Studies on the regulation of ornithine decarboxylase in yeast: Effect of deletion in the *MEU1* gene

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Methylthioadenosine is formed during the biosynthesis of spermidine and of spermine and is metabolized by methylthioadenosine phosphorylase, an enzyme missing in several tumor cell lines. In *Saccharomyces cerevisiae*, this enzyme is coded by the *MEU1* gene. We have now studied the effect of the *meu1* deletion on polyamine metabolism in yeast. We found that the effects of the *meu1Δ* mutation mostly depend on the stage of cell growth. As the cell density increases, there is a marked fall in the level of ornithine decarboxylase (ODC) in the *MEU1*⁺ cells, which we show is caused by an antizyme-requiring degradation system. In contrast, there is only a small decrease in the ODC level in the *meu1Δ* cells. The *meu1Δ* cells have a higher putrescine and a lower spermidine level than *MEU1*⁺ cells, suggesting that the decreased spermidine level in the *meu1Δ* cultures is responsible for the greater apparent stability of ODC in the *meu1Δ* cells. The lower spermidine level in the *meu1Δ* cells probably results from an inhibition of spermidine synthase by the methylthioadenosine that presumably accumulates in these mutants. In both *MEU1*⁺ and the *meu1Δ* cultures, the ODC levels were markedly decreased by the addition of spermidine to the media, and thus our results contradict the postulation of Subhi *et al.* [Subhi, A. L., *et al.* (2003) *J. Biol. Chem.* 278, 49868–49873] of a novel regulatory pathway in *meu1Δ* cells in which ODC is not responsive to spermidine.

methylthioadenosine | methylthioadenosine phosphorylase | polyamines | spermidine | putrescine

Ornithine decarboxylase (ODC) is the key regulatory enzyme of the polyamine biosynthetic pathway and catalyzes the decarboxylation of ornithine to 1,4-diaminobutane (putrescine). Putrescine is then converted to spermidine and spermine by the addition of aminopropyl groups from decarboxylated *S*-adenosylmethionine (1). Methylthioadenosine is formed as a byproduct of these aminopropyl transferase reactions (spermidine synthase and spermine synthase) (2–4) and is then converted to adenine and to methionine by the “methionine salvage” pathway (5–9). The first step in the methionine salvage pathway is the phosphorolysis of methylthioadenosine to methylthioribose-1-phosphate and adenine by methylthioadenosine phosphorylase (EC 2.4.2.28). The metabolism of methylthioadenosine in mammalian cells has been of special interest because methylthioadenosine phosphorylase is absent in many tumors (10–15).

Thomas *et al.* (16) and Subhi *et al.* (17) have shown that, in *Saccharomyces cerevisiae*, methylthioadenosine phosphorylase is coded by the *MEU1* gene and have described *meu1Δ* mutants. The availability of these yeast mutants deleted in the methylthioadenosine phosphorylase gene permits more extensive studies on the role of this gene and the methionine salvage pathway in polyamine metabolism in yeast, and a comparison of these effects in yeast with those reported in mammalian cells. Therefore, as an extension of our work on the regulation of ornithine decarboxylase and of polyamine pools in yeast (18), we have now studied the effect of the *meu1Δ* deletion on these parameters. Our findings were different from those reported by Subhi *et al.*

(17) on the effects of this mutation, and data are presented on the probable reasons for some of these differences.

Materials and Methods

Strains, Media, and Growth. The yeast strains used in this study are listed in Table 1. All strains were maintained in YPAD (1% yeast extract/2% peptone/2% agar/2% dextrose) medium, except strains Y622 and Y623, which were grown on SD-Ura-Leu plates, with additions of the required supplements. For most of the experiments, SD medium [6.7 g of yeast nitrogen base/20 g of dextrose/0.75 g of complete supplement mixture, pH 5.2 (Qbiogene, Irvine, CA)] was used. Yeast cultures were inoculated from plate to liquid SD medium to make the primary inocula, and, after growth, these inocula were further diluted into fresh SD medium and incubated at 30°C with shaking for 16–24 h. Cultures were harvested at different optical densities and washed with PBS (for polyamine estimation) or with enzyme extraction buffer (for enzyme assays) and stored at –80°C. Optical density was measured at 600 nm in a Genesis2 spectrophotometer (Thermo Spectronic, Rochester, NY); an optical density of 1.0 represented 1×10^7 cells per ml.

For experiments with cycloheximide, cultures were grown to an OD₆₀₀ of 1.5, and cycloheximide (final concentration 200 μg/ml culture) was added from a stock solution in ethanol.

Enzyme Assay and Protein Estimation. ODC was measured by using L-[1-¹⁴C]ornithine (Amersham Pharmacia, Cat. no. CFA491, 57 mCi/mmol) (1 Ci = 37 GBq) as a substrate (19, 20). The harvested cells were resuspended in cold extraction buffer [50 mM Tris-HCl, pH 8.0/1 mM DTT/0.1 mM EDTA/0.05% Tween 80/50 μM pyridoxal phosphate/1 mM phenylmethylsulfonyl fluoride (Sigma) and 1× “protease halt,” (Pierce)]. The suspensions were transferred to 2-ml screw cap tubes (Sarstedt) together with equal amounts of glass beads (425–600 μm, Sigma). The cultures were broken in a MiniBead Beater (Bio-spec Products, Bartlesville, OK) with two 1-min bursts, cooling the suspensions in ice between bursts. The cell lysates were centrifuged at 12,000 rpm in a Sorvall RC5C, SS34 rotor (DuPont) at 4°C for 20 min, and 10 μl of the clarified supernatant was used for each assay in duplicate in a total volume of 125 μl (50 mM Tris-HCl, pH 8.0/400 nCi of L-[1-¹⁴C]ornithine/100 μM cold ornithine/0.5 mg/ml BSA/50 μM pyridoxal phosphate/5 mM DTT). ODC activity was expressed as nmol of ¹⁴CO₂ released from ornithine per mg of protein per h at 37°C.

Spermidine synthase was assayed with some modifications of the method reported previously (4). The yeast cultures were grown to OD₆₀₀ of 0.8 and were washed with extraction buffer (50 mM Tris-HCl, pH 8.0/1 mM DTT/0.1 mM EDTA/1 mM phenylmethylsulfonyl fluoride). The protein was extracted as above. The spermidine synthase assay mixture contained 0.1 M Hepes-KOH (pH 7.3), 5 mM DTT, 50 nCi of [1,4-¹⁴C]putrescine (Amersham Pharmacia, Cat. no. CFA301, 108 mCi/mmol), 100 μM unlabeled putrescine, 100 μM decarboxylated *S*-adenosyl-

Abbreviations: ODC, ornithine decarboxylase; MTOB, 4-methylthio-2-oxobutanoic acid.

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Table 1. Strains and genotypes of the yeast cells used in this study

Strain names	Genotypes	Source
Y534	Mata <i>his3 leu2 met15 ura3</i>	<i>S. cerevisiae</i> deletion bank*
Y606	Mata <i>his3 leu2 met15 ura3 meu1Δ::KANMX</i>	<i>S. cerevisiae</i> deletion bank*
Y610	Matα <i>his3 leu2 lys2 ura3</i>	<i>S. cerevisiae</i> deletion bank*
Y607	Matα <i>his3 leu2 lys2 ura3 meu1Δ::KANMX</i>	<i>S. cerevisiae</i> deletion bank*
Y619	Matα <i>his3 leu2 ura3 ade2 trp1 ado12-1</i>	Surdin-Kerjan (16), CY85-3C [†]
Y620	Matα <i>his3 leu2 ura3 ade2 trp1 ado12-1 meu1Δ::LEU2</i>	Surdin-Kerjan (16), CY85-8B [†]
Y622	Matα <i>ura3 met15 leu2 ade2 meu1Δ::LEU2 pMEU1</i>	Kruger (17), AS24-1b, (pMEU1) [†]
Y623	Matα <i>ura3 met15 leu2 ade2 meu1Δ::LEU2 pRS316</i>	Kruger (17), AS24-1b, (pRS316) [†]
Y651	Matα <i>his3 leu2 lys2 ura3 oaz1Δ::KANMX</i>	<i>S. cerevisiae</i> deletion bank*

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methionine (a gift from G. Stramentinoli, Bio Research Co., Research Laboratories, Liscate, Milan, Italy), and 3 μ l of enzyme extract in a total volume of 10 μ l. The mixture was incubated at 37°C for 30 min, and the reaction was terminated by adding 10 μ l of ice cold 10% trichloroacetic acid. Five microliters of the trichloroacetic acid extract was spotted on thin layer chromatography (Polygram Ionex-25 SA-Na, Macherey-Nagel, Düren, Germany) along with radioactive standards for putrescine and spermidine. The chromatograms were developed with 2 M KCl and exposed to a Fuji BAS-MS imaging plate; after scanning, the [¹⁴C]spermidine formation was quantitated by IMAGE GAUGE software (Fuji). To test its inhibitory effect, methylthioadenosine at different concentrations was added in a total volume of 1 μ l per 10 μ l of reaction mixture, and incubated as above. Protein concentrations of the cell lysates were measured with the Bradford reagent (Bio-Rad), by using BSA as the standard (21).

Estimation of Polyamines. Polyamines were assayed by a modification of the HPLC chromatography procedure previously described (4). The amines in the cells were extracted with 5 ml of 10% perchloric acid per gram wet weight of cells. We applied 30–60 μ l of the extract to a sulfonated cation exchange resin (A5-2806, St. John Associates, Beltsville, MD), packed in a 4.5 $\times$ 150-mm column, which was maintained at 70°C. The eluted amines were measured fluorometrically after reaction with *o*-phthalaldehyde. The eluting buffer was modified to contain 112 g of KCl, 24 g of tripotassium citrate·H₂O, 24 ml of 2 M HCl, and 29.2 g of NaCl per liter.

Western Blot Analysis. The same protein extracts (20 μ g) that were used for ODC assays were fractionated on 10% NuPAGE Novex Bis-Tris SDS gel (Invitrogen) in a minigel apparatus (Invitro-

gen). Immunoblotting was performed on poly(vinylidene difluoride) (PVDF) membrane (Invitrogen). Immunodetection was carried out by using an antibody (1:5,000 dilution) raised against recombinant yeast ODC protein (18). Immunospicificity of the antibody was enhanced by prior adsorption of the antibody with an *spe1Δ* extract that does not contain ODC protein. For chemiluminescence assay, Western Breeze immunodetection kit (Invitrogen) was used, and the immunoreactive bands were detected and scanned with a Bio-Rad Scanner (GS-800).

Results

***meu1* Deletion Does Not Affect the Growth Rate.** We have measured the growth rate of four different isogenic *meu1Δ* and *MEU1*⁺ pairs of strains and have found essentially no effect of the *meu1Δ* on the growth rate. The results are presented along with polyamine content in Table 2.

Effect of *meu1* Deletion on ODC Activity and Polyamine Levels in Logarithmically Growing Cells. As shown in Fig. 1, we found a small effect of the *meu1* deletion on the amount of ODC in early logarithmically growing cells (OD = 0.4) when measured either by assay of ODC activity or by Western blot. With either assay, the ODC level of the *meu1Δ* cells was 30–60% higher than that found with the *MEU1*⁺ cells. The same results were obtained with four different isogenic pairs of *MEU1*⁺ and *meu1Δ* cultures (Fig. 1, Sets A–D). Set C contains the results we obtained with the strains used by Subhi *et al.* (17) and shows a much smaller increase in the ODC level in the *meu1Δ* strain than the 800% increase reported by these investigators.

Polyamine assays on perchloric acid extracts of the above cultures (Table 2) showed a 3- to 5-fold elevation in the putrescine level and a small decrease in the spermidine level,

Table 2. Polyamine content of different pairs of isogenic strains of *MEU1*⁺ and *meu1Δ* cells harvested at 0.4 OD₆₀₀

Set	Strain	Relevant genotypes	Putrescine, nmol/mg wet weight	Spermidine, nmol/mg wet weight	Spermine, nmol/mg wet weight	Putrescine: Spermidine	Doubling time, h
A	Y534	<i>MEU1</i> ⁺	0.2	1.8	0.3	0.1	1.7
	Y606	<i>meu1Δ</i>	1.0	1.4	0.1	0.7	1.7
B	Y610	<i>MEU1</i> ⁺	0.3	2.1	0.2	0.14	1.6
	Y607	<i>meu1Δ</i>	0.8	1.7	0.1	0.5	1.6
C	Y622	<i>MEU1</i> ⁺	0.1	0.7	0.1	0.15	2.9
	Y623	<i>meu1Δ</i>	0.4	0.9	0.1	0.45	2.8
D	Y619	<i>MEU1</i> ⁺	0.06	1.3	0.1	0.05	2.2
	Y620	<i>meu1Δ</i>	0.3	1.0	0.1	0.3	2.1

Fig. 1. Level of ODC in logarithmically growing *MEU1*⁺ and *meu1Δ* cultures (OD = 0.4). Each set represents a different pair of *Meu1*⁺ and *meu1Δ* isogenic strains. (Upper) The assays for ODC activity expressed in nmol of ¹⁴CO₂ released per mg of protein per h. (Lower) The assays for ODC protein (Western blot) with the same extracts (20 μg).

which resulted in an increase in the putrescine to spermidine ratios in *meu1Δ* cells compared with *MEU1*⁺ cells.

Effects of Cell Density on ODC Levels of *MEU1*⁺ and *meu1Δ* Cultures.

Because Subhi *et al.* (17) might have used cells from an overgrown culture, we then repeated the ODC assays with cultures grown to higher optical densities to see whether the cell concentration might account for the differences between our results and theirs. As shown in Fig. 2A, when the ODC measurements were carried out on extracts of cells harvested at higher optical densities, the ODC levels were lower in both the *MEU1*⁺ and *meu1Δ* cells but the decrease was much less pronounced in the *meu1Δ* cultures. When the measurements were made on nearly stationary phase cultures (OD₆₀₀ 1.5–2.0), the *MEU1*⁺ cells showed an 80–90% decrease in activity, whereas the *meu1Δ* cells showed only a 30–40% decrease in ODC activity. Thus, when measured in overgrown cultures, the residual activity in the *meu1Δ* cells was 3- to 4-fold higher than found in the *MEU1*⁺ cultures. Western blot analysis showed a similar effect of cell density, and indicated that the decrease in ODC was due to a decrease in the ODC protein (Fig. 2B). Completely stationary

Fig. 2. Effect of increase in optical density of the cultures on ODC activity (A) and ODC protein (Western blot) (B). For these experiments, Y610 (*MEU1*⁺) and Y607 (*meu1Δ*) cells were used. The result with a *spe1Δ* extract (rightmost lane, Western blot) shows the specificity of the ODC antibody.

Fig. 3. Cell density does not affect the ODC activity or ODC protein (Western blot) in the absence of antizyme (*MEU1*⁺ *oaz1Δ* strain, Y651).

phase cultures had very little ODC activity both in *MEU1*⁺ and *meu1Δ* cells (OD = 4.4, Fig. 2A).

The recent availability of an *oaz1Δ* mutant that lacks antizyme (22) permitted us to study whether the loss of ODC activity in cultures grown to a high cell density was the result of an effect on enzyme synthesis or of enzyme degradation via the well-established antizyme system (22–27). For this purpose, we repeated the above experiment on the effect of cell density on ODC activity and protein level in a *MEU1*⁺ *oaz1Δ* strain (isogenic with Y610, except for the *oaz1* deletion). As shown in Fig. 3, the cell density did not affect the level of ODC in this strain, indicating that the loss of ODC seen in Fig. 2 was due to the action of the antizyme-mediated degradation system of ODC.

ODC Is More Stable in *meu1Δ* Cultures than in *MEU1*⁺ Cultures. The above experiments (Fig. 2) showed that the ODC level in the *meu1Δ* cultures did not decrease as rapidly as the enzyme level in the *MEU1*⁺ cultures, as the cell density increased. To test whether this difference in the behavior of the two strains was the result of increased stability of the ODC in the *meu1Δ* cultures, we treated both *MEU1*⁺ and *meu1Δ* cultures (OD = 1.5) with cycloheximide to inhibit protein synthesis, and followed the decay in ODC. We chose the culture with an OD₆₀₀ of 1.5, because we found that, at this optical density, the ODC activities of *MEU1*⁺ and *meu1Δ* cells showed the largest difference (Fig. 2A). As shown in Fig. 4, in *MEU1*⁺ cultures, ODC decreased with a half-life of 1.5 h. In contrast in *meu1Δ* cells, there was almost no decrease in ODC activity or protein in 3 hours.

Because we know that the level of ODC is markedly affected by the spermidine concentration in the yeast cell (18, 22, 28, 29), we next determined the level of polyamines in the *meu1Δ* and *MEU1*⁺ cultures grown to the same cell density as in the above experiment. These assays (Fig. 5) showed that the spermidine level was at least 50% less in *meu1Δ* cells than in *MEU1*⁺ cells and showed a 3.5-fold increase in putrescine content in *meu1Δ* cells compared with *MEU1*⁺ cells.

We then tested whether the higher spermidine level in *MEU1*⁺ cells was sufficient to explain the decreased level of ODC seen in these cells compared with the *meu1Δ* cells. We found that this was indeed the case. Thus, if the *meu1Δ* cells were grown in 5×10^{-6} M spermidine, the spermidine level of the cells increased from 1.4 to 2.6 nanomoles per milligram wet weight, and this increase in the intracellular spermidine level resulted in a 90% decrease in ODC and a 91% decrease in the putrescine level.

The decrease in spermidine concentration found in the *meu1Δ*

Fig. 4. ODC is more stable in *meu1Δ* (Y607) cells than in *MEU1*⁺ (Y610) cells. ODC was measured in *MEU1*⁺ and *meu1Δ* cultures after cycloheximide (CHX) treatment. Cultures were grown to OD₆₀₀ of 1.5, and, at time zero, CHX was added at 200 $\mu\text{g}/\text{ml}$. Aliquots of the cultures were harvested at 1-hour intervals and assayed for ODC activity (A) and ODC protein (Western blot) (B).

cells could have been due to inhibition of spermidine synthase by methylthioadenosine as has been described for spermidine synthase from other sources (30–32). By using crude extracts from *MEU1*⁺ and *meu1Δ* cultures, we found that this was indeed the case (Fig. 6). Even 0.0025 mM methylthioadenosine showed 30–40% inhibition; higher concentrations (0.25–2.5 mM) caused nearly complete inhibition of spermidine synthase activity.

Spermidine Addition Causes a Rapid Decrease in ODC Levels in both *MEU1*⁺ and *meu1Δ* Cells in both Logarithmically Growing and Overgrown Cultures. In contrast to the results reported by Subhi *et al.* (17), we found that spermidine addition to the cultures caused a rapid fall in the ODC activities in both the *MEU1*⁺ and the *meu1Δ* cells (Fig. 7). Incubation of logarithmically growing cultures (final OD = 0.4) with 100 μM of spermidine for 4 h resulted in 95% decrease of ODC activity in both strains. A similar decrease in ODC activity was found when spermidine was added to a more dense culture (final OD = 2.5). The decrease in enzyme activity was due to a decrease in ODC protein as measured by Western blot (result from a logarithmically growing culture is shown in Fig. 7 Lower).

Effect of 4-Methylthio-2-Oxobutanoic Acid (MTOB) Addition on the Level of ODC. We have found a difference in the effect of MTOB when tested in *MET15*⁺ or *met15Δ* strains. When tested with

Fig. 5. Polyamine levels of *MEU1*⁺ (Y610) and *meu1Δ* (Y607) cultures, harvested at an OD₆₀₀ = 1.5.

Fig. 6. Effect of methylthioadenosine on spermidine synthase activity. Spermidine synthase assays were carried out in extracts of *MEU1*⁺ (Y610) and *meu1Δ* (607) by using [^{14}C]putrescine and decarboxylated *S*-adenosylmethionine in the presence or absence of different concentrations of methylthioadenosine *in vitro*. Formation of [^{14}C]spermidine from [^{14}C]putrescine was measured, and 100% activity was considered as the amount of spermidine formed in the absence of methylthioadenosine.

MET15⁺ strains, we did not find any repression of ODC by either 1 mM or 0.1 mM MTOB in either *MEU1*⁺ (Y610) or *meu1Δ* (Y607) cultures (data not shown).

However, we did find a small effect of MTOB when added for 4 h to *met15Δ* cells. These cells were grown with methionine and washed twice in methionine-free medium before incubation with MTOB. When incubated in the absence of methionine, additions of 0.1 mM or 1 mM MTOB for 4 h or 20 h caused a 30–40% inhibition of ODC in *MEU1*⁺ cells (Y534) and 20–25% inhibition in *meu1Δ* cells (Y606). Additions of MTOB to cells supplemented with methionine showed no effect of MTOB. Interpretation of these results is complicated by slower growth rate of cells supplemented with MTOB alone compared with cells supplemented with both methionine and MTOB. These results are thus somewhat different from the findings of Subhi *et al.* (17) of a complete repression of

Fig. 7. ODC is markedly decreased by addition of spermidine to both *MEU1*⁺ (Y610) and *meu1Δ* (Y607) cultures. At time zero (OD₆₀₀ = 0.1 or OD₆₀₀ = 1.0), yeast cultures were divided into two parts, and spermidine at 100 μM final concentration was added in one part. Samples were harvested after 4 h and assayed for ODC. (Upper) In culture A, the initial OD = 0.1 and the final OD = 0.4. In culture B, the initial OD = 1.0 and the final OD = 2.5. (Lower) Western blot on extracts from culture A.

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