1 (○, □) or MPM1 (●, ■) in the presence of 0.3 mM ALA. In all cases, cells were transferred to fresh ALA-free medium and incubated (○, ●) or not (□, ■) for 2 h before ALA uptake measurements.

However, as ALA is incorporated by the GABA-specific permease Uga4 (9), its uptake is regulated as if ALA were a nitrogen source, which is not. GABA, although not essential for growth, can be used by yeast cells as a nitrogen source (26) and is taken up through strictly regulated permeases.

Because only mutants that are defective in ALA synthesis depend on ALA uptake for heme synthesis and a functional ALA transport system would not be necessary in a wild-type cell, it is not unexpected that there is no specific ALA permease. So, a key metabolite as it is ALA, would not have a transport system regulated according to its essential role.

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