ON THE SUBUNIT STRUCTURE OF CYTOCHROME OXIDASE FROM BEEF HEART MITOCHONDRIA

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1. Introduction

Cytochrome oxidase (EC 1.9.3.1) the terminal member of the mitochondrial electron transfer chain is a complex enzyme containing two heme groups, two copper atoms and a number of different polypeptide components [1]. The enzyme is an intrinsic part of the mitochondrial inner membrane, and as Complex IV is likely to be intimately associated with other electron complexes to facilitate electron transfer from DPNH at Complex I or succinate at Complex II to molecular oxygen [2]. Further, Complex IV must be associated with F_1 and coupling factors to allow ATP synthesis at this coupling site. The tight association between cytochrome oxidase and other components of the mitochondrial inner membrane has made isolation and purification of the enzyme difficult and a variety of preparations containing from 2 to 7 polypeptides with molecular weights varying between 8,000 and 80,000 have been reported [3–6]. Recently we have described a method of preparing cytochrome oxidase with a heme *a* content between 9.4 and 10.6 nmoles/mg protein [7]. In preparations with the highest heme *a* content, all the component polypeptides had molecular weights below 20,000 [7]. We have since been able to purify the enzyme further, taking advantage of the different solubility of cytochrome oxidase and its associated impurities in media containing potassium cholate and ammonium sulphate. This purification step yields an enzyme with a heme *a* content as high as 14.6 nmoles/mg protein. Further there are only two molecular weight species of polypeptides in the enzymic complex.

2. Experimental

2.1. *General methods*

Protein was determined by the method of Lowry et al. [8]. Phospholipid was determined by phosphorus analysis according to Chen et al. [9]. Heme *a* was estimated by the reduced versus oxidized alkaline pyridine hemochromogen difference spectral method as described by Williams using ϵ_{mM} (587–620 nm) = = 21.7 [10]. Cytochrome *c* oxidase activity was determined spectrophotometrically at 38° by the method of Smith [11] in a medium 0.1 M in sodium phosphate buffer pH 7.0 and 0.25% in Tween 20. The initial concentration of reduced cytochrome *c* was 22 μM.

Gel electrophoresis was performed and gels were fixed and stained as described by Fairbanks et al. [12] except that 3% sodium dodecyl sulphate and 5 mM β-mercaptoethanol were used to solubilize the sample. Densitometric tracings of the gels were performed with a Gilford linear scanning attachment to a Beckman DU spectrophotometer using quartz cells. Molecular weights were calculated from the migration of several proteins of known molecular weight subjected to electrophoresis in parallel with the cytochrome oxidase samples [13]. The calibration curve for 10% gels used in this study is shown in fig. 1.

2.2. *Preparation of mitochondria*

Beef heart mitochondria were prepared by the method of Crane et al. [14] except that 10 mM Tris-HCl buffer (pH 7.8) replaced phosphate as a buffer.

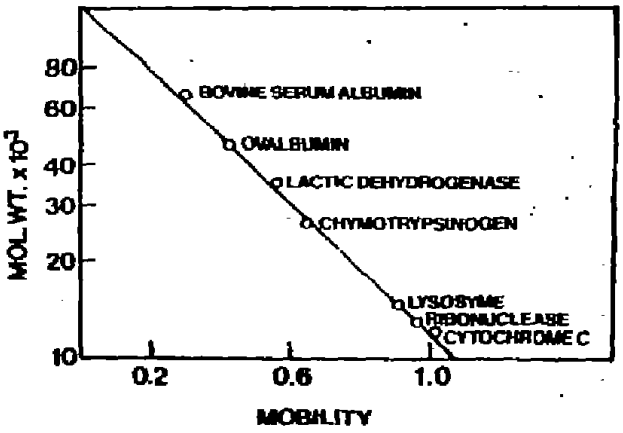

Fig. 1. Standard curve, constructed from the mobilities of a number of proteins of known molecular weight, run in parallel with cytochrome oxidase on 10% polyacrylamide gels in sodium dodecyl sulphate.

2.3. *Preparation and further purification of cytochrome oxidase*

Cytochrome oxidase prepared as described by Capaldi and Hayashi [7], was washed twice with 0.1 M potassium phosphate buffer (pH 7.4). This precipitated the enzyme which was collected by centrifugation at 78,000 *g* for 15 min. The pellet was washed once with a solution of potassium cholate (1% w/v) and ammonium sulphate (0.1 M) in the phosphate buffer. In this solution of low ammonium sulphate concentration, cytochrome oxidase was less soluble than associated impurities and small amounts of these impurities were removed in the supernatant. The enzyme was then suspended in 0.1 M potassium phosphate (pH 7.4) containing potassium cholate (1 mg/mg protein) and precipitated with ammonium sulphate (45% saturation). The supernatant was discarded and the pellet was extracted with 0.1 M potassium phosphate buffer containing potassium cholate (1%) and ammonium sulphate (25% saturation). Under these conditions cytochrome oxidase was more soluble than associated impurities and the insoluble material separated by centrifugation at 78,000 *g* for 15 min (P_1), was rich in impurities. Addition of ammonium sulphate to a final concentration of 45% saturation then precipitated a highly purified cytochrome oxidase (P_2), as detailed in the following sections. P_1 and P_2 were resuspended in 0.1 M potassium phosphate buffer pH 7.4 and stored at −20°.

Table 1
The characteristics of purified cytochrome oxidase.

Sample	Specific activity (sec^{-1}/mg protein in 1 ml volume)	Heme *a* (nmoles/mg protein)	Phospholipid (mg/mg protein)
Starting material	2.6–3.5	9.3–10.6	0.17–0.25
P_2	4.0–6.1	13.2–14.6	0.12–0.18

3. Results and discussion

The activity, heme *a* content and phospholipid content of the starting material i.e., cytochrome oxidase, as purified by the method of Capaldi and Hayashi [7] and P_2 are listed in table 1. Heme *a* was concentrated in the P_2 fraction and this material had enhanced enzymic activity.

The polypeptide components in each sample are shown by the electrophoretic patterns in fig. 2. Crude cytochrome oxidase with a heme *a* content of 9.4 nmoles/mg protein showed six bands in polyacrylamide gels; two major components of 14,000 and 11,500 daltons and four minor components of 36,000 19,000, 12,800 and 10,000 daltons. The 36,000 and 19,000 dalton polypeptides are significant components of the enzymic complex in preparations with this low heme *a* content, as reported previously [7].

Interestingly, the two major bands had a reddish-purple fluorescence when gels stained with Coomassie blue were examined under an incandescent lamp. None of the minor components showed this property. Baum et al. [15] have reported similar fluorescence of certain hemoprotein–amido black complexes in phenol–acetic acid–urea gels while Clayton and Haselkorn [16] have observed fluorescence of some components of bacterial photosynthetic membranes stained with Coomassie blue. Baum et al. [15] suggested that this fluorescence is a property of heme bearing polypeptides.

The electrophoretic patterns of P_1 and P_2 are shown in traces b and c of fig. 2. Concomitant with the increased purity of the enzyme in fraction P_2, there was a great reduction in the amount of many of the polypeptides as compared with the starting

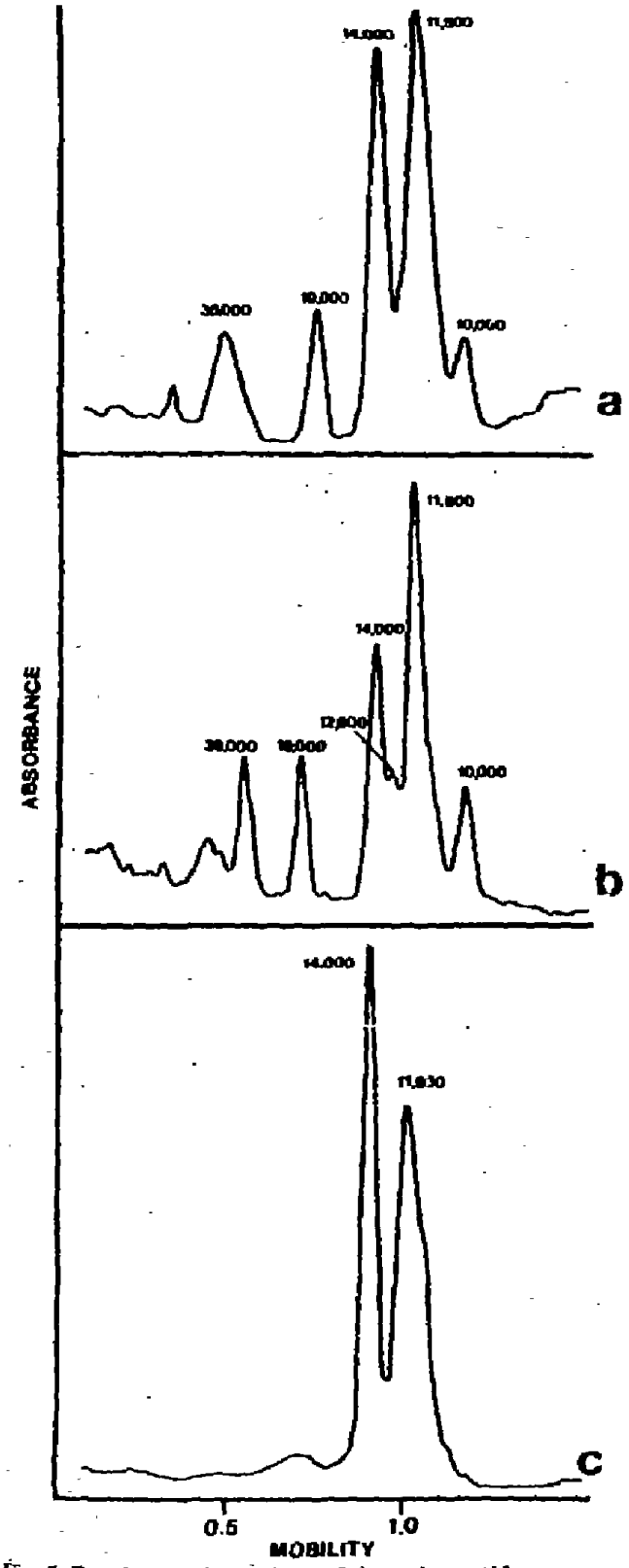

Fig. 2. Densitometric tracings of the polypeptide components in various fractions obtained during the purification of cytochrome oxidase from beef heart mitochondria. Trace 1: enzyme prepared as described by Capaldi and Hayashi [7]. 9.3 nmoles heme *a*/mg protein. Trace 2: P_1. Trace 3: P_2 14.6 nmoles heme *a*/mg protein.

material (trace a). The 36,000 dalton polypeptide was completely absent. Components of 19,000, 12,800 and 10,000 daltons were drastically diminished. These polypeptides were all concentrated in fraction P_1 (trace b). Only the 14,000 and 11,500 molecular weight species were present in significant amounts in the purified cytochrome oxidase. This is not to say that the minimum complex competent to catalyze the oxidation of ferrocytochrome *c* has only two different polypeptide components. In fact, the asymmetric nature of the 11,500 dalton band is indicative of this band being constituted from two or more different polypeptides with fairly similar molecular weights.

In summary therefore, we report a method of preparing cytochrome oxidase which yields an enzyme of heme *a* content up to 14.6 nmoles/mg protein. The minimum molecular weight (69,000), calculated on the basis of this heme *a* content is close to that reported for the molecular weight of the monomer form of the enzyme by Criddle and Bock [17]. This enzyme preparation contains only two molecular weight species of polypeptide of 14,000 and 11,500. The 11,500 dalton band is probably derived from at least two different polypeptides. Finally, it is worthy of note that the molecular weight species we have determined to constitute cytochrome oxidase " in the narrowest sense", that is the minimum complex containing heme *a* and copper that can catalyze oxidation of ferrocytochrome *c* by molecular oxygen, are not synthesized in the mitochondrion [18, 19], thus ending speculation that cytochrome oxidase is wholly or in part synthesized in this organelle.

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