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THE BIOGENESIS OF MITOCHONDRIA

Transcriptional, Translational and Genetic Aspects

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Contents

CONTENTS

NASCENT POLYPEPTIDE CHAINS ON MITOCHONDRIAL RIBOSOMES AND THEIR INTEGRATION INTO THE INNER MITOCHONDRIAL MEMBRANE

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Little is known about the mechanism how mitochondrial translation products are transferred from their site of synthesis to their site of function, *i.e.* from the mitochondrial ribosomes into enzyme complexes of the inner mitochondrial membrane (1-4). The peculiar nature of the mitochondrial translation products may play a distinct role in this process. Therefore we have studied some properties of mitochondrial translation products before and after their integration into the membrane.

RESULTS.

Properties of nascent translation products on mitochondrial ribosomes. After labelling whole *Neurospora* cells with radioactive amino acids in the presence of cycloheximide (CHI), it is possible to isolate mitochondrial ribosomes in which the nascent peptide chains are radioactively labelled, whereas the ribosomal proteins are not. This is due to the fact, that the proteins of mitochondrial ribosomes are formed at the CHI sensitive cytoplasmic ribosomes (5-7). By this way, we have an experimental system in which the nascent translation products of mitochondria can easily be identified and analysed.

A) Mitochondrial ribosomes carrying nascent peptide chains.

Fig. 1 shows the result of density gradient centrifugation of mitochondrial ribosomes from cells exposed

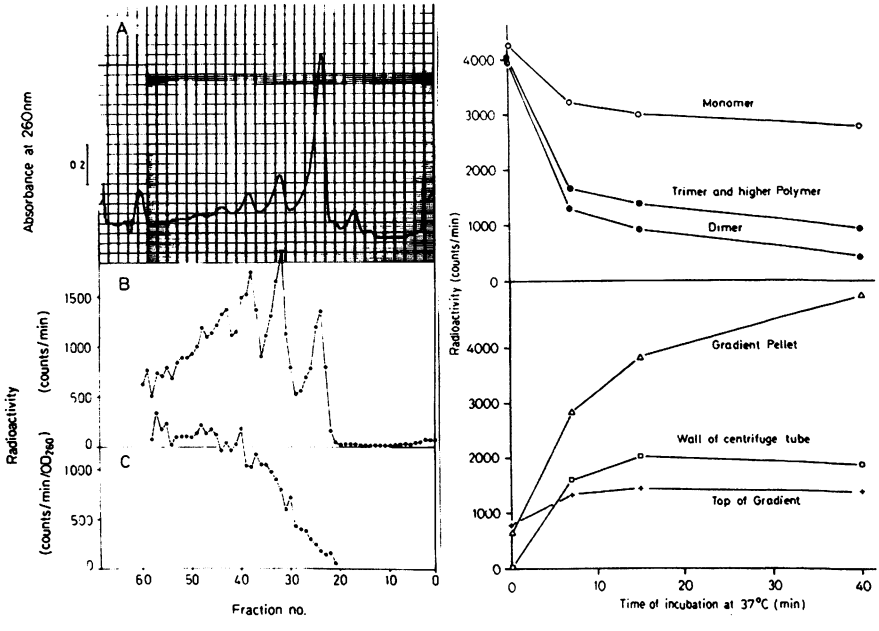

Fig. 1. Sucrose density gradient centrifugation of mitochondrial ribosomes with radioactively labeled nascent peptide chains. A: Optical density pattern (260nm); B: Radioactivity pattern; C: Specific radioactivity related to absorbance at 260 nm. Cells were incubated with cycloheximide (100 µg/ml) for 2.5 min, [^{3}H]leucine was added (1 µCi/ml) and after 1.5 min the cells were rapidly cooled to 0° C. Mitochondrial ribosomes were isolated and subjected to gradient centrifugation (9). Fraction 1 is top, and fraction 60 bottom of the gradient.

Fig. 2. Effect of incubation of mitochondrial ribosomes in AMT buffer at 37° C on the distribution of radioactivity in the sucrose density gradient. Mitochondrial ribosomes were labeled as described in Fig. 1. They were kept at 37° C in AMT buffer (0.1 M NH_4Cl, 10 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.5) for the time periods indicated in the figure. In the case of the radioactivity associated with the wall of the centrifuge tube the radioactivity in the control sample (8250 counts/min) was subtracted from all values.

to a pulse of radioactive leucine in the presence of CHI. In the optical density pattern (Fig. 1A) the peak of the monomeric ribosomes is prominent. A considerable amount of polymeric ribosomes (ca. 45 % of total ribosomes) is present. In the labelling pattern (Fig. 1B) monomeric, dimeric and higher polymeric ribosomes also can be distinguished. However, the majority of the radioactivity (ca. 85 %) is found associated with polymeric ribosomes. Accordingly, the specific radioactivity, related to A_{260nm} (Fig. 1C) is about five times higher in the polymeric ribosomes. This difference in specific radioactivity of monomeric and polymeric ribosomes suggests, that monomers are not merely breakdown products of polymers. This is in contrast to cytoplasmic ribosomes, where monomers and polymers have the same specific radioactivity after short pulse labelling.

The amount of total radioactivity in the polymer region is subject to large alterations which depend on the preparation conditions of the ribosomes. However, the less the amount of radioactivity in the polymer region is, the more radioactivity appears in the pellet of the gradient. The yield of radioactivity at the monomer is rather constant. This leads us to suppose that the polymeric ribosomes have a high tendency to aggregate.

In order to test this, mitochondrial ribosomes were kept in Mg containing buffer at 37° C for different time periods and then subjected to gradient centrifugation. In Fig. 2 the radioactivity found in the different fractions of the gradient is shown. During the time period of incubation the radioactivity associated with the monomer remains constant after a slight initial decrease. In contrast, the radioactivity at the dimer and higher polymers decreases strongly. The radioactivity disappearing from this region does not appear at the top of the gradient, but can be traced in the pellet and at the wall of the centrifuge tube.

This selective tendency of polymeric ribosomes to aggregate and form heavy particles gives rise to the

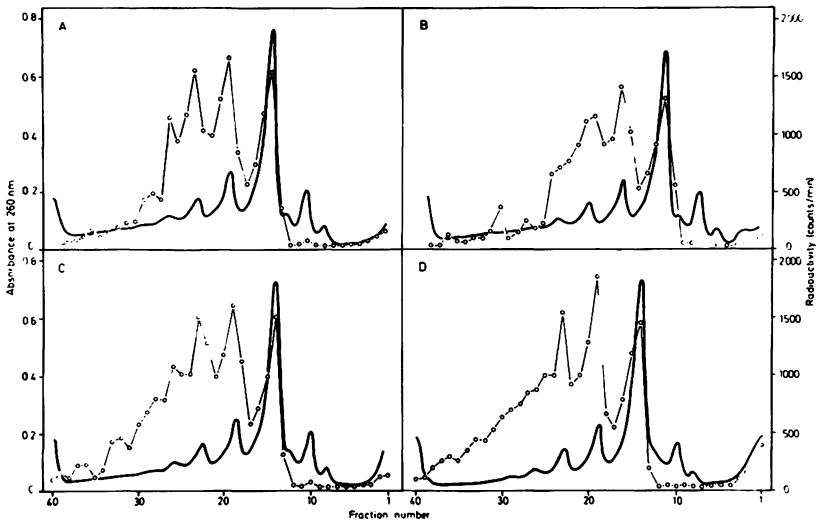

Fig. 3. Treatment of mitochondrial ribosomes with ribonuclease. Ribosomal suspensions in AMT buffer were kept at 0° C for 60 min with and without pancreatic ribonuclease, and then subjected to gradient centrifugation. o——o : radioactivity; ——: absorbance at 260 nm. A, control (without ribonuclease); B, 1 µg/ml ribonuclease; C, 4 µg/ml ribonuclease; D, 20 µg/ml ribonuclease.

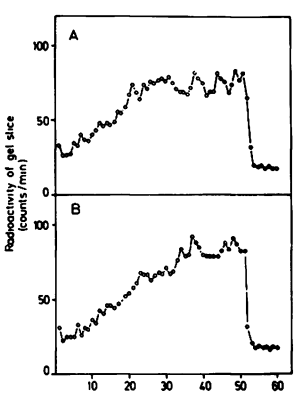

Fig. 4. Gel electrophoretic analysis of radioactively labeled nascent peptide chains associated with mitochondrial polymeric ribosomes. A, Ribosomes dissolved in 0.1 M Tris-HCl, 0.5 % SDS, pH 8, and kept for 1 h at 37° C; B, Ribosomes dissolved as in A, plus 20 µg/ml pancreatic ribonuclease.

question whether the polymeric ribosomes are real messenger-ribosome complexes or aggregates themselves. Treatment with ribonuclease is one way to test this. Cytoplasmic polymeric ribosomes are already converted to monomers to a considerable extent by incubation with 0.5 µg/ml ribonuclease for 60 min at 0° C. This conversion is complete at a concentration of 20 µg/ml ribonuclease. In contrast, the optical density pattern of mitochondrial ribosomes is not changed by incubation with corresponding ribonuclease concentrations (Fig. 3). Also no change is observed in the radioactivity pattern. This suggests that mitochondrial polymeric ribosomes are aggregates. In agreement with this is the observation that after treatment of isolated mitochondria with puromycin, aggregation of ribosomes is almost completely abolished, Therefore it appears that nascent chains are responsible for this aggregation. Similar observations and conclusions were made by Ojala and Attardi (8).

B) Properties of nascent translation products.

On the basis of these findings we are led to conclude that the peptide chains on mitochondrial ribosomes have certain peculiar properties. This is underlined by the observation that these peptides in the form of peptidyl-transfer-RNA cannot be separated from the ribosomal proteins by treatment with phenol, unless SDS is present. If SDS is removed from SDS solubilized ribosomes, the nascent peptides become insoluble.

In order to further elucidate these properties, it was examined whether the nascent peptide chains can be removed from the mitochondrial ribosomes as peptidyl-puromycin and as peptidyl-tRNA. To control the experimental setup, first cytoplasmic ribosomes were tested. In this case, treatment of ribosomes with puromycin, GTP and G-factor leads to the release of the nascent chains in a soluble form. Also, upon exposure of cytoplasmic ribosomes to EDTA, dissociation into subunits and release of peptidyl tRNA occurs. In contrast, when mitochondrial ribosomes are treated with puromycin, GTP and G-factor, the radioactive nascent

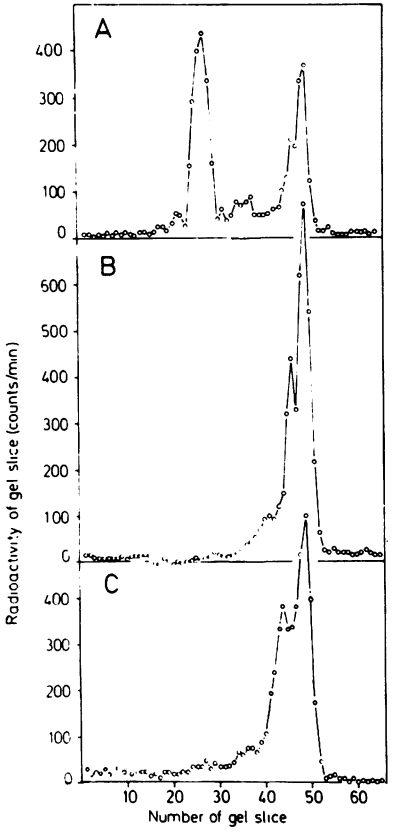

Fig. 5. Gel electrophoretic analysis of radioactively labeled nascent peptide chains associated with mitochondrial monomeric ribosomes. A, Ribosomes pretreated as described for Fig. 4A; B, Ribosomes dissolved in 0.1 M phosphate buffer, pH 11, and kept for 1 h at 37° C; C, Ribosomes pretreated as described for Fig. 4B.

chains disappear from monomers and polymers in the gradient, however they are not released in a soluble form, but are found at the wall and in the pellet of the tube after centrifugation. Treatment with EDTA dissociates the mitochondrial ribosomes but the radioactive chains remain associated with the large subunits and with ribosomal structures which cannot be clearly identified on the basis of their sedimentation behaviour. They probably represent aggregates of the large subunit (9). It appears from these observations that the nascent chains on mitochondrial ribosomes are not watersoluble but aggregate in aqueous solutions.

For further analysis of the nascent chains the ribosomes were dissolved in SDS containing buffer and subjected to gel electrophoresis in the presence of SDS. This was done separately for poly- and monomeric ribosomes. When nascent chains on cytoplasmic ribosomes were studied they showed a scattered distribution of apparent molecular weights (AMWs). No significant difference between chains of monomeric and polymeric ribosomes is seen (9). In Fig. 4A gel electrophoresis of radioactively labelled chains on mitochondrial polymeric ribosomes is shown. The AMWs also appear to be quite spread here. Fig. 4B represents the distribution after treatment of the SDS solubilized polymeric ribosomes with ribonuclease. This was done to degrade peptidyl tRNA which might still be present after incubation of the ribosomes at pH 8 and 37° C for one hour. The minor changes compared to Fig. 4A and the observation that tRNA on this gel migrates corresponding to an AMW of ca. 15,000 suggest that hydrolysis of the peptide-tRNA bond has already occurred to a large extent.

In Fig. 5A an electrophoretic separation of chains associated with mitochondrial monomeric ribosomes is presented. A peak with an AMW of 27,000 is prominent in addition to a double peak with an AMW in the range of 8,000 - 12,000. If the ribosomes are immediately subjected to gel electrophoresis after having been dissolved, mainly the first peak is present. Prolonged incubation of the ribosomes results in a decrease

of the first peak and in an increase of the second one. At pH 6 this process is slower than at pH 8, and at pH 11 (Fig. 5B) it is faster. Treatment of SDS solubilized ribosomes with ribonuclease causes the disappearance of the 27,000 AMW peak. At the same time the double peak with the lower AMW increases (Fig. 5C).

Since determinations of AMWs are difficult or even impossible in the low molecular weight range by gel electrophoresis, gel chromatography was carried out. The chains on the monomeric ribosomes are eluted from the column (Sephadex G 200) together with the marker cytochrome *c*. After digestion with trypsin smaller products appear. These are eluted together with the marker leucine. The chains at the polymeric ribosomes show a very similar behaviour.

These results suggest that the 27,000 AMW peak represents peptidyl tRNA and that the peptides have AMWs of 8,000 - 12,000. This is substantiated by the observation already mentioned that transfer RNA migrates with an AMW of about 15,000.

On the basis of the results obtained by gel chromatography, the AMW of the polymer product is in the same range as that of the monomer product. This can only be reconciled with the electrophoretic data, if we assume that on the gel the polymer chains aggregate either with each other or with the hydrophobic gel material. This again would be in accordance with the selective tendency of polymeric ribosomes to aggregate.

Translation products after integration into the membrane. What do the translation products on the monomeric ribosomes which display a rather uniform AMW represent ? If they are completed translation products, then it should be possible to find them in the mitochondrial membrane. To follow this question, *Neurospora* cells were labelled under the following conditions: CHI was added to the culture, after 2.5 min radioactive leucine and after further 2 min a chase of unlabelled leucine was given. After 45 min the cells were cooled, mitochondrial membranes were prepared and subjected to gel electrophoresis. The dis-

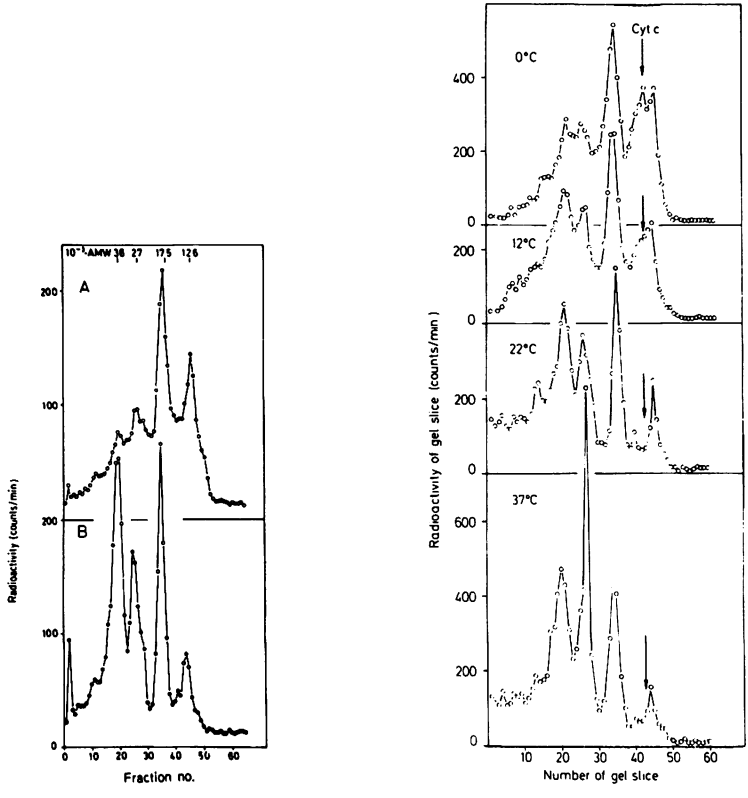

Fig. 6. Gel electrophoretic analysis of mitochondrial membranes after pulse labeling with radioactive leucine *in vivo* in the presence of cycloheximide. After 2.5 min preincubation of *Neurospora* cells with cycloheximide, a 2 min pulse of [^{3}H]-leucine was given, followed by a chase of unlabeled leucine. In (A) the cells were immediately cooled after having given the chase, in (B) the chase lasted for 45 min at growth temperature (25° C).

Fig. 7. Gel electrophoretic analysis of mitochondrial membranes after pulse labeling with [^{3}H]leucine *in vivo* in the presence of cycloheximide followed by a 1 h chase at different temperatures. The temperature during chase is given in the figure.

tribution of radioactivity on the gel is shown in Fig. 6B. Peaks are prominant at AMWs of 36,000, 27,000 and 18,000, and a small one at about 12,000. If all these protein bands were original translation products, then we would have to conclude that we do not recover completed translation products on the monomeric ribosomes. However, if we stop the labelling procedure immediately after having added the chase, a quite different labelling pattern of the membrane is observed (Fig. 6A). A large part of the radioactivity co-migrates with cytochrome *c* or is even faster. The high molecular weight peaks are present only to a low extent, the 18,000 AMW peak to a similar extent as after a long chase. The background in the high molecular weight region is very high and it looks like low molecular weight material is tailing. The total radioactivity in the membrane does not change, so it must be conluded that a conversion of translation products with lower AMWs to such with higher AMWs takes place in the membrane. If chase times in between those shown in Fig. 6 are investigated, a gradual shift of the radioactivity is observed.

This process was studied in a further experiment. Labelling was performed here as described for Fig. 6, with the modification that after giving the chase, the culture was divided into four equal portions and these were rapidly adjusted to 0, 12, 22 and 37° C, respectively. Incubation at these temperatures was carried on for one hour. Fig. 7 shows the labelling patterns of the mitochondrial membrane. A chase performed at 0° C does not change the labelling pattern of the unchased cells (*cf.* Fig. 6A). At higher temperatures the conversion takes place. It is the faster the higher the temperature is.

DISCUSSION.

1. The rather uniform apparent molecular weight of the nascent chains at the mitochondrial monomeric ribosomes may have two possible reasons: a) It is an artifact, because nascent chains are cut down to a certain length; b) the monomers carry completed chains.

Possibility a) does not appear to be likely, since no indication for such a breakdown was found under the different conditions of isolation and separation of mitochondria and ribosomes. Furthermore, the observed conversion of translation products in the membrane could explain that no larger peptide chains are found at the mitochondrial ribosomes.

The presence of completed polypeptide chains at the monomeric ribosomes is related to the problem, how mitochondrial translation products reach the inner membrane. They are obviously so hydrophobic that it seems highly improbable that they are released from the ribosomes into the matrix in a soluble form. So we can speculate that the ribosome leaving the messenger-RNA does not immediately release the product and dissociate, but rather dissociates only after having transported the completed chain to the membrane. For discussion of the alternative mechanism that ribosomes are bound to the inner membrane, see ref. 9.

2. Experiments presented here suggest that the mitochondrial translation products found in enzyme complexes such as cytochrome aa_3, cytochrome b, and ATPase (10-13) are not original translation products, but are rather generated by conversion of peptides with lower apparent molecular weights. After pulse labelling, the mitochondrially synthesized subunits of cytochrome c oxidase appear in the enzyme protein with a time course similar to that described for the 36,000, 27,000 and 18,000 AMW peaks in the membrane (14). It is possible that for each subunit this conversion process is the rate limiting step.

3. It is concluded in this report that mitochondrial translation products exhibit a strong hydrophobic character. This is substantiated by amino acid analyses of subunits of cytochrome c oxidase formed inside the mitochondria (15). On the basis of these data we are led to suggest that in mitochondria a system of transcription and translation had to be maintained (if we follow the endosymbiont theory) or had to be created (if we follow some other theory (16), because the translation products are so hydrophobic that they can-

not be transported through the cytoplasm and the intercristae space. They rather have to be delivered to the inner membrane directly, *i.e.* from the matrix side.

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