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Inhibition of the Assembly of Cytochrome Oxidase in *Neurospora crassa* by Chloramphenicol

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Cytochrome oxidase was prepared from *Neurospora crassa* by chromatography on oleyl polymethacrylic acid resin and separated into seven polypeptides by polyacrylamide gel electrophoresis in the presence of sodium dodecylsulfate. Incorporation of labelled amino acids into the single polypeptides was investigated after a pulse labelling in the absence and presence of chloramphenicol, and after washing out the inhibitor.

Chloramphenicol (4 mg/ml) inhibited amino acid incorporation into all polypeptides 90—95 %, while labelling of the whole membrane protein was inhibited only 30 %. After washing out the inhibitor and further growth of the cells, the four smaller polypeptides were highly labelled, whereas the other polypeptides showed only a small increase in radioactivity. It is concluded that the four small-sized polypeptides of cytochrome oxidase are synthesized but not integrated into the functional enzyme under the action of chloramphenicol.

In a previous paper it was shown that the apoprotein of a cytochrome oxidase preparation from *Neurospora crassa* is comprised of several polypeptides, one with an apparent molecular weight of 18000 showing an elevated level of cycloheximide-resistant incorporation of radioactive amino acids [1]. This result suggested that part of cytochrome oxidase is provided by the mitochondrial protein-synthesizing system.

In order to prove that the polypeptide represented by this radioactivity is an essential component of the complex enzyme, the inhibition of amino acid incorporation by chloramphenicol was investigated. In contrast to cycloheximide this antibiotic inhibits mitochondrial but not extramitochondrial protein synthesis [2—4].

In the pulse labelling experiments to be described here, chloramphenicol inhibited amino acid incorporation not only into the polypeptide with a molecular weight of 18000 but also into all other polypeptides. This offered the possibility of studying the assembly of the protein part of cytochrome oxidase.

MATERIALS AND METHODS

Neurospora crassa, wild-type 74 A, was grown in Vogels minimal medium [5] supplemented with 2 % sucrose under aeration [1]. The growth times of the cultures and the labelling procedures are described in the legends to the tables.

D-(—)Chloramphenicol (Bayer, Leverkusen, Germany) was dissolved in 50 % ethanol and 10 ml of the solution was added to 1 l of culture. The final concentrations are given in the legends to the tables. For washing out the chloramphenicol the hyphae were filtered by suction and resuspended in fresh culture medium. This procedure was repeated five times. The specific radioactivities of the ^{3}H and ^{14}C-labelled L-amino acids were 150—1000 Ci/mol.

Cytosolic proteins, mitochondrial membrane proteins and cytochrome oxidase were prepared as described [1]. The purity of cytochrome oxidase was checked by measuring the absorbancies at 280 nm of the oxidized form and at 443 nm of the dithionate-reduced form. The ratio of these absorbancies was 2.1—2.4. The heme *a* contents were determined from difference spectra (reduced minus oxidized) using a millimolar absorbance coefficient $\Delta\varepsilon_{605\,nm} = 24\,mM^{-1}\,cm^{-1}$ [18].

Polyacrylamide gelelectrophoresis in sodium dodecylsulfate was performed as described [1], with the following modifications. A 15 % gel was polymerised with 3 % crosslinker. The samples were dissolved by incubating the protein for 2 h at 0 °C in 5 % sodium dodecylsulfate, 5 % mercaptoethanol and 0.1 M Tris-HCl, pH 8.5, at a concentration of 3 mg/ml. The gel was loaded with 30 to 50 µg protein.

The apparent molecular weights of the proteins were determined by running in parallel on the same gel slab a mixture of bovine serum albumin, lactate dehydrogenase, triosephosphate isomerase, lactalbumin and cytochrome *c*, with molecular weights of 68000, 34000, 27000, 18500 and 11700 respectively.

Protein and radioactivity were determined as described [1].

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

RESULTS

Double Labelling

Throughout the experiments double-labelling procedures were used. Generally, the hyphae were incubated for 1 h with [^{14}C]leucine, [^{14}C]isoleucine and [^{14}C]phenylalanine. Thereafter the same amino acids labelled with ^{3}H were incorporated under special conditions. The general ^{14}C-label of the proteins is found to be stable. The specific ^{14}C radioactivity decreases only due to dilution by newly synthesized proteins during growth. Even in the presence of cycloheximide no measurable breakdown of proteins takes place [6]. After a certain time of labelling, whole cytosolic protein and whole mitochondrial membrane protein of the exponentially growing cells gain the same specific ^{14}C radioactivity. Deviations from this specific radioactivity occur with proteins, which have a large pool of precursor protein. This has been found for some polypeptides of cytochrome oxidase [6]. Nevertheless, in a first approximation the ^{14}C label may be assumed to be proportional to the protein content. In consequence the calculated ^{3}H/^{14}C ratios should have a similar meaning to specific radioactivity.

Kinetics of Amino-Acid Incorporation in the Presence of Chloramphenicol

As seen in Fig. 1, a pulse labelling of the cellular proteins is obtained after adding low concentrations of radioactive leucine, isoleucine and phenylalanine to the culture. The radioactive amino acids are rapidly taken up into the fast-growing cells of *Neurospora crassa* [7], and the cellular pools of these amino acids are low [8].

Labelling of the whole cytosolic proteins is finished after 10 min. After 2 min the label has reached 85% of the final value.

In the absence of chloramphenicol similar labelling kinetics of the cytosolic proteins are found. Here it was shown, that already after 5 min only 2.5% of the added radioactivity is present in the cellular amino acid pool and in the culture medium [6].

By the action of chloramphenicol the labelling of whole mitochondrial membrane protein is reduced compared to the cytosolic proteins. This has been described already for other organisms and has been explained by inhibition of synthesis of the products of mitochondrial protein synthesis [9].

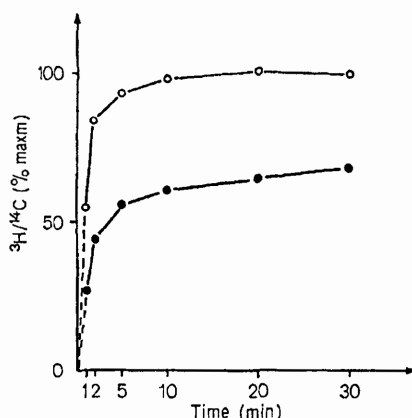

Fig. 1. *Labelling kinetics of cytosolic protein and mitochondrial membrane protein in the presence of chloramphenicol.* Hyphae grown for 17 h were incubated for 1 h with [^{14}C]leucine, [^{14}C]isoleucine and [^{14}C]phenylalanine. Then 4 mg chloramphenicol per ml was added and 5 min later [^{3}H]leucine, [^{3}H]isoleucine and [^{3}H]phenylalanine. At the times indicated, aliquots were mixed with ice and the cytosolic protein (○) and the whole mitochondrial membrane protein (●) were isolated. All ^{3}H values were related to the ^{14}C label, and the maximal ^{3}H/^{14}C ratio of the cytosolic protein was taken as 100%

Inhibition of the Labelling of Cytochrome Oxidase in the Presence of Different Concentrations of Chloramphenicol

In the absence of chloramphenicol (Table 1, first column), after 30 min cytosolic and mitochon-

Table 1. *Labelling of cytosolic protein, mitochondrial membrane protein and cytochrome oxidase in the presence of different concentrations of chloramphenicol*

Hyphae grown for 17 h in a 4 l culture were divided into four 1-l cultures and each was incubated for 1 h with 12.5 μCi [^{14}C]leucine, [^{14}C]isoleucine and [^{14}C]phenylalanine each. Thereafter chloramphenicol was added in the concentrations indicated. 5 min later, to each flask 250 μCi [^{3}H]leucine [^{3}H]isoleucine and [^{3}H]phenylalanine each were added. 30 min after the addition of the ^{3}H-labelled amino acids the cultures were poured on ice and the different fractions were isolated. The specific ^{14}C radioactivities of the cytosolic protein and whole the mitochondrial membrane protein were 85000 counts × min^{-1} × mg^{-1} at all chloramphenicol concentrations. The ^{14}C radioactivities of cytochrome oxidase were 8600, 8100, 7900 and 8200 counts × min^{-1} × nmol^{-1} heme *a* at the chloramphenicol concentrations of 0, 0.5, 1.0 and 2.0 mg/ml respectively

Cellular fraction	^{3}H/^{14}C ratios at chloramphenicol concn of			
	0	0.5	1.0	2.0 mg/ml
Cytosolic protein	6.25	6.3	6.5	6.45
Whole mitochondrial membrane protein	6.3	5.9	5.7	5.5
Total protein of cytochrome oxidase	5.4	3.1	2.1	0.8
Polypeptides of cytochrome oxidase with molecular weights				
36000	5.5			0.31
28000	6.3			0.62
18000	6.4			0.72
13000	3.0			0.61
11000	2.1			0.59
8000	5.3			0.79

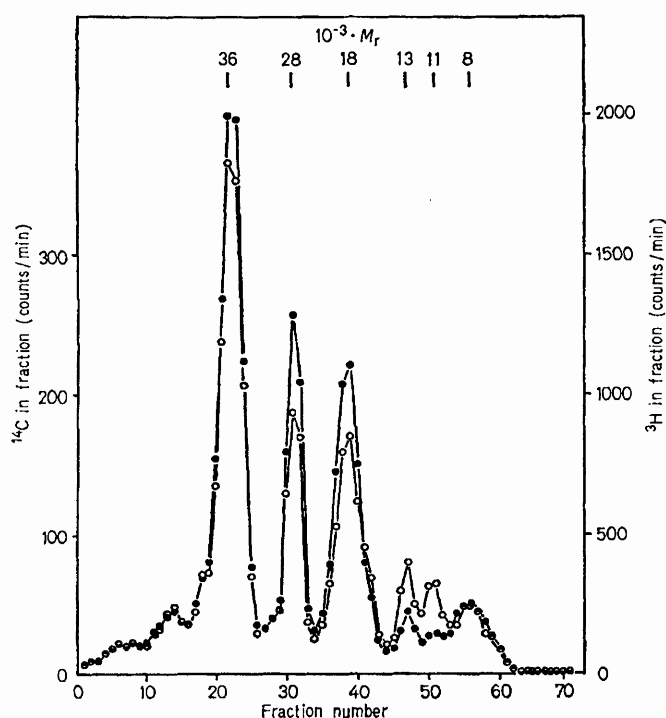

Fig. 2. *Distribution of control label among electrophoretic fractions of cytochrome oxidase.* The cytochrome oxidase preparation was separated as described in the first column of Table 1. (○) ^{14}C radioactivity; (●) ^{3}H radioactivity incorporated during a 30 min labelling period

drial membrane proteins are labelled to the same extent. This is not the case with cytochrome oxidase. To determine the $^{3}H/^{14}C$ ratio of the single polypeptides, cytochrome oxidase was subjected to gel electrophoresis. The distribution of radioactivities among the electrophoretic fractions is shown in Fig. 2. The $^{3}H/^{14}C$ ratios calculated from Fig. 2 are presented in Table 1 (first column). Only the polypeptides with molecular weights of 28000 and 18000 reach the $^{3}H/^{14}C$ ratio of the whole mitochondrial membrane protein, whereas the ratio of the polypeptides with molecular weights of 13000 and 11000 are lower by a factor of about 2. The inhomogeneous labelling of the different polypeptides may be explained by different pool sizes for the different precursor proteins, as described elsewhere [6].

With increasing concentrations of chloramphenicol the labelling of the cytosolic proteins is not affected, while incorporation into the mitochondrial membrane protein is slightly diminished. In contrast, incorporation into cytochrome oxidase is inhibited to a large extent. At a concentration of 2 mg chloramphenicol per ml, labelling of cytochrome oxidase is inhibited 85%. A still higher inhibition is found for the single polypeptides, because in the whole preparation a low percentage of uninhibited protein is present as contamination.

Recovery of Polypeptides of Cytochrome Oxidase with Chloramphenicol-Resistant Labelling, after Washing out the Inhibitor and Further Growth of the Cells

At a concentration of 4 mg chloramphenicol per ml labelling of cytochrome oxidase is inhibited 95%, as seen in Table 2 (first column). The distribution of the ^{14}C and ^{3}H radioactivities among electrophoretic fractions of this preparation are shown in Fig. 3A. The $^{3}H/^{14}C$ ratios of the individual polypeptides calculated from Fig. 3A are presented in Table 2. The inhibition by chloramphenicol is nearly equal for the different polypeptides.

After washing out the chloramphenicol and further growth of the hyphae, the absolute values of the specific ^{14}C radioactivities of the cytosolic and the membrane protein decrease somewhat, as shown in the legend of Table 2. The $^{3}H/^{14}C$ ratios remain nearly constant. In contrast, the $^{3}H/^{14}C$ ratio of cytochrome oxidase increases more than three-fold.

Table 2. *Labelling of cytochrome oxidase in the presence of 4 mg chloramphenicol per ml and changes in the labelling pattern after washing the cells free of the inhibitor and further growth for 1 h*

A 4-l culture of hyphae grown for 17 h was labelled for 1 h with 50 μCi [^{14}C]leucine, [^{14}C]isoleucine and [^{14}C]phenylalanine each. Thereafter 4 mg chloramphenicol per ml was added and 5 min later 1 mCi [^{3}H]leucine, [^{3}H]isoleucine and [^{3}H]phenylalanine each. 30 min after adding the ^{3}H-labelled amino acids 2 l of the culture were poured one ice. The other 2 l were washed free of the chloramphenicol and incubated for another hour before isolation of the different fractions. The specific ^{14}C radioactivity of the cytosolic protein and the whole mitochondrial membrane protein was 80000 cpm/mg before and 68000 counts × min^{-1} × mg^{-1} after washing out the inhibitor. The ^{14}C radioactivities of the cytochrome oxidase were 7800 counts × min^{-1} × nmol heme a^{-1} before and 7100 counts × min^{-1} × nmol heme a^{-1} after washing out

Cellular fractions	$^{3}H/^{14}C$ ratios	
	before washing out of chloramphenicol	after washing out of chloramphenicol
Cytosolic protein	5.0	5.3
Whole mitochondrial membrane protein	3.5	3.7
Total protein of cytochrome oxidase	0.26	0.85
Polypeptides of cytochrome oxidase with molecular weights		
36000	0.2	0.3
28000	0.18	0.4
18000	0.21	0.35
17000	— [a]	2.0
13000	0.25	2.2
11000	0.26	2.3
8000	0.34	1.5

[a] The polypeptide with molecular weight of 17000 is seen clearly only in the wash-out experiment.

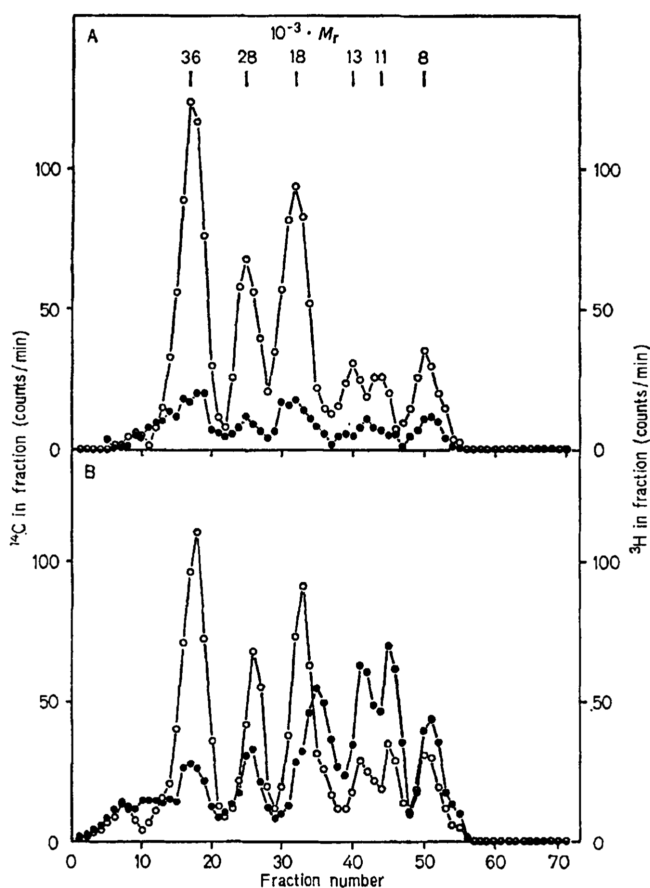

Fig.3. *Distribution of radioactivity among electrophoretic fractions of cytochrome oxidase before and after washing out of chloramphenicol.* The cytochrome oxidase preparations were separated as described in Table 2. (○) ^{14}C radioactivity; (A) (●) ^{3}H radioactivity incorporated during a 30 min labelling period in the presence of 4 mg chloramphenicol per ml; (B) (●) ^{3}H radioactivity after washing out of chloramphenicol and further growth for 1 h

This increase is caused mainly by the five to ten-fold increased ^{3}H label of the small-sized polypeptides (Table 2, second column). The $^{3}H/^{14}C$ ratios of the individual polypeptides were calculated from Fig.3 B, where the distribution of the ^{3}H and ^{14}C radioactivities among the electrophoretic fractions of this cytochrome oxidase preparation are shown. The label of the large polypeptides remains nearly unchanged. The small enhancement may be explained by traces of ^{3}H-labelled amino acids still present in the cells after washing out of chloramphenicol.

The fraction with the molecular weight of 18000 contains two polypeptides. One of them shows an increased label after washing out the chloramphenicol, while the label of the other remains reduced. The smaller polypeptide appears as a shoulder also in the pherogram shown in Fig.1.

DISCUSSION

Some results shown in this report lead to the supposition, that the polypeptides comprising the apoprotein of the membrane-bound enzyme cytochrome oxidase may have different pool sizes of precursor proteins. The problems of these precursor pools will be described in detail elsewhere [6]. The aim of the present study is to show which part the products of mitochondrial protein synthesis may play in the assembly of the functional enzyme.

Chloramphenicol is known to inhibit mitochondrial protein synthesis *in vitro* [2,3,10], as well as cycloheximide-resistant amino acid incorporation *in vivo* [9,11,12]. It is further known that chloramphenicol *in vivo* inhibits the formation of cytochrome oxidase [4,13,11]. The antibiotic simulates the phenotype found in the petite mutants of yeast [14] and the *mi-1* mutant of *Neurospora crassa* [15]. It was therefore concluded that chloramphenicol inhibits the biosynthesis of cytochrome oxidase by inhibiting mitochondrial protein synthesis. Until now, however, it is an unresolved problem how mitochondrial protein synthesis exerts this control on the formation of the membrane-bound enzyme.

Recently we reported that radioactive amino acids are incorporated into a polypeptide of a cytochrome oxidase preparation by the mitochondrial protein-synthesizing system [1]. This finding suggested, that mitochondrial protein synthesis may control the formation of cytochrome oxidase by contributing an essential component to the complex enzyme.

Remarkably, in the short-time experiments described here, chloramphenicol inhibited amino acid incorporation not only into the polypeptide showing cycloheximide-resistant labelling, but into all polypeptides of a functional cytochrome oxidase. It was highly improbable that the synthesis of the polypeptides with extramitochondrial origin was repressed by a regulation process, because the radioactive amino acids were nearly completely incorporated into protein 10 min after addition of chloramphenicol. Indeed under the action of chloramphenicol the accumulation of polypeptides of extramitochondrial origin could be demonstrated. After washing out the inhibitor, cytochrome oxidase became labelled, even though no more radioactive precursor amino acids were present. This shows clearly that under the action of chloramphenicol at least part of the polypeptides of extramitochondrial origin are synthesized but not integrated into the functional oxidase.

This effect can hardly be explained by chloramphenicol inhibition of the synthesis of a catalytic protein active in heme synthesis or in another reaction necessary for the assembly of the cytochrome oxidase. In our experiments the time of exposure to chloramphenicol was only 35 min. Within this time the labelling of all polypeptides of the functional oxidase is nearly completely inhibited. Therefore,

such a postulated catalytic protein should have an extremely fast turnover.

In consequence, it seems reasonable to conclude that chloramphenicol inhibits the assembly of cytochrome oxidase by inhibiting the synthesis of an essential component provided by the mitochondrial protein-synthesizing system.

The inhibition of the labelling of all polypeptides of cytochrome oxidase is explained by a low pool of precursor protein of this component. Such a low pool has been demonstrated for the polypeptide with the molecular weight of 18000 [6], which is labelled in the presence of cycloheximide [1]. After washing out the chloramphenicol, this polypeptide is synthesized in unlabelled form, and it combines with the labelled precursor proteins of extramitochondrial origin, which have accumulated during the 30-min labelling period in the presence of chloramphenicol.

An accumulation of a mitochondrial protein in the cytosol has been described. Mitochondrial ATPase was found in soluble form, when yeast cells were incubated with chloramphenicol during glucose derepression [16]. In the cytoplasm of *Neurospora crassa* crystals of an unknown protein were found, when the cells were grown in the presence of ethidium or euflavine [17].

For the cytochrome oxidase preparation, as described in the present report, the site of synthesis of the polypeptides with molecular weights of 18000, 17000, 13000, 11000 and 8000 is established by positive results. The first one becomes labelled in the presence of cycloheximide [1] and the four others are labelled in the presence of chloramphenicol. The conclusions drawn in the previous paragraphs apply at least to these five polypeptides, which amount to 50% of the total protein of the preparation. Cytochrome oxidase as defined in the literature (see [1]) contains about 10 μmol heme *a* per g protein. The cytochrome oxidase preparation described in this report fulfills this criterian of purity [1]. Therefore, it seems likely that these polypeptides are an integral part of the enzyme.

The site of synthesis of the polypeptides with molecular weights of 36000 and 28000 is still uncertain. The inhibition of labelling by chloramphenicol suggests a mitochondrial origin, but the labelling in the presence of cycloheximide varies and is never as high as the labelling of the polypeptide with the molecular weight of 18000. If these polypeptides are synthesized on extramitochondrial ribosomes inhibition of labelling in the presence of chloramphenicol could be explained on the basis of the following assumptions: either their synthesis may be repressed by a fast regulatory process after inhibition of mitochrondial protein synthesis, or they may be synthesized in the presence of chloramphenicol, but the accumulated polypeptides cannot be utilized after restoration of normal growth. Alternatively, these components may be synthesized on mitochondrial ribosomes. Inhibition of labelling in the presence of cycloheximide does not necessarily disprove a mitochondrial origin of these polypeptides. It has been shown, that the two components have a larger pool of precursor polypeptides than the component with the molecular weight of 18000 [6]. In the enzyme complex label will appear more slowly, because the newly synthesized polypeptides are diluted by a larger pool. Possibly the cycloheximide-resistant protein synthesis stops before these two components in the enzyme complex are significantly labelled. In yeast cytochrome oxidase prepared by immunoprecipitation, cycloheximide-resistant labelling was reported for three polypeptides with molecular weights of 42000, 34500 and 23000 [19]. By this technique also labelled precursor polypeptides might be precipitated in addition to the completed cytochrome oxidase. This would explain the difference in the labelling patterns of a cytochrome oxidase prepared by chromatography [1] and by immunoprecipitation, provided that the two large polypeptides of the yeast enzyme correspond to the two large polypeptides of the *Neurospora* enzyme.

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