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Precursors of Cytochrome Oxidase in Cytochrome-Oxidase-Deficient Cells of *Neurospora crassa*

Comparison of the Nuclear Mutant *cni-1*, the Cytoplasmic Mutant *mi-1*, and Copper-Depleted Wild Type

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Three different cell types of *Neurospora crassa* deficient in cytochrome oxidase were studied: the nuclear mutant *cni-1*, the cytoplasmic mutant *mi-1* and copper-depleted wild-type cells.

1. The enzyme-deficient cells have retained a functioning mitochondrial protein synthesis. It accounted for 12–16% of the total protein synthesis of the cell. However, the analysis of mitochondrial translation products by gel electrophoresis revealed that different amounts of individual membrane proteins were synthesized. Especially mutant *cni-1* produced large amounts of a small molecular weight translation product, which is barely detectable in wild-type.

2. Mitochondrial preparations of cytochrome-oxidase-deficient cells were examined for precursors of cytochrome oxidase. The presence of polypeptide components of cytochrome oxidase in the mitochondria was established with specific antibodies. On the other hand, no significant amounts of heme *a* could be extracted.

3. Radioactively labelled components of cytochrome oxidase were isolated by immunoprecipitation and analysed by gel electrophoresis. All three cell types contained the enzyme components 4–7, which are translated on cytoplasmic ribosomes. The mitochondrially synthesized components 1–3 were present in *mi-1* mutant and in copper-depleted wild-type cells. In contrast, components 2 and 3 were not detectable in the nuclear mutant *cni-1*. Both relative and absolute amounts of these polypeptides in the enzyme-deficient cells were quite different from those in wild-type cells.

4. The components of cytochrome oxidase found in the enzyme-deficient cells were tightly associated with the mitochondrial membranes.

5. Processes, which affect and may control the production of enzyme precursors or their assembly to a functional cytochrome oxidase are discussed.

The assembly of cytochrome oxidase in the inner mitochondrial membrane involves polypeptide precursors, heme *a*, copper and phospholipids [1–5]. The production of these components depends on two distinct genetic systems and takes place in different compartments of the cell [2, 6–9]. Delicate regulation mechanisms are required to control the assembly process. Information on these mechanisms may be obtained from cells in which the close cooperation of nuclear and mitochondrial genes is disarranged or blocked. Such a situation may occur in mutants in which one of the two genetic systems is altered, in cells in which one system is blocked by specific inhibitors of transcription or translation, and in cells in which specific cofactors of cytochrome oxidase are missing. The impaired assembly of cytochrome oxidase in these cells can be studied on the level of precursors of the enzyme. In a preceeding report it has been shown that antibodies to complete cytochrome oxidase and to individual subunits are excellent tools for the determination of polypeptide components of cytochrome oxidase [10].

Using these antibodies we have focused our studies on three different cell types of *Neurospora crassa:* the

Enzyme. Cytochrome oxidase or ferrocytochrome c: O_2 oxidoreductase (EC 1.9.3.1).

nuclear mutant *cni-1* [11], the cytoplasmic mutant *mi-1* [12], and copper-depleted wild-type cells [13]. All these cells exhibit normal growth and are characterized by a deficiency in cytochrome oxidase.

The present communication is concerned with the following questions. Is the lack of cytochrome oxidase associated with alterations in mitochondrial protein synthesis? Do the enzyme deficient mitochondria contain precursors of cytochrome oxidase; and if precursors are present, do they accumulate in the organelles? Is it possible to correlate the nature of the defect with particular alterations in synthesis and assembly of individual precursors?

MATERIALS AND METHODS

Strains of Neurospora crassa

Wild-type (74 A re) and extrachromosomal mutant *mi-1* (3627-4A) were obtained from the Fungal Genetics Stock Center (FGSC) (Arcata, California). The chromosomal mutant *cni-1 (inos)* was kindly supplied by Dr D. Edwards (Department of Biological Sciences, State University of New York, Albany, N.Y.).

Growth Conditions

Hyphae were grown in aerated liquid cultures at 25 °C in Vogel's minimal medium [14] plus 2% sucrose. In the case of *cni-1* mutant, the medium was supplemented with inositol (50 µg/ml) [11]. Copper-free growth medium was prepared as described by Schwab [13]. The standard inoculum was 1×10^6 conidia/ml. For copper-limited growth it was reduced to 2.5×10^4 conidia/ml. Hyphae of mutants *cni-1* and *mi-1* were harvested during early log phase, those of wild-type during mid log phase of growth.

Cytochrome Spectra

Difference spectra of mitochondrial suspensions were recorded with a Cary spectrophotometer (model 118 C). The test sample was reduced with sodium dithionite, the reference sample was left in oxidized state.

Quantitative Determination of Mitochondrial Protein Synthesis

The contribution of the mitochondrial protein synthesizing system was measured by following the cycloheximide-resistant incorporation of radioactive leucine into cell proteins [15,16]. For a quantitative evaluation the specific radioactivity of the intracellular leucine pool must be known. In the experiments described here, the amount of radioactive leucine added after administration of cycloheximide, exceeds by far the initial amount of intracellular leucine.

Enough external leucine is taken up within 5 min to ensure that the dilution by endogenously produced leucine may be neglected. Thus, during the cycloheximide labelling period the specific radioactivity of the intracellular leucine is equal to that of the added leucine [17].

The total cellular protein of exponentially growing cells was labelled by addition of L-[^{14}C]leucine (10 µCi/l; specific activity 311 mCi/mol) to the culture medium. After 30 min the culture was divided into two equal portions. To one portion cycloheximide (0.1 mg/ml) was added. The other portion remained untreated, serving as a control. 2 min after addition of the inhibitor, L-leucine (final concentration 1 mM) and L-[^{3}H]leucine (1 mCi/l; specific activity 52 Ci/mmol) were given to both portions of the culture. Samples were withdrawn at distinct intervals. Cells were harvested by rapid filtration and immediately frozen by liquid nitrogen. Mitochondrial membranes were prepared from the samples and washed twice with trichloroacetic acid (6% in water).

The precipitated protein was dissolved in 1 N NaOH; radioactivity and protein content were measured. The rates of leucine incorporation into mitochondrial membranes were calculated from the labelling kinetics.

Labelling Procedures

To achieve a continuous labelling of cell proteins, hyphae were cultured in the indicated growth media, which were supplemented with L-leucine (0.5 mmol/l) and L-[^{3}H]leucine (3.8 mCi/l). Under copper-limited growth conditions radioactivity was added only 10 h after inoculation. At the time of harvesting of the cells 20–25% of the added leucine were incorporated.

Labelling of cells in the presence of cycloheximide or chloramphenicol was performed as described previously [10]. Cells were harvested 35 min after addition of the inhibitors. Preparation of mitochondria and mitochondrial membranes has been reported elsewhere [10].

Immunological Procedures

Preparation of immunoglobulins, techniques of immunoprecipitation, and of electrophoresis have been described earlier [10].

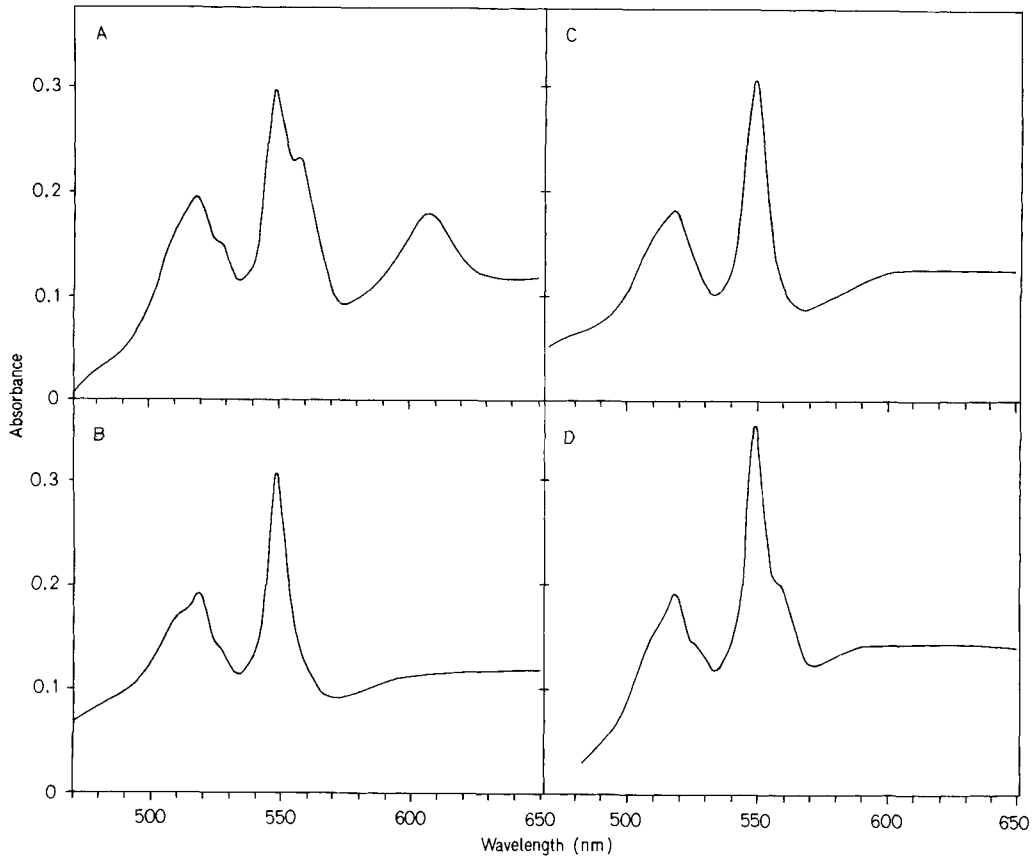


Fig. 1. *Absorption spectra of mitochondria from wild-type (A)*, cni-1 *(B)*, mi-1 *(C), and copper-depleted wild-type cells (D).* The test sample was reduced with dithionite, the reference sample was left in oxidized state. Protein concentrations of mitochondrial suspensions: (A) 7.2 mg/ml, (B) 2.9 mg/ml, (C) 6.1 mg/ml, (D) 6.3 mg/ml. The spectra were recorded at room-temperature

Determination of Heme a

Mitochondrial preparations (10–20 mg) were washed three times with neutral acetone–water (8:2, v/v). The residues were extracted four times with HCl–acetone mixtures [18]. The combined extracts were evaporated to dryness *in vacuo*. The residues, containing protoheme and heme *a*, were taken up with pyridine–water (3:1, v/v, adjusted to pH 11 with NaOH) and spectra of the resulting pyridine hemochromogens were recorded. The concentration of heme *a* was calculated using the following absorption coefficients:

$$\varepsilon^{\text{heme } a}_{587\,\text{nm}} = 27.4\ \text{mM}^{-1} \times \text{cm}^{-1}\ [18],$$

$$\varepsilon^{\text{heme } a}_{557\,\text{nm}} = 8.4\ \text{mM}^{-1} \times \text{cm}^{-1}\ [19],$$

$$\varepsilon^{\text{protoheme}}_{587\,\text{nm}} = 1.3\ \text{mM}^{-1} \times \text{cm}^{-1}\ [20],$$

$$\varepsilon^{\text{protoheme}}_{557\,\text{nm}} = 34.0\ \text{mM}^{-1} \times \text{cm}^{-1}\ [20].$$

RESULTS

Cytochrome Spectra of Mitochondria from the Various Cell Types

Fig. 1 shows room-temperature spectra of mitochondrial cytochromes present in wild-type, *cni-1*, *mi-1* and copper-depleted wild-type cells. In wild-type mitochondria there is a distinct band at 605 nm, which can be attributed to cytochrome aa_3. This band is absent in the three other cell types. *b*-type cytochromes are present in wild-type and in copper-depleted wild-type cells as indicated by the shoulder at 558 nm. In *cni-1* and *mi-1* mitochondria no *b*-type cytochromes were detected with the applied technique. Mitochondria of all cells contained cytochrome *c* (549 nm).

Mitochondrial Protein Synthesis in Cytochrome-Oxidase-Deficient Cells

All three cell types deficient in cytochrome oxidase, have retained the ability to translate proteins on mito-

Table 1. *Rate of leucine incorporation into mitochondrial membranes of wild-type cells and of various types of cytochrome-oxidase-deficient cells in the absence and presence of cycloheximide*
Radioactive leucine (specific activity 1.0 mCi/mmol) was added to cultures of exponentially growing cells to a concentration of 1 mM. The rates of leucine incorporation were obtained by dividing the labelling rates through the specific radioactivity of the added leucine

Cell type	Rate of leucine incorporation		Proportion of cycloheximide resistant incorporation
	in the absence of cycloheximide	in the presence of cycloheximide	
	nmol × mg protein^{-1} × min^{-1}		%
Wild-type	3.5	0.54	15
cni-1	2.9	0.35	12
mi-1	2.0	0.26	13
Copper-depleted wild-type	3.3	0.54	16

chondrial ribosomes. In order to compare the activities of mitochondrial protein synthesis in the various cell types, exponentially growing cells were incubated with radioactive leucine in the absence and presence of cycloheximide. The amino acid was added to the incubation medium to a high concentration (1 mM) in order to maintain a constant intracellular leucine pool after administration of cycloheximide (see Methods).

The results are listed in Table 1. In the absence of the inhibitor cells of *cni-1* mutant and of copper-starved wild-type showed similar rates of leucine incorporation as wild-type cells. *mi-1* mutant incorporated the amino acid into its cellular protein more slowly, in agreement with the slower growth rate of this strain. Cycloheximide-insensitive protein synthesis accounted for 12–16% of the total cellular protein synthesis in the various cytochrome-oxidase-deficient cells. In wild-type a similar proportion was observed. Obviously in the enzyme-deficient cells mitochondrial protein synthesis is operating at a similar rate as in wild-type cells. However, there may be differences in the various cell types concerning the relative proportions of the individual proteins or the species of proteins made in the organelles.

To study this problem, the cycloheximide-resistant labelling patterns of mitochondrial membranes from the cytochrome-oxidase-deficient cells were compared to those from wild-type cells (Fig. 2). [^{3}H]Leucine was used to label the enzyme-deficient cells in the presence of cycloheximide. [^{14}C]Leucine was incorporated into wild-type cells under the same conditions. The cultures were harvested and mitochondrial membranes were prepared. Then, each of the ^{3}H-labelled membrane preparations was mixed with ^{14}C-labelled membranes and subjected to dodecylsulfate-gel electrophoresis.

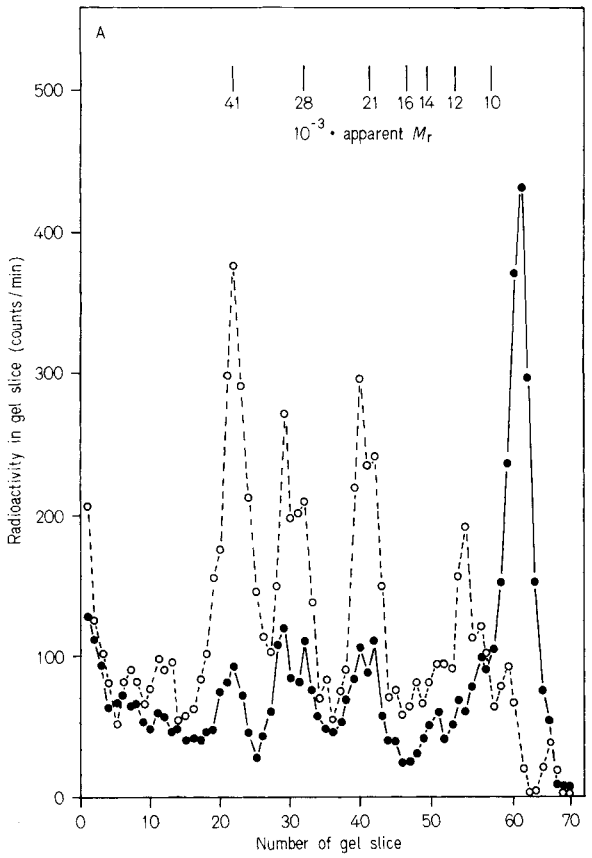
Mitochondrial membranes of the nuclear mutant *cni-1* (Fig. 2A) exhibited a labelling pattern quite different from that of wild-type membranes. High molecular weight components were present only in small quantities, whereas a large amount of low molecular weight polypeptides was found. The major part of this fast migrating material did not coincide with the smaller polypeptides present in wild-type. In contrast, labelled proteins in the cytoplasmic mutant *mi-1* (Fig. 2B) showed the same electrophoretic mobility as the labelled proteins in wild-type. However, the relative peak areas of the individual proteins were altered. Compared to wild-type membranes, the synthesis of polypeptides with apparent molecular weights of 41000, 32000 and 28000 were significantly reduced. On the other hand, the formation of the polypeptide of molecular weight 21000 and of part of the smaller components was enhanced. The cycloheximide-resistant labelling pattern of mitochondrial membranes from copper-starved wild-type cells (Fig. 2C) was very similar to that of wild-type cells, grown in a copper-sufficient medium.

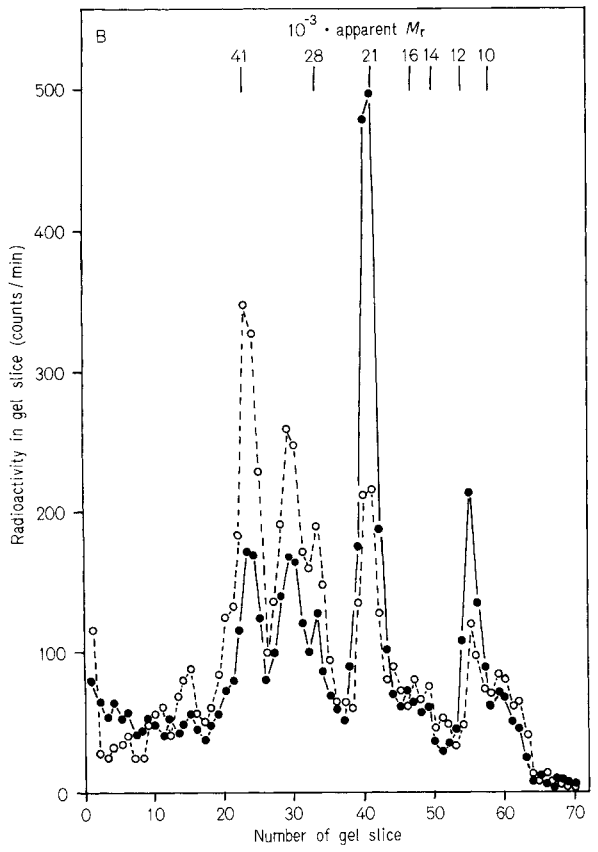
In order to prove that mitochondrial translation products were completely recovered in the membrane fraction, whole cells were solubilized in dodecylsulfate and subjected to gel electrophoresis. This procedure yields the same labelling patterns as the analysis of isolated mitochondrial membranes.

Analysis of Polypeptide Components of Cytochrome Oxidase

To detect possible precursor proteins of cytochrome oxidase, cells were labelled continuously with radioactive leucine. This labelling procedure ensures that all proteins become labelled, irrespective of their pool sizes and half-lives [10].

Mitochondria from the various cytochrome-oxidase-deficient cells (labelled with [^{3}H]leucine) were mixed with mitochondria from wild-type cells (labelled with [^{14}C]leucine) to provide cytochrome oxidase as a carrier and as a marker. The suspensions were solubilized with Triton X-100 and immunoglobulin to cytochrome oxidase [10] was added. The resultant immunoprecipitates were dissolved in dodecylsulfate and submitted to gel electrophoresis. Fig. 3 shows the distribution of radioactivity on the gels. Fig. 3A represents the analysis of the immunoprecipitate obtained with the nuclear mutant *cni-1*. Each of the





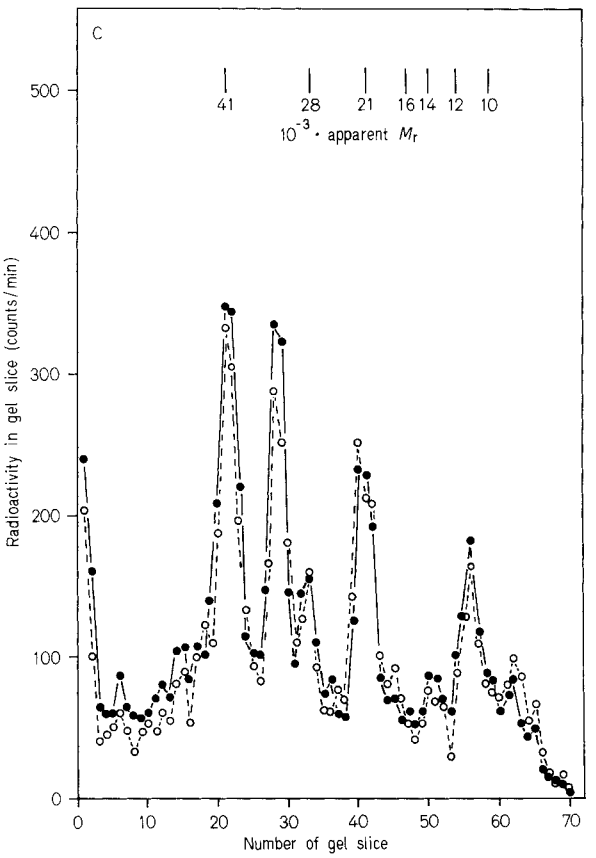
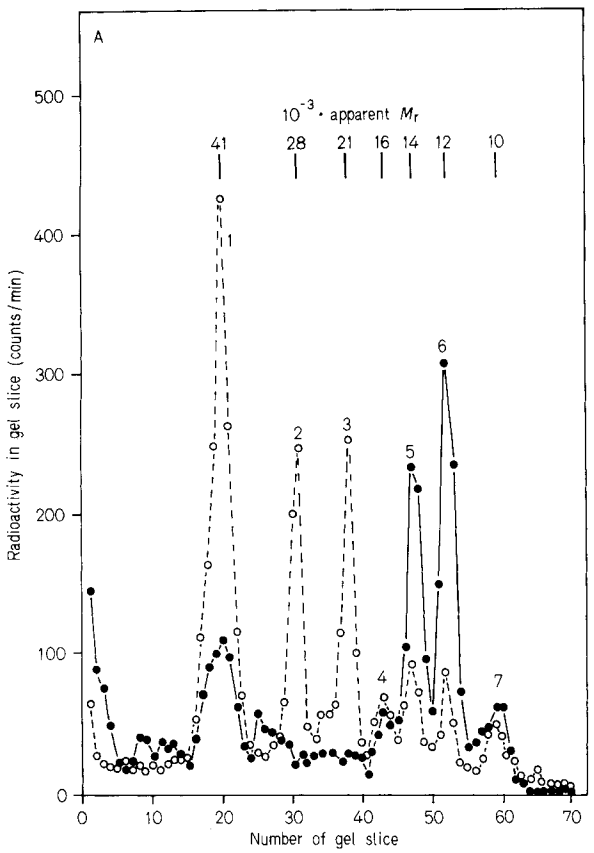
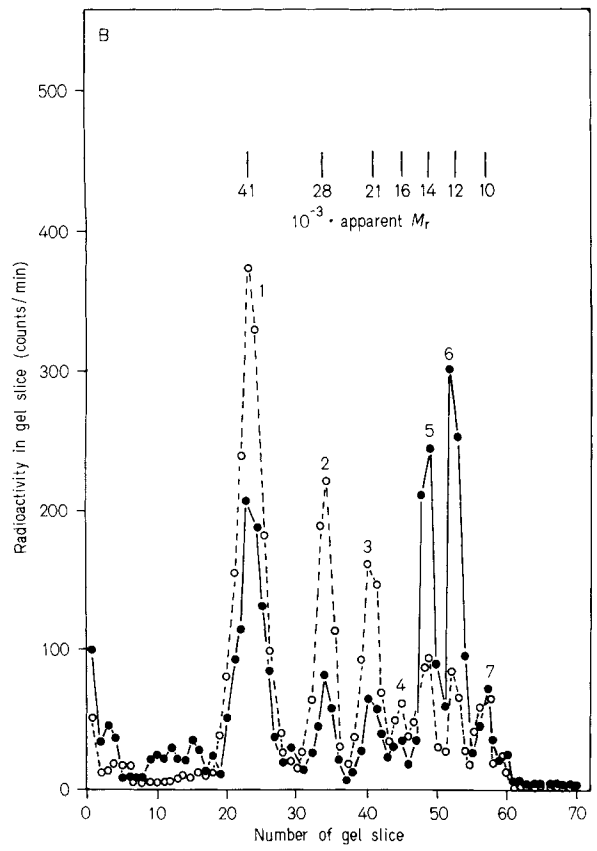


Fig. 2. *Gel electrophoretic analysis of mitochondrial translation products in wild-type and various types of cytochrome-oxidase-deficient cells.* Normal wild-type cells and cells lacking cytochrome oxidase were labelled in the presence of cycloheximide with [^{14}C]leucine and [^{3}H]leucine, respectively. Mitochondrial membranes were prepared, mixed and subjected to dodecylsulfate-gel electrophoresis. (A) *cni-1* mutant + wild-type, (B) *mi-1* mutant + wild-type, (C) copper-depleted wild-type + wild-type. Gels were calibrated with subunits of cytochrome oxidase. (O----O) ^{14}C radioactivity, (●——●) ^{3}H radioactivity

^{3}H-labelled proteins on the gel coincided with one of the ^{14}C-labelled marker subunits of cytochrome oxidase. The same holds for the proteins present in the *mi-1* mutant (Fig. 3B) and in copper-depleted wild-type cells (Fig. 3C). Furthermore, in all three cell types components 4, 5, 6 and 7 (apparent molecular weights 16000, 14000, 12000 and 10000) were present. In contrast, marked differences were found in the labelling patterns of the components 1, 2 and 3 of cytochrome oxidase (apparent molecular weights 41000, 28000 and 21000). In the *cni-1* mutant only component 1 could be detected, while *mi-1* cells and copper-depleted wild-type cells contained all of these three components.

Table 2 lists the amounts of radioactivity found in component 1 and in components 4–7 (taken together)





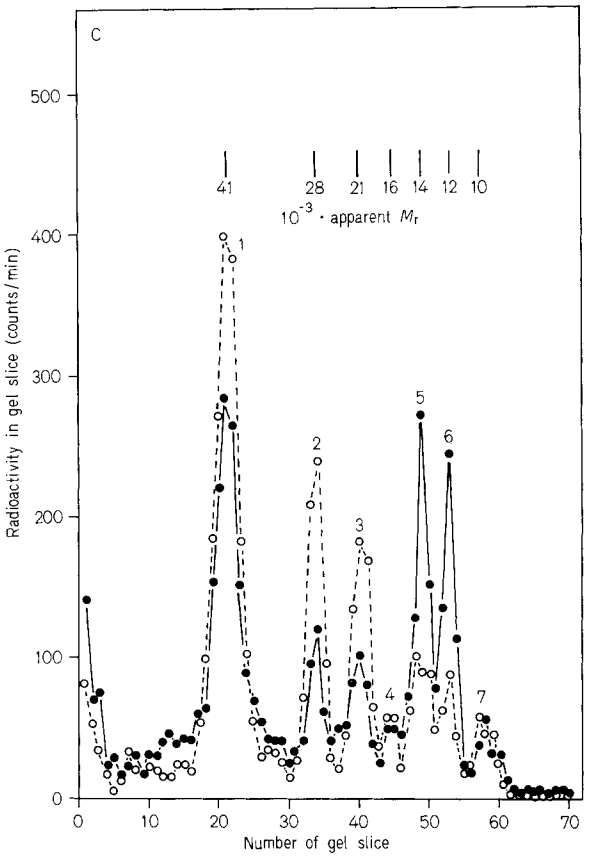


Fig. 3. *Electrophoretic analysis of polypeptide components of cytochrome oxidase present in various types of cytochrome-oxidase-deficient cells.* The enzyme deficient cells were labelled continuously with [^{3}H]leucine. Mitochondria were isolated. To each of these ^{3}H-labelled mitochondrial preparations ^{14}C-labelled wild-type mitochondria were added. The suspensions were solubilized with Triton X-100 and treated with immunoglobulin to cytochrome oxidase. The resultant immunoprecipitates were subjected to dodecylsulfate-gel electrophoresis. (A) *cni-1* mutant, (B) *mi-1* mutant, (C) copper-depleted wild-type. (O----O) ^{14}C radioactivity, (●——●) ^{3}H radioactivity

related to the amount of radioactivity of total mitochondrial protein. As described earlier [10] (see also Method section) the applied technique of continuous labelling ensures that all cellular proteins obtain the same specific radioactivity. Thus, the data in Table 2 represent approximate quantities of cytochrome oxidase components in mitochondria of the various cell types. As reported previously [10], the antibody used in this experiment did not precipitate isolated component 3; the ability of the antibody to react with the isolated subunit 2 has not yet been tested. Consequently values for components 2 and 3 were not evaluated.

Table 2 also lists the amount of that portion of components 4–7 in wild-type, which is not integrated

Table 2. *Quantities of cytochrome oxidase components in various cell types determined by immunoprecipitation*
Values were calculated on the basis of radioactive leucine found in the different electrophoretic fractions of the immunoprecipitates. The amount of radioactivity in the peaks (see Fig. 3) was related to the amount of radioactivity in the mitochondrial preparations, from which the components were precipitated

Cell type	Component 1	Components 4–7
	% total mitochondrial protein	
Wild-type	1.45	1.1 (0.20)[a]
cni-1	0.08	0.28
mi-1	0.17	0.32
Copper-depleted wild-type	0.19	0.24

[a] Not integrated into the enzyme complex (precursors).

into cytochrome oxidase (precursors). This value was obtained using specific antibodies to components 4–7, which make it possible to discriminate between precursors and complete cytochrome oxidase (S. Werner, unpublished results).

Characterization of Polypeptides of Cytochrome Oxidase by means of Selective Inhibitors of Protein Synthesis

Cytochrome-oxidase-deficient cells were incubated with chloramphenicol prior to application of radioactive amino acids. In the immunoprecipitates obtained with antibodies to cytochrome oxidase components 4, 5, 6 and 7 were selectively labelled (not

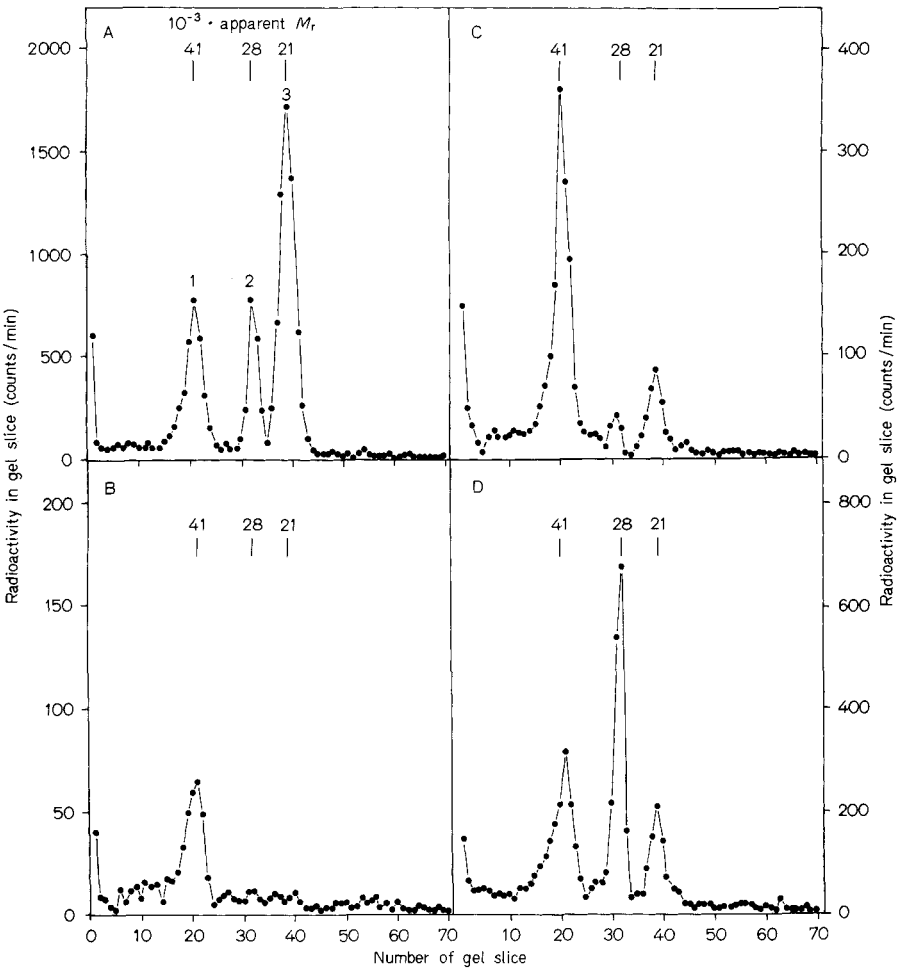


Fig. 4. *Electrophoretic analysis of mitochondrially synthesized components of cytochrome oxidase present in wild-type and in various types of cytochrome-oxidase-deficient cells.* Cells were labelled with [^{3}H]leucine in the presence of cycloheximide. Mitochondria were isolated. Mitochondria from the enzyme-deficient cells were mixed with unlabelled wild-type mitochondria. Immunoprecipitation was carried out with immunoglobulin to cytochrome oxidase as described in legend to Fig. 3. (A) Wild-type, (B) *cni-1* mutant, (C) *mi-1* mutant, (D) copper-depleted wild-type

shown). This demonstrates the translation of these components on cytoplasmic ribosomes.

In order to identify mitochondrially synthesized polypeptides the enzyme-deficient cells were labelled with [^{3}H]leucine in the presence of cycloheximide. Mitochondria were prepared and mixed with unlabelled wild-type mitochondria. The suspensions were solubilized with Triton X-100 and treated with antibodies to cytochrome oxidase. The immunoprecipitates were analysed by dodecylsulfate-gel electrophoresis (Fig. 4). In wild-type mitochondria (Fig. 4A) polypeptides 1, 2 and 3 were labelled. Most of the radioactivity was incorporated into component 3; the amount of radioactivity found in components 1 and 2 was much smaller. Quite different labelling patterns were observed in cells lacking cytochrome oxidase. Only component 1 was observed in the immunoprecipitate of the *cni-1* mutant (Fig. 4B). In the labelling pattern of the *mi-1* mutant all three components were present (Fig. 4C). Most of the radioactivity was found in component 1. Also, in the immunoprecipitate from copper-depleted wild-type all three components were observed (Fig. 4D). In this case, incorporation occurred predominantly into components 1 and 2.

Separate Analysis of Component 3 of Cytochrome Oxidase

Immunoglobulin to complete cytochrome oxidase did not precipitate component 3, which was isolated from cytochrome oxidase [10]. Consequently it was inferred that this immunoglobulin is not suitable to detect free component 3. Therefore, the presence of this component in cytochrome-oxidase-deficient cells was examined by means of a subunit specific antibody [10]. Cells were labelled in the presence of cycloheximide as described above. Mitochondria were prepared, solubilized with Triton X-100 and treated with subunit-specific immunoglobulin from rabbit. Then, a second antibody, immunoglobulin from sheep to rabbit γ-globulin was added. This "sandwich technique" is suitable for the isolation of minimal amounts of precursor proteins [10]. The immunoprecipitates were analysed by gel electrophoresis. The results are shown in Fig. 5. From the nuclear mutant *cni-1* no polypeptide could be precipitated (Fig. 5A). In contrast, the cytoplasmic mutant *mi-1* as well as copper-depleted wild-type cells contained the polypeptide of molecular weight 21 000 (Fig. 5B, C).

These findings suggest that in mutant *cni-1* at least component 3 of cytochrome oxidase is not synthesized. However, it cannot be excluded that this polypeptide is produced, but rapidly degraded by proteolytic enzymes, because its integration into the membrane is blocked.

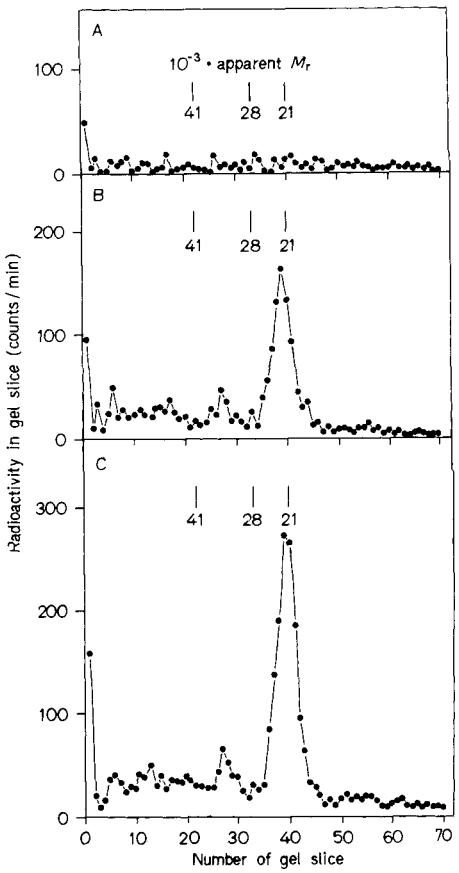


Fig. 5. *Separate analysis of component 3 in various types of cytochrome-oxidase-deficient cells.* Mitochondria from cells labelled with [^{3}H]leucine in the presence of cycloheximide (same preparations as in Fig. 4) were solubilized with Triton X-100. They were treated successively with anti-subunit 3 γ-globulin from rabbit and anti-rabbit γ-globulin from sheep. The resultant immunoprecipitates were analysed by dodecylsulfate-gel electrophoresis. (A) *cni-1* mutant, (B) *mi-1* mutant, (C) copper-depleted wild-type cells. Gels were calibrated with subunits of cytochrome oxidase

Binding of Cytochrome Oxidase Polypeptides to the Mitochondrial Membrane

Preparations of mitochondria from *cni-1*, *mi-1* and copper-depleted wild-type cells were submitted to sonic irradiation in buffers of high ionic strength [10]. After centrifugation, the cytochrome oxidase components were completely recovered in the insoluble fraction. Obviously, these polypeptides are tightly associated with mitochondrial membranes.

Heme a *Content of Cytochrome-Oxidase-Deficient Cells*

Since polypeptide components of cytochrome oxidase are present in cells lacking this enzyme, the question arises, whether these cells do also contain

heme *a*. From wild-type mitochondria 0.50 nmol heme *a* per mg mitochondrial protein were extracted. The amount of heme *a* found in mitochondria of mutants *cni-1* and *mi-1* was extremely low (0.02 and 0.03 nmol per mg mitochondrial protein, respectively). From mitochondria of copper-depleted wild-type cells no heme *a* could be extracted at all.

DISCUSSION

In this report it is shown that antibodies to cytochrome oxidase precipitate several polypeptides present in mitochondria from cytochrome-oxidase-deficient cells. The cross-reacting polypeptides are closely related to polypeptide subunits in cytochrome oxidase. This is concluded on the basis of the following criteria: specificity of the applied antibodies, translation of the polypeptides at mitochondrial or cytoplasmic ribosomes and coincidence of apparent molecular weights.

One might assume that the precipitated polypeptides are subunits of a small amount of residual cytochrome oxidase, still present in these cells. The following observations are not compatible with this assumption. Firstly, the quantity of precipitated protein components could not be correlated with the very small amount of heme *a* extracted from these cells. Secondly, the relative amounts of the individual polypeptides precipitated from the enzyme-deficient cells were quite different from the relative amounts of the corresponding subunits present in the cytochrome-oxidase complex of wild-type cells. Thus, it is evident that the polypeptides precipitated from cytochrome-oxidase-deficient cells are precursors of the enzyme.

The absolute amount of the precipitable polypeptides in the enzyme-deficient cells was small compared to that of polypeptides in the enzyme complex of wild-type cells.

Concerning the cytoplasmically synthesized components 4–7, their amount in the enzyme-deficient mitochondria was in the same range as the amount of the precursor proteins of components 4–7 in wild-type mitochondria (0.2% of total mitochondrial protein). The same result was obtained, when specific antibodies to isolated cytoplasmic subunits of cytochrome oxidase were applied (unpublished results). From these data we conclude that the cytoplasmic precursors of the enzyme are not accumulated to a large extent in cytochrome-oxidase-deficient mitochondria. Possibly, mitochondrial membranes possess a limited number of receptor sites for these precursors.

The amount of mitochondrially synthesized polypeptides (components 1–3) precipitable with an antibody to complete cytochrome oxidase was also relatively small. However, these components may be underestimated, because of incomplete precipitation as already pointed out in Results. It is possible that only that part of the components 1, 2 and 3 was precipitated, which was tightly associated with cytoplasmically synthesized components.

On the other hand, it seems clear that at least component 3 was not accumulated in the enzyme-deficient cells. This is concluded from experiments in which a specific antibody to component 3 was employed, and in which mitochondrial translation products were selectively labelled in the presence of cycloheximide.

In these experiments no radioactivity could be precipitated from *cni-1* mitochondria and as little as 1–2% of the label present in *mi-1* and copper-depleted wild-type mitochondria. These findings were not immediately expected from the labelling patterns of the mitochondrial translation products in the various cell types (see Fig. 2). In all cases a considerable amount of radioactivity was incorporated into the fraction of molecular weight 21000. This is most conspicuous with copper-depleted wild-type cells, which contained nearly the same amount of radioactivity in this fraction as normal wild-type cells.

There are several explanations for this discrepancy. Firstly, the membrane fraction with the apparent molecular weight of 21000 contains not only component 3 of cytochrome oxidase, but also other mitochondrial translation products, *e.g.* a component of ATPase. Secondly, the major part of the label in this membrane fraction represents precursors of subunit 3 of cytochrome oxidase, but the antibody recognizes only a minor part of the precursors. This could be due to different molecular conformations. Our present knowledge does not permit to discriminate between these possibilities.

Do the residual cytochrome oxidase polypeptides combine to protein complexes? As mentioned above, antibodies to complete cytochrome oxidase do not precipitate isolated component 3 of the enzyme. However, a tight association of this polypeptide with other precursors would explain why part of it is precipitated by antibodies to the complete enzyme in the mutant *mi-1* and in copper-depleted wild-type cells.

The idea of such an incomplete or altered assembly process is supported by the labelling experiments with cycloheximide-poisoned cells. The individual polypeptides 1–3 were labelled to quite different extents in the various cytochrome oxidase deficient cells (see Fig. 4). This may be an expression of the different compositions of "pre-cytochrome oxidase" complexes, precipitated by the antibody. Altered pool sizes of

individual precursors may further complicate the situation.

All components of cytochrome oxidase were found to be tightly associated with the mitochondrial membranes in the enzyme-deficient cells. In contrast, Ebner *et al.* [21] reported that sonication of mitochondria from a cytoplasmic *petite* mutant of yeast completely solubilized the residual components 4 and 5 of cytochrome oxidase. This cytoplasmic *petite* mutant lacked all three mitochondrially synthesized polypeptides. It is tempting to speculate that at least one mitochondrial component is required for a tight binding of the cytoplasmic precursor polypeptides to the mitochondrial membranes.

What is known about the primary defects or mechanisms which lead to the loss of cytochrome oxidase in these cells?

The most puzzling feature of the nuclear mutation in *cni-1* is the alteration in the synthesis of mitochondrial translation products. A low molecular weight polypeptide was synthesized in mitochondria of mutant *cni-1*. This component accounted for about 40% of the cycloheximide-resistant label in the membrane. The significance of this component is not known.

The analysis of the immunoprecipitable precursors of cytochrome oxidase demonstrated that the nuclear mutant lacks at least one mitochondrially synthesized component of the enzyme. A similar situation was recently found in nuclear mutants of *Saccharomyces cerevisiae*, which were also deficient in cytochrome oxidase [21,22]. The authors reported that these mutants lacked cytochrome oxidase polypeptides, which in the wild-type are synthesized on mitochondrial ribosomes. Like mutant *cni-1* of *Neurospora* the yeast mutants contained the four components of cytoplasmic origin.

These findings in yeast and in *Neurospora* further support the hypothesis that nuclear genes code for a component which influences the production of mitochondrial components of cytochrome oxidase or their combination with cytoplasmic enzyme precursors.

Concerning the cytoplasmic mutant *mi-1*, Rifkin and Luck [23] and Neupert *et al.* [24], have shown that these cells are deficient in functional mitochondrial ribosomes, due to a lack of small ribosomal subunits. This led to the suggestion that the absolute rate of protein synthesis in mitochondria is reduced. The present results confirm this assumption. Furthermore, it is shown that the species of mitochondrial products translated in *mi-1* and in wild-type, are identical with reference to their apparent molecular weights. The complete set of cytochrome oxidase polypeptides is synthesized in *mi-1*. One may conclude that in this mutant the proper assembly of cytochrome oxidase is limited by the low quantities of mitochondrially synthesized precursors which are available.

It is well known that mycelia of the *mi-1* mutant in late log phase contain a considerable amount of enzymatically active cytochrome oxidase. A similar phenomenon was found in the nuclear mutant *cni-1*. Edwards and coworkers have shown that *cni-1* mutant which was grown into stationary phase did contain functional cytochrome oxidase (personal communication). Recent experiments in our laboratory revealed that the two polypeptides which were not present in mitochondria from *cni-1* in the early log phase appear in the late log phase of growth (unpublished results). Hence, the deficiency in both mutants is a transient one. These cells may recover almost completely at the end of growth. This would suggest that the underlaying mutations affect regulatory rather than structural genes.

Copper-limited growth of cells is associated with a specific deficiency of cytochrome oxidase [25,13]. Nevertheless, the pattern of mitochondrial translation products could not be distinguished from that of wild-type cells, grown in a copper-sufficient medium. Moreover, all cytochrome oxidase polypeptides were present in copper-depleted cells, yet in different relative amounts compared to normal wild-type. It seems reasonable to assume that copper deficiency leads to an impairment of the assembly to the functional enzyme on a post-translational level.

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