STRUCTURE OF BOVINE CYTOCHROME OXIDASE

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1. Introduction

The structure of mitochondrial cytochrome *c* oxidase (EC 1.9.3.1) is being very actively studied. Although a variety of different methods are used to study the isolated [1–3] as well as the membrane-bound [4,5] enzyme, no conclusive results on the quaternary structure have been presented. It remains unknown, for example, which of the polypeptides present in preparations of isolated enzyme are components of the functional unit, i.e. of cytochrome aa_3 (see discussion in [6]).

One source of problems in the case of the mammalian enzyme is polydispersity of the enzyme preparations [6]. We have been trying to overcome this problem by further purification of a more 'uniform' enzyme out of the 'conventional' preparation. Previously this was done on an analytical scale [6], and in this report we describe a preparative electrophoretic procedure for the same purpose.

Characterization of the homogeneous cytochrome oxidase shows that maximally six different polypeptides are required to assemble the enzyme. These are present in one-to-one stoicheiometry, and one molecule of haem A is present per one set of six subunits. Cytochrome aa_3 is thus composed of two sets of six different polypeptides, two haems and two coppers.

2. Materials and methods

Cytochrome oxidase was isolated from bovine heart mitochondria [7] essentially as in [8] but using cholate exclusively as the solubilising detergent. The isolated enzyme was stored in 0.5 ml aliquots at 77°K. For further purification the enzyme was dialysed into 38 mM Tris-glycinate (pH 8.3) containing 0.1% (w/v) Triton X-100 (BDH). Discontinuous electrophoresis (see [6] for the buffer system) was performed in the presence of 0.1% Triton X-100 in a cylindric gel of 1% Agarose C (Pharmacia) with dimensions 1.1 × 20 cm. The gel was cast into a water-cooled column in an apparatus similar to the one in [9]. 3 mg of the 'crude' enzyme was applied for a run. Electrophoresis was routinely carried out for 15–16 h at 200 V. After the run, the clearly recognizable green band in the gel was cut off and the enzyme was eluted with the running buffer and concentrated by ultrafiltration. 1.0–1.5 mg of purified enzyme could be obtained from a single run.

Amidination of the polypeptides was performed with methyl acetimidate. The ^{14}C-labelled reagent (≥0.2 mCi/mol) was synthesised as in [10]. The enzyme was denatured in 1% SDS (1 h at 37°C) and dialysed into 0.1 M sodium borate, 0.1% SDS (pH 9.0). Solid [^{14}C]methyl acetimidate was added into 1 ml protein solution (2 mg of protein/ml), which was constantly stirred at 20°C. Three additions of 2 mg each with 30 min intervals was found to result in complete reaction. The excess of reagent was removed by dialysis. Incorporation of radioactivity to the polypeptides was determined after electrophoretic separation in SDS/urea-PAGE [1,6]. The gels were fixed in 30% (v/v) methanol–12% (w/v) trichloracetic acid and stained with Coomassie Blue, the protein bands were cut off and digested with hydrogen peroxide in the presence of ammonia [11] and counted in 5 ml of Lipoluma–Lumasolve–water (100:10:2) with a Rackbeta (LKB) liquid scintillation counter.

Haem A concentration was measured using $\Delta\epsilon_{605}^{red\text{-}ox}$ = 12 mM/cm [12]. Protein was determined

Abbreviations: SDS- and SDS/urea-PAGE, polyacrylamide gel electrophoresis in the presence of dodecyl sulphate and dodecyl sulphate plus urea, respectively

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by a modified Lowry procedure [13]; cytochrome oxidase stock, standardized with the biuret method and with bovine serum albumin as reference, was diluted in 0.1% Triton X-100 and used as standard. The protein determination was confirmed by amino acid analysis to give reliable results within ± 10%. Analytical ultracentrifugation was carried out with Spinco Model E centrifuge equipped with a photoelectric scanner.

3. Results

The six polypeptides of the electrophoretically purified bovine cytochrome oxidase are compared with the polypeptide composition of a conventionally isolated enzyme in fig.1. High molecular weight impurities and most of the contaminants a–c [6] are separated from the enzyme in the preparative electrophoresis (cf. trace A and B in fig.1). Occasionally,

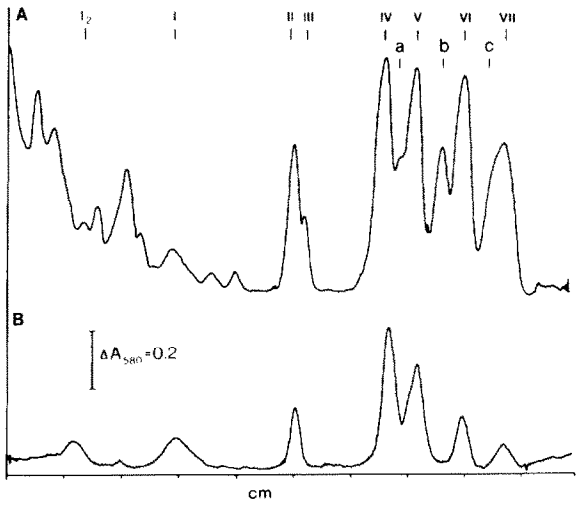

Fig.1. Polypeptide composition of 'crude' and purified bovine cytochrome oxidase. SDS-PAGE of a conventionally isolated (according to [8]) and an electrophoretically purified cytochrome oxidase was carried out in a Neville-type discontinuous system [14]; see also [6]. Total acrylamide concentration of the gels was 10.0% (w/v) of which 5% was methylenebisacrylamide. ~100 μg of the 'crude' and 40 μg of the purified enzyme were applied to the gels. The gels were stained with Coomassie Blue and scanned with an ISCO model AU-5 absorbance scanner. Trace A shows the isolated and trace B the purified enzyme. Nomenclature of the subunits I-VII and of the 'contaminants' a–c is identical to [6], which has been checked with SDS/urea-PAGE in the second dimension. I_2 refers to a dimer of subunit I occasionally produced under the alkaline SDS-PAGE conditions. Migration proceeds from left to right.

part of subunit I was found to migrate as a dimer under the electrophoretic conditions used in fig.1 (see trace B). The purified preparations do not contain the so-called subunit III [1], as already reported [6].

Stoicheiometry of the polypeptides in 'crude' and purified cytochrome oxidase was determined by labelling all the amino groups. At pH 9 imidate is a highly specific reagent for ε-amino groups of the lysyl residues and for the free N-termini [15,16]. In addition this reaction is quantitative and does not change the migration rates of proteins in SDS-PAGE. The extent of labelling in our experiments was determined to be 102 ± 3% of the total lysines indicating that all reactive groups including the N-terminal amines were modified. When the number of reactive sites in each individual polypeptide is known, the relative incorporation of radioactivity can be used for resolving their stoicheiometry. This analysis showed that the stoicheiometry of the six polypeptides in the purified enzyme was close to one-to-one (table 1). Roughly the same stoicheiometry was also found in the case of the 'crude' enzyme but some components (subunit III protein as well as subunit IV) were present in lower amounts. The slightly higher relative stoicheiometries of subunits VI and VII are due either to underestimation of reactive sites or presence of contaminating proteins under these bands.

Sequence data [17–21] together with estimations on SDS-PAGE [1,22] have shown that the expected sum of the MW of the six subunits (I,II,IV–VII) equals 105–110 kd. The haem A/protein ratio of the purified enzyme was determined to be 9.76 ± 0.59 nmol/mg corresponding to a minimal MW of 102 ± 5 kd. This suggests that one set of the subunits is present per one haem A in cytochrome oxidase.

In sedimentation velocity runs the purified enzyme formed a biphasic boundary (fig.2). However, the rapidly sedimenting species represented only about 10% of the total enzyme and was probably due to an oligomeric (dimeric?) form of cytochrome aa_3. The slower boundary was symmetric showing that the purified enzyme is mainly composed of hydrodynamically homogeneous particles. The apparent sedimentation coefficient of this Triton-oxidase complex is 7.7 S. In sedimentation equilibrium studies the MW of the protein in this complex was determined to be ~210 kd, after correction for bound detergent (Saraste, M., Penttilä, T. and Wikström, M., in preparation). Thus the homogeneous 7.7 S enzyme corresponds to monomeric cytochrome aa_3.

Table 1
Stoicheiometry of the polypeptides in cytochrome oxidase preparations

Subunit	Expected number of reactive sites	A		B	
		Cpm/reactive site	Stoicheiometry relative to subunit II	Cpm/reactive site	Stoicheiometry relative to subunit II
I	9	319 ± 6	0.98	214 ± 5	1.01
II	6	327 ± 4	1.00	212 ± 16	1.00
III	7	75 ± 10	0.23	(not present)	
IV	19	229 ± 2	0.70	221 ± 13	1.04
V	8	332 ± 13	1.02	221 ± 12	1.04
VI	6	383 ± 33	1.17	256 ± 16	1.21
VII	5	398 ± 45	1.22	295 ± 36	1.40

Amidination of and subsequent incorporation of radioactivity in the 'crude' (A) and purified (B) enzyme were performed as described in section 2. 108 and 62 μg of protein were applied to electrophoresis in (A) and (B), respectively. The expected numbers of reactive sites (lysines and free N-termini) were calculated from published sequences of subunits II,IV,V,VI and VII [17–21], respectively, and from the amino acid composition and estimated MW of subunits I and III [1,22,23]. Standard deviations are calculated from five gels run in parallel. Relative stoicheiometry of the subunits is presented with respect to subunit II. For location of the subunit I–VII bands in the electrophoretic pattern of the 'crude' enzyme, see fig.3 in [6]

4. Discussion

Results reported briefly in this paper suggest that mammalian cytochrome oxidase is composed of twelve polypeptides per two haems and two coppers. The six different subunits are each present in two copies in cytochrome aa_3. The purified enzyme has a higher catalytic activity than the isolated 'crude' enzyme [6] and Penttilä, T., (in preparation). Moreover, preliminary experiments with the six-subunit enzyme incorporated into the membranes of liposomes have shown that respiratory control is generated

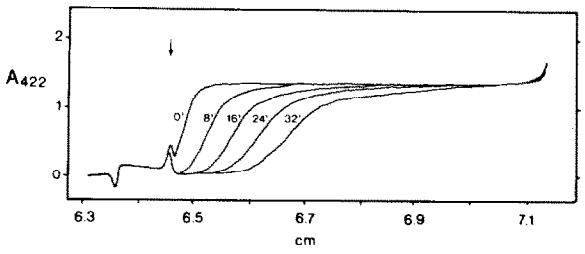

Fig.2. Sedimentation velocity of purified cytochrome oxidase. Sedimentation velocity of the purified cytochrome oxidase (1.2 mg of protein/ml) was measured in 20 mM Tris–HCl (pH 9.0), 90 mM NaCl at 44 700 rev./min. The scanning curves (at 422 nm) were recorded 0,8,16,24 and 32 min after reaching the speed. Sedimentation from left to right; the arrow locates the enzyme solution meniscus. The distance from the rotation centre is given by the abscissa.

and that the enzyme catalyses proton translocation [24]. These data indicate that the purified enzyme is fully functional. However, since recent experiments [25] have suggested that dicyclohexylcarbodiimide may inhibit the oxidase proton pump when bound to the subunit III protein which is not present in the purified preparation, a role of this protein in oxidase function cannot be completely ignored and further experimentation is required to settle this apparent discrepancy.

Our data suggest, however, that the subunit III protein seen in 'crude' preparations, is not an integrated part of the enzyme. In our preparations it is present in far too low amounts to be a stoicheiometric component of the oxidase (table 1). Recently Fry et al. [26] have demonstrated that a protein of this size (21 kd) is produced by degradation of subunit I. It seems probable that the subunit III protein is either such a cleavage product of a true subunit, or then this mitochondrial protein may be vital outside the direct electron transfer and proton translocation mechanism of cytochrome oxidase. It may be noted that proteins of similar MW copurify with the F_o portion of the mitochondrial oligomycin-sensitive ATPase (e.g. [27]).

We have previously discussed alternative possibilities of arrangement of the subunits in active cytochrome oxidase [6,28]. The model that we arrive at here could be called a 'protomer model' [28] in the

sense that there are two copies of each subunit and of haem A and copper in the enzymatically active monomer. However, there is no doubt today that the two haems as well as the two coppers (haems *a* and a_3; coppers Cu_A and Cu_B, [29]) have very different properties in situ. Hence it seems likely that the assembly of the two a priori identical ('protomeric') sets of subunits into monomeric cytochrome aa_3 is also asymmetric so that their identity is broken. Assembly of two nominal 'protomers' can lead to an asymmetric entity even in the case of soluble enzymes [28]. This possibility may be much enhanced by incorporation of the enzyme into a phospholipid bilayer. Electron microsmicroscopic studies have not indicated any internal symmetry in the cytochrome aa_3 molecule [5].

In spite of differences in subunit composition, the mammalian enzyme (six different subunits) and the *Neurospora* or *Saccharomyces* enzymes (seven different subunits [3,30]) may yet turn out to be similar at the level of their quaternary structure. The work by Weiss and Kolb [3] has shown that *Neurospora* cytochrome oxidase contains two times seven different subunits per two haems A in a 320 kd molecule. Hence this enzyme also consists of two 'protomers'. In contrast, Ludwig and Schatz suggest, in a recent report on *Paracoccus* cytochrome aa_3 [31] that this bacterial enzyme contains only two different subunits per two haems. However, a final conclusion on this interesting enzyme must await more data, particularly on the MW as measured hydrodynamically.

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