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Analytical characterization of cytochrome oxidase preparations with regard to metal and phospholipid contents, peptide composition and catalytic activity

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A number of preparations of cytochrome oxidase have been analyzed for metals by energy dispersive X-ray fluorescence spectrometry. The EPR characteristics, the peptide compositions, the protein and phospholipid contents as well as the catalytic constants of the samples have also been determined. It is confirmed that the enzyme functional unit contains three copper atoms and one zinc atom in addition to two iron atoms. On the basis of the parameters determined for the different samples it is suggested that a high catalytic activity of a preparation can be correlated to a number of other analytical characteristics.

Introduction

It has long been thought that the functional unit of cytochrome *c* oxidase, the terminal enzyme of the mitochondrial respiratory chain, contains two heme irons and two protein-bound copper ions [1]. More recently, however, it has been discovered that it has one strongly bound zinc ion and one magnesium ion as well [2,3], and it has been reported that the number of copper ions is three [4,5]. The functional significance of these 'extra' ions has been doubted [6]. Also, that all preparations contain three copper ions, can be questioned, as four electrons only can be added to the enzyme anaerobically [7,8] and as no other copper EPR signals than those from Cu_A and small, non-stoichiometric amounts of extraneous copper have been observed [7,9]. In fact, the investigators discovering the zinc and magnesium found only 2.5 copper ions in their preparation [3].

As we have a large number of separate preparations of cytochrome oxidase in our laboratory and the availability of a sensitive, nondestructive analytical method for heavy elements in the form of energy dispersive X-ray fluorescence [10,11], we have analyzed 11 samples of bovine-heart cytochrome oxidase for metals and have looked for correlations with the catalytic activity. We have also studied the copper EPR characteristics of these samples. As a decreased specific activity may be caused by the presence of contaminants as well as by loss of functional components, we have determined the peptide composition and the protein concentration normalized to heme content. False correlations with the catalytic activity could be derived from variations in phospholipid content, as this influences the activity [12], for which reason we have, in addition, analyzed the samples for phosphorus.

Leaving out two samples which were obviously poor by other criteria, we find an average of 2.8 Cu and 1.0 Zn when the data are normalized to 2.0 Fe. There is no obvious correlation of the small variations observed with the catalytic activity, even if the lowest activity was found in two samples which were low in both Cu (2.6) and Zn (0.86), and the best preparation in most respects contained 3.0 Cu and 1.05 Zn. A summary of all our analytical results suggests criteria for good oxidase preparations in addition to the spectrophotometric ones commonly used.

Abbreviations: EDXRF, energy dispersive X-ray fluorescence; EPR, electron paramagnetic resonance; SDS, sodium dodecyl sulfate.

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Materials and Methods

Reagents and proteins. All chemicals used were of highest possible quality. Cytochrome *c* was obtained from bovine hearts according to the method of Brautigan et al. [13] and then further purified by ion-exchange chromatography. Beef-heart cytochrome oxidase was prepared essentially as described by Van Buuren [14]. Concentrations were determined spectroscopically with the absorption coefficients listed in Ref. 15.

Catalytic activity. The catalytic constant k_0 of the different cytochrome oxidase preparations was determined by a spectrophotometric method [16]. The slope of the first-order plots for cytochrome *c* oxidation was determined by logarithmic regression and the limiting rate estimated from Hanes plots, as described earlier [17].

Metal analysis. The metal concentrations in the different oxidase preparations were determined by EDXRF spectroscopy [10,11]. The spectrometer has a secondary target arrangement, in which the primary radiation from the X-ray tube, the exciting radiation from the secondary target and the fluorescent radiation from the preparations form three mutually orthogonal samples. This geometry gives high sensitivity. The quantitative evaluation was made by using the intensities in the fluorescence peaks together with that of the scattered radiation from the sample. The information from the scattered radiation is necessary to determine the attenuation of the radiation and thus to obtain the total mass of the sample. The oxidase preparations were positioned on thin polypropylene foils which were placed directly in the EDXRF spectrometer. The thickness of the preparations was in the order of 1–10 mg/cm^2.

The concentrations of cytochrome *a*, Cu_A, and extraneous copper were estimated by integrations of EPR spectra recorded at 15 K[18]. The uncertainty in such integrations is about 10%.

Peptide composition. A modification of the method of Laemmli [19] was used for the SDS-gel electrophoresis. Glass plates, notched according to Studier [20], were made into cassettes, and the gradient gels were prepared according to Pharmacia Laboratory Separation Division (Uppsala, Sweden). The light and heavy separation gels contained 5 M urea, 0.1% SDS, 0.375 M Tris (pH 8.8), and 12 or 22% acrylamide/bisacrylamide, respectively [21].

Cytochrome oxidase solutions were diluted to give a protein concentration of 2–3 μg/μl. The samples were allowed to stand overnight at room temperature to assure good subunit dissociation, and a small amount of 2-mercaptoethanol was added 1–2 h prior to electrophoresis [22]. Samples containing 15–25 μg of oxidase were applied to the gels.

Protein and phospholipid contents. Protein concentrations were determined by the bicinchoninic acid method following the procedure of Smith et al. [23] with bovine serum albumin as standard.

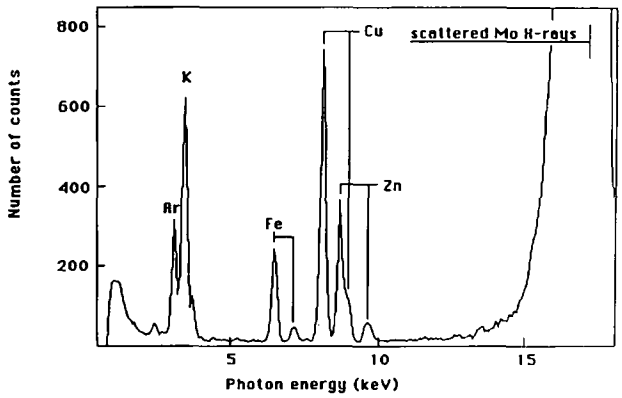

Fig. 1. EDXRF spectrum of cytochrome oxidase.

Phospholipid contents were estimated by phosphorus analysis with a modified Fiske-SubbaRow method, as described by Bartlett [24]. Solutions of KH_2PO_4 were used as standards, but the procedure was also tested by analysis of solutions of glycerate 3-phosphate.

Results

An EDXRF spectrum of one of our cytochrome oxidase samples is shown in Fig. 1. This demonstrates that, apart from P, S, Ar and buffer components the

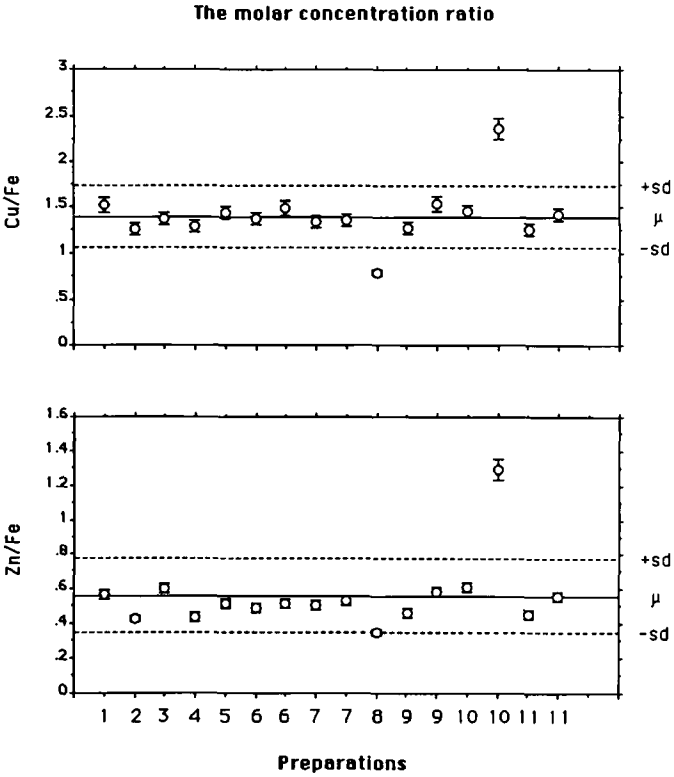

Fig. 2. The Cu/Fe and Zn/Fe mole ratios determined by EDXRF spectroscopy. The middle line represents the mean value and the other two lines ± 1 S.D.

TABLE I

Analytical characteristics of cytochrome oxidase preparations

Prep. No.	Protein concn. chem./spect. [a]	P content [b] (mole fraction)	Cu_A [c] $g3$	k_0 (s^{-1})	Remarks [d]
1	1.10	–	1.12	57.0	–
2	0.92	–	1.15	13.7	Low III, impure Cu_A
3	–	–	1.22	–	Impure Cu_A
4	1.06	15.4	1.08	18.8	Extra bands, $g4$ (denat.)
5	1.04	10.5	1.04	18.6	Ditto
6	1.19	12.9	0.97	81.9	Few heavy bands
7	0.98	7.74	0.98	20.8	Ditto
8	2.41	6.43	1.08	18.1	Many heavy bands, strong $g4$ (denat.)
9	–	–	1.10	–	–
10	–	–	0.88	–	–
11	1.13	13.9	1.02	44.4	Few heavy bands

[a] The ratio of the concentrations determined chemically and spectrophotometrically.
[b] Preparations 1 and 2 were in phosphate buffers and could thus not be analyzed for P.
[c] Ratio of the integrated EPR signal.
[d] Bands refer to gel electrophoresis patterns (Fig. 3) and Roman numerals refer to subunits.

preparations contain no elements with an atomic weight greater than 30 other than Fe, Cu and Zn. The peak heights are not directly proportional to the concentrations, as the fluorescence yields of the elements differ. Proper corrections have been applied to give the molar concentration ratios Cu/Fe and Zn/Fe shown in Fig. 2 for 16 samples, derived from 11 different oxidase preparations, numbered chronologically. The mean ratio Cu/Fe is 1.42 ± 0.30 and Zn/Fe 0.518 ± 0.094. Leaving out the two samples that have values far outside the standard deviations, the ratios are 1.39 ± 0.08 and 0.512 ± 0.051, respectively. Normalized to 2.00 Fe, the enzyme thus contains 2.78 ± 0.16 Cu and 1.02 ± 0.10 Zn.

An example of our SDS-gel electrophoresis results is shown in Fig. 3. Twelve clearly separated peptides representing subunits of cytochrome oxidase, as well as numerous bands from contaminating material in some preparations, can be seen. Preparation 8 displays stronger bands from heavy components than any other sample. Preparation 2 contains very little of subunit III (Fig. 3). Some remarks about the gel patterns of the different preparations are included in Table I, which also gives the results from protein and P analysis, EPR spectra and the activity measurements.

Discussion

Our results clearly show that good preparations of cytochrome oxidase contain close to 3 Cu and 1 Zn per 2 Fe, i.e., in its functional cytochrome aa_3 unit. As the variations are small, except for preparations 8 and 10, it is not possible to make any correlations with the catalytic activity, which, in fact, varies much more than any other parameter (Table I). Preparation 8 is obviously contaminated (Fig. 3), possibly with complex I, and is also partially denatured according to the EPR results (Table I), so it is not surprising that it has the second lowest activity as well as low Cu and Zn contents. Samples with low Cu/Fe ratios, e.g. preparations 2 and 4, do have low activities, but they also have low Zn/Fe ratios, suggesting that they are contaminated with other Fe-containing proteins.

The highest value of EPR detectable copper in our preparations is about 1.2 per cytochrome *a* ($g3$) (Table I), and in samples with high values the signal around $g2$ always shows the presence of extraneous Cu(II) [9]. Thus, the third Cu ion in cytochrome oxidase is must likely in the Cu(I) state, which would be consistent with the redox titrations [7,8]. The only other possibility would appear to be a magnetic coupling of Cu(II) to an unidentified paramagnetic ion, but the absence of contributions from any other metals than Fe, Cu and Zn in the spectrum of Fig. 1 speaks against such a situation.

Cytochrome oxidase investigators do not use a standard set of criteria for the quality of their enzyme preparations. Most often the only purity criteria offered in cytochrome oxidase papers are the spectrophotometric ones. In our opinion, the key property of any enzyme preparation should be a high catalytic activity. A scrutiny of all our analytical data suggests that a high activity can be correlated with certain other parameters, which may thus be useful for the characterization of different oxidase preparations. A limitation in our study is, of course, that all our preparations have been made by a single method [4].

All samples with high activity also have a high phospholipid content (preparations 6 and 11) but this is not a guarantee for high activity. All samples with a low phospholipid content, on the other hand, do have a low activity (preparations 7 and 8).

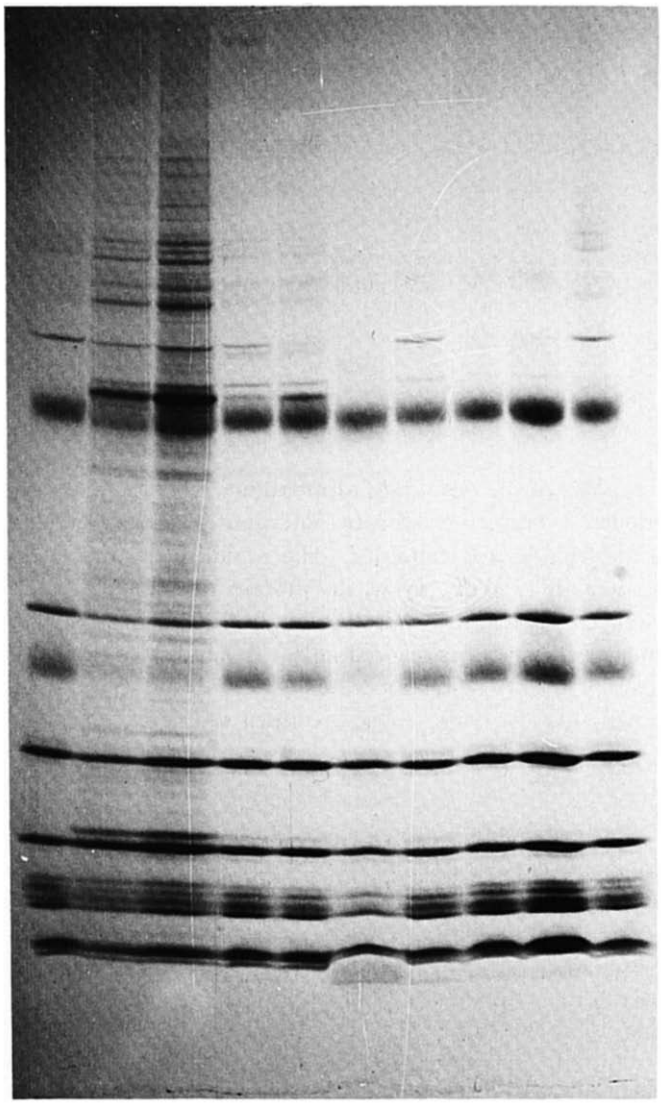

Fig. 3. SDS-gel electrophoresis patterns of some preparations of cytochrome oxidase. From left to right (cf. Table I): preparations 4, 8 (12 μg), 8 (24 μg), 7, 5, 2, 3, 1 (22 μg), 1 (36 μg) and 4.

Samples with high $Cu_A/g3$ ratios generally have extraneous Cu(II) (preparations 2 and 3). Samples with a high protein concentration compared to the concentration determined from heme absorption generally show strong bands from heavy components in gel electrophoresis and also display EPR signals from denaturation ($g4$) (preparation 4 and 8). Such preparations have low activity. Highly active preparations often display weak extra bands in the peptide maps, however, suggesting that attempts to remove all contaminating peptides may lead to denaturation. It may, on the other hand, be noted that our second most active sample (preparation 1) has a very clean peptide map (Fig. 3).

In summary, we conclude that a good cytochrome oxidase preparation, i.e., one with high catalytic activity, will also be characterized by a high phospholipid content, a clean Cu_A EPR signal and the absence of a $g4$ signal as well as the lack of strong bands from heavy components in the peptide maps. They should, in addition, have Cu/Fe and Zn/Fe ratios close to 1.5 and 0.5, respectively.

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