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The Polypeptide Composition of Subunit I of Beef Heart Cytochrome Oxidase

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SUMMARY

It has been shown that subunit I of cytochrome oxidase (∿ MWt. 40,000) can be resolved into a number of smaller polypeptides. This resolution apparently occurs through two stages with the generation of polypeptides of approximate molecular weights of 20,000 and 8,500.

INTRODUCTION

The biosynthesis of cytochrome oxidase (EC 1.9.3.1) depends on the close cooperation of two genetic systems: the three largest polypeptides are apparently coded for by mitochondrial DNA while the smaller polypeptides are synthesised on cytoplasmic ribosomes (1-4). However, the major products of mitochondrial protein synthesis appear to be small molecular weight polypeptides (approx, mol.wt. 10,000) which can form stable heavy aggregates under normal conditions of sodium dodecyl sulfate gel electrophoresis (5-7). Treatment of such aggregates with performic acid frequently produces small molecular weight polypeptides (5) and Kuntzel et. al. (8) have shown that subunit I of cytochrome oxidase from Neurospora crassa can be dissociated in this manner to polypeptides of apparent molecular weight 10,000.

This genetically dichotomous nature of cytochrome oxidase can be readily exploited and we have described how the enzyme can be resolved into two segments, one containing the mitochondrially synthesised polypeptides and the other the polypeptides of cytoplasmic origin (9,10). Through numerous large scale preparations of cytochrome oxidase from beef heart mitochondria we have observed from gel electrophoretic profiles of the enzyme that subunit III is frequently present in non-stoichiometric amounts. Indeed, treatment of cytochrome oxidase

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by repeated ammonium sulfate precipitation from cholate solution can almost completely remove subunit III from the gel profile of the enzyme. However, on resolution of the refractionated enzyme in an acid-alcohol mixture (9), the amount of subunit III is apparently increased and may even be comparable to the amount of subunit I present. These observations prompted us to investigate the relationship between subunits I and III in the acid-insoluble fraction (9) of cytochrome oxidase. Our results have shown that, under appropriate conditions, purified subunit I can be resolved into a number of smaller polypeptides indicating a polymeric structure for this subunit. One such polypeptide derived from subunit I is electrophoretically indistinquishable from subunit III of native cytochrome oxidase.

EXPERIMENTAL PROCEDURES

Beef-heart cytochrome oxidase was prepared by the method of Fowler et. al. (11) as modified by Capaldi and Hayashi (12) and further purified according to Fry et. al. (9). Cytochrome oxidase was resolved in an acid-alcohol mixture (9) to give an acid-insoluble fraction containing predominantly subunits I and III and lesser amounts of subunit II (10). Amino acid analyses were made on subunits I and III resolved by chromatography from such a fraction.

The method developed by Swank and Munkres (13) for sodium dodecyl sulfate urea/gel electrophoresis (SDS-PAGE) in highly cross-linked gels was employed for analysis of subunit composition. Protein samples were solubilized in dissociating buffer (8M urea, 4% SDS, 40mM dithiothreitol, 0.01M sodium phosphate, adjusted to pH 10.5 by addition of 10M NaOH) so that the final SDS concentration was at least 2%. Samples were heated to 37°C for 1 hr. Approximately 25 µg. of protein were applied directly to each gel and electrophoresed at a constant current of 2.5 mA per gel for 20-22 hr. Densitometric traces of gels were made at 550nm in a Gilford linear transport and recorder apparatus.

Protein was subjected to performic acid oxidation according to Hirs (14). Following oxidation, protein was precipitated by addition of 100 vols. of 50mM Tris HCℓ, pH 8.5, containing ammonium sulfate to 50% saturation and centrifuged at 30,000 rpm for 30 min. in a Spinco N^{o} 30 rotor. The protein was further washed twice with the Tris-HCℓ buffer and solubilized for gel electrophoresis in the alkaline urea/SDS reagent.

Acid-insoluble protein (pelleted from an aqueous suspension) could be completely solubilized in 100 mℓ. of chloroform/methanol (2:1), 2% (v/v) in 10M NaOH. This mixture was allowed to stand at 25°C for 5 hr, after which it was evaporated to dryness under vacumn and the protein washed twice by resuspending and centrifuging from 50 mℓ. of distilled water.

The individual polypeptides of the acid-insoluble fraction of cytochrome oxidase were purified by high pressure liquid chromatography (HPLC) and by gel filtration on Sephadex G-150 according to Yu and Yu (15). HPLC was carried out on a 9 mm preparative ODS column (Whatman). An acid-insoluble fraction of cytochrome oxidase was solubilized and eluted in chloroform/methanol (1:2)

containing 0.3M potassium acetate, pH 6·5, 1% benzene and 7.5% H_2O, at a flow rate of 2 mℓ/min and 1800 psi. Peak assignments were made on the basis of urea/SDS-PAGE. For chromatography on Sephadex G-150, an acid-insoluble protein fraction was solubilized in 5% SDS in the presence of 40mM dithiothreitol and 50 mM phosphate buffer, pH 7·0, at 37°C for 2 hr. before applying to the column (5 x 80cm). The column was eluted with 0.1% SDS in 50 mM phosphate buffer, pH 7·0, at a flow rate of 0·4 mℓ/min, and 10 mℓ fractions were collected. Peak fractions were pooled for subunits I and III. Subunits purified by HPLC or filtration through Sephadex G-150 were judged to be electrophoretically pure upon urea/SDS-PAGE of up to 50 µg. of purified subunit/gel tube.

RESULTS

Cytochrome oxidase, dialyzed free of cholate and ammonium sulfate and suspended in 0·1M potassium phosphate buffer, pH 7·4, can be resolved in an acid-alcohol mixture into an acid-insoluble and acid-soluble fraction (9, 10). The acid-insoluble fraction consists predominantly of subunits I and III whereas the acid-soluble fraction contains subunits II and IV-VII of cytochrome oxidase (Fig. 1). The ratio of subunit I to subunit III (on the basis of relative peak areas) is significantly decreased on going from the intact enzyme complex to the resolved acid-insoluble fraction (2·6:1 to 1:1·5 - average of 10 experiments).

Amino acid analyses of subunits I, II and III purified from an acid-insoluble fraction of cytochrome oxidase by HPLC (Fig. 2) or of subunits I and III from

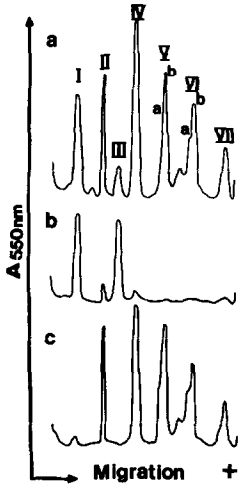

Fig. 1. Densitometric traces of urea/SDS-PAGE of (a) cytochrome oxidase, (b) acid-insoluble fraction, and (c) acid-soluble fraction.

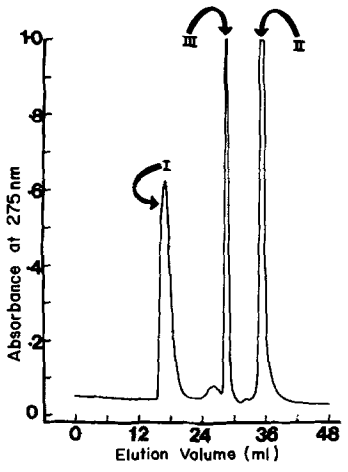

Fig. 2. Resolution of subunits I, II and III by HPLC on a 9mm preparative ODS column. An acid-insoluble fraction of cytochrome oxidase was solubilized and eluted in chloroform/methanol (1:2) containing 0.3M potassium acetate, pH 6.5, 1% benzene and 7.5% H_2O, at a flow rate of 2ml/min and 1800 psi. Peak assignments were made on the basis of urea/SDS-PAGE.

gel filtration on Sephadex G-150, indicated a very similar amino acid composition for subunits I and III although that of subunit II was clearly different (Table 1).

Acid-insoluble protein was subjected to performic acid oxidation for different times of incubation at either 4°C or 37°C (Fig. 3). As performic acid oxidation proceeded the amount of subunit I diminished with the appearance of low molecular weight polypeptides (∿ MWt. 8,500). Prolonged oxidation at the higher temperature resulted in complete loss of subunits I and III with a large diffuse staining band in the 8,500 MWt. range.

Similar gel profiles were obtained with performic acid oxidation of purified subunit I (purified on Sephadex G-150). Fig. 4 shows how subunit I can be dissociated in two stages, firstly through a polypeptide of apparent molecular weight 20,000 (identical in gel position to that of subunit III of unresolved cytochrome oxidase) and eventually with complete dissociation to low molecular weight polypeptides. The low molecular weight polypeptides derived by complete oxidation of either subunit I or III were indistinquishable by gel electrophoretic examination, both giving diffuse staining bands in the 8,500 MWt. range.

Table 1. Amino acid compositions of purified subunits I, II, and III isolated from an acid-insoluble fraction of cytochrome oxidase.

Residue	Mole %				
	I		II	III	
	a	b	a	a	b
ASP	6.76	6.84	7.41	7.00	7.01
THR	8.91	8.89	8.61	8.77	8.84
SER	6.62	6.84	9.38	6.89	6.58
GLU	4.14	4.32	8.60	4.46	4.31
PRO	5.95	5.85	6.65	5.93	5.89
GLY	9.93	10.32	7.27	10.51	10.42
ALA	7.81	7.95	5.66	8.53	7.95
VAL	6.19	6.00	4.45	5.65	6.20
MET	5.77	5.64	6.23	5.51	5.73
ILE	5.39	5.33	3.78	5.175	5.41
LEU	12.51	12.89	12.59	14.00	13.11
TYR	3.87	2.89	4.49	2.68	2.85
PHE	8.19	8.11	4.68	7.51	7.88
HIS	4.18	4.10	4.27	4.06	4.12
LYS	1.82	1.93	2.94	1.32	1.75
ARG	1.99	2.10	2.98	2.02	1.95

a - subunits purified by HPLC. b - subunits purified on Sephadex G-150.

Upon solubilization in and subsequent removal from alkaline chloroform/methanol, urea/SDS-PAGE indicated the almost complete dissociation of subunit I from an acid-insoluble protein fraction with a concomitant increase in the amount of subunit III and low molecular weight polypeptides (Fig. 5).

Following treatments with performic acid or alkaline chloroform/methanol it was found necessary to solubilize proteins in alkaline urea/SDS for gel electrophoresis, otherwise the use of neutral pH urea/SDS reagent could result

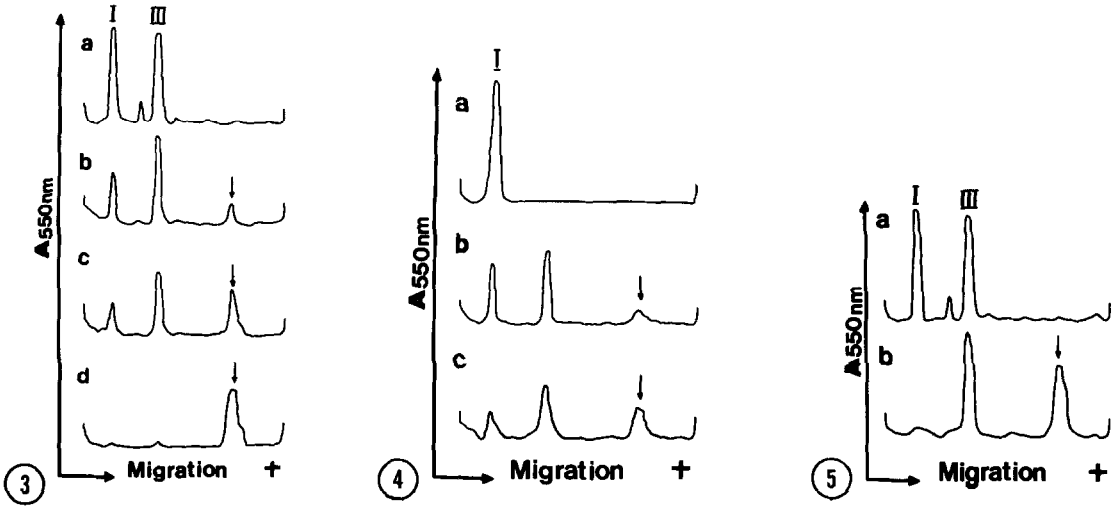

Fig. 3. Densitometric traces of urea/SDS-PAGE of an acid-insoluble protein subjected to performic acid oxidation. (a) un-oxidized protein, (b) after 2 1/2 hr at 4°C, (c) after 5 hr at 4°C, and (d) after 2 hr. at 37°C. Arrow shows appearance of polypeptides of ~ mol. wt. 8500.

Fig. 4. Densitometric traces of urea/SDS-PAGE of purified subunit I subjected to performic acid oxidation. (a) un-oxidized subunit I, (b) after 2 1/2 hr at 4°C, and (c) after 5 hr. at 4°C. Arrow shows appearance of polypeptides of ~ mol.wt. 8500.

Fig. 5. Densitometric traces of urea/SDS-PAGE of an acid-insoluble protein fraction subjected to alkaline chloroform/methanol treatment. (a) untreated protein, and (b) treated protein.

in reaggregation of small polypeptides. The use of alkaline urea/SDS did not in itself change the relative proportions of subunit I and III in an acid-insoluble protein fraction.

DISCUSSION

The present results demonstrate the ability of certain procedures, particularly performic acid oxidation, to dissociate the largest subunit of cytochrome oxidase (subunit I, MWt. 40,000) into smaller polypeptides of apparent MWt. 8,500. Dissociation apparently proceeds through an intermediate polypeptide of apparent MWt. 20,000. This polypeptide is referred to as subunit III, on the basis of its gel position on urea/SDS-PAGE, and we do not infer that this polypeptide is the same as the subunit III generally recognized in unresolved preparations of cytochrome oxidase. Indeed, our results suggest that this polypeptide is generated by procedures that result in the dissociation of subunit I. Amino acid analyses of subunits I and III from an acid-insoluble

fraction of cytochrome oxidase (a procedure which apparently leads to dissociation of subunit I) support the idea that the subunit III polypeptide is derived from subunit I. The amino acid composition of subunit III given in Table I is necessarily a composite of that polypeptide III generated from subunit I and of subunit III originally present in cytochrome oxidase, assuming the two polypeptides are different. Yu and Yu (15) have determined the amino acid composition of subunit III of beef heart cytochrome oxidase and shown it to be quite different from subunit I, particularly with regard to a high lysine and glutamic acid content. We find no such amino acid differences between subunits I and III from which we conclude that the major contribution to the subunit III fraction, isolated from an acid-insoluble fraction of cytochrome oxidase, has been derived from dissociation of subunit I, particularly given the small proportion of subunit III in the original cytochrome oxidase preparation. Generation of a subunit III polypeptide from subunit I has been confirmed by oxidative treatment of purified subunit I.

Thus, the present investigation demonstrates that subunit I of cytochrome oxidase is an assembly of small molecular weight units that may be visualized by the successive dissociation of the parent subunit by an oxidative procedure that does not result in the splitting of peptide bonds (14).

We have recently shown (10) that subunit I of cytochrome oxidase (but not subunit III) has ion-channel properties mediating the rapid influx of cations into liposomes. These, and the present investigation, are aimed at gaining a clearer understanding of the functional and structural role that this polypeptide assembly may perform in the cytochrome oxidase complex.

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