THE POLYPEPTIDE COMPOSITION OF CYTOCHROME OXIDASE FROM BEEF HEART MITOCHONDRIA

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1. Introduction

Cytochrome oxidase, the terminal member of the mitochondrial electron transfer chain, has been studied extensively but the subunit structure of the enzyme has not yet been clearly defined. In this paper we describe a preparation of the enzyme which is active, has a high heme content, is devoid of other cytochromes, and has only a small number of polypeptide components which are low in molecular weight.

2. Experimental

2.1. *General methods*

Protein concentrations were determined by the method of Lowry et al. [1] using bovine serum albumen as a standard. Phospholipid was determined by phosphorus analysis according to Chen et al. [2]. An average phospholipid molecular weight of 775 was assumed. Heme *a* was estimated by the pyridine hemochromogen difference spectral method described by Williams, using an extinction coefficient E 587–620 = 21.7 [3]. Cytochrome *c* oxidase activity was determined by the method of Smith [4]. Spectra were obtained by the method of Chance and Williams [5] using a Cary 14 spectrometer.

2.2. *Gel electrophoresis*

Samples were prepared for polyacrylamide gel electrophoresis by being dissolved in a solution which was 3% in sodium dodecyl sulphate (SDS) and 5 mM in β-mercaptoethanol. Complete dissolution was obtained by heating to 100° for 1 min. Gel electrophoresis using 10% polyacrylamide gels was performed according to the method of Dunker and Reuckert [6]. 20–50 μg samples of membrane protein were loaded onto gels, which were subsequently fixed and stained with Coomassie Blue. Gels were calibrated as described by Weber and Osborn [7] using α-chymotrypsinogen, lysosyme, pepsin, ribonuclease and cytochrome *c* as standards. Densitometric measurements of the gels were performed at 550 nm with a Gilford linear scanning attachment to a Beckman DU spectrometer, using 5 mm × 10 cm quartz cuvettes.

2.3. *Preparation of mitochondria*

Beef heart mitochondria were prepared by the method of Crane et al. [8] except that pH 7.8 Tris-HCl (10 mM) replaced phosphate as buffer.

2.4. *Preparation of cytochrome oxidase*

Cytochrome oxidase was prepared by the method of Fowler et al. [9] incorporating the following modifications. The green pellet obtained after the first deoxycholate treatment was suspended in phosphate buffer (0.1 M potassium phosphate, pH 7.4) rather than Tris-sucrose-histidine. Potassium cholate (20% solution, pH 7.4) was then added to a concentration of 1 mg/mg protein and this solution was maintained at 0° while saturated ammonium sulphate was added slowly with stirring to a final conc. of 25% v/v. The mixture was allowed to stand at 0° for 1 hr before being centrifuged at 105,000 *g* for 15 min in a No. 40 rotor. The supernatant, which contained the cytochrome oxidase, was decanted and kept at 4°. Saturated ammonium sulphate (9 ml/100 ml of solution) was added and the solution was allowed to stand for 30 min before being

Table 1
Characteristics of the purified enzyme.

Activity	6.4 – 9.3	μmoles cytochrome *c* oxidized/min/mg protein
Heme *a*	9.4 –10.6	μmoles/mg protein
Phospholipid	0.17– 0.25	mg/mg protein

The data is given as the range of values obtained in 5 separate preparations.

centrifuged at 105,000 *g* for 15 min. The heavy white precipitate was discarded. Two further ammonium sulphate fractionations were performed on the supernatant, using firstly 8 ml/100 ml of solution which precipitated a reddish-brown material and then an additional 12 ml/100 ml of solution. After the last fractionation a yellow supernatant, a floating precipitate and a dark green pellet were obtained. The supernatant and floating material were discarded and the pellet was suspended in phosphate buffer (0.1 M potassium phosphate, pH 7.4). This suspension was purified cytochrome oxidase. Cholate and ammonium sulphate were removed by dialysis against phosphate buffer or 10 mM Tris-HCL, pH 7.4 for 48 hr in the cold (4°), with regular changes of dialysate.

The enzyme was stored at –20° at a protein concentration of 20 mg/ml.

3. Results and discussion

The activity, heme *a* content and phospholipid to protein ratio of our cytochrome oxidase preparation are listed in table 1 and the visible spectrum is shown in fig. 1. A peak at 421 nm in the oxidized state which shifts to 442 nm in the reduced state, and a peak at 605 nm generated in the oxidized *vs.* reduced difference spectrum, are both characteristic of this enzyme. There are no absorption maxima of other cytochromes in the spectrum of the reduced state, indicating the absence of these potential contaminants.

The different polypeptide components in our preparation are indicated by the electrophoretic pattern in fig. 2. There are only three major bands in the gels, migrating with molecular weights of 11,500, 14,000 and 19,000 daltons. Two of these bands of 11,500 and 14,000 daltons predominate and adsorb over 80% of

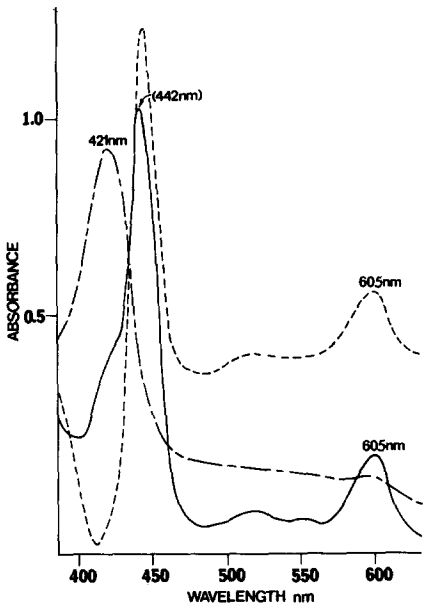

Fig. 1. The absorption spectrum of oxidized, and dithionite-reduced cytochrome oxidase. Also shown is the difference spectrum of oxidized *vs.* reduced samples. (- — -) Oxidized sample; (——) reduced sample; (- - -) difference spectrum.

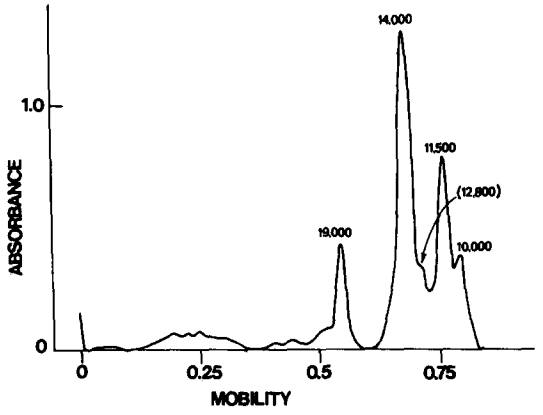

Fig. 2. A densitometric tracing of the component polypeptides of beef heart cytochrome oxidase observed after polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate. The preparation had a heme content of 10.6 μmoles heme *a*/mg protein.

the stain in the gel. Three other minor bands of molecular weights 10,000, 12,800 and 36,000 daltons are also apparent. The 36,000 dalton band was virtually absent in preparations of high heme *a* content but was a significant component of our preparations with low heme *a* content. Thus it is likely that this component is a contaminant, only removed under optimum conditions of fractionation.

Our results do not confirm the subunit structure of beef heart cytochrome oxidase proposed by either Chuang and Crane [10], who observed only two polypeptides in their gels of 26,500 and 54,000 daltons, or the findings of Shakespeare and Mahler [11] who estimated five major components ranging in size from 9,000 to 55,000 daltons. The data are however compatible with those reported by Kierns et al. [12] who identified components of 10,000, 14,000, 19,000 and 37,000 daltons in their beef heart cytochrome oxidase, prepared by a modification of the method of Yonetani [13]. Agreement in the size of the various components is particularly good considering the inaccuracies of the gel electrophoretic method for estimating molecular weights.

We conclude from the gel electrophoretic pattern of our preparation of cytochrome oxidase that there are only three major components of this enzyme, all with molecular weights less than 20,000 daltons. It remains to be determined which of these polypeptides bear the heme and copper moieties.

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