Mitochondrial gene expression in *Saccharomyces cerevisiae*

Proteolysis of nascent chains in isolated yeast mitochondria optimized for protein synthesis*

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We demonstrate here that mitochondrial translation products synthesized by isolated yeast mitochondria are subject to rapid proteolysis. The loss of label from mitochondrial peptides synthesized *in vitro* comes from two distinct pools of peptides: one that is rapidly degraded ($t_{1/2}$ of minutes) and one that is much more resistant to proteolysis ($t_{1/2}$ of hours). As the length of the incubation period increases, the percentage of labelled peptides in the rapidly-turning-over pool decreases and cannot be detected after 60 min of incubation. This proteolysis is inhibited by chloramphenicol and is dependent on the presence of ATP. The loss of label during the chase occurs from fully completed translation products. The proteolysis observed here markedly affects measurements of rates of mitochondrial protein synthesis in isolated yeast mitochondria. In earlier work, in which proteolysis was not considered, mitochondrial translation was thought to stop after 20–30 min of incubation. In the present study, by taking proteolysis into account, we demonstrate that the rate of translation in isolated mitochondria is actually constant for nearly 60 min and then decreases to near zero by 80 min of incorporation. These findings have allowed us to devise a procedure for measuring the ‘true’ rate of translation in isolated mitochondria. In addition, they suggest that mitochondrial translation products which normally assemble with nuclear-encoded gene products into multimeric enzyme complexes are unstable without their nuclear-encoded counterparts.

INTRODUCTION

The biogenesis of a mitochondrion is a complex process requiring efficient co-operation between the nuclear–cytoplasmic genomic system and the mitochondrial genomic system (for reviews, see [1–3]). The mitochondrial system in yeast is responsible for encoding and synthesizing eight peptides essential for mitochondrial oxidative metabolism. These peptides are: subunits I, II and III of cytochrome *c* oxidase; apo-cytochrome *b* of the *bc*₁ complex; subunits 6, 8 and 9 of the *F*₀-ATPase; and Var1, a protein of the small ribosomal subunit [1–3]. The nuclear–cytoplasmic genomic system is responsible for encoding and synthesizing the remaining 200–300 proteins of the mitochondrial compartment. All of the mitochondrial translation products assemble into multimeric enzyme complexes that include nuclear-encoded cytoplasmically synthesized subunits. We are interested in how the yeast cell regulates the expression of the mitochondrial genome and thus the production of these proteins. To facilitate studies on the regulation of mitochondrial gene expression we developed an isolated mitochondrial system from baker's yeast (*Saccharomyces cerevisiae*) that was capable of all, or nearly all, steps in mitochondrial gene expression in the absence of any other cellular fraction [4–6]. We previously reported the optimization of this system for mitochondrial protein synthesis and demonstrated that certain mitochondrial parameters, such as energy charge and availability of oxidizable substrates, are potential regulators of yeast mitochondrial translation [4,5]. In subsequent work [6] we demonstrated that isolated yeast mitochondria are capable of high levels of both initiation and elongation, and we rigorously established that the products of mitochondrial translation made *in vitro* were labelled to the same relative extent as, and were identical with, the mitochondrial translation products labelled *in vivo* in the presence of

cycloheximide, an inhibitor of cytoplasmic protein synthesis. Additional work showed that these mitochondrial preparations were also capable of transcription and transcript processing [7]. Finally, it was demonstrated that the addition of a dialysed yeast cytosolic extract to the isolated mitochondrial system resulted in a 1.5-fold stimulation of synthesis.

One of the hallmarks of yeast mitochondrial translation is the short duration of amino acid incorporation, namely 20–40 min (for reviews, see [1,2]). This has been observed in studies in intact cells labelled in the presence of cycloheximide as well as in isolated mitochondria. The short apparent duration of synthesis was originally interpreted by many investigators as evidence that mitochondrial translation was in some way tightly coupled to cytoplasmic protein synthesis [1,2,8]. Two types of models have been proposed: (i) the loss or inactivation of a cytoplasmically derived protein might directly turn off mitochondrial translation or (ii) cytoplasmically synthesized products may be required for assembly with their mitochondrially synthesized counterparts in order for mitochondrial translation to occur [8]. More recent studies have shown that the products of yeast mitochondrial protein synthesis, labelled *in vivo* in the presence of cycloheximide, are subject to rapid proteolysis [9,10], both *in vivo* and in the subsequently isolated labelled mitochondria. It has been proposed that this degradation is triggered by an abnormal accumulation of free mitochondrial subunit pools that are unable to assemble into enzyme complexes because their cytoplasmically synthesized subunit counterparts are limiting [9,10]. Thus an alternative explanation for the short duration of mitochondrial translation discussed above is that translation does not stop, but that a steady-state is reached in which the rate of label entering protein via synthesis is balanced by the amount of label leaving protein via proteolysis.

A major goal in developing an isolated yeast mitochondrial

Abbreviation used: PSM, protein-synthesis medium.

* This is part III of a series of papers on this topic; parts I and II are [5] and [6].

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protein-synthesizing system has been to study its potential regulation by cytoplasmic factors and/or proteins. To accomplish this goal it is clear that a better understanding of the mechanisms leading to the cessation of incorporation in this system is needed. Thus the purpose of the present study was to investigate the extent of proteolysis of nascent peptide chains synthesized in an isolated yeast mitochondrial system optimized for protein synthesis, and to determine the role of proteolysis relative to the several hypothesis presented above.

We have found that the cessation of net protein synthesis that occurs after 30 min of incubation is not caused by a cessation of translation, but is caused by a steady-state balance of incorporation into, and proteolytic loss of label from, mitochondrial translation products. This proteolytic loss of label occurs primarily from fully completed normal mitochondrial translation products that exist in at least two different pools: one that is proteolytically very sensitive and one in which the peptide products are much more stable. Proteolysis from the sensitive pool is ATP-dependent and is inhibited by chloramphenicol. Lastly, from these data a technique is presented in which a much better estimate of the 'true' rate of mitochondrial translation in isolated yeast mitochondria can be made.

MATERIALS AND METHODS

Yeast strains and growth conditions

The laboratory strain D273-10B (*rho*⁺) of *Saccharomyces cerevisiae* was used in the present study. Growth conditions have been previously described [5]. Cells were harvested during the mid-exponential growth phase.

Isolation and incubation of mitochondria

Mitochondria were isolated and incubated in a protein synthesizing medium as described previously [5]. Pulse-labelling of the isolated mitochondria was done with [³⁵S]methionine (0.15 mCi/ml) and 0.01 mM-unlabelled methionine. Chase

experiments were initiated with the addition of unlabelled methionine to a final concentration of 10 mM. The kinetics of incorporation of label into and out of proteins were estimated by measuring the amount of label incorporation into trichloroacetic acid precipitates with a filter-paper-disc assay [5]. Labelled mitochondrial translation products were prepared, separated by SDS/PAGE and revealed by autoradiography [5].

RESULTS

Mitochondrial protein synthesis is not limited by components of the protein-synthesizing medium

Recognizing that mitochondrial protein synthesis is being measured in an 'in vitro' system, one potential reason for the relatively rapid cessation of translation might be related to a limitation of the protein-synthesis medium (PSM). Thus, before examining the role of proteolysis in this system, it was important to rule out the possibility that a component of the PSM becomes limiting during the incubation, resulting in a reduction or a cessation of incorporation. To accomplish this goal, mitochondria were incubated with [³⁵S]methionine in fresh PSM (Fig. 1) and the kinetics of incorporation of [³⁵S]methionine were determined. Aliquots were removed at 10 and 40 min and centrifuged (2 min, 13000 g) to separate the mitochondria from the PSM. Fresh additions of mitochondria were added to this previously incubated (conditioned) PSM, whereas the previously incubated (conditioned) mitochondrial pellets were resuspended in fresh PSM with fresh [³⁵S]methionine. These tubes were re-incubated, and the kinetics of label incorporation were determined again. The expected time course of incorporation is observed in the control, namely that a plateau is reached in 30 min (Fig. 1a). However, mitochondria removed at 40 min from this incubation fail to incorporate any additional label when resuspended and incubated in fresh PSM with fresh [³⁵S]methionine (Fig. 1b). Since the mitochondria contained in the 10 min aliquot continue to incorporate label when

Fig. 1. Mitochondrial protein synthesis is not limited by components of the PSM

Mitochondria were isolated and incubated in PSM with [³⁵S]methionine under the conditions described in the Materials and methods section to measure mitochondrial protein synthesis. Aliquots of the incubated sample were removed at 10 and 40 min of incubation, and the mitochondria were pelleted from the medium in each aliquot by centrifugation for 2 min in a Fisher Microfuge. The mitochondrial pellet (conditioned mitochondria) from each of these time points was resuspended in fresh PSM with fresh [³⁵S]methionine and incubated again (b). In turn, fresh aliquots of mitochondria were added to each of the PSM supernatants from these centrifugations (conditioned PSM) and incubated again (c). Additional aliquots were removed as indicated from the original sample (a) and from the re-incubated samples to determine the level of incorporation of labelled methionine. The results shown are expressed as methionine radioactivity incorporated per mg of mitochondrial protein and are from a representative experiment repeated three times with equivalent results. In (a) the results are expressed as the mean $\pm$ S.E.M. from three separate determinations of methionine incorporation from a single preparation of mitochondria.

resuspended and incubated in fresh PSM (Fig. 1*b*), this failure to incorporate new label is not a function of the centrifugation and resuspension procedures. On the other hand, a normal time course of incorporation is observed when fresh mitochondria are added to either of the 10 min or the 40 min-previously-incubated PSMs (Fig. 1*c*). Labelled mitochondrial translation products were collected at the end of each of the above incubations and shown to be normal by SDS/PAGE and autoradiography (results not shown). These data demonstrate that the PSM is capable of supporting at least two rounds of mitochondrial protein synthesis, and that the cessation of incorporation is a function of the mitochondria and is not caused by a limiting component in the PSM.

Assessing the extent of proteolysis in isolated yeast mitochondria

The loss of label caused by proteolysis during isolated mitochondrial translation experiments was assessed in pulse-chase experiments. Mitochondria were incubated with [³⁵S]methionine in PSM for various periods, a 1000-fold excess of unlabelled 'chase' methionine was added, and the kinetics of incorporation before and after the chase were monitored. The results of this experiment (Fig. 2) demonstrate that the loss of labelled methionine is extensive and occurs throughout the time course of protein synthesis. This loss of label is not caused by a reduction in non-specific adsorption related to the addition of a large bolus of unlabelled methionine, since the amount of non-specific adsorption as measured by the level of incorporation observed in mitochondria incubated with chloramphenicol (an inhibitor of mitochondrial protein synthesis) is negligible and does not change in the presence of the methionine addition (results not shown). Thus the loss of trichloroacetic acid-precipitable label appears to be caused by protein degradation of labelled peptides. Note that more label is lost during the first few

Fig. 2. Proteolysis causes the rapid decay of incorporated label in pulse-chase experiments in isolated mitochondria

Mitochondria were isolated and incubated in protein synthesis medium with [³⁵S]methionine as described in the Materials and methods section. Chase methionine was added (10 mM final) to individual samples after 6, 10, 20, 40, 60 and 80 min of labelling. Aliquots were removed before and after the addition of chase to measure the level of incorporation of labelled methionine as described in the Materials and methods section. Results are expressed as pmol of methionine incorporated/mg of mitochondrial protein, by dividing the radioactivity incorporated/mg of mitochondrial protein by the specific radioactivity of the methionine pool, as previously described [5]. Results shown are means $\pm$ S.E.M. for six to ten independent determinations; S.E.M. bars not shown are within the symbol size.

Fig. 3. Determinations of protein degradation rate constants and pool sizes

The calculation of degradation rate constants from semi-logarithmic plots was described previously [12]. The data from each chase time point shown in Fig. 2 was recalculated by assigning the incorporation level at the beginning of each chase as 100% and expressing the decay of incorporation at each subsequent point as a percentage of this value. These data were plotted as the means $\pm$ S.E.M. of the log(percentage of initial incorporation level) versus time after the addition of chase. Since the decay data from the 10, 20 and 40 min chase tubes were quite similar when expressed in this way, these data were pooled into one plot. This plot and the 60 min chase plot could not be described by a single straight line, as would be expected if degradation was occurring from a single pool. However, these plots could be closely fitted (by inspection) using two straight lines, suggesting that the decay of label occurred simultaneously from two pools of labelled proteins; one turning over more slowly (broken line, *a*), and one turning over rapidly (*b*). The relative sizes of these two pools were determined by the intercepts of the lines. All data were taken from Fig. 2. $\circ$, 10–40 min chase; $\triangle$, 60 min chase; $\square$, 80 min chase.

minutes of each chase period than at subsequent times, suggesting that the pool of labelled products becomes more resistant to degradation with time. As expected, the size of this pool of resistant products increases with the length of the labelling period. Since protein degradation is a first-order process, it is possible to obtain a better understanding of the rate of proteolysis in this system by plotting the logarithm of the decay of incorporation observed at each chase time point against time [12]. In such a plot the slope of the decay line is equal to the apparent rate constant of degradation, K_d (Fig. 3) [12]. In this analysis, the level of incorporation observed when the chase is initiated is considered to be 100%, and the amount of incorporation observed at each subsequent point on the decay curve is expressed as a percentage of this value. Since the decay curves for chase time points between 10 and 40 min were quite similar when treated in this way, these data were pooled into one curve (Fig. 3). As predicted from Fig. 2, two lines are required to fit the data for the average 10–40 min chase curve and the 60 min chase curve, indicating that the loss of label occurs from two pools of labelled peptides: one pool that is rapidly degraded ($t_{1/2} < 10$ min; Fig. 3*b*), and one pool that is significantly more resistant to proteolysis ($t_{1/2} > 3$ h; Fig. 3*a*). The intercepts of the lines used to fit the curves can be used as an estimate of the relative size of each pool at each time point. Note that, because

the size and proteolytic sensitivity of the slowly-turning-over pool increases with time, decay from the rapidly-turning-over pool can no longer be observed in the 80 min chase curve (Fig. 3). By using the pool sizes and decay rate constants obtained from these data, it is possible to calculate the rate of loss of labelled methionine at various points during incorporation (Fig. 5a below). The shape of this curve indicates that the loss of [35 S]methionine from protein increases to a maximum between 40 and 60 min of incubation and then decreases rapidly. The increase in the rate of loss of label during the first 40 min of incubation is caused primarily by an increase in the amount of label in the rapidly-turning-over pool of products. The subsequent decrease is caused by the decrease and eventual disappearance of the rapidly-turning-over pool by 80 min of incubation.

Analysis of labelled mitochondrial proteins before and after proteolysis

Several questions must be considered in light of the proteolytic data presented above. What is the nature of the labelled products that are being degraded and what accounts for the existence of two distinct pools of labelled proteins? At least two interpretations are possible.

(1) The rapid decay of acid precipitable incorporation is due to the presence of abnormally synthesized or prematurely terminated translation products that are rapidly degraded. Rapid proteolysis of such products have been reported in many systems [13,14]. The slower decay of incorporated label would represent decay from *bona fide* fully completed mitochondrial translation products.

(2) Alternatively, full-length normal mitochondrial translation products may exist in at least two forms (pools): nascent chains that have not assembled with any other peptides and are very sensitive to protein degradation (free subunit pool) and nascent chains that have assembled, or partially assembled with other peptides (or themselves), into a more proteinase-resistant form. In this latter case it is possible that the step, or steps, in the process leading to proteinase resistance may proceed more slowly *in vitro* because of the lack of new cytoplasmically synthesized proteins with which to assemble. Thus the decay of label caused by proteolysis would be observed *in vitro* because the rate of translation exceeds the rate of assembly, a condition that may, or may not, occur *in vivo*.

To distinguish between these alternative possibilities, we have examined, by SDS/PAGE and autoradiography, the labelled mitochondrial translation products obtained from different labelling conditions before and after the chase. The specific radioactivity of mitochondrial proteins prepared for electrophoresis agreed well with the specific radioactivity of mitochondrial proteins determined from the incorporation assay; thus it is unlikely that significant degradation of products occurred during the preparation of samples for electrophoresis. In Fig. 4(a), the patterns of mitochondrial translation products pulsed from zero time for 5, 10 and 20 min and collected without chase (lanes 1, 2 and 3 respectively) are compared with products pulsed for 20 min and chased for 40 min (lane 4). The pattern of bands in all these lanes are typical for yeast mitochondria and demonstrate that *bona fide* mitochondrial translation products were primarily synthesized [5]. It is noteworthy that the pattern of labelled bands in Fig. 4(a), lanes 3 and 4, are similar, even though 40% of the radioactivity is lost during the chase period (see Fig. 2). A comparison of these lanes by densitometry confirms this point (results not shown). If the loss of label is occurring preferentially from prematurely terminated products of all sizes, a significant reduction in the background area of the autoradiogram should be observed. Earlier work with this system

Fig. 4. Autoradiogram of labelled mitochondrial translation products obtained after different periods of labelling and chases in isolated mitochondria

Mitochondria were isolated and incubated in PSM as described in the legend of Fig. 2, except that [35 S]methionine and chase methionine were added at various times. At the end of each labelling regimen, the labelled mitochondrial translation products were isolated, dissociated and separated by electrophoresis on SDS/12%-(w/v)-polyacrylamide gels [5]. The gels were fixed, stained, dried and exposed to Kodak SB5 film. Mitochondrial products were labelled from zero time (a), and after 40 (b), 60 (c) and 80 (d) min of incubation. In (a), products were prepared after 5, 10 and 20 min of labelling (no chase) (lanes 1, 2 and 3 respectively), and after 20 min of labelling and 40 min of chase (lane 4). In (b), (c) and (d), products were prepared after 10 and 20 min of labelling (no chase) (lanes 1 and 2) and after 20 min of labelling and 40 min of chase (lane 3). A total of 10^8 d.p.m. of radioactivity was added to each lane. The autoradiogram shown is a composite autoradiogram from several identically run SDS/polyacrylamide gels. The products were identified by comparison with other gels [5]. The abbreviations are as follows: Var I, protein of the small ribosomal subunit; Cyt ox I, II and III, subunits I, II and III of cytochrome *c* oxidase; Cyt b, apocytochrome *b* of the *bc*₁ complex; ATPase 6, subunit 6 of the *F*₀ portion of the ATP synthase complex. Subunit 9 of the *F*₀ portion of the ATP synthase complex (ATPase 9) was not identified on these gels.

[6] has shown that losses of incomplete products much smaller than 40% of the total can be readily observed as reductions in the background of autoradiograms. This suggests that the loss of label occurs more or less equally from all translation products and not preferentially from prematurely terminated, or otherwise abnormal, mitochondrial products. The pattern of labelled products obtained before and after chase at other time points were also similar (results not shown).

These data suggest that mitochondrial translation products, synthesized *in vitro*, exist as two pools with respect to sensitivity to proteolysis. As Fig. 2 and 3 show, the relative amounts of these two pools change during incubation. Thus an additional method to demonstrate that the labelled translation products in both pools are identical is to compare the pattern of translation products labelled at intervals of incubation in which the relative sizes of the two pools are different. The patterns of mitochondrial products pulsed from 40, 60 and 80 min are shown in Figs. 4(b), 4(c) and 4(d) respectively. Lanes 1 and 2 in each panel were obtained after 10 and 20 min of labelling, whereas lane 3 in each panel was labelled for 20 min and chased for 40 min. Note that the amount of the larger-molecular-mass products [cytochrome oxidase subunit I (Cyt ox I) and Var I] produced after 10 min of labelling become progressively reduced at the later intervals (compare lanes c1 and d1 with a2), but approach normalcy with prolonged incubation (lanes c2 and d2). This suggests that

normal translation products are synthesized throughout the time course of protein synthesis, but that the rate of elongation may decrease with time. Since the pattern of products eventually labelled at these later intervals (Figs. 4*b*, 4*c* and 4*d*) are similar to the products labelled from zero time (Fig. 4*a*), both pools of products (once dissociated for SDS/PAGE) are composed of the same translation products. Further, as in Fig. 4*a*, there were no differences in the patterns of products observed before and after the chase in Figs. 4*b*, 4*c* and 4*d*, demonstrating again that the loss of label occurs more or less equally from normal translation products. Lastly, it is noteworthy that normal mitochondrial translation products were still made after the 'apparent' synthesis of mitochondrial proteins had stopped, as measured by 'net' incorporation of [³⁵S]methionine (Figs. 4*b*, 4*c* and 4*d*).

Fig. 5. Rates of methionine loss due to proteolysis and comparison of 'net' and 'total' rates of methionine incorporation

The absolute rate of methionine loss at each point of incubation (*i*) was calculated from the rate constants of degradation and the pool sizes of labelled proteins determined from the plots in Fig. 3 and the following equation:

$$(\text{pmol of methionine/mg of protein})_i (\text{relative size of fast pool})_i [K_d(\text{fast pool})(\text{min}^{-1})]_i + (\text{pmol of methionine/mg of protein})_i (\text{relative size of slow pool})_i [K_d(\text{slow pool})(\text{min}^{-1})]_i = (\text{pmol of methionine lost/mg of protein} \cdot \text{min})_i$$

The rate of methionine loss calculated from this equation is shown in (a). The 'net' rate of translation (b) was determined at each time point by determining the amount of methionine incorporated between that time point and the preceding time point and dividing by the time interval. This value reflects the balance of methionine entering protein via protein synthesis and the loss of methionine from protein via proteolysis. At the plateau region of incorporation (Figs. 1 and 2), this value is zero. The calculated 'total' incorporation of methionine was determined at each time point by adding the rate of methionine lost at each time point (a) to the 'net' rate of methionine gained (b). This value, in theory, reflects the total rate of methionine incorporation (b). The measured total rate of incorporation of methionine (broken line, b) was obtained by measuring the initial rate of incorporation of [³⁵S]methionine over very short intervals (< 5–10 min) after various lengths of incubation period. This approach minimizes the loss of methionine caused by proteolysis. Note that the measured total incorporation rates agree well with the calculated total incorporation rates.

Effect of proteolysis on measurement of rates of translation in isolated mitochondria

It is clear from the experiments described above that the loss of label caused by proteolysis occurs throughout the entire time course of mitochondrial protein synthesis in yeast mitochondria optimized for protein synthesis. Thus the amount of [³⁵S]methionine incorporated at any moment represents the difference between the amount of label entering protein via synthesis and the amount of label leaving protein via degradation. Clearly, once the loss of label leaving protein becomes significant, the rate of protein synthesis is substantially underestimated by this technique. As discussed above, it is possible from the data in Fig. 3 to calculate the amount of label leaving protein at any moment of incubation (Fig. 5*a*), and by adding this amount to the net amount of label incorporated, it is possible to calculate the total amount of methionine incorporated. The curve generated from this calculation is shown in Fig. 5*b* (total incorporation) and compared with the rate of net incorporation. It is clear that there is a large difference between the rate of total incorporation of methionine and the rate of net incorporation of methionine. Presumably the rate of total incorporation provides a much more realistic indication of the true rate of mitochondrial translation. To confirm this point, a technique of measuring synthesis that would minimize the effect of proteolysis was developed. This was accomplished by incubating mitochondria in a complete synthesizing medium (containing unlabelled methionine) and adding [³⁵S]methionine at various times and measuring the initial rate of incorporation over a very short period (< 10 min), before the loss of label from protein became significant. The experimentally determined rates of protein synthesis from this approach are shown in Fig. 5 and agree well with the total rates of methionine incorporation calculated from net incorporation and the loss of label caused by proteolysis. The discrepancy observed at 60 min of incubation is probably related to the rather rapid decrease in translation observed between 60 and 80 min, i.e. the measured rate of total methionine incorporation at 60 min equals the calculated rate of total methionine incorporation at 70 min. This is well within reasonable agreement for different mitochondrial preparations.

Characterization of the proteolytic activity

The effects of ATP depletion and chloramphenicol (a mitochondrial translational inhibitor) on the proteolysis of mitochondrial translation products labelled *in vitro* were determined. This was accomplished as described above for the experiments illustrated in Fig. 3, except that, in one set of chase tubes, chloramphenicol was added with the chase methionine, and, in another, oligomycin (to block synthesis of ATP) and glucose and hexokinase (to deplete medium ATP) were added with the chase methionine. The addition of chloramphenicol ('CAP') during the chase results in near-complete inhibition of the decay of label from the rapidly-turning-over pool, whereas the rate of label decay from the slowly-turning-over pool is unaffected (Fig. 6). This result agrees well with the results of Kalnov *et al.* [10], who demonstrated that chloramphenicol inhibited the proteolysis of mitochondrial translation products labelled *in vivo* in yeast. However, the mechanism of this effect is unknown. Chloramphenicol is known to inhibit mitochondrial protein synthesis by binding to ribosomes and blocking elongation. Our data, as well as that of others [10,15], suggest that the most recently synthesized products released from the ribosome are the products most susceptible to degradation. Thus one effect of chloramphenicol may be to block the release of labelled, but incompletely elongated, peptide chains from the ribosome, protecting these labelled peptides from degradation. It

Fig. 6. Chloramphenicol and ATP depletion inhibit proteolysis of mitochondrial translation products labelled in isolated yeast mitochondria

Mitochondria were isolated, incubated and labelled and chased as described in the legend of Fig. 2, except that chloramphenicol (2.5 mg/ml) was added to some of the tubes immediately before the addition of chase methionine (Δ). In several experiments ATP was depleted in tubes immediately before the addition of chase, as described in the Materials and methods section ($\square$). Data were calculated as described in Fig. 3, and as was the case in Fig. 3, the 10, 20 and 40 min chase data were quite similar and were pooled to obtain the plot shown. The broken lines represent the size and degradation rate constant of the slowly-turning-over pools. The lines without symbol represent the 10–40 min chase curve from Fig. 3 and is provided for comparison. Chloramphenicol and ATP depletion appear to dramatically decrease the size of the rapidly-turning-over pool. The modest difference in the ATP depletion curve and the chloramphenicol curve between 6 and 20 min is not significant.

is noteworthy that cycloheximide, which inhibits cytoplasmic protein synthesis by blocking elongation on cytoplasmic ribosomes, is also known to inhibit protein degradation in mammalian tissues [13]. This has led to the hypothesis that inhibiting protein synthesis may in turn inhibit proteolysis [13]. However, puromycin, which inhibits mitochondrial protein synthesis by causing the immediate release of incompletely elongated peptide chains, did not inhibit proteolysis of yeast mitochondrial products labelled *in vivo* [10]. This suggests that the inhibition of proteolysis is not related simply to an inhibition of mitochondrial translation, although the inhibition of proteolysis could still be mediated via certain types of translational blocks. On the other hand, both cycloheximide and chloramphenicol have been reported to inhibit lysosomal proteinases in cell-free incubations [16]; thus an additional possibility is that chloramphenicol may directly inhibit mitochondrial proteinase(s).

ATP-dependent proteolysis has been shown to be important in the degradation of denatured or abnormal proteins in *Escherichia coli* [17] and has also been shown to be important in degrading mitochondrial translation products in isolated rat liver mitochondria [14,15]. The data in Fig. 6 suggest that the proteinase(s) responsible for the decay of label from the proteinase-sensitive pool of mitochondrial products labelled *in vitro* is also ATP-dependent. One interpretation of these data is that the peptides in the rapidly-turning-over pool are newly synthesized free subunits that accumulate in this pool *in vitro* because their rate of synthesis exceeds their rate of assembly into enzyme complexes, a potential result of the absence of cytoplasmic subunit counterparts for the assembly process. This peptide pool may not exist under normal conditions *in vivo*, and it is possible that the proteins of this pool are recognized as abnormal proteins and are rapidly removed from the system by an ATP-dependent proteolytic system. However, it should be noted that a significant

amount of mitochondrially synthesized subunits made *in vitro* do become relatively resistant to proteolysis. Although it is not yet known if this resistant pool represents a passage of the labelled peptides from a free subunit pool into an initial assembly complex, preliminary data has demonstrated that as much as 50% of the label incorporated into the mitochondrially synthesized subunits of cytochrome *c* oxidase during a 40 min incubation *in vitro* can be immunoprecipitated by antiserum to a cytoplasmically synthesized subunit, suggesting some form of association [18]; C. L. Moore, E. E. McKee & R. O. Poyton, unpublished work). It should also be noted that the depletion of ATP in this system (like chloramphenicol) inhibits mitochondrial protein synthesis, which, as noted above, may play some role itself in the reduction of proteolysis.

DISCUSSION

In earlier work we described an isolated intact mitochondrial system from yeast that could be used to measure rates of yeast mitochondrial translation [4,5,6]. The purpose of the experiments in the present study was to determine the extent of proteolysis in this system. As Figs. 2 and 3 show, the loss of label caused by proteolysis in this system is significant and occurs throughout the time course of protein synthesis, thereby causing the rate of mitochondrial protein synthesis to be seriously underestimated. Further, the loss of label from mitochondrial peptides synthesized *in vitro* appeared to come from two distinct pools of peptides: one that was rapidly degraded ($t_{1/2}$ of minutes) and one that was much more resistant to proteolysis ($t_{1/2}$ of hours). As the length of the incubation period increased, the percentage of labelled peptides in the rapidly-turning-over pool decreased and could not be detected after 60 min of incubation. On SDS/polyacrylamide gels the dissociated products observed at all time points, before or after chase, appeared to be identical (Fig. 4). These results demonstrated that normal mitochondrial products were synthesized at all time points of incubation and that the loss of label during the chase occurred from fully completed translation products as opposed to incompletely elongated or abnormally synthesized products. These data, taken together, suggest that there is no difference in the dissociated peptide products between the rapidly-turning-over pool of proteins and the proteolytically resistant pool of proteins. Thus the resistance to proteolysis is likely to be a function of the association of the nascent peptide subunits with other proteins (perhaps other subunits on the assembly pathway) that protect the nascent peptide subunits from degradation. In the first 40–60 min of translation in isolated yeast mitochondria, the rate of synthesis may exceed the rate of the nascent proteins to form these proteolytically stable associations, giving rise to a pool of free protein subunits that are rapidly degraded. At later intervals of incubation, the rate of translation decreases and eventually falls below that at which proteolytically stable associations form and the rapidly-turning-over pool disappears. Although we have not here presented evidence for the nature of these proteolytically stable associations, earlier work demonstrated that mitochondrially synthesized subunits of cytochrome *c* oxidase synthesized in isolated yeast mitochondria can be precipitated with antibodies to the cytoplasmically synthesized subunits of this complex, suggesting the proteins synthesized *in vitro* associate with nuclear-encoded subunits [18]; C. L. Moore, E. E. McKee & R. O. Poyton, unpublished work). It is not clear whether this represents a one-for-one exchange of a nascent radiolabelled subunit with its unlabelled counterpart in the holoenzyme or represents assembly *de novo* from a presumably small pool of unassembled nuclear-encoded subunits. It is also possible that some protection from proteolysis might also be gained by the

newly synthesized mitochondrial peptides by association among themselves; i.e. perhaps subunits I, II and III of cytochrome *c* oxidase associate with each other. This may, or may not, be on the normal assembly pathway.

The physiological significance of the high rate of proteolysis under these conditions *in vitro* is not completely clear, since the synthesis of mitochondrial proteins in the absence of cytoplasmic protein synthesis does not occur under normal conditions of growth. However, the proteinases that make up this system are, nevertheless, present in mitochondria isolated from yeast in the mid-exponential growth phase and presumably have a function *in vivo*. Luzikov [19] has proposed that one function of this proteinase system may be the elimination, by a performance criterion, of non-functional mitochondrial enzyme complexes.

Since mitochondrial subunits are synthesized *in vitro* in the absence of synthesis of their cytoplasmic counterparts, it seems likely that the normal processes of assembly of the holoenzymes in question would be significantly compromised. This may lead to the accumulation of a pool of free (unassociated) mitochondrial subunits that are perceived by the proteolytic system as abnormal peptides and are rapidly degraded. This accumulation of free subunits may not occur under normal '*in vivo*' conditions of growth. However, clearly a significant number of mitochondrially synthesized peptides become resistant to proteolysis and, as noted above, some appear to associate with nuclear-encoded subunits.

The loss of label caused by proteolysis markedly affects measurements of rates of mitochondrial protein synthesis. This is expressed best in Fig. 5, in which the rate of synthesis is plotted with, and without, taking proteolysis into account (total incorporation and net incorporation respectively). Note that the calculated rate of total incorporation remains constant for at least 40 min of incubation, whereas the net rate of incorporation plunges to near zero by 40 min of incubation. In earlier work the cessation of incorporation observed in mitochondria after 40 min of incubation was interpreted as a block in synthesis brought about by the apparently close dependence of mitochondrial translation on concomitant cytoplasmic protein synthesis. It is clear from the present study that mitochondrial protein synthesis proceeds far longer than 40 min, but does eventually decrease to low levels by 80 min. This might also be related in some way to the lack of cytoplasmic protein synthesis, but may also be related to the stability of the mitochondrial preparations.

As indicated above, a goal in developing an isolated intact mitochondrial system from yeast was to measure rates of mitochondrial translation. From the present study it became apparent that techniques of measuring synthesis needed to be developed that would minimize the effect of proteolysis. This was accomplished by measuring the initial rate of methionine incorporation over very short incubation periods (< 10 min). This yielded results in good agreement with the total rate of in-

corporation calculated by adding the rate of degradation to net incorporation (Fig. 5, broken line).

Lastly, we have shown, as have others [10], that the loss of label caused by proteolysis of the newly synthesized mitochondrial translation products in yeast is inhibited by chloramphenicol and appears to be dependent on ATP. The mechanism of the effect of chloramphenicol on proteolysis is unknown and will require further work. Puromycin, which inhibits protein synthesis by prematurely terminating and releasing peptides from the ribosome, does not appear to inhibit proteolysis [10]. An ATP-dependent mitochondrial proteinase has been isolated from rat liver mitochondria [14]. Additional work must be done to determine whether the proteinase in the present yeast system is similar to the rat liver proteinase.

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