THE NUMBER OF SUBUNITS IN BOVINE CYTOCHROME *c* OXIDASE

Timo PENTTILÄ, Matti SARASTE and Mårten WIKSTRÖM
Department of Medical Chemistry, University of Helsinki, Siltavuorenpenger 10 A, Sf-00170 Helsinki 17, Finland

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1. Introduction

Cytochrome *c* oxidase (EC 1.9.3.1) is usually isolated and purified by ammonium sulphate fractionation of detergent-solubilised mitochondrial proteins. Although spectroscopically pure, i.e., free or other cytochromes to the limit of detection, the enzyme preparations are polydisperse as judged from sedimentation analyses [1,2]. These preparations contain 6–9 different 'major' polypeptide bands as determined by SDS–PAGE [3–9], and often as many as 12–14 different polypeptides may be distinguished by using an electrophoretic system with high resolving power [4]. In contrast to bovine cytochrome *c* oxidase, the enzyme preparations from *Saccharomyces cerevisiae* or from *Neurospora crassa*, which are purified by procedures also involving chromatographic steps [10–12], have an apparently more uniform polypeptide composition [9,11,12]. The hypothesis that cytochrome oxidase consists of 7 different subunits was developed on the basis of studies on the microbial enzymes [11,12] and was subsequently extended to include the mammalian enzyme [4,13].

The heterogeneity of mammalian cytochrome *c* oxidase preparations may be due to co-purification of other mitochondrial proteins, and/or partial splitting of the multisubunit enzyme to subfragments upon exposition to detergent (especially cholate or deoxycholate) and high salt concentration.

Here we describe a simple method that can be used to detect polydispersity in cytochrome *c* oxidase preparations and to analyse the reasons for such polydispersity. It is a two-dimensional system which combines electrophoresis in porous acrylamide gel in the presence of TX-100 with electrophoresis in highly crosslinked gel in the presence of SDS and urea. In the first dimension, protein complexes that are stable in the nonionic detergent solution are fractionated. In the second dimension the polypeptide composition of these complexes is determined. With preparations of bovine heart cytochrome *c* oxidase, the major band resolved in the first dimension contains an enzymatically active species which apparently lacks the subunit III described in [4]. This band is highly purified from contaminating polypeptides present in the original enzyme preparation and contains 6 distinct polypeptides corresponding to numbers I, II, IV–VII in the terminology of [4].

Abbreviations: SDS, sodium dodecylsulphate; TX-100, Triton X-100 (*t*-octylphenylpolyoxyethylene ethanol); SDS–PAGE, SDS/urea–PAGE and TX–PAGE, polyacrylamide gel electrophoresis in the presence of SDS, SDS and urea and TX-100, respectively. *T*, total acrylamide concentration in electrophoresis gels; *C*, percentage of methylenebisacrylamide in *T*.

2. Methods and materials

Cytochrome *c* oxidase was isolated and purified from bovine-heart mitochondria [14] essentially as in [1] but using cholate as the only detergent in the solubilisation steps. The preparations had a haem/protein ratio between 8–10 nmol/mg, haem *a* being determined using a molar absorptivity $\Delta\epsilon = 13.5\ mM^{-1}\ cm^{-1}$ for dithionite-reduced minus oxidised enzyme at the wavelength couple 605 nm minus 630 nm, and protein being determined by the biuret method [15] using bovine serum albumin as standard. The enzymatic activity was measured polarographically [16,17] in 1% (w/v) Tween-80, 50 mM Tris–PO_4 (pH 6.8) at 20°C. The electron-donating system consisted of ascorbate (7.4 mM) and horse-heart cytochrome *c* (44 μM).

TX–PAGE was carried out in a discontinuous system [18] as detailed in [19]. The gels were composed of 4.0% acrylamide (T) containing 4.0% methylenebisacrylamide (C). Both gel buffer (0.375 M Tris–HCl, pH 8.9) and reservoir buffer (0.038 M Tris–glycinate, pH 8.3) contained 0.1% (w/v) TX-100. The gels were polymerised into cylinder tubes. The runs took 4–5 h at 20°C at a constant 100 V. SDS–PAGE was performed as in [20] using an alkaline gel buffer (23.7 N HCl–1.3147 M Tris, pH 9.81). The gel concentration was 10.0 × 5.0 (T × C). SDS/urea–PAGE was carried out in 12.5 × 10.0 (T × C) gels [4]. The dimensions of cylinder gels were 0.6 × 10 cm, and of slab gels 0.3 × 16 × 16 cm.

After electrophoresis the gels were fixed in 12.5% (w/v) trichloroacetic acid–30% (v/v) methanol for 1 h, stained with 0.5% (w/v) Coomassie brilliant blue G-250 in the same solution for 1 h and destained in 7.5% (w/v) acetic acid–5% (v/v) methanol. Cylinder gels were scanned at 580 nm with an ISCO model AU-5 absorbance scanner. Staining for the presence of haem was performed with 3,3′,5,5′-tetramethylbenzidine as in [21].

3. Results

The separation and distribution of protein complexes of a cytochrome oxidase preparation using

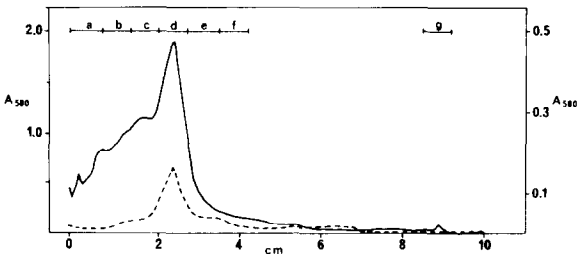

Fig.1. Electrophoresis of cytochrome c oxidase in the presence of Tx-100. TX–PAGE was performed as in section 2. Gels run in parallel with 40 μg and 60 μg protein were stained for protein and haem, respectively. The absorbance scales on the left and right are for the protein-stained and haem-stained gels, respectively.

TX–PAGE is shown in fig.1. The main protein component (fraction d, fig.1) was followed by a broad 'multicomponent' band (fractions a–c, fig.1) and also had a 'trailing' front edge (fractions e,f). Thus most protein complexes migrated more slowly than the main component with the exception of a minor component running in the front of the main peak. Most but not all of haem a comigrated with the main protein component (fig.1, lower curve). Therefore, the haem/protein ratio varies strongly along the TX-gel.

Figure 2 shows analyses of TX-gels in the second

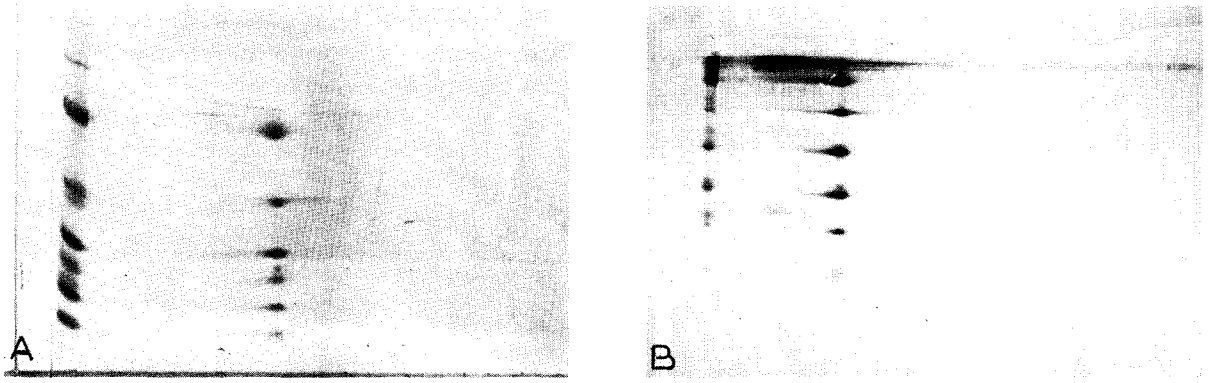

Fig.2. Two-dimensional electrophoresis of cytochrome c oxidase. TX-gels similar to one in fig.1 but containing 100 μg protein were incubated after electrophoresis in 2% SDS plus the appropriate SDS– or SDS/urea–PAGE buffer for 1 h at 37°C, and sealed on the top of the second dimension slab gels (with 1% agarose in A and 5 × 5 (T × C) acrylamide in b). The electrophoreses were carried out for 6 h (A) and 20 h (B) at 20°C. The slab gels were fixed and stained as in section 2. O and F indicate the origin and front of the TX-gels. The numbering of the subunits is shown on the left side of the original samples (R) electrophoresed in the slab gels for reference.

dimension under two different conditions, those in [20] (fig.2A) and those in [4] (fig.2B). Both procedures yielded similar results. The main component in TX–PAGE apparently contains only 6 polypeptide bands, which can be identified as subunits I, II, IV–VII using the numbering in [4]. The subunit III (see [4]) was distributed along the broad band preceding the main component in the TX-gels (fig.2B, and see below).

A more detailed analysis of the distribution of polypeptides in TX–PAGE was performed as follows: A TX-gel was sliced as indicated by the lettering in fig.1, and the slices were incubated in 1 ml 2% SDS, 8 M urea, 0.1 M Tris–PO_4 (pH 6.8) at 37°C for 45 min, then applied on top of cylindric SDS/urea gels and electrophoresed. Figure 3 shows the scanning curves of these gels revealing the polypeptide composition of the slices a–g (see fig.1) of the TX-gel. This may now be compared with the electrophoretic pattern of the original enzyme preparation (fig.3, top trace). Bands are denoted according to [4], designating the 'major' bands I–VII and 'major' impurities a–c.

From fig.3 it is clear that the impurity denoted b, band III, and a large fraction of impurity c, run in slices a–c before the main protein component d. The only fraction in which the cytochrome oxidase protein appears as a reasonably homogenous entity is the 6 polypeptide complex under the main peak (fig.3d). This fraction contains only small amounts of high molecular weight aggregates and 'impurities' a and c. Note that it does not contain 'subunit' III, which runs in fractions a–c, nor the contaminant b, which dominates the 'subunit VI region' of the original enzyme (fig.3, top trace).

It should be noted that cytochrome oxidase subunit proteins were present in all fractions (except g), but that their relative stoichiometries vary strongly

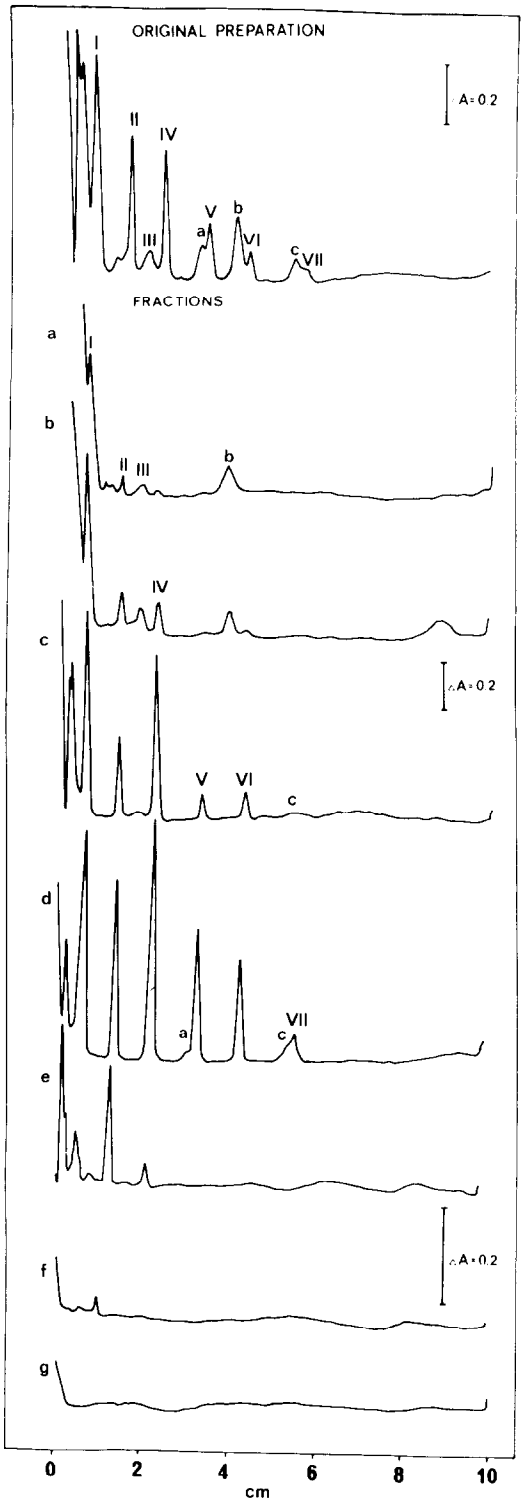

Fig.3. Polypeptide compositions of TX-gel slices. The slices a–g were cut off from a TX-gel (100 μg protein) as indicated in fig.1. SDS/urea–PAGE of the original preparation (curve on the top) and of the slices a–g was performed as in section 2. The absorbance scale of curves a, b, e, f and g is 2-times that of curves c and d (the different scales are indicated on the right).

from one fraction to another. Moreover, in some slices certain subunits were entirely absent while others were present. Thus subunits V–VII were absent in slice a, whereas subunit VII is absent in slice c. Also there seemed to be free subunit II in slices e and f.

In all SDS/urea gels there was apparent high molecular weight protein (aggregates) present stacking on top of the gels (fig.2B, fig.3). However, as also seen in fig.2B,3, this material was mainly concentrated in the early segments a–c of the TX-gel, very little being present in the main fraction d (fig.3, trace d). It is interesting that under alkaline conditions (fig.2A), the high molecular weight material was much less pronounced and yet, the polypeptide composition of the main TX-gel fraction d is very similar. It therefore seems reasonably certain that the polypeptide composition of fraction d (fig.3) has not been significantly upset due to the occurrence of high molecular weight complexes.

Small amounts of the 6 polypeptide fraction (fraction d, fig.3) were purified electrophoretically. Fractions d of parallel TX-gels were sliced off, and several slices were applied on top of a 0.6 × 2 cm TX-gel. The protein was electrophoresed into a 0.2 ml cavity under the gel which was sealed with a dialysis membrane [11]. The activity of the preparation recovered from this cavity was determined polarographically (see section 2). As a control the original oxidase preparation was diluted to correspond to the concentration of the combined fractions d with 0.1% TX-100. The enzyme preparations (and TX-100) were diluted 36-fold in the polarographic assay system. Molecular activities were calculated on the basis of the molar absorptivity of reduced enzyme of 100 mM^{-1} cm^{-1} at 445 nm [22]. In all experiments the molecular activity of fraction d was ⩾ 2-times higher than the activity of the original preparation. In one particular experiment the molecular activity of fraction d was determined to be 27 s^{-1}, while that of the original oxidase preparation was 14 s^{-1}. These activities are effected by the presence of TX-100, which inhibits the enzyme [2]. However, the residual amount of detergent was similar in both cases so that a comparison between the original and the purified preparations seems justified. The molecular activities reported were not extrapolated to infinite cytochrome *c* concentration [16] but are those obtained under the conditions described (see section 2).

4. Discussion

Data from immunological [8,23] and inhibition-inducing specific labelling studies [24] is the only source of evidence that may be thought to distinguish between non-subunit and subunit proteins in cytochrome *c* oxidase preparations. Co-precipitation of 7 polypeptides from a yeast enzyme preparation by an antibody against one subunit [23] supports the hypothesis that the 7 polypeptides are knit into a physical entity, and are not merely united by 'artefactual togetherness' [25]. The 7 subunit hypothesis was proposed also for the bovine enzyme after finding of an additional 'major' band in SDS/urea–PAGE [4]. Although criticised as theoretically unsound for molecular weight determinations [26], the SDS/urea–PAGE yields, in practice, the highest resolution of different polypeptides in cytochrome oxidase preparations [4,27]. It is therefore the system of choice for classification of subunit candidates, unless experimental hazards are detected. A puzzling feature of SDS/urea–PAGE is the apparent aggregation of some polypeptides (cf. fig.2A,B).

4.1. *The uniform cytochrome oxidase fraction*

A two-dimensional system involving electrophoretic protein analysis under non-denaturing (first dimension) and denaturing (second dimension) conditions [28] is in the present case essentially an extraction procedure in which a uniform species of the enzyme is freed of accompanying 'heterogeneities' and further analysed for its polypeptide composition. The 6 polypeptides present in the purified fraction correspond most closely to subunits I, II, IV–VII [4]. The impurities, some of which are remarkably enriched in the subunit V–VII region of the original preparation (see [29] and fig.3, top trace), are almost completely absent in the purified fraction. Moreover, the proposed subunit III clearly migrates among the impurities in fractions a–c (fig.3). Hence, this polypeptide is not associated with the uniform and enzymatically active enzyme species tightly enough to remain attached to it in TX-100. A mild non-ionic detergent such as TX-100 is not expected to dissociate a protein complex in which the individual polypeptides have any affinity to stick together [30], as they should have in a functionally active enzyme. The uniform and enzymatically active fraction d is also remarkably

effectively freed from apparent high molecular weight 'aggregates' that are visualised in SDS/urea–PAGE with the original enzyme preparation.

4.2. *Polydispersity of cytochrome oxidase preparations*

In our enzyme preparation, which is not significantly different from preparations of other laboratories (data not shown), much of the polydispersity seems to be due to partial splitting of the multisubunit enzyme during purification. This phenomenon may be a consequence of the purification procedure in which both ionic detergents and high salt concentrations are employed. From the pattern shown in fig.3 it may be concluded that the original preparation contains protein complexes in which cytochrome oxidase subunits are present in odd stoichiometries. It is obvious that such a relatively large proportion of 'non-stoichiometric' protein complexes in the original enzyme preparation may effectively disturb studies on both enzyme chemistry and other aspects of structure and function.

The second reason for the heterogeneity is the presence of true impurities as described above. In this context we are particularly concerned about time-consuming sequencing studies of polypeptides in the subunits 'V–VII' area (fig.3, top), where 'major bands' often represent impurities rather than true subunits as shown for the peak b in fig.3. Our results clearly stress the importance of careful purification of a homogeneous enzyme preparation in further investigations of the structure of cytochrome oxidase.

4.3. *Arrangement of the subunits into the functional enzyme unit*

Knowledge of the number and molar stoichiometries of the different subunits is obviously fundamental for the understanding of the structure of the enzyme. We have proposed [31] on the basis of hydrodynamic studies [2] and the apparent molecular weights of the subunits [3,4,27], that the enzyme could be composed of (i) two identical protomers and/or (ii) that it may contain nonequal molar amounts of subunits. On the other hand, according to the most common present view the enzyme consists of a single set of subunits per two-haem functional unit. Although our present results do not provide conclusive proof for either view, some considerations may be worthy of mention at this stage.

The ratios of integrated areas under the six peaks of curve d (fig.3), when converted into arbitrary molar units using the apparent molecular weights, are 1.0/1.2/2.5/2.0/2.1/1.8 for subunits I, II, IV–VII, respectively. The molar absorptivities of Coomassie blue-stained polypeptides cannot, however, be assumed to be equal [32,33]. In fact, it was reported [33] that the heavy cytochrome oxidase subunits stain much less intensely than the smaller hydrophilic subunits. Our result is therefore not inconsistent with the idea that all 6 subunits may be present in 1:1 stoichiometry. The sum of the apparent molecular weights of these 6 subunits is ~100 000, which is close to the minimum molecular weight per haem and fits with the mol. wt ~200 000 of monomeric cytochrome aa_3 [2].

As seen in fig.1, the haem is enriched in fraction d. Hence the haem/protein ratio is higher in the 6 polypeptide complex as compared to the original preparation. By integrating the areas under the main protein and haem peaks of fig.1 we arrive at an increase by a factor of ~1.2 in the heam/protein quotient (since a large-scale purification of the homogeneous enzyme has not yet been achieved, this ratio has not so far been determined directly). In the experiment of fig.1 this increment leads to a haem/protein quotient of the order of 10 nmol/mg protein for th homogeneous preparation, again suggesting a minimal mol. wt ~100 000 haem. The present data therefore agree with the hypothesis (see [31] and above) that cytochrome aa_3 may consist of two sets of 6 different subunits. On the other hand, if the enzyme is assembled of one set of subunits per two haems *a*, the haem/protein ratio should be two times higher. Our results clearly do not support this idea.

The above conclusion is much strengthened if subunit III is indeed not part of the enzyme. Since reconstitution experiments (see [34]) have not yet been possible with the homogeneous enzyme, we cannot exclude the possibility that subunit III might have a role in proton-pumping catalysed by the enzyme. It seems clear, however, that this polypeptide is not required for electron transport between cytochrome *c* and oxygen. Since this means a departure from the situation with the yeast enzyme [35], but is consistent with studies of the cytochrome *c*-reactive site on the bovine enzyme [36–38], and since sub-

unit III appears to be very loosely connected with the other subunits (present results), we tend to favour the idea that subunit III may not be a native part of mammalian cytochrome oxidase.

5. Conclusion

The hypothesis that there are seven different subunits in mammalian cytochrome *c* oxidase has to be rechecked. The most homogeneous fraction of the enzyme, which can be resolved from impurities and heterogeneous polypeptide complexes by electrophoresis in non-ionic detergent, contains only six different polypeptides and retains enzymatic activity.

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