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Action of Intracellular Proteinases on Mitochondrial Translation Products of *Neurospora crassa* and *Schizosaccharomyces pombe*

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Summary: Gel electrophoretic analysis of mitochondrial membranes from *Neurospora crassa* shows the presence of a polypeptide fraction with apparent molecular weights of 7 000 - 12 000, which is synthesized on mitochondrial ribosomes. This fraction comprises between 10 and 50% of total mitochondrial translation products. Evidence is presented that the major part of this fraction is derived from components with higher apparent molecular weights by proteolytic activity. The proteolytic activity is located in vesicles which are co-isolated with mitochondria upon differential centrifugation. The activity is strongly enhanced by application of detergents such as sodium dodecylsulfate and Triton. Proteins synthesized on mitochondrial as well as cytoplasmic ribosomes are subject to proteolytic breakdown. This proteolysis can be blocked by addition of inhibitors such as diisopropylfluorophosphate to isolated mitochondria. Similar observations were made with *Schizosaccharomyces pombe*. In *Neurospora*, the amount of mitochondrial translation products with apparent molecular weights of less than 12 000 is low in mitochondria from cells treated with cycloheximide for 1 h and high in mitochondria from cells treated with cycloheximide for 5 min. This observation is explained by the finding that proteinase activity in mitochondrial preparations decreases exponentially with a $t_{1/2}$ of 20 min during preincubation of cells with cycloheximide. Procedures are described to remove or block contaminating proteinase activity. The results appear to be relevant for the interpretation of many data obtained from experiments in which this puzzling kind of artifact has not been sufficiently considered.

Wirkung intrazellulärer Proteasen auf mitochondriale Translationsprodukte von Neurospora crassa und Schizosaccharomyces pombe

Zusammenfassung: Gelelektrophoretische Analyse der mitochondrialen Membranen von *Neurospora crassa* zeigt die Anwesenheit einer Polypeptidfraktion mit scheinbarem Molekulargewicht von 7 000 - 12 000, die an den mitochondrialen Ribosomen gebildet wird. Bezogen auf die gesamten mitochondrialen Translationsprodukte, macht diese Fraktion zwischen ca. 10 und 50% aus. Es wird gezeigt, daß der größte Teil dieser Fraktion aus Komponenten mit höheren scheinbaren Molekulargewichten durch proteolytische Aktivität entstanden ist. Die proteolytische Aktivität ist in Vesikeln lokalisiert, welche bei differentieller Zentrifugation zusammen mit den Mitochondrien

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Abbreviation: DFP = diisopropylfluorophosphate.

isoliert werden. Durch den Einsatz von Detergentien wie Natriumdodecylsulfat oder Triton wird die Proteaseaktivität stark erhöht. Sowohl Proteine, die an den mitochondrialen Ribosomen gebildet werden als auch solche, die an den cytoplasmatischen Ribosomen gebildet werden, sind von dem proteolytischen Abbau betroffen. Dieser Abbau kann durch Zugabe von Inhibitoren wie Diisopropylfluorophosphat zu den isolierten Mitochondrien geblockt werden. Ähnliche Beobachtungen wurden mit *Schizosaccharomyces pombe* gemacht. In *Neurospora* ist der Anteil der mitochondrialen Translationsprodukte mit apparenten Molekulargewichten von weniger als 12 000 niedrig in den Mitochondrien aus Zellen, die 1 h mit Cycloheximid behandelt wurden, und hoch in Mitochondrien aus Zellen, die 5 min mit Cycloheximid behandelt wurden. Die Erklärung für diesen Effekt wird durch den Befund geliefert, daß die Proteaseaktivität in Mitochondrienpräparationen bei Vorinkubation der Zellen mit Cycloheximid exponentiell mit einer Halbwertszeit von ca. 20 min abnimmt. Es werden Verfahren beschrieben, um kontaminierende Proteaseaktivität zu entfernen oder zu inhibieren. Die Ergebnisse, die hier dargestellt werden, erscheinen relevant für die Interpretation einer Reihe von Ergebnissen aus Experimenten, in denen diese verwirrende Quelle von Artefakten nicht ausreichend beachtet wurde.

Key words: Mitochondrial translation products; intracellular proteinases, cycloheximide, *Neurospora.*

Investigations of cellular reactions involving the subfractionation of cells are often subject to experimental artifacts which are caused by the activation of intracellular degradative enzymes. This is especially relevant for proteolytic enzymes which become redistributed during cell disruption. In many types of cells, proteolytic enzymes are compartmented in special organelles such as lysosomes or proteinase vacuoles (for reviews see[1,2]). These vesicles are often co-isolated with mitochondria and their hydrolytic activity may have serious damaging effects when reactions in mitochondria are studied after subfractionation of the organelles. We have been confronted with this kind of artifact in our studies on the synthesis and structure of mitochondrial translation products in *Neurospora crassa.* There are many reports in the literature which describe the occurrence of mitochondrial translation products with apparent molecular weights of less than 12 000[3-14]. These low molecular weight products are found in addition to the well described high molecular weight mitochondrial translation products, which are components of the mitochondrial membrane complexes cytochrome oxidase, cytochrome b and ATPase[15,16]. Several different explanations have been offered for the occurrence of the low molecular weight polypeptides[3-18]. Generation of these polypeptides by a physiological or artifactual action of intracellular proteinases was investigated as one possible explanation. In this report experiments are presented which suggest that these polypeptides are mainly generated by the artifactual action of intracellular proteinases.

Material and Methods

Growth conditions

Neurospora hyphae (wild-type 74 A re) were grown in aerated liquid cultures at 25 °C in Vogel's minimal medium[19] supplemented with 2% sucrose. The inoculum was 5×10^5 conidia per m*l*. The cells were harvested after 18 h growth (mid-log phase) by filtration. *Saccharomyces cerevisiae* (D-1827 leu^-) and *Schizosaccharomyces pombe* ($32\ h^-\ leu^-$) were grown in complete medium supplemented with 3% glycerol into log phase and harvested by centrifugation[20].

Isolation of mitochondria and gradient centrifugation

Mitochondria from *Neurospora crassa* were prepared as described by Weiss et al.[21], mitochondria from *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* according to the method described by Balcavage and Mattoon[22].

Centrifugation of mitochondrial preparations on linear sucrose gradients was performed for 1 h at 78000 × *g* in a Beckman Spinco, rotor SW 25.2. Sucrose concentrations were 20 - 60% (w/v) in 2mM EDTA, 10mM Tris/HCl, pH 7.4.

Extraction of whole cells with sodium dodecylsulfate-containing buffer

0.1 - 0.2 g of *Neurospora* hyphae (wet weight) were transferred to a mortar, frozen with liquid nitrogen and ground under liquid nitrogen for 2 min. After evaporation of the nitrogen, 0.2 m*l* of 0.1M Tris/HCl, 1% sodium dodecylsulfate, pH 8.0, was added and grinding was continued at 0 °C for a further 2 min. The resulting slurry was transferred to centrifuge tubes and centrifuged for 4 min at 17 000 x *g* at 0 °C. The supernatant was collected, dialysed for 2 h against 0.1M Tris/HCl, 1% sodium dodecylsulfate, pH 8.0 at 4 °C and then subjected to gel electrophoresis.

Labeling procedures

After 16 h growth, [^{14}C]L-leucine (spec. radioact. 254 mCi/mmol) (Radiochemical Centre, Amersham, England) was added to the cultures (50 μCi/*l*). Two h later, cycloheximide was added (0.1 g/*l*; C. Roth OHG, Karlsruhe, Germany) and after another 2.5 min [^{3}H]L-leucine (spec. radioact. 53 Ci/mmol; NEN Chemicals GmbH, Dreieichenhain, Germany) was added (1 mCi/*l*). Further incubation was carried out for the time periods indicated in the individual experiments. At the end of incubation, cells were chilled and harvested by filtration or centrifugation.

Determination of protein and radioactivity

Protein was estimated with the biuret method[23]. Procedures for determination of radioactivity and for performance of gel electrophoresis were as described before[6].

Determination of proteinase activity

Proteinase activity was determined by following the hydrolysis of azo-casein according to the method described by Hazen[24]. The test volume was reduced to 1 m*l*.

Results

Proteolytic degradation of mitochondrial proteins

Mitochondria were isolated from cells in which total cellular proteins were homogeneously labeled with [^{14}C]leucine and mitochondrial translation products specifically labeled in the presence of cycloheximide with [^{3}H]leucine for 5, 20 and 60 min. The radioactivity patterns obtained upon gel electrophoresis after dissolving in sodium dodecylsulfate-containing buffer are shown in Fig. 1 a - c. After the 5 min labeling period, the ^{14}C- und ^{3}H-radioactivities are found in material essentially with molecular weights in the range of 7 000 to 15 000. After a labeling period of 20 min, more of the radioactivity is found associated with bands of higher molecular weights (20 000 - 80 000) and less radioactivity in the low molecular weight range (Fig. 1 b). When incorporation was allowed to proceed for 60 min in the presence of cycloheximide, the homogeneous label was found to be associated mainly with bands corresponding to molecular weights of 20 000 to 80 000. The cycloheximide resistant label shows definite bands with apparent molecular weights of 40 000, 30 000 and 20 000. Low molecular weight material is present only in minor amounts.

One possible explanation for this observation is that under the conditions of the experiment massive proteolytic breakdown of mitochondrial and cytoplasmic translation products takes place in mitochondria after short (5 min) but not after long (60 min) cycloheximide treatment of cells. In order to verify this explanation, samples of mitochondria from the same respective batches were first treated with the proteinase inhibitor diisopropylfluorophosphate (DFP) before dissolving the mitochondria in dodecylsulfate. Then the same procedure was followed as with the samples not treated with DFP. The result of this experiment is shown in Fig. 1 d - f. In all DFP treated samples irrespective of the period of incubation in the presence of cycloheximide, the homogeneous label (^{14}C) displays mainly bands with high molecular weights and the pattern is quite similar to that seen in Fig. 1 c. Also, in the cycloheximide resistant (^{3}H) labeling patterns of the three samples, the components with higher molecular weights are predominant. Low molecular weight material is only observed after 5 min incorporation in the presence of cycloheximide and the pattern appears somewhat diffuse. In this case, the inhibitory effect of DFP may not be complete and/or there may be some breakdown even before sodium dodecylsulfate is added.

When gels were stained with Coomassie Brillant Blue a protein distribution was found which coincided with the homogeneous ^{14}C label under all the different conditions described in Fig. 1.

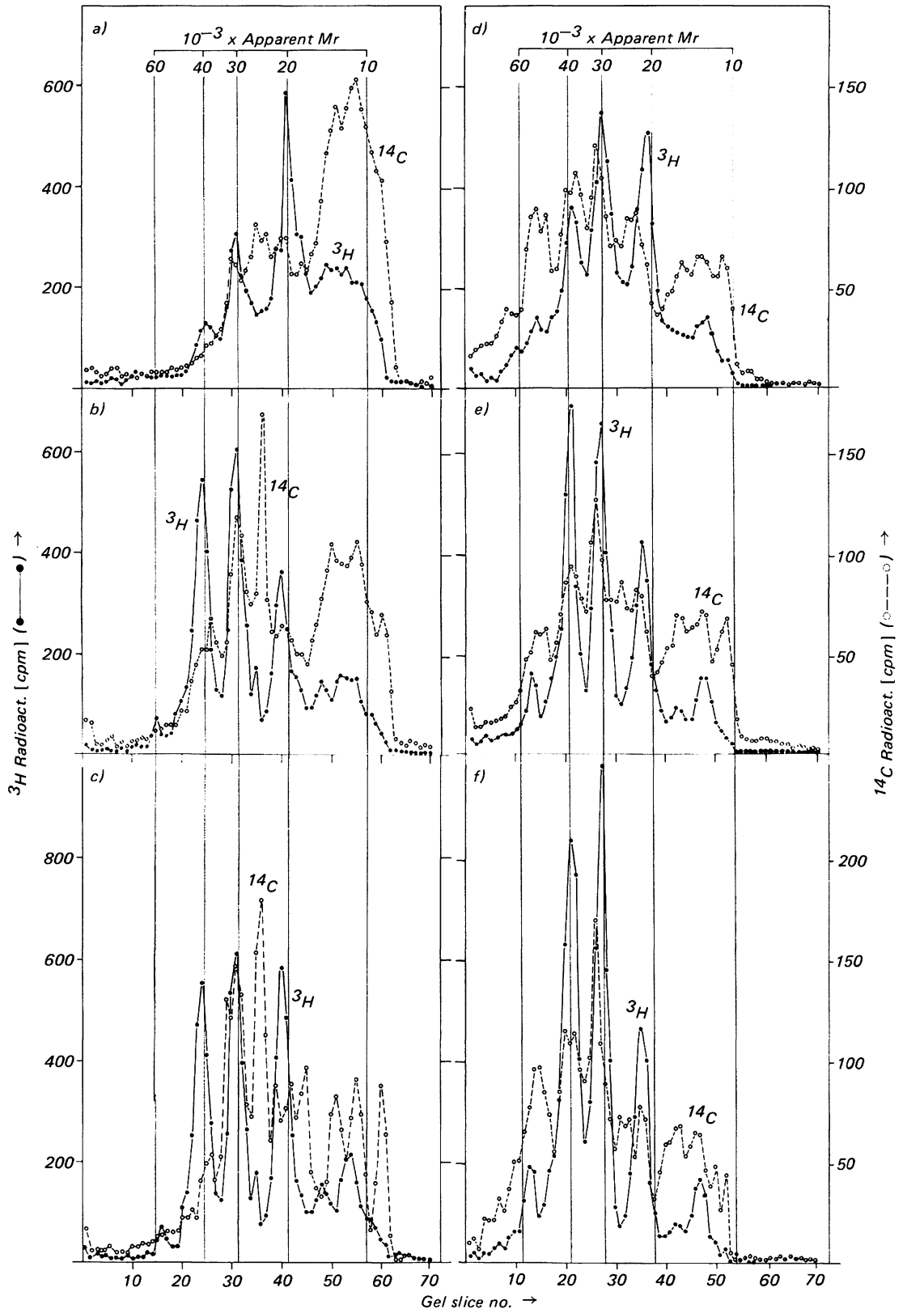
a)
b)
c)
d)
e)
f)
10^{-3} x Apparent Mr
60
40
30
20
10
^{14}C
^{3}H
600
400
200
800
150
100
50
10
20
30
40
50
60
70
^{3}H Radioact. [cpm] (●——●) →
^{14}C Radioact. [cpm] (○——○) →
Gel slice no. →

Fig. 1. Gel electrophoretic analysis of *Neurospora* mitochondrial proteins after different periods of labeling of cells with [^{3}H]leucine in the presence of cycloheximide.

Cells were homogeneously labeled with [^{14}C]leucine and specifically labeled with [^{3}H]leucine after addition of cycloheximide for 5 min (a, d), 20 min (b, e) and 60 min (c, f). Mitochondrial preparations were dissolved in sodium dodecylsulfate-containing buffer without adding DFP (a, b, c) (left column) and after adding DFP (1mM) (d, e, f) (right column). o----o Homogeneous label (^{14}C); •——• specific (cycloheximide resistant) label (^{3}H).

These findings suggest:

a) that preparations of *Neurospora* mitochondria contain proteolytic enzyme activity and that this activity decreases with increasing periods of cycloheximide treatment of cells;

b) that proteolysis takes place after dissolving the mitochondria with sodium dodecylsulfate;

c) that mitochondrial translation products are more resistant to proteinase than cytoplasmic translation products, especially those with apparent molecular weights of about 20 000.

In order to check whether activation of this proteolytic activity is also produced by Triton X-100, mitochondria from cells labeled homogeneously with [^{14}C]leucine as described for Fig. 1 and then labeled with [^{3}H]leucine for 15 min in the presence of cycloheximide were exposed to Triton X-100 under the following conditions (Fig. 2): First, as a reference, to one sample DFP was added and the mitochondria were kept for 1 h at 0 °C. Then Triton was added and immediately thereafter sodium dodecylsulfate. DFP was added to exclude proteolytic breakdown during the 1 h period and after dodecylsulfate treatment of mitochondria. Then gel electrophoresis was carried out (Fig. 2a). Both the ^{14}C- and ^{3}H labeling patterns indicate that no proteolytic breakdown occured (cf. Fig. 1). When mitochondria were first dissolved in Triton X-100, kept for 1 h at 0 °C, 22 °C and 32 °C, then treated with DFP, dissolved in sodium dodecylsulfate and subjected to gel electrophoresis, radioactivity patterns were obtained which are shown in Fig. 2b, c and d. The ^{3}H pattern in Fig. 2b (0 °C) is similar to that of the reference (cf. Fig. 2a). The homogeneous label however shows an increase of material in the low molecular weight region. Treatment of mitochondria at 22 °C (Fig. 2c) gives rise to a considerable breakdown of the homogeneously labeled proteins. Also, the cycloheximide-resistant label shows degradation of mitochondrial translation products. After incubation of mitochondria with Triton X-100 for 1 h at 32 °C (Fig. 2d), considerable quantities of both homogeneous and cycloheximide-resistant label are found in the low molecular weight range. Again, the cycloheximide-resistant label appears to be less affected compared to the homogeneous label. It is concluded from the data in Fig. 2 that after solubilisation of mitochondria with Triton X-100 breakdown of mitochondrially and cytoplasmically synthesized proteins does occur.

To determine whether proteinase also displays activity when mitochondria are kept in isolation medium, the following experiment was carried out. Mitochondria were incubated in the absence and presence of DFP in isolation medium for 30 min at 22 °C and then sodium dodecylsulfate gel electrophoresis was carried out. Mitochondrial translation products did not show indications of breakdown, however, cytoplasmic translation products with apparent molecular weights of 40 000 to 80 000 were broken down to a large extent.

Cellular localisation of proteinase activity

In order to test whether the proteolytic activity, effective in the experiments in Fig. 1 - 2, is associated with mitochondria or whether it just represents contaminating material, mitochondrial preparations were subjected to sucrose gradient centrifugation. After centrifugation mitochondria were seen as a turbid band in the lower third of the tube. Furthermore a pellet was obtained at the bottom of the tube. The gradient, divided into 10 fractions, and the pellet were monitored for protein content and for proteinase activity. The protein profile shows a peak at fract. 7. It represents mitochondria. About 10% of the total protein was found in the pellet fraction (Fig. 3a).

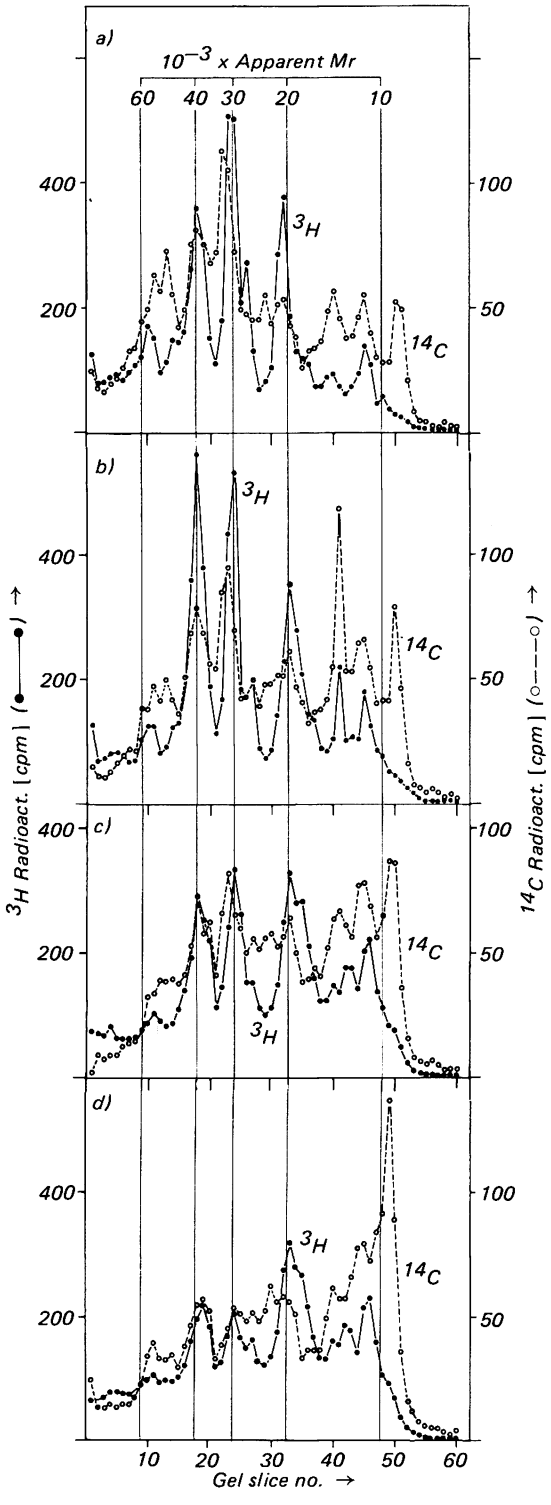

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Fig. 2. Gel electrophoretic analysis of *Neurospora* mitochondrial proteins after solubilization with Triton X-100 under different conditions.

Cells were homogeneously labeled with [^{14}C]leucine and specifically labeled with [^{3}H]leucine for 15 min after addition of cycloheximide. The mitochondrial preparation was resuspended in 0.1 M Tris/HCl, pH 8.0, portions were treated with Triton X-100 under the following conditions and then subjected to gel electrophoresis.

a) DFP was added to a final concn. of 5mM and the sample incubated for 1 h at 0 °C. Triton X-100 and sodium dodecylsulfate were then added to final concns. of 1%, followed by additional DFP (1mM). b) Triton X-100 was added to a final concn. of 1% and the sample was incubated at 0 °C for 1 h. DFP was then added to a final concn. of 1mM, followed immediately by sodium dodecylsulfate to a final concn. of 1%. c) Same as b) except that the incubation was carried out at 22 °C. d) Same as b) except that the incubation was carried out at 32 °C. ○- - - -○ Homogeneous label (^{14}C); ●——● specific (cycloheximide resistant) label (^{3}H).

When assayed for proteolytic activity, virtually none was detected in the mitochondrial fraction but a high activity in the pellet fraction (Fig. 3b). This suggests that the proteolytic enzyme activity – at least that which is detected with this special test – is concentrated in particles which have a higher density than mitochondria. The existence of high density proteinase-containing vesicles in *Neurospora* has been described by Matile et al.[25]. Moreover, Hasilik et al.[26] have shown that proteinases A, B and C in *Saccharomyces cerevisiae* are mainly found in the vacuole fraction. When mitochondria were isolated from cells pretreated with cycloheximide for 1 h and subjected to the same centrifugation procedure, a practically identical protein profile was observed (Fig. 3a). Again no proteolytic activity was found in the mitochondrial fraction. In the pellet fraction, however, this activity related to the total amount of mitochondrial protein is much lower (about one fourth) if compared to preparations from cells not treated with cycloheximide (Fig. 3b). It is concluded from this observation that *Neurospora* cells in the presence of cycloheximide lose proteinase containing vesicles. This is in agreement with the findings presented in Fig. 1, that proteolytic breakdown of mitochondrial proteins is

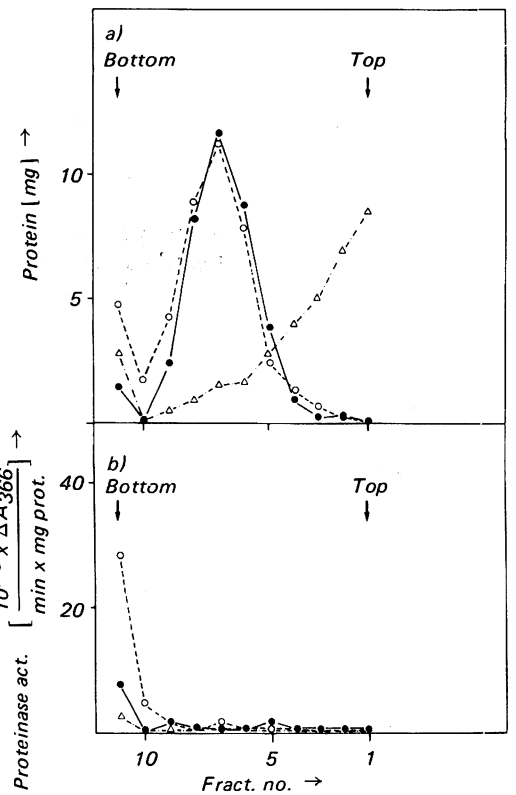


Fig. 3. Distribution of protein and proteinase activity in sucrose density gradient fractions after centrifugation of *Neurospora* mitochondrial preparations from cells treated in different ways.

The gradients were divided into 10 fractions and into the pellet fraction. ○----○ Cells grown for 18 h (control); ●——● cells grown for 17 h, then treated with cycloheximide for 1 h; △----△ cells grown for 18 h, then frozen with liquid nitrogen. a) Protein distribution; b) proteinase activity after solubilisation in Triton X-100.

much less expressed when cells from which mitochondria are isolated are pretreated with cycloheximide for 1 h.

In order to further prove that the degradative processes are actually caused by proteinase from contaminating vesicles, mitochondria were purified by density centrifugation. In this case, degradation after gel electrophoresis is strongly reduced with mitochondria from cells pretreated with cycloheximide for only 5 min.

A quite different way to remove the proteinase vesicles from mitochondrial preparations is suggested by the following experiment. After harvesting, *Neurospora* cells were rapidly frozen with liquid nitrogen and then thawed. Mitochondria were isolated as described for Fig. 1. Under these conditions, proteolytic breakdown after short and long treatment with cycloheximide is very low. An explanation for this effect is afforded by Fig. 3. Fig. 3a demonstrates that mitochondria isolated from cells frozen and thawed in this way do not form a distinct band upon sucrose gradient centrifugation. A considerable amount of protein, obviously representing broken mitochondria, remains at the top and partly smears to the bottom of the gradient. Determination of the proteinase activity in the fractions of the gradient reveals that in mitochondria from frozen cells practically no activity is found in the gradient fractions but that also in the pellet fraction proteinase activity is very low.

The simplest explanation for this effect is that in the freezing-thawing process not only mitochondria are disrupted but also the proteinase vesicles. The liberated proteinase is then removed from the mitochondrial membranes upon washing, which is carried out in the course of isolation of mitochondria. When proteinase activity is measured in a preparation of mitochondria resuspended in isotonic sucrose, a rather low activity is found. Furthermore, the activity increases during the test period. When mitochondria are lysed with Triton or subjected to sonication, the proteinase activity is several fold higher and no lag in the activity can be seen.

In the same test, sensitivity of the proteinase to various inhibitors was determined. The inhibitory effect of phenylmethylsulfonylfluoride and diisopropylfluorophosphate (final concentration 1mM) is about 90 to 95%. The sulfhydryl-group containing compound 2-mercaptoethanol (final concentration 10mM) is less effective, leaving 10 - 20% of the control activity uninhibited. The sulfhydryl inhibitor iodoacetamide (final concentration 1mM) does not inhibit the proteinase activity whereas the sulfhydryl sulfuric inhibitor *p*-chloromercuribenzoate (final concentration 1mM) causes a very strong inhibition of the proteinase activity (93%).

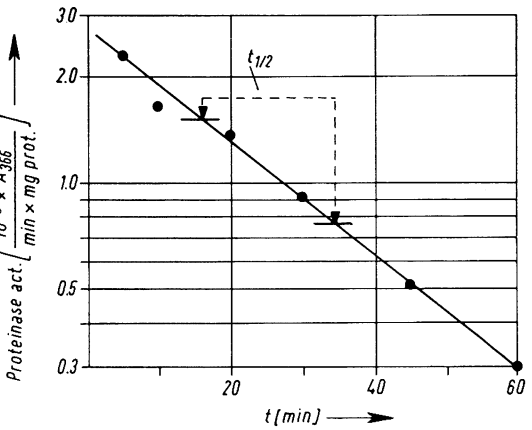


Fig. 4. Proteinase activity in *Neurospora* mitochondrial preparations obtained from cells treated with cycloheximide for various time periods.

Specific proteinase activity (A_{366}/(min x mg protein)) was determined after solubilizing mitochondria with Triton X-100. Abscissa: time of cycloheximide treatment.

Changes of proteinase activity during cyclohexmide treatment of whole cells

Mitochondrial preparations were obtained from cells which were exposed to cycloheximide for increasing time periods up to 2 h. After solubilizing the mitochondrial preparations with Triton X-100, specific proteinase activity was determined (see Fig. 4). The activity was plotted on a logarithmic scale versus time of cycloheximide treatment. The straight line indicates an exponential decrease of proteinase activity in cells treated with cycloheximide. A half life of about 20 min can be calculated from these results.

Two conclusions may be drawn from these data:

a) The proteinase which is detected by the in vitro test is subject to a rapid turnover. This turnover may either mean continuous synthesis and degradation of proteinase, or, more likely continuous synthesis and continuous excretion of the proteinase into the culture medium[27].

b) The decreasing degradation of mitochondrial and cytoplasmic translation products with increasing time of cycloheximide treatment of cells (cf. Fig. 1) appears to be an expression of the contamination of the mitochondrial preparation with proteinase vesicles.

Analysis of mitochondrial and cytoplasmic translation products without subfractionation of cells

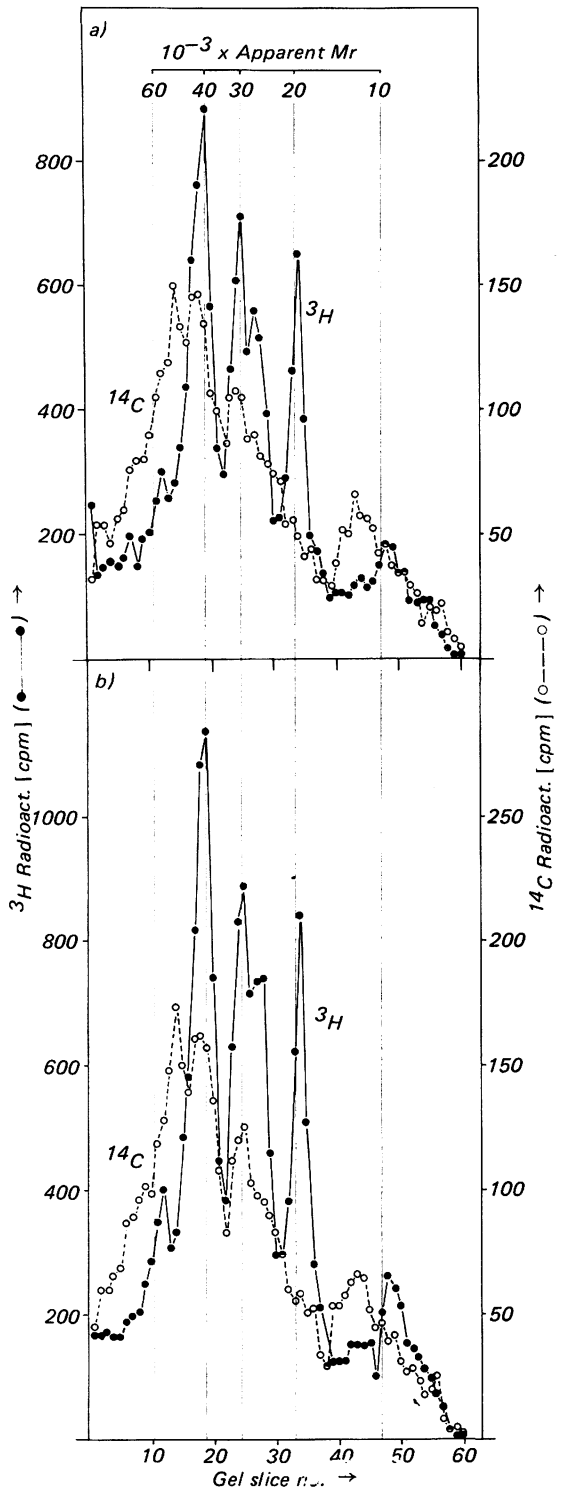
Since mitochondrial and cytoplasmic translation products can be selectively labeled it is possible to analyse them without separating cell fractions. This was done by simply freezing the cells with liquid nitrogen, breaking the cells by grinding at this temperature and extracting whole cellular proteins with a buffer containing sodium dodecylsulfate. A very similar procedure was described by Ebner et al. for yeast[28]. The results after short term and long term labeling in the presence of cycloheximide are shown in Fig. 5. In this case, gel electrophoretic analysis shows practically identical homogeneous and specific labeling patterns with cells labeled for 5 and 60 min in the presence of cycloheximide. No proteinase inhibitor was applied in these experiments. Obviously, under the conditions of the experiment, the proteinase present in the cell is not active. This effect can be explained by the presence of a proteinase inhibitor in the cytoplasm of the cell. It inhibits the proteinase set free from the vacuoles. It must be assumed that upon isolation of mitochondrial fractions, the cytoplasmic inhibitor is removed and the proteinase can be active. The existence of proteinase inhibitors in *Neurospora* and yeast has actually been described[27,29-31].

Comparison of Neurospora with yeast (Schizosaccharomyces pombe and Saccharomyces cerevisiae)

In order to examine whether the degradation of proteins under the conditions described is a phenomenon which also occurs with other fungi, experiments similar to those described with *Neu-*

Fig. 5. Gel electrophoretic analysis of *Neurospora* mitochondrial translation products in extracts of whole cells. ▷

Cells were homogeneously labeled with [^{14}C]leucine and specifically labeled with [^{3}H]leucine in the presence of cycloheximide for 5 min (a) and 60 min (b). They were then frozen with liquid nitrogen. After breaking cells by grinding under liquid nitrogen, total cellular proteins were extracted with sodium dodecylsulfate-containing buffer, dialysed and subjected to gel electrophoresis. ○----○ Homogeneous label (^{14}C); ●——● specific (cycloheximide-resistant) label (^{3}H).



rospora were carried out with yeast. Yeast is widely employed for studies on the biogenesis of mitochondrial proteins. As with *Neurospora*, cells were homogeneously labeled with [^{14}C]leucine and specifically labeled in the presence of cycloheximide with [^{3}H]leucine. The mitochondrial fractions were isolated after breaking the cells with a glass bead homogenizer and gel electrophoresis was carried out after dissolving mitochondrial fractions in sodium dodecylsulfate without and with added DFP. The results with *Schizosaccharomyces pombe* are shown in Fig. 6. Without added DFP, ^{14}C- and ^{3}H radioactivities are broadly distributed over a molecular weight range of 8 000 to 45 000. Bands with definite molecular weights are not seen. If DFP is added before dissolving mitochondrial fractions in sodium dodecylsulfate, the cycloheximide-resistant label shows definite bands with apparent molecular weights between 20 000 and 40 000. In contrast to the sample not treated with DFP, the background is low and less radioactivity is seen at molecular weights of 8 000 to 20 000. The homogeneous label is distributed mainly between apparent molecular weights of 20 000 to 80 000. The electrophoretic pattern of mitochondrial translation products is very similar to that described by Ebner et al. for *Saccharomyces cerevisiae*[28].

Further experiments with *Schizosaccharomyces* have shown that degradation is caused by proteinase activity which cannot be separated from mitochondria by sucrose density centrifugation. Furthermore, the proteinase activity appears not to be dependent on cycloheximide treatment of whole cells.

In the case of *Saccharomyces*, proteolysis of mitochondrial and cytoplasmic translation products was not observed under conditions which lead to the production of low molecular weight components in *Neurospora crassa* and *Schizosaccharomyces pombe*. Addition of DFP does not cause significant changes of the labeling patterns. Probably, the rather crude procedure for breaking the yeast cells leads to disruption of vacuoles and removal of proteinases during the isolation procedure of mitochondria. It might well be that under different conditions, such as isolation of mitochondria after preparation of spheroplasts, vacuoles are preserved and show degrading activity during subfractionation of mitochondria.

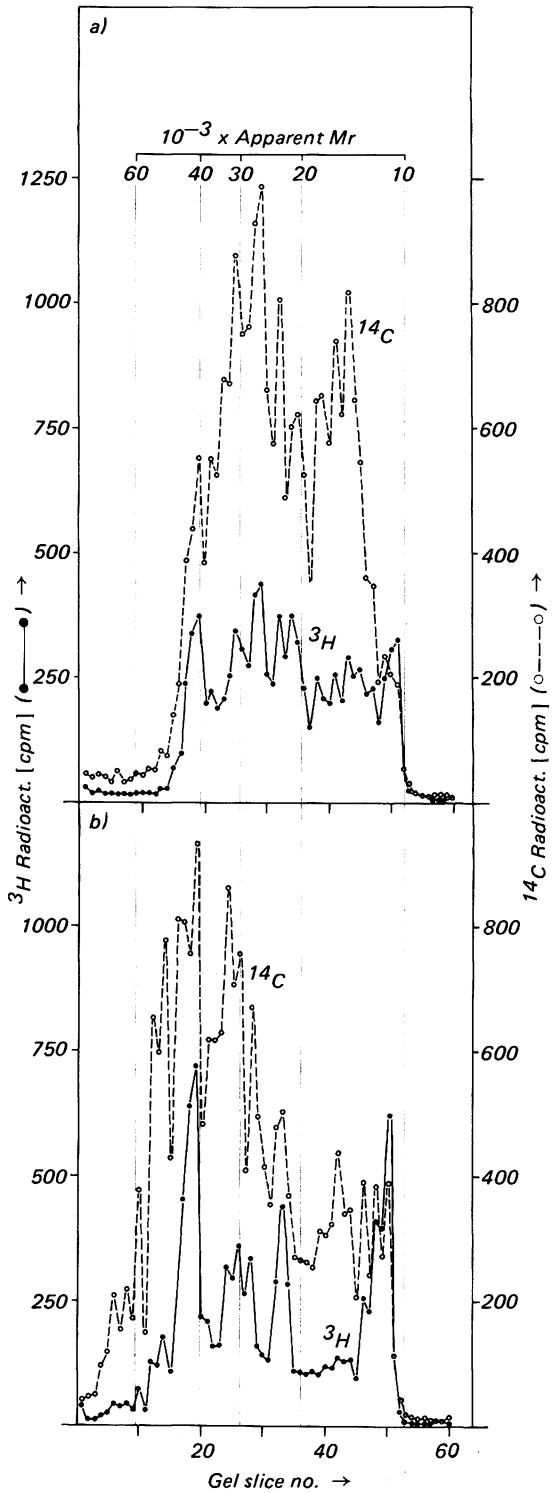


Fig. 6. Gel electrophoretic analysis of *Schizosaccharomyces pombe* mitochondrial proteins after solubilization with sodium dodecylsulfate in the presence or absence of proteinase inhibitor (DFP).

Cells were homogeneously labeled with [^{14}C]leucine for 1.5 h and specifically labeled with [^{3}H]leucine in the presence of cycloheximide for 15 min. Samples of the mitochondrial preparations were dissolved in sodium dodecylsulfate-containing buffer without (a) and with added DFP (1mM) (b) and subjected to gel electrophoresis. ○————○ Homogeneous label (^{14}C); ●————● specific (cycloheximide resistant) label (^{3}H).

Discussion

This study on proteolytic activities associated with mitochondrial preparations from *Neurospora* and yeast was undertaken to clarify the significance of mitochondrial translation products with apparent molecular weights of less than 12000. The data obtained strongly suggest that under a variety of conditions the major part of the low molecular weight translation products is generated by the artifactual action of proteinases. The proteolytic activity which may represent several different enzymes[32] is located in proteinase vesicles which are isolated together with mitochondria upon differential centrifugation. The activity becomes apparent when vesicles are opened. Breakdown of mitochondrial proteins therefore occurs essentially during procedures which involve the use of detergents. Disturbing effects of contaminating proteinases upon gel electrophoresis on isolated proteins or organelles from microorganisms have already been reported[10,33]. In the experiments described here, the critical steps are those after solubilization of the mitochondria with sodium dodecylsulfate or Triton for the further subfractionation of mitochondrial membranes, especially by gel electrophoresis. Mitochondrial translation products appear to be less affected compared to cytoplasmic translation products.

As described earlier, the proportion of low molecular weight mitochondrial translation products is high, if mitochondria are isolated from cells which are not, or only for short time, exposed to cycloheximide, and low in mitochondria from cells exposed to cycloheximide for 1 h and more[6]. This

observation led to some confusion. As a possible explanation we suggested that low molecular weight translation products are converted to high molecular weight translation products in the presence of cycloheximide[6,34]. Similar observations and considerations were made by others, partly on the basis of different experimental approaches[3,10,12,18]. The data presented in this report show that the interpretation is not correct, at least not for the results from experiments with cells exposed to cycloheximide for different periods. The real cause for the apparent shift of molecular weights from low to high during cycloheximide treatment is the decrease of proteinase activity. *Neurospora* cells grown on minimal medium into the early log phase possess proteinase vesicles which may represent proteinase storage and transport organelles. They contain proteinases probably destined for export into the extracellular space[27]. Apparently the inhibitor of cytoplasmic protein synthesis, cycloheximide, blocks the production of new vesicles and the existing ones leave the cell with a half life of about 20 min, which is about 1/12 of the doubling time of the cells. It remains to be checked whether the artifacts resulting from proteinase activity play a similar role in the experiments of other authors, in which large amounts of low molecular weight translation products were observed and which led to speculations on a conversion of these products.

The variation of proteinase activity in cells treated with cycloheximide is just one example of the rather disturbing and not easily comprehensible effects of unspecific proteinases. Slight differences in the preparation of cell fractions may lead to the removal of proteinase vesicles to different degrees. After employment of detergents for the separation of membrane proteins, activation of proteinases may occur to varying extents. Action of cytoplasmically located inhibitors may lead to confusing results in experiments which are slightly different in their design. Cells in different phases of growth may have different levels of proteolytic enzymes.

In order to avoid artifacts, the application of suitable proteinase inhibitors such as diisopropylfluorophosphate or phenylmethylsulfonylfluoride appears to be very useful. However, their efficiency must be established for the individual experimental conditions. e.g. phenylmethylsulfonylfluoride does not eliminate proteinase activity if it is just added during the isolation of mitochondria.

The observations described here may be of general importance for a broad variety of microorganisms which possess proteinase vacuoles and which are able to secrete proteinases into the extracellular space. Moreover, in higher organisms lysosomes may play a similar role[35]. Furthermore, there may be large differences between closely related organisms, as described here for *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*.

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