M_r-VALUES OF MATURE SUBUNITS I AND III OF BEEF HEART CYTOCHROME *c* OXIDASE IN RELATIONSHIP TO NUCLEOTIDE SEQUENCES OF THEIR GENES

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1. Introduction

Several hydrophobic polypeptides of the electron-transport and ATP-synthesizing complexes are coded for in the mitochondrial genome and synthesized on mitochondrial ribosomes. For example, cytochrome *c* oxidase (EC 1.9.3.1) is a complex containing 3 mitochondrially synthesized polypeptides (subunits I–III) and 4 cytoplasmically synthesized components (IV–VII) [1–3].

The sequences of both the human [3] and bovine (S. Anderson, personal communication) mitochondrial genomes have been determined. From these sequences, the amino acid sequences of the large subunits of cytochrome *c* oxidase have been deduced [3]. The genes for subunits I and III of the mammalian cytochrome *c* oxidases code for polypeptides of much higher M_r-value than had been expected from their migration on sodium dodecyl sulphate (SDS)–polyacrylamide gels. There are 3 possible explanations for this:

(i) Subunits I and III could be made as large precursors which are then processed to smaller polypeptides.
(ii) The genes for subunits I and III could be shorter than now thought. There is no obvious termination codon for any of the mitochondrial genes and therefore the end of any gene is difficult to predict [3].
(iii) The subunits could be as large as predicted from their gene sequences but behave anomolously on SDS–polyacrylamide gel electrophoresis.

Here we describe studies to decide among these possibilities. Our results indicate that there is little or no processing of these polypeptides. Rather, subunits I and III are much larger than indicated by SDS–polyacrylamide gel electrophoresis.

2. Materials and methods

Cytochrome *c* oxidase was prepared from beef heart as in [4]. 2-Nitro-5-thiocyanatobenzoic acid (NTCB) was synthesized as in [5]. This reagent was reacted with denatured cytochrome *c* oxidase (30 μM total heme) at a molar ratio of 4:1 (NTCB:aa_3) for 72 h in 8 M urea, 5% SDS 0.1 M Tris-acetate (pH 8.5) at 37°C.

Labelling with iodo[^{14}C]acetamide (400 μM, 0.16 Ci/mol) was conducted on denatured cytochrome *c* oxidase (5 μM total heme) for 30 min at room temperature: the reaction was then quenched with 1 mM 2-mercaptoethanol. Samples (~100 μg) were electrophoresed on a 16% SDS–urea polyacrylamide gel with a stacking gel. This was stained for protein, sliced and prepared for counting as in [6]. The subunits are numbered according to [6] after identification on the basis of iodo[^{14}C]acetamide labelling [7].

The amino acid composition of subunit III was determined after acid hydrolysis for 44 h. The concentration of Trp and Tyr was calculated by the method in [8].

3. Results and discussion

The M_r-values of subunits I and III predicted from the DNA sequence are 57 057 and 29 945, respectively (S. Anderson, personal communication). Their app. M_r-values from SDS–polyacrylamide gel electrophoresis are 35 700 and 21 000, respectively [7]. This

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raises the possibility that both subunits are made as large precursors which are subsequently processed to smaller, mature polypeptides. Such processing cannot occur at the N-terminus because the mature subunits each retain an *N*-formyl-methionine group [9]. Experiments were therefore aimed at determining whether any processing occurred from the C-terminus.

The DNA sequence of the bovine cytochrome *c* oxidase subunit I gene predicts the presence of 1 cysteine residue only, positioned 17 amino acids from the C-terminus (S. Anderson, personal communication). We therefore used cysteine specific reagents to probe the C-terminal segment of this subunit. Fig.1A shows the results of iodo[^{14}C]acetamide labelling of denatured cytochrome *c* oxidase. Subunits I–III, V and VII are labelled as well as contaminants *a* and *c*. Subunit IV which does not contain any cysteine residues is not labelled [10]. Studies with *N*-ethylmaleimide [11] and *N*-(1-anilinonapthyl-4)maleimide [12] also showed labelling of subunit I. The demonstration of a cysteine residue in mature subunit I shows that this polypeptide must be at least 498 residues long [3]. To determine the length of the polypeptide chain following this cysteine we used NTCB, a reagent which cleaves the polypeptide chain specifically at sulphydryl groups [5]. Fig.1B shows that subunit I has been cleaved by NTCB resulting in a product 1000–2000 M_r smaller than native subunit I. The size of the cleaved product correlates well with loss of a seventeen amino acid polypeptide. We conclude therefore that the M_r of subunit I is close to that predicted by the DNA sequence of the gene.

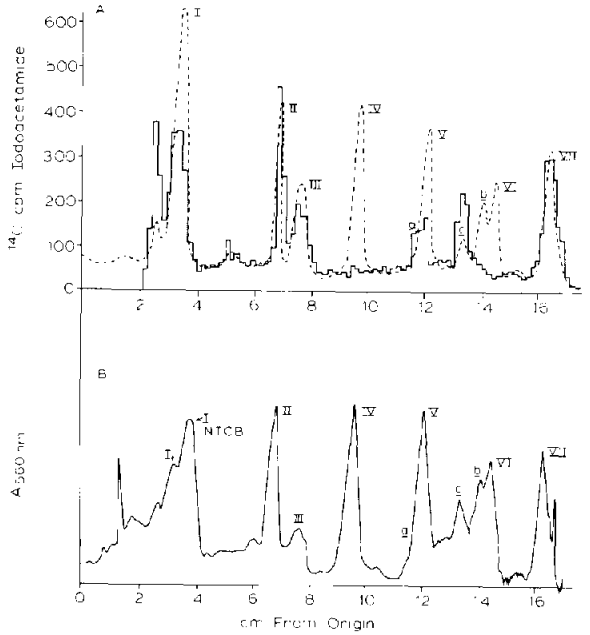

Fig.1. (A) Labelling of cytochrome *c* oxidase with iodo[^{14}C]-acetamide. Cytochrome *c* oxidase (5 μM total heme) was denatured prior to labelling with iodo[^{14}C]acetamide. After quenching the reaction with 1 mM 2-mercaptoethanol, the sample was electrophoresed on a 16% SDS–urea polyacrylamide gel. The densitometric trace of a channel stained for protein [6] is shown (. . .) with the counts/mm gel slice of an identical channel (— —). (B) Cleavage of subunit I with NTCB. Cytochrome *c* oxidase (15 μM total heme) was denatured at pH 8.5 in 8 M urea, 5% SDS of 0.1 M Tris–acetate (pH 8.5). NTCB was added to a final concentration of 250 μM and the sample incubated at 37°C for 72 h. The cleaved product of subunit I is labelled 1-NTCB.

Subunit III does not have a distribution of cysteine residues which would allow the same approach as described above for subunit I. However, the distribution of several amino acids is such that loss of any significant portion of the C-terminus will be reflected in the amino acid composition. Table 1 shows a typical experiment in which the molar ratios of selected amino acids in mature subunit III have been determined by amino acid analysis and for Trp and Tyr spectrophotometrically. The Met content has been set out at 11.0 for the amino acid analysis since all of the Met residues are present in the N-terminus to residue 183. Subunit III, consisting of residues, 1–183, has $M_r \sim 21\,000$ which is the value obtained by SDS–polyacrylamide gel electrophoresis [6,7]. The amino acid composition calculated for this polypep-

Table 1
Comparison of the amino acid composition of mature subunit III with compositions predicted from the DNA sequence

Amino acid	Experimental		Theoretical amino acid composition for residues:		
	Amino acid analysis	A_{280}	1–183	1–245	1–261
Met[a]	11.0		11	11	11
Pro	13.4		11	12	12
Val	17.9		8	13	16
Tyr[b]	12.5	10.0	7	9	11
Phe	26.7		12	23	24
Trp[b]		12.0	7	9	12
His	17.5		11	17	17

[a] All compositions are expressed as mol/mol subunit III based on a Met content of 11.0
[b] Determined spectrophotometrically (see text)

tide contains only 50% of the Val and Phe determined experimentally for mature subunit III (table 1). Clearly processing cannot have occurred to this extent. A polypeptide containing the first 245 residues still has significantly less Val and Trp than determined for mature subunit III. This limits any processing to the C-terminal 16 amino acids.

The fact that the M_r-values of subunits I and III are larger than indicated by SDS–polyacrylamide gels has important implications for the minimum M_r of cytochrome *c* oxidase (i.e., the no. g protein which contain 1 mol heme *a*). It has been a frequent practice to assume a one-to-one stoichiometry of subunits and simply sum them to determine a theoretical minimum M_r-value. This M_r may then be compared to independent determinations of the heme-to-protein ratio and serve as the basis for conclusions on the purity of preparations and subunit composition. Since the values of M_r for the subunits taken from gels are in error, such calculations have been misleading.

Assuming a one-to-one stoichiometry of subunits I–VI and 3 copies of subunit VII [13] and using the M_r of subunits II, IV, V, VII_{Ser}, VII_{Ile} determined from their amino acid sequences, subunits I and III from the DNA sequence of the genes, and subunits VI and VII_{Phe} from gels [7] we calculate the maximum theoretical heme-to-protein ratio to be 12 nmol heme *a*/mg protein.

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