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SUBCELLULAR LOCATION OF *S*-ADENOSYLMETHIONINE:Δ^{24} STEROL METHYLTRANSFERASE IN *SACCHAROMYCES CEREVISIAE*

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SUMMARY

Adaptation of anaerobically grown yeast cultures to oxidative conditions produces a rapid burst of ergosterol synthesis with concomitant formation of mitochondria. Mitochondrial enzymes are derepressed and synthesized de novo either on cytoplasmic or mitochondrial ribosomes during adaptation, and are subsequently incorporated into developing mitochondrial structures. The dependence of mitochondrial formation and respiratory competence on the presence of ergosterol has indicated a close relationship between the development of the two systems. Properties of *S*-adenosylmethionine:Δ^{24} sterol methyltransferase (EC 2.1.1.41), an enzyme involved in the terminal stages of ergosterol biosynthesis, have been examined in yeast during aerobic adaptation. The subcellular location of this enzyme is the promitochondria or mitochondria. The enzyme is synthesized on cytoplasmic ribosomes and coded for in nuclear DNA. The enzyme is present at low levels during anaerobic growth and is not subject to repression by high levels of glucose.

INTRODUCTION

Aerobically growing cultures of *Saccharomyces cerevisiae* produce up to 10% of their total dry weight as ergosterol [1]. Conversely, sterols can not be synthesized anaerobically because the cyclization of squalene requires molecular O_2 [2]. Ergosterol is, however, necessary for anaerobic growth [3]. Although a precise function for sterols has not yet been elucidated, ergosterol has been shown to be required for the acquisition and maintenance of respiratory competence by yeast [4, 5].

Since formation of mitochondria during aerobic adaptation is subject to repression by glucose, many of the mitochondrial functions are absent when yeast are grown in a glucose-containing medium [6–8]. Although sterols are not synthesized anaerobically, their synthesis is not subject to repression by glucose [9].

The biogenesis of yeast mitochondria during aerobic adaptation has been studied extensively and many inducible proteins have been identified [6, 10–12]. Some of these proteins appear to be synthesized exclusively on cytoplasmic ribosomes and

then incorporated into developing mitochondrial structures [7, 8, 13], while parts of others are synthesized on both cytoplasmic and mitochondrial ribosomes [8, 14-16].

There have been many suggestions that sterol synthesis and respiratory adaptation are closely linked. However, no one has yet shown a direct involvement between the two. Starr and Parks [17] have shown that ergosterol prevents petite induction at high temperatures and have described and characterized sterol transmethylation systems during aerobic adaptation [18]. Adams and Parks [19] also have shown that chloramphenicol inhibits the synthesis of sterols during aerobic adaptation suggesting that certain proteins involved in sterol synthesis may be mitochondrial in origin. Woods [20] has isolated a sterol mutant *nys*-3 which lacks ergosterol but produces high levels of the zymosterol-like compound $\Delta^{8(9),22}$ ergostadiene 3-β-ol [21, 22]. This organism does not grow well in ethanol medium suggesting a direct involvement between the sterol alteration and certain mitochondrial functions. Additional evidence for sterol–mitochondrial interactions stems from the presence of high levels of sterols associated with mitochondrial enzymes such as cytochrome oxidase [23, 24].

Although several enzymes involved in sterol synthesis have been studied in yeast, few attempts have been made to determine their location within the yeast cell. Progress has been made in determining the subcellular location of enzymes which function in the early stages of sterol biosynthesis [25, 26], but to date terminal enzymes in this pathway have not been examined. The present paper describes the subcellular location, glucose effects, and site of synthesis of *S*-adenosylmethionine:Δ^{24} sterol methyltransferase (a terminal enzyme in sterol synthesis).

MATERIALS AND METHODS

Organisms and cultural conditions

S. cerevisiae, strain 3701B, a haploid uracil-requiring organism was used principally throughout this study. Strain 5015D, a haploid auxotroph requiring methionine, adenine, uracil and tryptophan, and a mitochondrial DNA-less petite (EB-5) derived from 5015D were also employed.

The organisms routinely were grown with shaking in broth medium composed of 1% tryptone, 0.5% yeast extract and 2% glucose. The glucose was either replaced with 1.9% ethanol (v/v) for mitochondrial development, or was increased to 10% for complete repression of mitochondria. Cells were grown anaerobically in 2-l Erlenmeyer flasks filled with 1800 ml of a 1% yeast extract, 2% glucose, 0.1% NH_4Cl, 0.1 M phosphate buffer, pH 6.5 medium, and equipped with Bunsen valves. A 10-ml inoculum was added and the flasks were sparged through the cotton plugged arm of the Bunsen valve for 5 min with N_2 to remove residual O_2. 200 ml of 20% glucose were prepared separately and added to each flask after autoclaving. Anaerobically grown cells were adapted to aerobic conditions in Wickerham's complete medium [27] plus 5% glucose. All cultures were incubated at 30 °C.

Isolation of mitochondria

Cultures of 3701B were grown in the ethanol medium to late log phase and harvested by centrifugation. The cells were washed once in cold distilled water and resuspended in a 0.5 M sucrose, 0.1 M Tris–HCl, pH 7.5 buffer to a final concen-

tration of 1 g cells/ml. 25 ml of the cell suspension were added to a 75-ml Duran flask and broken with a 45-s burst of a Bronwill MSK tissue homogenizer using 40 g of 0.25-mm glass beads. Unbroken cells and other cellular fragments were removed by centrifugation at 2500 × *g* for 20 min. The mitochondria-containing supernatant was then centrifuged at 25 000 × *g* for 20 min. The mitochondrial pellet from this centrifugation was washed twice in the same buffer and recollected by centrifugation each time. The final pellet was resuspended to 40 mg protein per ml in the buffer and 1.0 ml was layered on linear 20–70% sucrose gradients. The sucrose gradient was also 0.02 M in Tris–HCl, pH 7.0, and 2 mM in EDTA. The mitochondria were centrifuged for 3 h at 60 000 × *g* (center of tube) in a Beckman SW 25.1 rotor. The gradients were fractionated by punching a hole in the bottom of the tubes and collecting 1-ml aliquots. Density of the gradients was determined with a Zeiss hand sugar refractometer model 0/30/0e. Mitochondria were also isolated from spheroplast preparations as described by Shimizu et al. [26]. The spheroplasts were lysed, and mitochondria separated by differential centrifugation as described above.

Digitonin fractionation of mitochondria

The mitochondria were isolated as detailed above and suspended in 0.5 M sucrose, 0.1 M Tris–HCl buffer, pH 7.5, at a concentration of 15 mg protein/ml. The digitonin method [28] for mitochondria fractionation was used with slight modifications. Aliquots of ice-cold digitonin (10%, w/v) were added to the mitochondria to a ratio of 1.1 mg digitonin per 10 mg mitochondrial protein. This suspension was incubated for 20 min at 4 °C, and then centrifuged at 15 000 × *g* for 10 min. The supernatant was decanted and saved. The pellet was washed with 10–15 ml of the 0.5 M sucrose buffer, recentrifuged, and this supernatant added to the first. The combined supernatants were centrifuged at 105 000 × *g* for 1 h. The pellet from the ultracentrifugation (outer membrane) was resuspended in 0.1 M Tris–HCl buffer, pH 7.5. Care was taken to exclude the white digitonin button at the bottom of the tube. The supernatant (inter-membrane soluble fraction) was dialyzed overnight at 4 °C against 100 vol. of 0.1 M Tris–HCl buffer, pH 7.5.

The pellet from the first 15 000 × *g* centrifugation was resuspended to 15 mg protein/ml in distilled water and sonicated with a Branson sonic disruptor for 60 s (3 × 20 s) at 4 °C at a power output of 85 A. Digitonin was again added to the same ratio (1.1/10) and the suspension incubated 20 min at 4 °C. The suspension was centrifuged at 15 000 × *g* for 10 min. This supernatant was then centrifuged at 105 000 × *g* for 1 h to give the inner membrane portion in the pellet and the matrix portion in the supernatant. The inner membrane fraction was resuspended in 0.1 M Tris–HCl buffer, pH 7.5, and the matrix fraction was dialyzed overnight under the same conditions employed for the inter-membrane fraction.

Isolation of promitochondria

Cultures of 3701B were grown in 10% glucose medium to late log phase and harvested by centrifugation. The cells were washed in cold distilled water, resuspended in 0.1 M Tris–HCl, pH 7.5, and broken as above. Unbroken cells and other cell fragments were removed by a 20-min centrifugation at 25 000 × *g*. The supernatant was centrifuged at 105 000 × *g* for 1 h and the resulting supernatant discarded.

The pellet was resuspended to a final concentration of 40 mg protein/ml in 0.1 M Tris–HCl buffer, pH 7.5, and homogenized in a Potter–Elvehjem tissue homogenizer. 1.0 ml of the homogenized preparation was layered on top of a linear 20–70% sucrose gradient and centrifuged for 16 h at 60 000 × *g* (middle of tube) in a Beckman SW 25.1 rotor. The sucrose gradient was also 0.02 M in Tris–HCl, pH 7.0, and 2 mM in EDTA. 1-ml fractions were collected as above.

Aerobic adaptation experiments

Cells were grown anaerobically in 2-l Erlenmeyer flasks for 72 h and then chilled to 4 °C overnight. The medium was decanted and the cells harvested rapidly by centrifugation and resuspended in Wickerham's complete medium plus 5% glucose, or the same medium containing either chloramphenicol (4 mg/ml) or cycloheximide (0.5 mg/ml). The cultures were placed at 30 °C on a rotary shaker and 250-ml samples were removed from each at 2-h intervals for 10 h. The cells were collected by centrifugation, washed once in cold distilled water, resuspended in 0.1 M Tris–HCl, pH 7.5, and broken in the tissue homogenizer. Unbroken cells and other cellular fragments were removed by centrifugation at 2500 × *g* for 20 min. This low speed centrifugation step precludes the sedimentation of any mitochondria that may form during adaptation. The supernatant was then centrifuged at 105 000 × *g* for 1 h. The pellet was resuspended in the Tris–HCl buffer and used directly for enzyme assays.

Enzyme assays

S-Adenosyl-L-methionine:Δ^{24} sterol methyltransferase was assayed by measuring [^{14}C]methyl group incorporation from *S*-adenosyl[*Me*-^{14}C]methionine into the non-saponifiable lipid fraction. The assay tubes (25 cm × 150 mm screw-cap culture tubes) contained 3.0 ml of 0.1 M Tris–HCl, pH 7.5, 10 μmoles $MgCl_2 \cdot 6H_2O$, 60 μmoles $KHCO_3$, 0.4 μmoles of zymosterol, and 1.0 ml of enzyme (the protein was diluted to 15 mg/ml prior to each assay). Protein was determined by the method of Lowry et al. [29]. The reaction was initiated by the addition of 0.25 μCi of *S*-adenosyl-[*Me*-^{14}C]methionine diluted with carrier *S*-adenosylmethionine. A final concentration of 400 nmoles of *S*-adenosylmethionine was added to each reaction. The tubes were incubated at 30 °C with gentle shaking for 30 min after which the reaction was stopped by the addition of 2 ml of 60% KOH. The mixture was saponified for 1 h and the non-saponifiable lipids removed by two 10-ml extractions with *n*-hexane [30]. The hexane was placed directly into scintillation vials, evaporated to dryness under N_2, 10 ml of scintillation cocktail (0.3% 2,5-diphenyloxazole and 0.01% 1,4-bis-(2-5-phenyloxazolyl)benzene in toluene) added, and the radioactivity determined in a Packard Tri-Carb 3214 scintillation spectrometer. All counts were corrected to 100% by the channels ratio method [31]. The incorporation of the (*Me*-^{14}C) group of *S*-adenosylmethionine by cell-free extracts into the non-saponifiable lipid fraction was linear for at least 45 min. Zymosterol was always suspended in absolute ethanol just prior to addition to the reaction mixture.

Cytochrome oxidase was assayed spectrophotometrically by following the oxidation of cytochrome *c* at 550 nm [24].

Malic dehydrogenase was assayed spectrophotometrically by measuring the oxidation of NADH at 340 nm [32].

Substrates and isotopes

Zymosterol was isolated from Fleishman's dry yeast. The yeast powder was saponified in a 50% methanol, 10% KOH, 0.5% pyrogallol solution for 24 h with refluxing. The non-saponifiable lipids were extracted into *n*-hexane, dried under N_2, and the ergosterol–maleic anhydride adduct formed according to Schwenk et al. [33]. The remaining sterols were extracted into alkaline methanol (10% KOH), partially dried under N_2 and then extracted into *n*-hexane and evaporated to dryness. The sterols were resuspended in a known volume of absolute ethanol–acetone (1:1, v/v) and then precipitated by the addition of an equal volume of 0.5% digitonin in 50% ethanol. The precipitate was allowed to form overnight at 4 °C and was collected by centrifugation and washed twice with acetone–ether (1:1, v/v). The digitonide was split with dimethylsulfoxide [34], extracted into hexane, and evaporated to dryness under N_2. The zymosterol was dissolved in hot methanol and allowed to recrystallize at 4 °C. Purity of the zymosterol was determined by the presence of a single spot on thin-layer chromatography (R_F 0.32) [22] and a melting point of 107 °C which closely approximates reported literature values [33, 35].

S-Adenosyl-L-[*Me*-^{14}C]methionine (Spec. Act. 52 Ci/mole) was purchased from International Chemical and Nuclear Company. Unlabeled *S*-adenosyl-L-methionine was obtained from Boehringer–Mannheim. Cytochrome *c* (Type III), β-glucuronidase (Type H-2), chloramphenicol, oxalacetate and NADH were purchased from Sigma Chemical Company, and digitonin and cycloheximide were purchased from Calbiochem.

RESULTS

Previous studies on the sterol methyltransferase in yeast were performed on cultures growing in glucose medium. We found, as have others [34], that over 95% of the methyltransferase activity in these cultures was associated with the "microsomal" fraction (105 000 × *g* pellet from a 25 000 × *g* supernatant). However, quite different results were obtained when the cells were cultured under conditions favorable for respiratory metabolism (ethanol medium). Table I shows partial cell fractionation data of a culture of 3701B grown in ethanol medium. The cells were harvested and fractionated according to the procedures for mitochondria isolation (see Materials and Methods). When the 25 000 × *g* supernatant (normally the fraction containing the methyltransferase enzyme [35]) was ultracentrifuged at 105 000 × *g*, less than 2% of the total methyltransferase activity was found in the pellet, and no activity was present in the supernatant. Instead the activity resided in the mitochondrial fraction. Cytochrome oxidase, a known mitochondrial enzyme [12], was also assayed in these preparations for comparative purposes. Table I shows that both cytochrome oxidase and the methyltransferase activities are associated primarily with the mitochondrial fraction. The results of the cellular fractionation were identical whether the cells were disrupted by the Bronwill tissue homogenizer or by spheroplast lysis. For convenience, all further mitochondrial preparations were prepared by tissue homogenization in the Bronwill MSK.

Sucrose gradient centrifugation was employed to determine the purity and sedimentation charactersitics of the mitochondrial fraction shown in Table I. Fig. 1 shows that the $A_{280\ nm}$, cytochrome oxidase, and methyltransferase activities all band-

TABLE I

THE DISTRIBUTION OF STEROL METHYLTRANSFERASE AND CYTOCHROME OXIDASE ACTIVITIES IN VARIOUS CELLULAR FRACTIONS OF YEAST

Cells were grown under aerobic conditions with ethanol as the principle carbon source. The cells were broken by tissue homogenization and the unbroken fragments removed by centrifugation at 2500 × *g* for 20 min. Aliquots of this supernatant were used directly for enzyme assays and the remaining portion was centrifuged at 25 000 × *g*. The pellet from this centrifugation contains the mitochondria. The 25 000 × *g* supernatant was recentrifuged at 105 000 × *g* for 1 h. The pellet from this centrifugation is the microsomal fraction.

Cellular fraction	Cytochrome oxidase ($\Delta A_{550\ nm}$/min)		Sterol methyltransferase (cpm)	
	Total activity	Total %	Total activity	Total %
2500 × *g* supernatant	3400	100	$4.0 \cdot 10^7$	100
25 000 × *g* pellet (mitochondria)	3300	97	$5.6 \cdot 10^7$	140*
105 000 × *g* pellet (microsomal fraction)	140	2	$1.0 \cdot 10^6$	2

* Increase in total activity is due to removal of enzyme inhibition by monovalent cations (following paper).

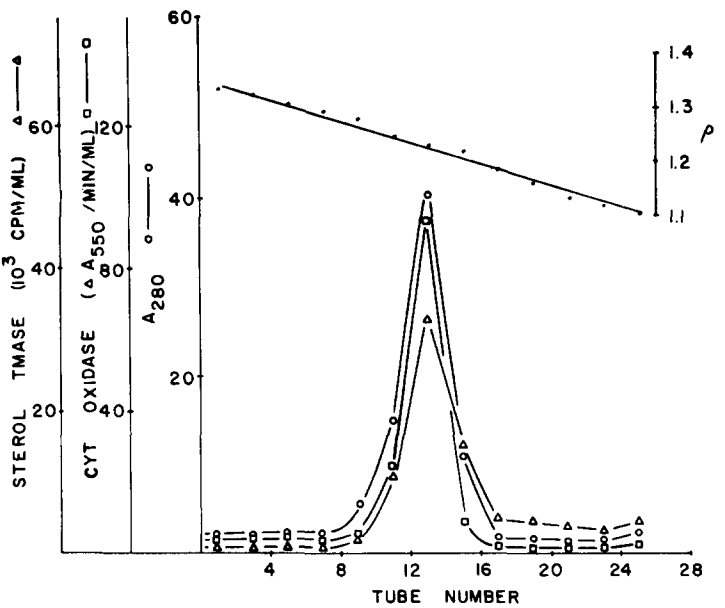

Fig. 1. Sucrose gradient profile of isolated yeast mitochondria. Cells were grown under aerobic conditions with ethanol as principal carbon source. Mitochondria were isolated by differential centrifugation and resuspended to 40 mg protein per ml in 0.5 M sucrose, 0.1 M Tris–HCl buffer, pH 7.5. 1.0 ml of the mitochondria were layered on the linear 20–70% sucrose gradient.

ed at a density of 1.22 under the conditions described in Materials and Methods.

Since the methyltransferase enzyme was present in the mitochondrial fraction of ethanol-grown cells, the logical location of the enzyme in glucose-repressed cells would be the promitochondria. Fig. 2 shows a sucrose density gradient profile of the homogenized promitochondrial fraction from cells grown aerobically in 10% glucose medium. As can be seen the cytochrome oxidase activity is completely repressed, but methyltransferase activity is still present. The buoyant density value obtained for promitochondria was 1.18 under the conditions described in Materials and Methods.

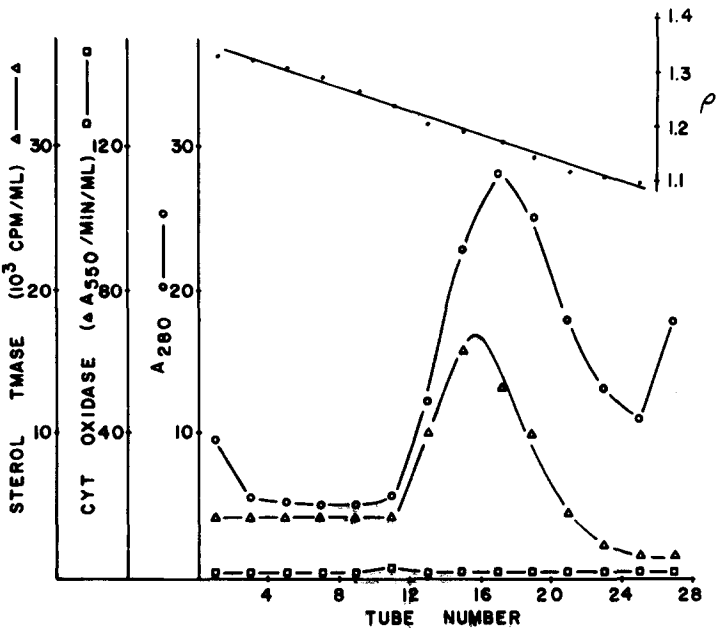

Fig. 2. Sucrose gradient profile of isolated yeast promitochondria. Cells were grown aerobically in 10% glucose medium to repress mitochondria formation. Promitochondria were isolated by differential centrifugation, resuspended to 40 mg protein per ml on 0.1 M Tris–HCl buffer, pH 7.5, and homogenized extensively in an iced Potter–Elvehjem. 1.0 ml of the promitochondria was layered on top of the linear 20–70% sucrose gradient.

Although published procedures exist for the purification of the methyltransferase from yeast promitochondria (previously identified as the microsomal fraction [35]), fractionation studies of the enzyme from mitochondria have not been performed. Table II shows the results of digitonin fractionation of isolated mitochondria. Again, the results obtained by this fractionation were identical whether the mitochondria were obtained from spheroplast lysates or homogenates from the Bronwill MSK. Marker enzymes cytochrome oxidase and malic dehydrogenase were assayed for comparative purposes. Approx. 86% of the cytochrome oxidase activity is associated with the inner membrane, while malic dehydrogenase activity is found mainly in the matrix. The sterol methyltransferase is apparently associated with both the inner membrane and the matrix portions of the mitochondria.

TABLE II

THE DISTRIBUTION OF STEROL METHYLTRANSFERASE, CYTOCHROME OXIDASE AND MALIC DEHYDROGENASE ENZYME ACTIVITIES IN FRACTIONATED YEAST MITOCHONDRIA

The digitonin method of mitochondria fractionation is described in the Materials and Methods section. All enzyme assays were also performed as described in the Materials and Methods section.

Mitochondrial fraction*	Cytochrome oxidase		Malic dehydrogenase		Sterol methyltransferase	
	$\Delta A_{550\ nm}$/min per mg	Total activity (percent)	$\Delta A_{340\ nm}$/min per mg	Total activity (percent)	cpm/h per mg	Total activity (percent)
Outer membrane	1.47	7.9	2.7	2.5	265	11.7
Inter-membrane soluble fraction	0.42	0.7	20.0	5.7	105	1.4
Inner membrane	3.44	86.2	3.6	15.8	190	39.8
Matrix	0.91	5.2	76.0	76.0	1010	47.1

* Designation of membrane fractions is based on a digitonin separation procedure described by Hoppel and Tomec [28].

Since the methyltransferase activity resides in the mitochondrial or promitochondrial structures of yeast, it was decided to determine the site of coding and synthesis. Strains 5015D and EB-5 were grown in 10% glucose medium, harvested, broken and the methyltransferase enzyme obtained by differential centrifugation as described in the promitochondria isolation section of Materials and Methods. The mitochontrial DNA-less mutant (EB-5) and its parental strain 5015D both possessed equivalent levels of methyltransferase activity showing the enzyme is not coded by mitochondrial DNA. To determine the site of methyltransferase synthesis, four 2-l flasks were inoculated and the cells grown anaerobically for 72 h. The flasks were placed at 4 °C overnight and the cells harvested rapidly and transferred to aerobic adaptation medium containing the various protein synthesis inhibitors described in Materials and Methods. Fig. 3 shows that cycloheximide completely shut off increased synthesis of the methyltransferase during aerobic adaptation. Chloramphenicol had no effect on the synthesis of the methyltransferase enzyme. A 5- to 6-fold increase in specific activity was obtained in both the chloramphenicol and control flasks during aerobic adaptation in the presence of high levels of glucose. That methyltransferase activity was present in anaerobically grown cells was demonstrated when 72-h anaerobic cultures were poisioned with cycloheximide prior to overnight cooling and the complete cell isolation done at 4 °C in the presence of the antibiotic. Methyltransferase levels in cultures treated in this manner were the same as control cultures harvested in the absence of the antibiotic.

DISCUSSION

The subcellular location of *S*-adenosylmethionine:Δ^{24} sterol methyltransferase has been determined. The enzyme has been described previously as a component of the "microsomal" fraction of yeast [35]. It now appears that the enzyme is located within the promitochondrial or mitochondrial structures of yeast.

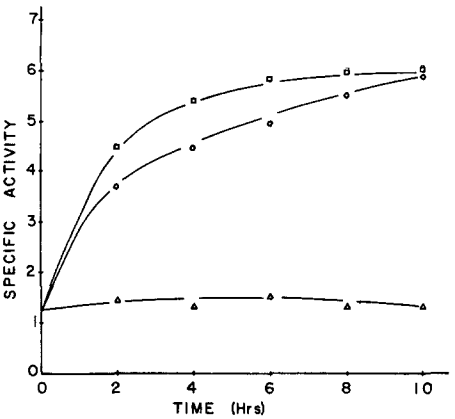

Fig. 3. Effects of protein synthesis inhibitors on the induction of sterol methyltransferase during aerobic adaptation. Anaerobically grown cells were harvested as in Materials and Methods and transferred to Wickerham's complete medium containing 5% glucose. Samples were collected by centrifugation, and broken in the Bronwill MSK homogenizer. The cellular fragments were removed by centrifugation at 2500 × *g* for 20 min and the supernatant was recentrifuged at 105 000 × *g* for 1 h. The ultracentrifuge pellet was resuspended to 3.0 ml and 1 ml used for the methyltransferase assay. Specific activity is reported as 10^2 cpm/30 min incubation per mg protein. Open circles represent the control flask without antibiotics. Open squares represent addition of 4 mg/ml chloramphenicol. Open triangles represent addition of 0.5 mg/ml cycloheximide.

The mitochondrial location of the methyltransferase is attractive considering the amount of published data suggesting a direct linkage between the synthesis of sterols and the acquisition of respiratory competence in yeast cultures [4, 5, 12]. Unlike most mitochondrial enzymes, sterol methyltransferase activity is always present in these cultures. The enzyme is not repressed completely during anaerobic growth even though zymosterol is not synthesized during this period [2]. Furthermore the enzyme is not subject to repression by glucose as are most mitochondrial enzymes [6–8].

Although sterol methyltransferase activity is present at low levels throughout anaerobic growth, a significant and rapid increase in activity occurs during aerobic adaptation (Fig. 3). This increase is inhibited by the cytoplasmic protein synthesis inhibitor cycloheximide [6, 36]. Chloramphenicol, which specifically inhibits mitochondrial protein synthesis [6, 36], does not affect the appearance of the methyltransferase enzyme during aerobic adaptation. Thus it appears that the enzyme is synthesized on cytoplasmic ribosomes and then incorporated into the promitochondria or mitochondrial structure. The incorporation is apparently entirely independent of mitochondrial protein synthesis. This is expected, however, since mitochondrial ribosomes are also subject to glucose repression [6, 12].

The coding for the methyltransferase appears to reside in nuclear rather than mitochondrial DNA. Most mitochondrial proteins are likewise coded for by this DNA [6, 12].

Recent publications [25, 26] describing the subcellular location of enzymes involved in mevalonate biosynthesis have shown these enzymes to be mitochondrial. However, mevalonate is converted to non-saponifiable lipids by cell-free extracts only when mitochondrial and cytoplasmic fractions are combined implying that some of

the sterol-synthesizing enzymes are not mitochondrial. Data presented here indicate that at least enzymes in the terminal portion of the ergosterol pathway are located within the mitochondria.

The location of the sterol methyltransferase enzyme within the mitochondria is not completely clear. Significant amounts of activity are found both in the inner membrane and matrix portions of the mitochondria as evidenced by association with known marker enzymes. Thus, the enzyme may be loosely associated with the inner membrane and found in the matrix due to the fractionation procedure, or the enzyme may be located chiefly in the matrix and bind to the inner membrane during isolation. Of importance is the fact that negligible amounts of the enzyme were found associated with the apparent outer membrane portions.

Factors controlling the synthesis and partial repression of sterol methyltransferase are yet to be described. It is apparent, however, that the enzyme is present during anaerobic growth, is actively synthesized during aerobic adaptation and unlike many other mitochondrial enzymes is not subject to glucose repression.

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