IS THE MITOCHONDRIALLY MADE SUBUNIT 2 OF CYTOCHROME OXIDASE SYNTHESIZED AS A PRECURSOR IN *NEUROSPORA CRASSA*?

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1. Introduction

Amino acid sequencing of subunits of cytochrome oxidase from beef heart has revealed that the mitochondrially made subunit II of the enzyme starts with formyl-methionine at the amino terminus [1]. This result is consistent with the identification of *N*-formyl-methionine as a residue present in nascent polypeptides, as well as in 'intrinsic' membrane proteins of yeast mitochondria [2,3]. No information is available indicating that, similar to bacteria, a post-translational removal of the formyl residues, or/and a subsequent processing of the amino ends of the polypeptide chains takes place in mitochondria prior to the integration into functional membrane complexes.

We have obtained strong evidence, both from kinetic experiments [4] and from amino acid sequence data (W.M., S.W., in preparation) that the mitochondrially synthesized subunit 1 of the *Neurospora* oxidase is formed from a larger precursor polypeptide extended at the amino terminus. Here we present the partial N-terminal sequence of subunit 2 of the *Neurospora* enzyme suggesting the existence of a higher molecular weight precursor also for this membrane component.

Abbreviations: SDS, sodium dodecylsulfate; PTH, phenylthiohydantoin; HPLC, high pressure liquid chromatography

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

This paper is dedicated to Professor Th. Bücher on the occasion of his 65th birthday

2. Experimental

2.1. *Cultivation of cells and mitochondrial preparations*

Neurospora crassa wild-type strain 74 A was grown under unsterile conditions on a large scale [5], or under sterile conditions in 100–250 ml cultures for labelling experiments [6], as described. The preparation of submitochondrial particles for the enzyme isolation [5] and of crude mitochondrial fractions for immunoprecipitations [7] have been reported.

2.2. *Preparation of subunit 2 from cytochrome oxidase*

Cytochrome oxidase was prepared by ammonium sulphate precipitation in the presence of potassium cholate. Subunit 2 (M_r 28 000) of the dissociated enzyme was separated from the other subunits on a Sephadex G-100 column, equilibrated with 2% SDS in water. Details will be published elsewhere (W.M. et al. in preparation).

2.3. *Isolation of radioactively labelled unassembled subunit 2*

Selective labelling of mitochondrial protein in the presence of cycloheximide was performed by addition of tritiated leucine, isoleucine and tryptophan, respectively, to the cultures [6]. For uniform labelling of cells [^{14}C]leucine was applied. Direct immunoprecipitation of cytochrome oxidase [6], the

isolation of unassembled subunit 2 by a protein A/Sepharose technique [8], as well as analytical procedures, such as polyacrylamide gel electrophoresis [5] and determination of radioactivity [7] have been detailed.

2.4. *Solid-phase sequence analysis*

Subunit 2 (100 nmol) was covalently attached to *p*-phenylene diisothiocyanate-activated aminopropyl glass via its lysine sidechains. Activated glass beads were prepared from controlled-pore glass (CPG 10–240) as in [9]. Coupling of the protein was performed in 2% SDS adjusted to pH 8–9 with solid $NaHCO_3$ for 15 h at 25°C. The immobilized protein was sequenced by automated Edman degradation in a non-commercial solid-phase sequencer [10]. The resulting PTH amino acids were identified in a high performance liquid chromatograph operating on-line with the sequencer [11].

Trace amounts of radioactively labelled unassembled subunit 2 were coupled and degraded after mixing with the unlabelled subunit 2 (100 nmol). The separated HPLC-peaks corresponding to PTH-tryptophan, PTH-isoleucine and PTH-leucine, respectively, were collected and radioactivity was determined by liquid scintillation counting.

3. Results and discussion

The N-terminal amino acid sequence obtained by solid-phase Edman degradation of subunit 2 isolated from *Neurospora* cytochrome oxidase is shown in fig.1. Residues 1–39 were identified unambigously from quantitated HPLC. An averaged repetitive yield of 93% was calculated from the in-step signals of Phe^{10} and Phe^{37} after background subtraction. The mean overlap from step *n* to step *n*+1 was 1.1% per step. Details will be presented elsewhere [11].

In the coupling technique used here, most of the N-terminal residue remains attached to the solid support [12]. This particular residue was identified from trace amounts of unbound PTH-aspartic acid found in the first degradation cycle and was confirmed by dansylation of the protein.

The complete amino acid sequence of subunit II (M_r 24 000) of cytochrome oxidase from beef heart has been published [1]. Residues 1–38 of this protein are included in fig.1 for comparison with the *Neurospora* sequence. In contrast to the corresponding beef heart protein, subunit 2 from *Neurospora* does not begin with *N*-formyl-methionine, but has an 'open' sequence with a N-terminal aspartic acid. No PTH-methionine was detected after incubation of the

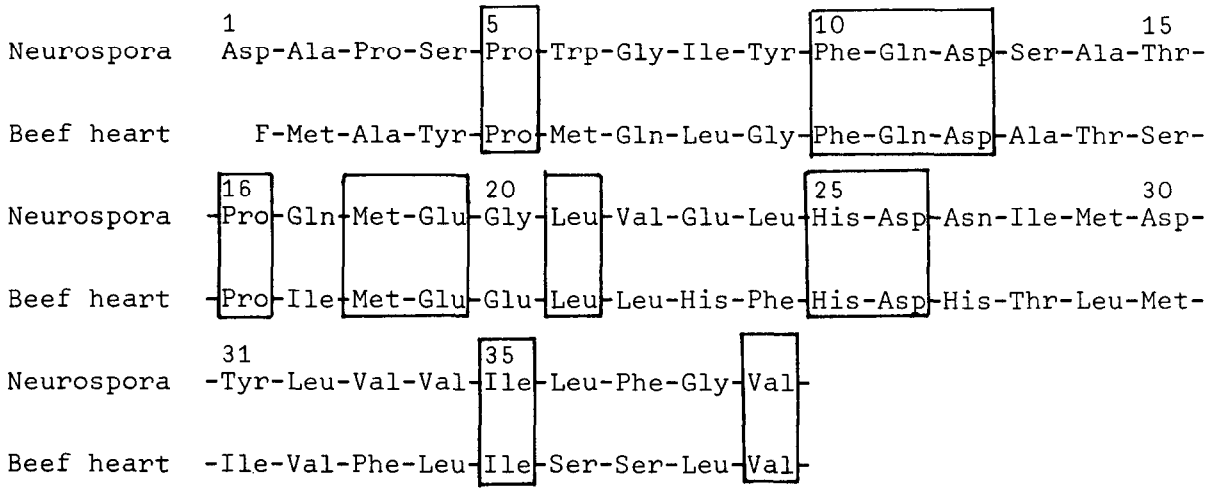

Fig.1. N-terminal amino acid sequences of subunit 2 of cytochrome oxidase from *Neuropora crassa* and of subunit II from the beef heart enzyme [1].

glass-bound protein in methanolic HCl under deformylating conditions [1]. Besides this essential difference, the partial sequence of the *Neurospora* subunit shows a significant homology to positions 1–38 of the beef heart protein. Identical residues are found in 12 out of 38 positions (fig.1).

These results suggest that *Neurospora* subunit 2 may be translated as an extended precursor protein beginning with *N*-formyl-methionine. The precursor could be processed into the mature subunit of the functional oxidase complex by the proteolytic release of its N-terminal fragment. This fragment may be solely one methionine residue as it has been shown for the ovalbumin precursor [13], or it may comprise a longer pre-sequence as described for the precursors of many secretory proteins, as well as bacterial membrane proteins [14].

At what stage of the subunit formation could a processing of the putative precursor occur in mitochondria? In order to reach a rough assessment of the timing of the assumed cleavage of the mitochondrial product, unassembled (free) subunit 2 was labelled in vivo with various amino acids in the presence of cycloheximide. Then, the polypeptide was isolated from the cell extracts using a subunit-specific antibody and protein A/Sepharose. Figure 2 shows the gel electrophoretic analysis of the unassembled subunit 2 obtained under these experimental conditions. The polypeptide migrates on the SDS–gel exactly at the position of the corresponding marker subunit derived from the immunoprecipitated holoenzyme. It is clear, however, that small differences in the apparent molecular weights cannot be detected by the applied technique. On the other hand, sequencing of the radioactively labelled unassembled polypeptide revealed that the incorporated [^{3}H]leucine, as well as [^{3}H]tryptophan and [^{3}H]isoleucine (see fig.3) were recovered at the same degradation steps as at those in which the corresponding amino acids of unlabelled subunit 2 from the holoenzyme were identified. This result supplies strong evidence for identical N-terminal sequences of both polypeptides. Thus, the predicted processing of a larger molecular weight precursor might occur not only very rapidly in the wild-type organelle, but also at a very early (possibly co-translational) stage of the subunit formation.

The idea of the existence of a higher molecular

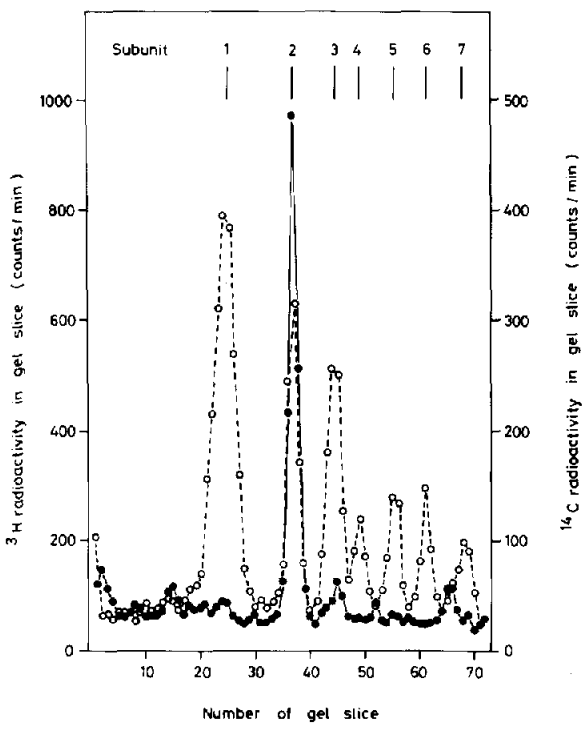

Fig.2. Gel electrophoretic analysis of unassembled subunit 2 of cytochrome oxidase. Cells were labelled in the presence of cycloheximide with [^{3}H]leucine. Mitochondria were isolated and solubilized with Triton X-100. The extract was treated successively with an antiserum to subunit 2 and protein A/Sepharose. The material released from the Sepharose support was mixed with cytochrome oxidase immunoprecipitated from cells labelled uniformly with [^{14}C]leucine and then applied to a 15% polyacrylamide gel containing SDS. (●——●) ^{3}H radioactivity; (○– – –○) ^{14}C radioactivity.

weight precursor for the enzyme subunit 2 is also supported by recent findings with the *mi-3* mutant of *Neurospora*. It has been shown that another mitochondrially made subunit, namely the oxidase subunit 1 (M_r 41 000), is synthesized in the form of a larger precursor polypeptide ($M_r \sim 45\,000$), which can be processed to a mature enzyme subunit [4]. This larger precursor polypeptide was isolated and subjected to amino acid sequence analysis. It was found that the, obviously 'blocked', N-terminal piece of the precursor protein is not identical with that of the mature subunit. On the other hand, similar to the observation with subunit 2, the mature subunit 1 also displays an

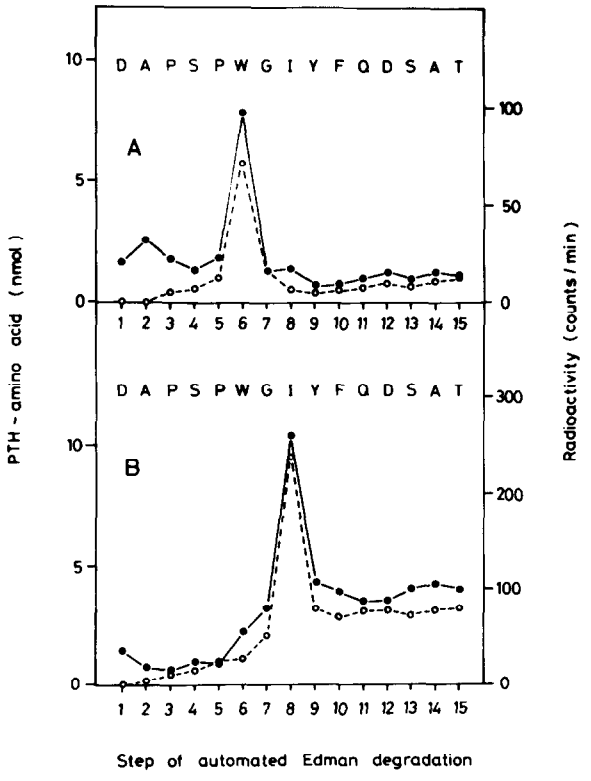

Fig.3. Yields of PTH-tryptophan (A) and PTH-isoleucine (B) obtained in solid-phase Edman degradation (step 1–15) of subunit 2 from *Neurospora* cytochrome oxidase. PTH yields (o---o) during the degradation of unlabelled subunit 2 isolated from the holoenzyme were calculated from the peak areas of HPLC. Radioactivity (•——•) resulting from the degradation of unassembled subunit 2 labelled with [^{3}H]tryptophan (A) and [^{3}H]isoleucine (B), respectively, was determined in the HPLC eluates corresponding to these peaks. The amino acid sequence of subunit 2 is depicted in the upper parts of the panels.

'open' sequence, beginning in this case with serine (W.M., S.W., in preparation).

The most rapid decision, whether or not the postulated precursor of subunit 2 is true in the proposed form, could arise from a comparison of the base sequence of the corresponding mitochondrial DNA fragment with the amino acid sequence data presented here.

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References

[1] Steffens, G. J. and Buse, G. (1979) Hoppe Seyler's Z. Physiol. Chem. 360, 613–619.
[2] Mahler, H. R., Dawidowicz, K. and Feldman, F. (1972) J. Biol. Chem. 247, 7439–7442.
[3] Feldman, F. and Mahler, H. R. (1974) J. Biol. Chem. 249, 3702–3709.
[4] Werner, S. and Bertrand, H. (1979) Eur. J. Biochem. in press.
[5] Werner, S. (1977) Eur. J. Biochem. 79, 103–110.
[6] Werner, S. (1974) Eur. J. Biochem. 43, 39–48.
[7] Bertrand, H. and Werner, S. (1977) Eur. J. Biochem. 79, 599–606.
[8] Werner, S. and Machleidt, W. (1978) Eur. J. Biochem. 90, 99–105.
[9] Machleidt, W. and Wachter, E. (1977) Methods Enzymol. 47, 263–277.
[10] Machleidt, W., Hofner, H. and Wachter, E. (1975) in: Solid-Phase Methods in Protein Sequence Analysis (Laursen, R. A., ed.), pp. 17–30, Pierce Chemical Co., Rockford, IL.
[11] Machleidt, W. and Hofner, H. (1979) in: Proc. 3rd Int. Conf. Solid Phase Methods in Protein Sequence Analysis, submitted.
[12] Laursen, R. A. (1977) Methods Enzymol. 47, 277–288.
[13] Palmiter, R. D., Gagnon, J. and Walsh, K. A. (1978) Proc. Natl. Acad. Sci. USA 75, 94–98.
[14] Austen, B. M. (1979) FEBS Lett. 103, 308–313.