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Different Pool Sizes of the Precursor Polypeptides of Cytochrome Oxidase from *Neurospora crassa*

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Pulse-labelling experiments with growing *Neurospora crassa* revealed that the polypeptides composing the protein moiety of a cytochrome oxidase preparation are derived from at least four independent pools of precursor polypeptides. The pool sizes range from 2% to 25% of the amount of the corresponding polypeptide present in cytochrome oxidase. The smallest pool is assigned to a polypeptide of mitochondrial origin. Serial pools were found for one of the polypeptides.

It has been recently shown that cytochrome oxidase prepared from *Neurospora crassa* by means of a chromatographic method [1] contains several polypeptides which are partly of cytoplasmic and partly of mitochondrial origin [2,3]. In this report it will be shown that after pulse-labelling of cells with radioactive leucine, the appearance of label follows a different time-course for each of these polypeptides. It is concluded that cytochrome oxidase is assembled from pools of precursor polypeptides of different sizes.

METHODS

Determination of Trichloroacetic-Acid-Insoluble Radioactivity

Neurospora crassa wild-type 74 A was cultured as described previously [4]. A 2-l culture of exponentially growing cells (approximately 1.6 g protein) was divided into 50-ml portions. To each portion, 2.5 µCi L-[U-^{14}C]leucine (330 mCi/mmol) was added. Cycloheximide (0.1 g/l) was added when indicated. After different times the cells were harvested by filtration and frozen immediately by pressing between two aluminium blocks precooled with liquid nitrogen.
The cells were ground in a mortar in the presence of liquid nitrogen. The frozen cell powder was mixed with 2 ml trichloroacetic acid (8% in 50% ethanol), the resulting slurry was warmed to 5 °C and centrifuged. The pellet was extracted another three times with trichloroacetic acid (6% in water), once with acetone, and dissolved in formic acid by means of sonication. Radioactivity was measured in this solution, and protein was determined by the Lowry method after evaporation of the formic acid.

Determination of the Size and the Radioactivity of the Leucine Pool

The trichloroacetic acid extracts were combined. Ethanol and the bulk of trichloroacetic acid were removed *in vacuo* over KOH. The amino acids were purified by means of a cation exchanger [5]. In the resulting mixture of amino acids, radioactivity was measured and the leucine content was determined chromatographically.

Double Labelling

To an 8-liter culture of cells (approximately 6 g protein), 250 µCi L-[U-^{14}C]leucine was added, followed after 200 min by 2 mCi L-[G-^{3}H]leucine (250 mCi/mmol). One-liter portions were taken from the culture at distinct intervals and were harvested and frozen as described above.

Isolation and Gel Electrophoresis of Cytochrome Oxidase

Procedures described elsewhere were applied [1–3].

Determination of Radioactivity

The counting efficiency of the liquid scintillation system used [2] was 27% for ^{3}H and 61% for ^{14}C. The ^{3}H/^{14}C ratio given in the results are uncorrected ratios of measured radioactivity.

Definition. A_{280} unit, the quantity of material contained in 1 ml of a solution which has an absorbance of 1 at 280 nm when measured in a 1-cm path-length cell.

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

RESULTS

Pulse Labelling of Proteins

Cells were incubated with 0.19 μmol [^{14}C]leucine/g protein. This quantity will add about 5% to the cellular free-leucine pool (4 μmol/g protein). The time-course of distribution of the label between the culturing medium, the leucine pool, and the total protein represented by the trichloroacetic-acid-insoluble material is shown in Fig. 1A. Within two minutes 95% of the label disappears from the culturing medium. The radioactivity present in the cellular pool of free leucine increases during the first minute and decreases subsequently to a negligible value (2% of the total radioactivity added) while the absolute amount of leucine present in the pool remains constant (Fig. 1B). The protein is rapidly labelled after a short lag of 0.5 min; after 5 min it contains 85% of the total radioactivity.

Obviously, the radioactive leucine which is taken up rapidly by an active transport mechanism [6] is constantly diluted by non-radioactive leucine produced by the cells, the leucine pool serving as a precursor pool for protein synthesis. The whole label is incorporated during a period of a few minutes, leading to a pulse-labelling of polypeptides.

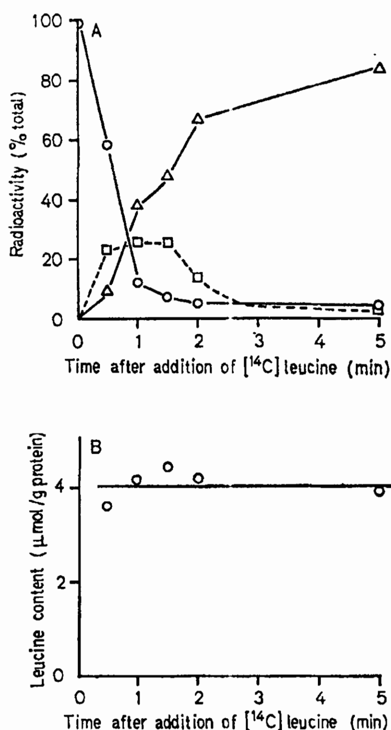

Fig. 1. *Pulse labelling of cellular proteins by addition of [^{14}C]-leucine to the culture medium.* (A) Time-course of radioactivity in the culture medium (O——O), in the cellular free-leucine pool (□- - - -□), and in the total protein (acid-insoluble radioactivity) (△——△). (B) Time course of the absolute size of the leucine pool

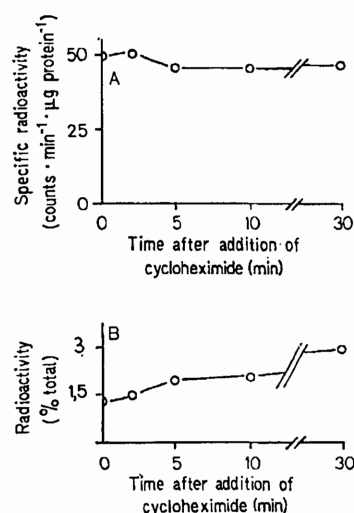

Fig. 2. *Stability of label of the cellular proteins in the presence of cycloheximide.* The drug was added 15 min after pulse-labelling of the proteins with [^{14}C]leucine. (A) Time-course of the specific radioactivity of total protein. (B) Time-course of radioactivity in the cellular free-leucine pool

In order to establish the stability of the label present in proteins, cells were incubated with cycloheximide 15 min after labelling with [^{14}C]leucine. Although protein synthesis is blocked to 98% by this antibiotic [2], the label present in the acid-insoluble material remains constant during 30 min (Fig. 2A). No significant amount of radioactivity is released into the free amino acid pool during this time (Fig. 2B). Consequently, protein breakdown is negligible in exponentially growing *Neurospora* cells.

Difference in Labelling Kinetics between Total Membrane and Cytochrome Oxidase

In the experiments described in this section, cells were treated first with a pulse of [^{14}C]leucine. Most of the ^{14}C label will be at its definitive position after 200 min. At this time, [^{3}H]leucine was added. In a first approximation, the ^{3}H/^{14}C ratios measured in proteins will correspond to relative specific ^{3}H radioactivities. The time-course of the ^{3}H/^{14}C ratios in the mitochondrial membranes and in the isolated cytochrome oxidase is presented in Fig. 3A. In the mitochondrial membranes, the ^{3}H/^{14}C ratio shows a rapid increase within the first 15 min after addition of the ^{3}H label and then remains at a constant level of 3.2. When this ratio is corrected for counting efficiencies (*cf.* Methods section), a value of 7.6 is obtained which is in accordance with the ratio of 8 of the ^{3}H and ^{14}C radioactivities added initially to the culture. In contrast to the average membrane protein, the cytochrome oxidase reaches the level of 3.2 only after 50 min.

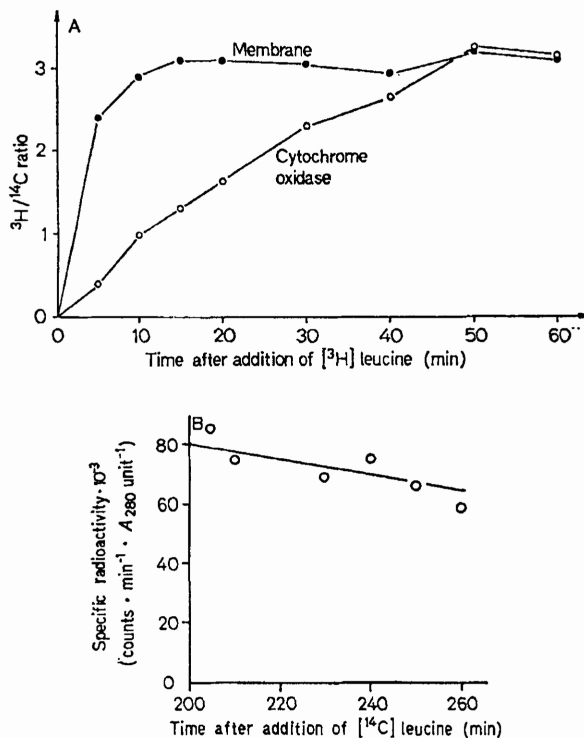

Fig.3. *Time-course of labelling of the total mitochondrial-membrane protein and of cytochrome oxidase.* [^{3}H]Leucine was added 200 min after the addition of [^{14}C]leucine. (A) $^3H/^{14}C$ ratio in the mitochondrial membranes (●——●) and in cytochrome oxidase (○——○). (B) Specific ^{14}C radioactivity of cytochrome oxidase. The circles represent the experimental data. The straight line represents the theoretical decrease calculated from the cellular growth rate (doubling-time 200 min)

During this time, the specific ^{14}C radioactivity of cytochrome oxidase decreases slowly (Fig.3B). As a consequence of growth, the total amount of cytochrome oxidase increases leading to a dilution of label. The decrease of specific radioactivity calculated from the doubling-time of cellular growth (200 min) is represented by the straight line included in Fig.3B. The coincidence of this line with the experimental points indicates that an eventual turnover of cytochrome oxidase is negligible.

Appearance of Labelled Polypeptides in Cytochrome Oxidase

In order to study the time-course of the appearance of labelled polypeptides in cytochrome oxidase, the enzyme preparations from the cells harvested at eight different times after addition of [^{3}H]leucine (*cf.* Fig.3) were submitted to gel electrophoresis. The distribution of radioactivity over the gels is shown in Fig.4 for three of these preparations. In this figure, the scales of ^{14}C are expanded relative to those of ^{3}H by a factor of 3.2, corresponding to the final $^3H/^{14}C$ ratio of the proteins (*cf.* preceding sections).

Six maxima of radioactivity can be distinguished corresponding to polypeptides with apparent molecular weights of 8000, 11000, 13000, 28000, and 36000. This is in agreement with earlier results [2], except the appearance of an additional polypeptide with an apparent molecular weight of 36000. This polypeptide can be detected only by dissolving the cytochrome oxidase in detergent solution at 0 °C instead of 37 °C before electrophoresis [3].

The distribution patterns of ^{14}C-radioactivity are similar after different times. This excludes the possibility that single polypeptides show a turnover comparable to their net increase due to the cellular growth.

Five minutes after addition of the [^{3}H]leucine (Fig.4A), the ^{3}H radioactivity is found predominantly in the 18000 molecular weight peak, which approaches the final $^3H/^{14}C$ ratio of 3.2 after 10 min (Fig.4B).

Within a 40-min period, this ratio is reached by four of the six polypeptides, as revealed by the coincidence of the ^{3}H and the ^{14}C radioactivity curves (Fig.4C).

The different shapes of the ^{3}H and the ^{14}C radioactivity curves in the 18000 molecular weight peak may be explained by the presence of a minor polypeptide with an apparent molecular weight of 17000 [3].

Half-Lives of Precursors

The time-courses of appearance of labelled polypeptides in the enzyme complex are summarized in Fig.5.

It may be assumed that all polypeptides have a common pool of precursor leucine. Under the conditions applied in the present experiments, where the amount of [^{3}H]leucine added to the culture (1.6 µmol/g protein) is of the same order of magnitude as the cellular leucine pool (4 µmol/g protein, see Fig.1B), half of the [^{3}H]leucine added to the culture will be integrated into polypeptides within 2—3 min. The label found in the mitochondrial membrane proteins is half-maximal after 3 min (Fig.3A). Hence the time needed for the synthesis of these proteins on the ribosomes and for their transfer to the mitochondrial membranes is less than 1 min. This is in accordance with the high translation rates reported for *Escherichia coli* [7] and for isolated reticulocytes [8,9]. It is reasonable to assume an equally high translation rate for the polypeptides of cytochrome oxidase. The slower labelling rate of this enzyme suggests an assembly from pools of precursor polypeptides.

The amounts of these precursors were estimated from the time-course of appearance of labelled polypeptides in the cytochrome oxidase. In the case that only one precursor is involved in the formation of a given polypeptide of cytochrome oxidase, the half-life of this precursor will be given by the time needed until the label present in the end product is half-maximal. In the calculation of the half-lives presented in Table 1, the effect of the leucine pool was accounted for by subtracting from this time the time needed for half-maximal labelling of the mitochondrial membrane proteins (3 min). The effect of precursor polypeptide pools may be neglected for these proteins, since the label appears in the mito-

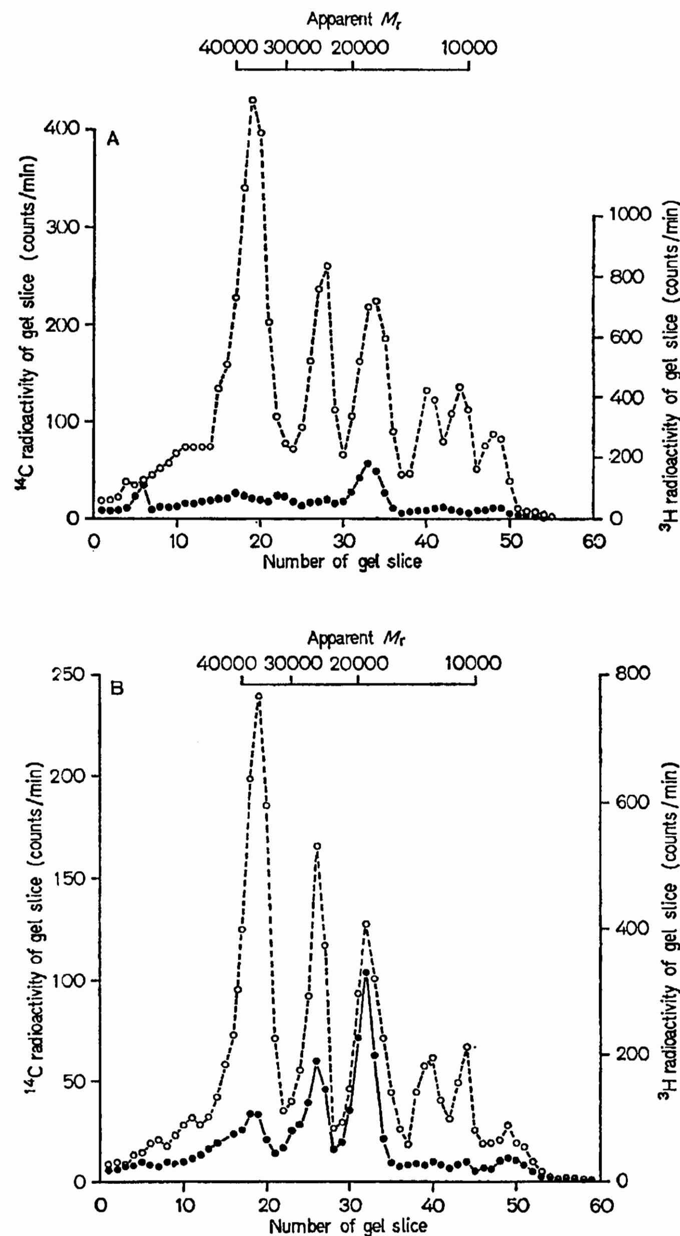

Fig. 4. *Distribution of ^{3}H (●——●) and ^{14}C (O----O) radioactivity over the gels after electrophoretic separation of cytochrome oxidase.* Three of the cytochrome oxidase preparations from Fig. 3 are shown, derived from cells harvested (A) 5 min, (B) 10 min, and (C) 40 min after addition of [^{3}H]-leucine. The ^{14}C scales are expanded relative to the ^{3}H scales by a factor of 3.2. The radioactivity scales were adjusted so that the areas under the ^{14}C curves are equal

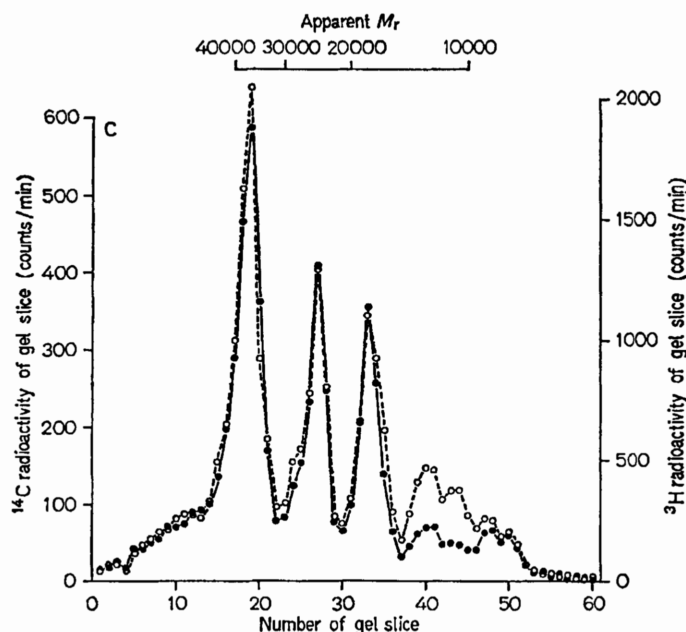

Fig. 4

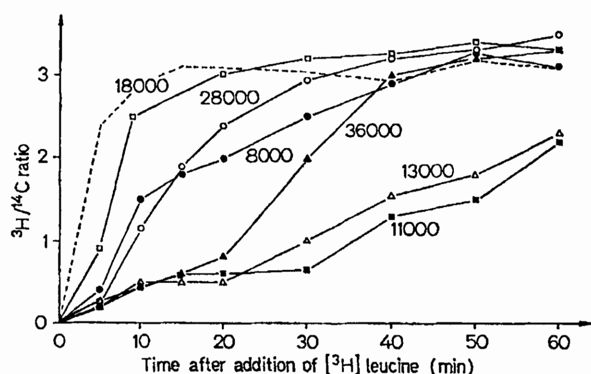

Fig. 5. *Time-course of the $^3H/^{14}C$ ratio in the polypeptides of cytochrome oxidase.* For labelling conditions see Fig. 3. The numbers indicate the apparent molecular weights of the polypeptides. The $^3H/^{14}C$ ratio of the mitochondrial membranes (*cf.* Fig. 3A) is included for comparison (----)

Table 1. *Half-lives and sizes of precursor pools of the polypeptides of cytochrome oxidase*
The pool sizes are given as a percentage of the amount of the corresponding polypeptide present in cytochrome oxidase

Apparent molecular weight	Half-life of precursor	Pool size of precursor
	min	%
8000	7	4
11000	47	25
13000	37	20
18000	3.5	2
28000	10	5
36000	(23)[a]	(10)[a]

[a] In the case of this polypeptide, two or more precursor pools are connected in series. The values given in the table correspond to the sum of half-lives or pool sizes.

chrondrial membrane with a delay of less than 0.5 min compared to the whole acid-insoluble material (unpublished observation).

The 8000 and the 28000 molecular weight polypeptides show similar time-courses of appearance of label and might therefore be derived from a common precursor pool. The same is true for the 11000 and the 13000 molecular weight polypeptides. In any case, at least four independent pools of precursor polypeptides exist. The sigmoid time-course of the appearance of labelled 36000 molecular weight polypeptide suggests that in this case, at least two pools of precursors in series are involved.

Pool Sizes of Precursors

As already mentioned, no protein breakdown and no turnover of cytochrome oxidase can be detected in the exponentially growing cells used in these experiments. Hence, the turnover rate of a precursor pool is equal to the rate of net increase of the product. The total cell mass doubles in approximately 200 min, and the total amount of cytochrome oxidase

doubles in the same time. The ratio of the amount of a precursor to that of the corresponding end product will then be equal to the ratio of the half-life of the precursor to the doubling-time of the whole culture. The effect of the net increase of the precursor pool may be neglected in this evaluation. The pool sizes are given in Table 1 as a percentage of the amount of the corresponding polypeptide present in cytochrome oxidase.

DISCUSSION

The results presented here verify the occurrence of at least four independent pools of precursor polypeptides in the biogenesis of cytochrome oxidase. From earlier results it follows that these precursors are synthesized in two different compartments, mitochondria and cytoplasm [2,3].

Due to the complexity of this enzyme protein it is probable that its assembly occurs in several steps. The precursors could therefore represent not only single polypeptides, but also molecules composed of several polypeptides, or modified forms. This view would include that several precursor pools in series may be involved in the formation of a given subunit. If these pools are of comparable size, a sigmoid time-course of appearance of label will result, as in found for the 36000 molecular weight polypeptide. In contrast, serial pools of largely divergent sizes would not be detected by the experimental methods applied. In any case, no information concerning the sequence of assembly can be obtained from kinetic data involving only the end product.

As shown previously [2], the polypeptide with an apparent molecular weight of 18000 is of mitochondrial origin. The low precursor pool size of this polypeptide would be consistent with a direct transfer of a newly synthesized peptide chain from the mitochondrial ribosomes to the mitochondrial membrane as the rate-limiting step in the assembly of cytochrome oxidase. Supposing that this polypeptide occurs once per heme group, the absolute pool size for this polypeptide calculated from a cytochrome aa_3 content of 0.24 μmol/g mitochondrial protein will be 0.01 μmol/g mitochondrial protein. This is of the same order of magnitude as the ribosome content of mitochondria, which is approximately 0.02 μmol/g mitochondrial protein (Neupert & Werner, personal communication).

The bulk of the mitochondrial membrane proteins are synthesized on the cytoplasmic ribosomes [10,11]. Although a direct interaction of these ribosomes with the mitochondrial membranes is improbable, most of the proteins of the mitochondrial membrane appear in a pulse-labelled form more rapidly than the fastest components appear in cytochrome oxidase. Thus, the transfer of polypeptides from the cytoplasmic ribosomes to the mitochondrial membrane is generally a rapid process and might not be the rate-limiting step for the assembly of cytochrome oxidase.

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