Effect of ethionine on the in vitro synthesis and degradation of mitochondrial translation products in yeast

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The effect of ethionine, an amino acid analog of methionine, has been studied in *Saccharomyces cerevisiae* in relation to cell growth, oxygen consumption, in vitro protein synthesis of mitochondrial translation products (MTPs) and the degradation of those mitoribosomally made proteins by an ATP-dependent process present within the organelle. Ethionine was found to increase the generation time of those cells already committed to cell division and to abolish the initiation of new cell cycles. Oxygen consumption of cultures grown in the presence of the analog was drastically reduced. Ethionine was also found to impair the incorporation of methionine and leucine into mitochondrial translation products, however the synthesis of proteins was not totally blocked and, apparently, mitochondria utilized ethionine as a precursor amino acid. MTPs synthesized by isolated mitochondria in the presence of ethionine were rapidly degraded inside the organelle at a faster rate compared with the normal proteins synthesized under identical conditions in the mitochondria. It is also shown that these in vitro synthesized proteins are degraded by an ATP-stimulated proteolytic system, as has been previously established.

Mitochondria *Ethionine* *Degradation* *ATP stimulation* *Mitochondrial translation product*

1. INTRODUCTION

The effect of ethionine, the *S*-ethyl analog of methionine, on protein synthesis, cell growth, transmethylation reactions, DNA and RNA biosynthesis has been investigated in a variety of biological systems [1].

In yeast, ethionine inhibits cell growth [2] and has been shown to be incorporated into proteins [3]. *S*-Adenosyl-L-ethionine is also formed and accumulated since the methyltransferases do not discriminate against the ethyl group [1]. Ethionine also acts as a repressor of normal methionine biosynthesis [4]. Synthesis of proteins is markedly reduced although the overall mechanism is relatively unaffected [2,5]. On the other hand, it has been shown in bacteria and reticulocytes that proteins containing amino acid analogs are degraded faster than those peptides made with normal amino acids [6]. This proteolytic process is stimulated by ATP in bacterial, reticulocyte and mitochondrial systems [7–9]. Most of the present information concerning the biochemical effects of ethionine has been obtained from bacteria and intact eucaryotic cells. Considerably less research has been carried out in isolated organelles. In an effort to understand the effect of ethionine on the synthesis and degradation of MTPs, we have studied the in vitro incorporation, in the presence of ethionine, of different amino acid precursors into mitoribosomal products. The degradation by isolated mitochondria of the proteins produced and the effect of ATP on this breakdown are analyzed. In addition, we report the effect of the analog on the growth and oxygen consumption of cultures of the yeast strain 273-10B.

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2. MATERIALS AND METHODS

2.1. *Growth of cells*

Saccharomyces cerevisiae D273-10B (ATCC 24657) was grown at 30 ± 2°C in a semisynthetic medium containing galactose as a carbon source as described [10]. Cell growth was monitored turbidimetrically at 540 nm.

2.2. *Isolation of mitochondria*

Yeast cells grown to a density of 8×10^7 cells/ml were harvested by centrifugation (2500 × *g* for 10 min) and immediately washed once by resuspension in sterile distilled water at 4°C. After washing, the cells were then resuspended in 100 mM Tris-SO_4, pH 9.0, 2.5 mM dithiothreitol, incubated at 30°C for 20 min with shaking (110 strokes/min), centrifuged and washed 3 times with sterile distilled water by centrifugation (5 min at 3000 × g_{av}). The washed cells were resuspended in 1.35 M sorbitol, 100 mM EDTA, pH 7.4 and converted to spheroplasts by incubation at 30°C with zymolyase 5000 or 60000 (Kirin Brewery, Japan) at a ratio of 1.6 and 1.0 mg/g wet cells, respectively. The spheroplasts were harvested by centrifugation, washed once with 1.35 M sorbitol, then resuspended at a ratio of 10–15 g/l in the original growth medium adjusted to 1 M sorbitol and shaken at 30°C for 30 min. This conditioning step allows the spheroplasts to recover metabolically [11]. The spheroplasts were then collected by centrifugation and washed twice with 1 M sorbitol. They were then lysed by osmotic shock in 0.6 M mannitol, 1 mM EDTA, pH 6.7, and subjected to differential centrifugation to obtain the mitochondria as described by Poyton and Kavanagh [10].

2.3. *Protein synthesis in vitro*

Isolated mitochondria (0.5 mg protein) in a final volume of 0.5 ml were incubated according to McKee and Poyton [11], with the following modifications: the Tris-Cl buffer was 50 mM and the amino acids tyrosine, asparagine and glutamine were not included in the protein-synthesizing mixture. The kinetics of incorporation of radioactive amino acids into mitochondrial proteins were determined by spotting 20-μl aliquots onto a Whatman 3 MM filter paper grid (2.5 cm diameter) followed by washing and counting as in [12].

2.4. *Protein degradation studies*

Mitochondria were labeled in vitro by incubating the isolated mitochondria in the protein-synthesizing mixture with [^{35}S]Met or [^{3}H]Leu in the presence or absence of ethionine. The radioactive mitochondria were washed twice by centrifugation (Eppendorf centrifuge model 5414) at room temperature for 3 min using a buffer containing 0.6 M mannitol, 2 mM sodium phosphate, pH 7.0 and 5 mM methionine or 5 mM leucine. At this point, an aliquot was removed and counted to obtain the total radioactivity. The preparation was then mixed with 2.5 mg non-radioactive mitochondrial protein as a carrier and suspended as explained in the legends of the respective figures. After the indicated time had elapsed, 2 aliquots of 100 μl each were withdrawn and centrifuged (Eppendorf centrifuge, model 5414) for 4 min at room temperature. 80 μl from each supernatant were carefully removed and an aliquot counted in a scintillation spectrometer using a commercially prepared scintillation cocktail (Research Products).

2.5. *Oxygen consumption*

The oxygen consumption was monitored with a Clark-type electrode using a Yellow Spring Instrument (model 53) at 25°C. The same buffer was used to measure oxygen consumption by whole cells and isolated mitochondria. Before any experiment with isolated mitochondria was conducted, the state of oxidation and coupling of the mitochondria were checked. 1 mg mitochondrial protein and a buffer composed of 0.65 M mannitol, 0.001 M potassium phosphate, 0.001 M Tris-maleate, 0.0001 M EDTA, and 0.01 M KCl, adjusted to pH 6.5 were used. α-Ketoglutarate (6 mM) was used as an oxidizable substrate. The degree of coupling was checked by the increment in oxygen consumption produced by the addition of 5–70 nmol ADP.

2.6. *Miscellaneous*

Mannitol, sorbitol, amino acids, phospho*enol*-pyruvate, pyruvate kinase (rabbit muscle type II), α-ketoglutarate, L-ethionine, ADP and ATP were obtained from Sigma (St. Louis, MO). [^{3}H]-Leucine (NET-460), [^{35}S]methionine (NEG-009 T) and $H_2{}^{35}SO_4$ (NEX-042) were obtained from New England Nuclear, Boston, MA. All other reagents

were of the highest grade available. Published procedures were followed for the measurement of protein, using bovine serum albumin as standard [10].

3. RESULTS

3.1. *Growth and oxygen consumption*

S. cerevisiae strain D273-10B continued growing for approximately one generation in the presence of L-ethionine and then the cell cycle arrested. This effect is dose dependent as seen in fig.1. At 10 mM ethionine the culture grew very slowly without doubling the cell number even after 13 h incubation. At concentrations 10- and 20-times lower the cultures doubled their density at 8 and 10 h, respectively, and became arrested. The control culture without ethionine grew with a generation time of 5 h without arresting even after 13 h culture. Thus, it appears that ethionine completely abolishes a critical event for the initiation of cell division but allows those cells already committed to mitosis to complete the cell cycle although at a slower rate. These results are in agreement with findings reported by Singer et al. [2] using *S. cerevisiae* GR2 (ura 1 his 6).

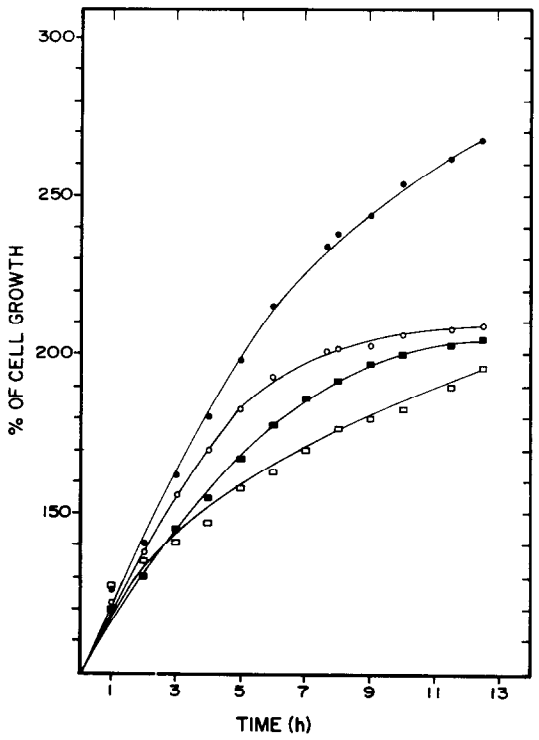

Fig.1. Effect of ethionine on the growth of *S. cerevisiae* strain 273-10B. Yeast cells were grown in 250-ml flasks containing 50 ml of medium (30°C, 300 rpm). At the indicated times, an aliquot was removed and the absorbance at 540 nm was determined. The values obtained are the light scattering at time *t*/initial light scattering value at time zero × 100. Culture grown with (●) 0 mM, (○) 0.5 mM, (■) 1 mM and (□) 10 mM ethionine.

Information about the influence of ethionine on oxygen consumption is surprisingly scarce. ATP levels of hepatic cells are markedly reduced by the analog [13]. In yeast, however, the ATP pool is not greatly altered, a finding that has been explained by the intense de novo synthesis of adenine groups elicited by ethionine [14]. Fig.2 shows the oxygen consumption of cells grown in the absence and presence of ethionine. The results are expressed in nmol O_2 consumed by 2.5×10^7 cells at different times of a growing culture. It is clear that in the absence of the analog the O_2 consumption capacity per cell increased, reaching a maximum around 6 h. At this point the oxygen consumption per cell is 3-times higher than that at zero time. However, in the presence of ethionine no increase in consumption was observed indicating that oxidative reactions are seriously impaired.

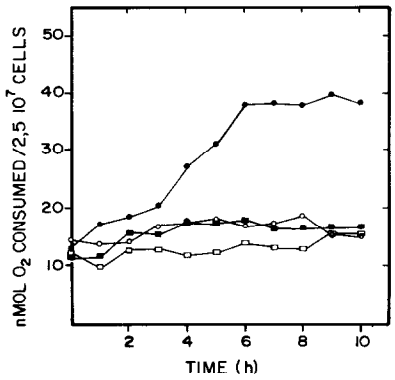

Fig.2. Effect of ethionine on the oxygen consumption of *S. cerevisiae*. Yeast cells were grown in 250-ml flasks containing 50 ml of media (30°C, 300 rpm). At the times indicated, an aliquot of the culture was removed and the absorbance at 540 nm was determined. The results are standardized to a constant number of cells (2.5×10^7). Culture grown with (●) 0 mM, (○) 0.5 mM, (■) 1 mM and (□) 10 mM ethionine.

3.2. *Synthesis and degradation of mitochondrial translation products*

Fig.3 shows the incorporation of [^{35}S]methionine (A) and [^{3}H]leucine (B) into proteins synthesized by isolated mitochondria in the presence and absence of ethionine. The presence of the analog caused a reduction in the incorporation of both labeled amino acids to about the same extent. The proteins synthesized under these conditions are identical with the non-ethionine control either having methionine or leucine as the radioactive tracer as determined by one-dimensional polyacrylamide gel electrophoresis (not shown). Thus, it appears that the reduction in the incorporation

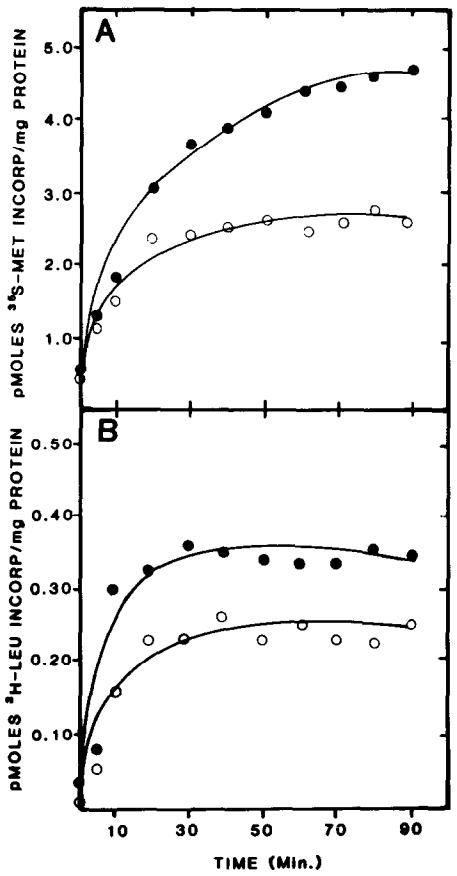

Fig.3. Incorporation of [^{35}S]methionine and [^{3}H]leucine into mitochondrial translation products in the presence and absence of ethionine. Mitochondria were incubated in the protein-synthesizing mixture (0.5 ml) containing 200 μCi/ml of [^{35}S]methionine (1223 Ci/mol) (A) or 80 μCi/ml of [^{3}H]leucine (1.23 Ci/mmol) (B). At the indicated times aliquots of 20 μl (0.01 mg mitochondrial proteins) were spotted on filter paper and processed as described in section 2. Control for both panels had no ethionine (●). Experiments had 1 mM ethionine final concentration (○).

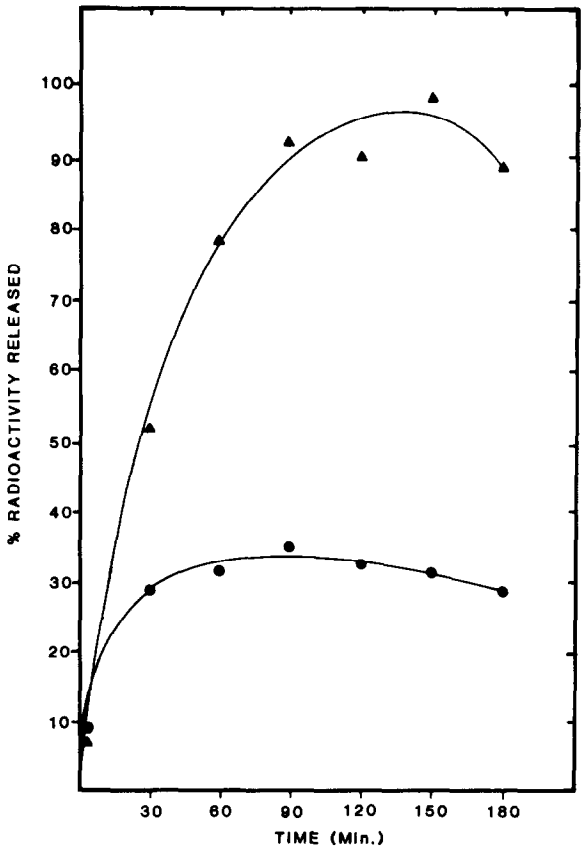

Fig.4. Effect of ATP on the degradation of ^{3}H-labeled mitochondrial translation products synthesized in vitro in the presence of ethionine. Mitochondria incubated in the protein-synthesizing system in the presence of 1 mM ethionine and [^{3}H]leucine (80 μCi/ml) followed by a wash (see section 2) were suspended in a medium containing, in a final volume of 1.5 ml, 0.6 M mannitol; 10 mM potassium phosphate, pH 7.2; 6 mM α-ketoglutarate; 1 mM ADP; 5 mM phospho*enol*pyruvate; 4 mM ATP; 10 U pyruvate kinase (▲). The control fractions contained neither α-ketoglutarate, ADP, ATP nor the ATP-generating system. 0.6 M mannitol was added to complete the volume to 1.5 ml (●). The incubation was performed in glass tubes (12 × 75 mm) at 30°C with shaking (100 strokes/min). The results are expressed as per cent of the radioactivity released to the supernatant, at the indicated times of incubation, of the total radioactivity initially present in the mitochondria suspension.

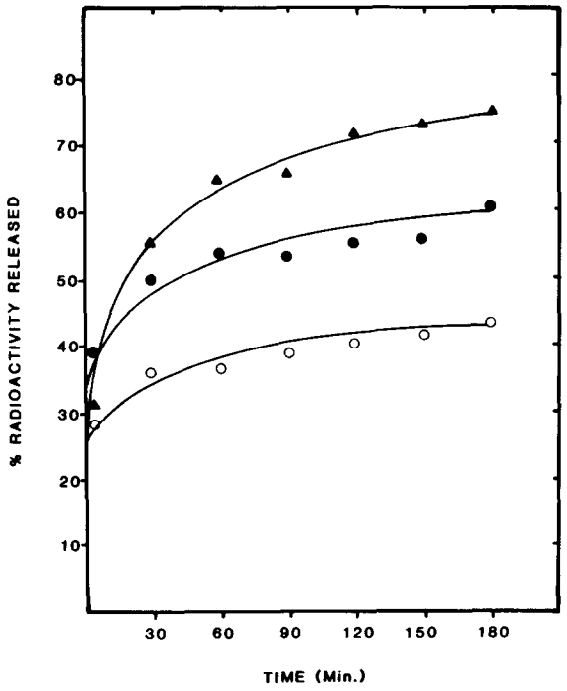

Fig.5. Degradation of [^{35}S]methionine-labeled mitochondrial translation products synthesized in vitro in the presence and absence of ethionine. Isolated mitochondria were programmed to synthesize proteins either in the presence of 1 mM ethionine or in its absence. A fraction (0.5 μg mitochondrial protein) where no ethionine was present was incubated in 0.6 M mannitol (no ATP, α-ketoglutarate, ADP and the ATP-generating systems) in a final volume of 1.5 ml (○). A fraction of the ethionine-containing mitochondria was incubated without the ATP, oxidizable substrate, and the ATP-generating system (●). A third fraction, where ethionine was present during the protein synthesis, was suspended in a medium containing ATP, ADP, α-ketoglutarate and an ATP-generating system (▲). For more details see legend to fig.4.

of radioactive methionine is not only due to substitution by unlabeled ethionine but also to inhibition.

It has been well established that MTPs synthesized in vivo [15,16] are degraded by an ATP-dependent proteolytic system. Therefore, it was of interest to study the degradation of MTPs synthesized in vitro in the presence and absence of ethionine and the influence of exogenously added ATP.

Fig.4 shows the release of trichloroacetic acid non-precipitable radioactive material from MTPs synthesized in the presence of ethionine using [^{3}H]leucine as radioactive precursor. It is clearly shown that the degradation of the ethionine containing MTPs is highly energy-dependent. The intactness of the organelle, monitored throughout the incubation period, by measuring the state of oxidation and the P:O ratio, showed no significant changes after 3 h incubation.

Fig.5 shows the release of non-precipitable radioactive material out of the mitochondria upon incubation of the organelle with and without ATP. Mitochondrial translation products synthesized in vitro in the absence of methionine using [^{35}S]methionine as a tracer are quite stable in the absence of ATP. However, MTPs made in vitro in the presence of ethionine are degraded faster even in the absence of ATP. This result would indicate that the mitochondria recognize these proteins as 'aberrants' and degrade them at a faster rate. Likewise, the MTPs synthesized in the presence of ethionine are degraded even faster when ATP is present. This result confirmed that of fig.4 even though a different amino acid tracer was used.

4. DISCUSSION

We have examined the effect of ethionine on various biological and biochemical processes in *S. cerevisiae* D273-10B. At the lowest concentration of ethionine used (5×10^{-4} M), the cell growth of this yeast strain stops after one generation and remains arrested for long periods of incubation. At higher concentrations of ethionine the generation time becomes proportionally longer and the cultures arrest slightly before doubling their cell density (fig.1). It appears, therefore, that ethionine decreases the rate of growth of yeast and completely abolishes the initiation of new cell cycles. These results of growth have been explained by a single or combined effect of various alterations known to take place in yeast treated with ethionine such as: a dramatic decrease of rRNA synthesis and processing [2], repression of the methionine biosynthetic pathway [17], inhibition of DNA replication [18], hypomethylation or ethylation of a sensitive component [19], alterations in the translational machinery [1]. To this list we might now add the possibility of a serious shortage in energy since we

have found a drastic decrease in oxygen consumption which, most probably, limits the rate of ATP production (fig.2).

As discussed by Singer et al. [2], ethionine-treated yeast cells arrest in G_1 at a step coincident with the 'start' period (α-pheromone-sensitive step). The arrest of cells in G_1 produced by α-pheromone appears to be mediated by inhibition of adenylate cyclase associated to a specific receptor [20]. Since ethionine seems to mimic the effect of α-pheromone it is not unlikely that one or more cAMP-dependent protein kinase reactions, involved in cell division, might be the critical point affected by the analog. This possibility is consistent with the finding that phosphorylation of eucaryotic ribosomal protein S6 is completely abolished after administration of ethionine to rats [20]. It is interesting that ethionine also inhibits cell division in procaryotic cells. Addition of the analog to a *met rel* strain of *E. coli* prolonged the generation time to 4–5 h. The residual growth, however, is entirely due to an increase in the size of the bacteria and not to cell division [17]. Nevertheless, despite the fact that ethionine produces a constellation of effects, none of them has been unequivocally related either to inhibition of growth or to the arrest of cell division. Obviously more experiments and data are needed to clarify the mechanisms involved in these 2 important effects.

The inhibition of protein synthesis produced by ethionine has been well established in various systems [1]. Ethionine can substitute for methionine as a substrate for protein synthesis but with a much lower efficiency, thus greatly limiting the overall process [17]. Moreover, administration of ethionine produces in procaryotic and eucaryotic cells disaggregation of polyribosomes, inhibition of and defects in the biosynthesis of ribosomal subunits, inhibition of methylations of both nucleic acids and proteins [1], and activation of an inhibitor of protein chain initiation similar to the hemin-controlled inhibitor found in reticulocytes [22]. Therefore there are several effects expected to decrease the rate of protein biosynthesis. No comparable data exist, however, for mitochondrial protein synthesis. We have found in isolated mitochondria that ethionine reduced the incorporation of radioactive methionine into MTPs in a dose-responsive manner (fig.3). This result was not just a dilution effect of the methionine pool by the added ethionine since a similar degree of inhibition was observed when [^{3}H]leucine was used as labeled precursor. Whether ethionine substitutes for methionine at the initiation step or is incorporated into internal positions or both, remains to be elucidated.

It is well known that proteins containing amino acid analogs are somewhat more labile and are degraded faster than native proteins [6,23]. On the other hand, a yeast mitochondrial ATP-dependent proteolytic system that degrades MTPs has been well established [15]. Therefore, it was of interest to us to determine the rate of degradation of MTPs synthesized in the presence and absence of ethionine by the mitochondrial proteolytic system. We found that the 'abnormal' MTPs made in the presence of the analog are degraded faster than their normal counterpart and that this degradation is stimulated by ATP (figs 4 and 5). Several energy-dependent proteolytic systems have been described in eucaryotic cells [24], particularly the ubiquitin-dependent system of reticulocytes where ATP is required to bind ubiquitin to the protein to be degraded [8] and the ATP-dependent, vanadate-sensitive proteolytic pathway of liver mitochondria reported by Desautels and Goldberg [9]. Since proteolysis is an exergonic process, energy dependence is not expected on thermodynamic basis. Therefore the requirement for energy observed in these systems as well as in the one reported here suggests that ATP is likely involved in an unknown mechanism, of recognition or control of proteolysis of the abnormal MTPs. Abnormal conformation of mitochondrial proteins may also result as a consequence of mutations, errors during transcription or translation and posttranslational alterations. It is possible that the energy-dependent yeast mitochondrial proteolytic activity described in this report might be the mechanism used by the mitochondria for a rapid disposal of all kinds of abnormal proteins synthesized in the organelle or transported into it.

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