THE SUBUNIT COMPOSITION OF BEEF HEART CYTOCHROME *c* OXIDASE

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1. Introduction

Beef heart cytochrome *c* oxidase (EC 1.9.3.1) preparations of 10 to 12 nmol heme aa_3/mg protein have previously been subjected to SDS PAGE [1–5]. Variations in both the number of bands (from four to six) and their staining intensities have been reported, leading to uncertainties concerning the subunit composition. In contrast we have found, by use of an SDS-urea system for PAGE [6], that different beef heart cytochrome *c* oxidase preparations contain constant amounts of eight subunits, ranging from 37 000 to 4500 daltons. Our results indicate that the failure of previous investigators to observe eight components in preparations at this level of purity was most likely due to the use of inadequate methods of electrophoresis.

The increased resolution of the SDS-urea system reveals as well the presence of minor amounts of at least four and possibly five higher mol. wt components in all five oxidases. By use of the SDS-urea procedure we have established that equilibration of an oxidase preparation with an NAD^+-affinity matrix results in the complete removal of the high mol. wt components. This occurrence is accompanied by the simultaneous binding by the matrix of the several NADH- or NADPH-linked reductase activities which we have reported to be consistent contaminants of oxidase preparations [7].

Two recent reports indicate that active cytochrome *c* oxidase preparations may consist of as few as two or three bands in SDS PAGE [8,9]. Such preparations have been derived by either extensive ammonium sulphate fractionation [8] or proteinase degradation [9] of preparations similar to those discussed above. If these reports prove correct, then it is our belief that cytochrome oxidase activity is quite reproducibly isolated as part of a basic membrane aggregate consisting of at least eight different component polypeptides.

2. Experimental

SDS-urea PAGE (8% gels) was performed as set forth by Swank and Munkres [6], with the following modifications. The acrylamide stock solution consisted of 16% recrystallized acrylamide and 0.54% of *N,N'*methylenebisacrylamide. The TEMED concentration of the buffer stock is adjusted from 0.6% to 4.8%. To make 28 ml of gel, 14.0 ml of the acrylamide stock; 3.5 ml of the buffer stock, and 13.5 g deionized urea are mixed and partially degassed. Then 2.8 mg ammonium persulphate dissolved in sufficient water to bring the final gel volume to 28 ml is added.

50–100 μg of lyophilized protein is dissolved in 0.05 ml of 0.01 M H_3PO_4 which contains 1% (w/v) recrystallized SDS : 1% (v/v) 2-mercaptoethanol : 8 M deionized urea : and adjusted to pH 6.8 with Tris. Dissociation is accomplished by heating at 70°C for 15 min and one drop of 50% glycerol and 3 μl bromphenol blue are added to the sample prior to its placement on the gel. Electrophoresis is carried out for 8 h at 2 mA/gel. Staining and destaining with Coomassie Blue R250 were performed as described by Swank and Munkres [6]. Isolation of subunits from SDS-urea gels was accomplished as described by Nelson et al. [10]. The non-urea SDS PAGE (10% gels) system utilized was that of Weber and Osborn [11].

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3. Results and discussion

Prior to adoption of the SDS-urea system for PAGE, our attempts at resolution of cytochrome *c* oxidase subunits in a non-urea system [11] were marked by incomplete penetration of the protein into the gel, no matter how stringent the denaturing conditions. The bands that did show up exhibited a lack of symmetry although the patterns generally resembled those in the literature. In contrast excellent resolution of highly symmetrical bands resulted when the SDS-urea system was employed. Fig.1 illustrates this point with densitometric traces of the five different preparations listed

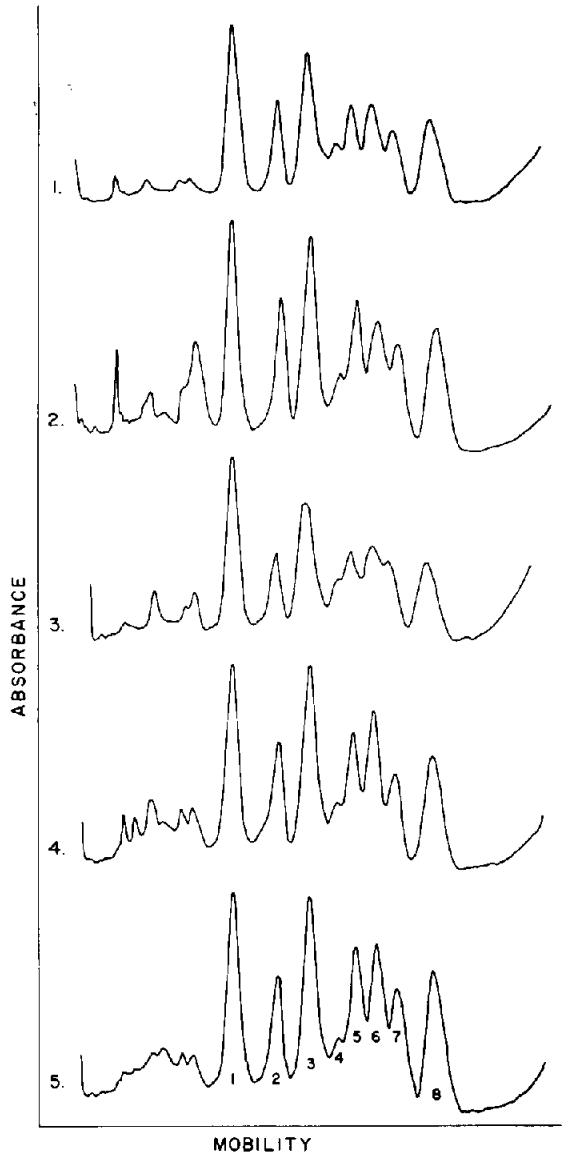

Fig.1. Densitometric traces of the five preparations of table 1 following electrophoresis in SDS-urea. Densitometry was performed with a Gilford Model 2000 Spectrophotometer after destaining had removed the diffusely stained SDS migration front.

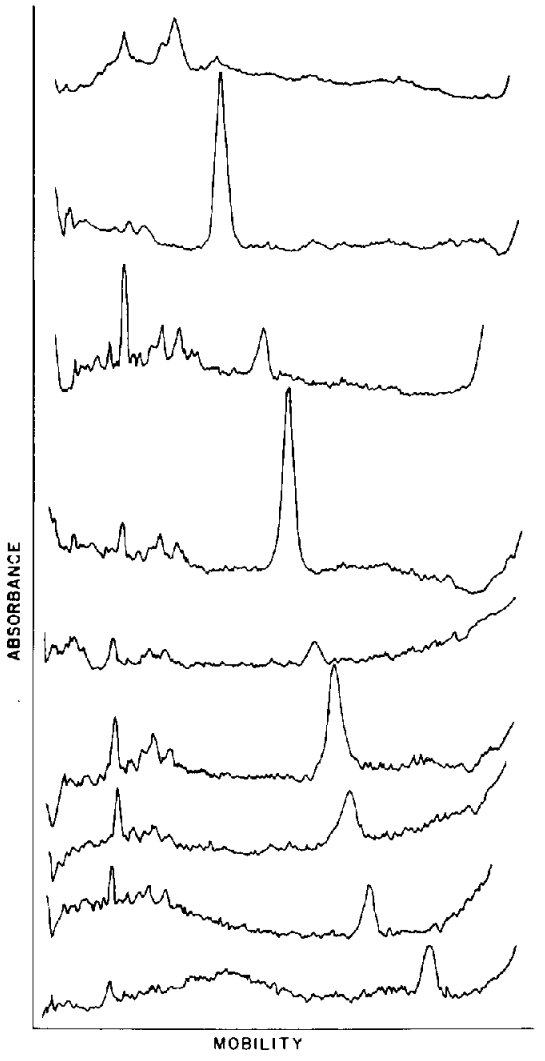

Fig.2. Isolated subunits of preparation #2 re-electrophoresed in SDS-urea PAGE. The uppermost trace is that derived for the 46 000 and 52 000 dalton components which were processed as a single fraction. Arrayed beneath it are the respective traces for subunits 1 through 8. They descend in order of decreasing apparent mol. wt.

Table 1
Heme to protein ratios and contents of contaminant reductases of five highly purified beef heart cytochrome *c* oxidase preparations

Preparation Number	Heme/protein	Reductase activities Substrate:	NADH	NADH	NADPH
		Acceptor:	$K_3Fe(CN)_6$	NBT	NBT
1 (17)[a]	10.3[b]		0.9	0.02	0.02
2 (17)	8.0		3.9	4.7	3.95
3 (18)	10.9		4.53	0.62	0.6
4 (1)	9.4		0.1	1.1	0.3
5 (1)	9.9		0	0.2	0.02

Heme aa_3 was estimated at 25°C by use of $\epsilon_{mM} = 16.5$ for the $A_{605} - A_{630}$ of reduced enzyme [14]. Proteins were determined by use of either the biuret [15] or the fluorescamine [16] procedures. In the latter instance a factor of 1.8 was employed to compensate for the difference in fluorescence yield of cytochrome *c* oxidase versus bovine serum albumin. All enzymes were assayed at 25°C utilizing a Beckman 25 spectrophotometer (1 cm cuvets), and results have been corrected for any non-enzymic contribution. NADH-ferricyanide reductase was assayed by addition of 300 nmol NADH to 35 mM phosphate, pH 7.3; 18 mM $K_3Fe(CN)_6$ and enzyme; results are expressed as μmoles $K_3Fe(CN)_6$ reduced · $minute^{-1}$ · mg $protein^{-1}$. NADH-NBT (nitrobluetetrazolium) and NADPH-NBT reductase activities were assayed by addition of either 300 nmol NADH or 150 nmol NADPH to 200 mM MES [2-(*N*-morpholino)ethanesulphonic acid] pH 6.4; 0.025% NBT, 1% Emasol 4130 and enzyme; results are expressed as units of ΔA_{510} · $minute^{-1}$ · mg $protein^{-1}$. The conditions employed yield zero order kinetics in the reduction of NBT.

[a]Numbers in parentheses indicate references to the preparative procedure.

[b]nmoles heme aa_3 · mg $protein^{-1}$.

in table 1. Instead of the four to six bands usually resolved by non-urea gels, all the preparations clearly show eight subunits between 40 000 and 4000 daltons. Not only are the patterns consistent in the number of bands but the apparent concentrations of each are strikingly similar as well.

To assure that none of the bands are possible artifacts of association, we have isolated each of the eight subunits below 40 000 daltons, and the adjacent bands at 46 000 and 52 000 from SDS-urea gels. These were then subjected to re-electrophoresis in both the SDS-urea and non-urea systems. In both systems, the subunits migrated as single bands; the results obtained in the SDS-urea system are presented in fig.2. The high mol. wt bands present in several traces are attributed to the aggregation of those individual subunits.

A comparison of the mobilities exhibited by each subunit in SDS-urea and non-urea gels is given in table 2. Inspection of the results reveals the explanation for the patterns of four to six bands which have been reported heretofore. In non-urea gels, the mobilities of subunits 3 and 4, and of subunits 5 and 6, are such that, when run as part of the complete oxidase, they will comigrate. The data also suggests that subunits 7 and 8 will, in non-urea gels, migrate slower than 5 and 6. While unusual, results such as these should not be unexpected; Swank and Munkres have amply demonstrated that many proteins of known mol. wt exhibit aberrant mobilities upon electrophoresis in SDS-urea [6].

The preparations show less similarity in the region above 40 000 daltons. While it is impossible to correlate any one specific reductase activity with a single protein band (table 1 and fig.1), the amount of material detectable in this region corresponds roughly to the amount of the contaminant reductase activities in a preparation. We have found that equilibration with an NAD^+-affinity matrix removes these activities quantitatively [12] while simultaneously removing all bands above 40 000 (fig.3). There are no discernible changes

Table 2
Mobilities and apparent molecular weights of isolated subunits of cytochrome *c* oxidase

Subunit	SDS PAGE (8 M Urea)		SDS PAGE	
	Mobility	Apparent mol. wt	Mobility	Apparent mol. wt
1	0.33	37 000	0.34	42 000
2	0.42	26 000	0.56	24 000
3	0.48	21 000	0.68	18 000
4	0.54	18 000	0.69	17 500
5	0.57	15 500	0.78	13 000
6	0.61	13 500	0.78	13 000
7	0.65	10 000	0.73	15 500
8	0.74	4500	0.76	14 500

Mobilities, calculated according to Weber and Osborn [11], were derived upon re-electrophoresis of each subunit in the presence and absence of urea. Standard curves for estimation of apparent mol. wts were prepared using the mobilities of Catalase, D-amino acid oxidase, Sperm whale myoglobin, lysozyme (Sigma Chemical Co.), Horse liver alcohol dehydrogenase (Worthington Biochemicals) and Cortrosyn (Organon), a synthetic 24 amino acid peptide. Subunits are numbered as in fig.1.

to the pattern below 40 000. Since the reductase activities can be recovered from the matrix, we believe that this procedure accomplishes a physical separation of these contaminants from the oxidase per se.

The similarity in apparent subunit composition of the five oxidases was mentioned earlier. At this point we cannot be certain that the intensities of staining of each of the subunits by Coomassie Blue reflect with accuracy their protein contents. Furthermore, slight variations in the staining of gels are unavoidable from one run to the next. However, despite these drawbacks, we believe it is valid to compare the staining patterns for a series of gels all of which have been processed simultaneously. Under such conditions the relative fraction of the total Coomassie Blue absorbance which resides in each band can be used to compare the subunit compositions of the oxidase preparations. For comparison purposes, the average fractional absorbances for each subunit were derived from traces of all five oxidases and are presented in table 3. Inasmuch as these values are averages, the coefficient of variation (SD/mean) reflects the degree to which the composition of any one band in any one oxidase differs from the mean fractional composition of that band in all

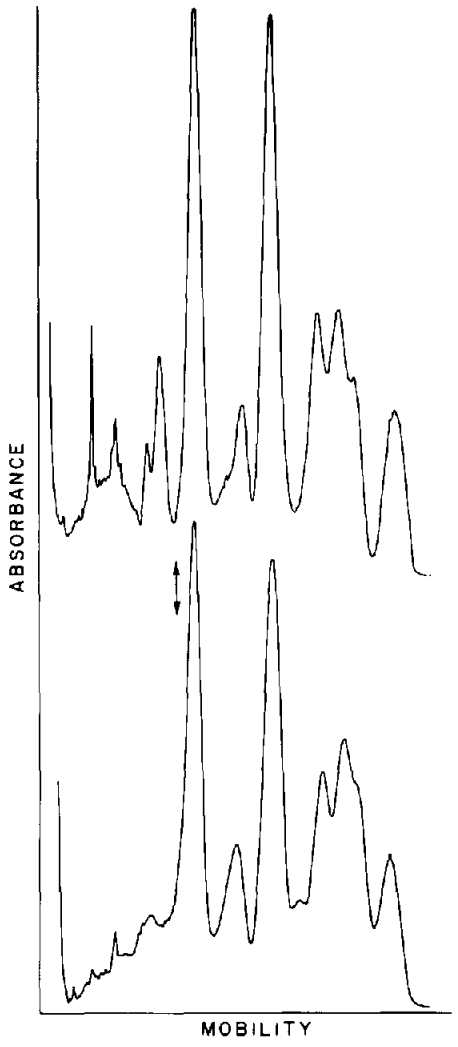

Fig.3. Densitometric patterns of cytochrome *c* oxidase preparation #2 before (top), and after (bottom) equilibrium with na NAD^+-affinity matrix (AGNAD Type 1, P.L. Biochemicals) by cyclic flow [12]. Matrix-equilibrated samples were treated with Bio-Beads SM-2 (Bio-Rad) to remove Tween 80 [19] before lyophilization. The arrow denotes the mobility equivalent to a mol. wt of 40 000. The apparent differences between the above patterns and those of fig.1 are due to two factors: a. the use of an inferior grade of bis-acrylamide which resulted in a loss of resolution of band 4; and b. the use of a different scale expansion during densitometry. However, it should be noted that under these conditions all five oxidases of table 1 retained their similarity in pattern below 40 000 daltons.

Table 3
Reproducibility of the subunit composition of cytochrome *c* oxidase preparations

Subunit	1	2	3	4	5	6	7	8
Fractional absorbance	16.4	10.0	18.3	7.4	12.3	14.6	9.0	11.5
Coefficient of variation	0.10	0.07	0.04	0.09	0.08	0.05	0.08	0.09

Conditions: Photocopies of densitometric traces of gel scans were augmented by completing Gaussian curves to the baseline for each peak. Such curves were then cut and weighed to determine their total weight and the fraction of the total contributed by each subunit. Averages for each subunit of the five oxidase preparations of table 1 are expressed below as fractional absorbances.

five oxidases. All coefficients are 0.1 or less, indicating the virtually identical nature of all five preparations. Since they were derived in three separate laboratories by modifications of either the Fowler or Yonetani methods, we submit that cytochrome *c* oxidase, as commonly prepared, contains eight component polypeptides. At this time it is impossible to assign a primary role, either catalytic or structural, to each component, insofar as cytochrome c oxidase activity is concerned. However, should reports that only two or three polypeptides are required for catalysis survive criticism [13] and prove to be correct, then preparations at a level of purity of 10–12 nmol heme aa_3/mg protein contain five or six polypeptides of unknown function. In this circumstance, we submit in addition that cytochrome *c* oxidase is purified by current procedures as part of an integral structure of the inner mitochondrial membrane. Whether this structure constitutes a portion or the entirety of Complex IV or, on the other hand, contains the vestiges of Site III of oxidative phosphorylation remains to be determined.

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