SPECTROSCOPIC AND CATALYTIC PROPERTIES OF CYTOCHROME *c* OXIDASE IN PHOSPHOLIPID VESICLES

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Received 15 October 1977

1. Introduction

Cytochrome *c* oxidase, the terminal component of the mitochondrial respiratory chain, contains two heme groups and two ions of copper in its functional unit [1]. An understanding of its catalytic mechanism requires a detailed description of the redox properties and kinetic behaviour of these four metal sites. Such information is generally derived by spectroscopic methods. Spectrophotometric measurements alone have been used in most investigations, which is unfortunate as other techniques, in particular EPR [2], are more discriminating.

For reasons of sensitivity and resolution the EPR method is most profitably applied to the purified oxidase. To be meaningful such studies require, however, that the essential structural and functional properties of the enzyme are not altered on removal from the ordered lipid environment in the mitochondrial membrane. While a number of observations indicate that structure and function is maintained in the isolated oxidase in detergent solution [1,3], the low specific activities of all purified preparations show this conclusion to be unwarranted [3]. Such low-activity oxidase can, however, be considerably activated by the addition of phospholipid [4,5], which suggests that maintenance of the functional conformation requires a membrane structure.

In this communication we demonstrate that purified cytochrome oxidase can be incorporated into the lipid bilayer of phosphatidylcholine vesicles prepared by sonication. Electron micrographs show that small (50–100 nm) vesicles can be obtained even with a weight of protein equal to that of the lipid, so that the oxidase concentration can be made high enough to permit transient kinetics and EPR studies. NMR measurements together with the results from electron microscopy indicate that the oxidase molecules span the lipid bilayer. The orientation appears to be statistical, however, as it was found that only half of the molecules are available for reaction with cytochrome *c*. Despite the resulting decrease in the effective oxidase concentration, there is a considerable increase in catalytic activity. This can be ascribed to an increase in the rate of intramolecular electron transfer from reduced cytochrome *a* to other electron acceptors. The EPR properties of the partially reduced enzyme are also changed compared to the same preparation in micellar detergent solution. The changes observed on incorporation into the phospholipid vesicles can, at least in part, by ascribed to an improvment in structural homogeneity.

2. Materials and methods

Cytochrome *c* oxidase was prepared from beef heart mitochondria according to a slight modification of the method [6]. L-α-Phosphatidylcholine from egg

yolk and cytochrome *c* (Type VI) were purchased from Sigma Chemical Company. Cytochrome *c* was further purified by ion-exchange chromatography on Amberlite CG-50. All other chemicals were of analytical grade.

For sample preparation, 0.05 g phosphatidylcholine was suspended in 2 ml 50 mM borate buffer, pH 7.7, containing 150 mM NaCl. The suspension was ultrasonically irradiated for 30 min at power level 5 with a Branson Sonifier Model B-12 equipped with a microtip. The sonication was carried out in a glass tube with a cone-shaped bottom cooled in an ice–water bath, with a constant stream of nitrogen into the tube. To remove undispersed phospholipid and any probe particles the solution was centrifuged for 60 min at 140 000 × *g* using an MSE Superspeed ultracentrifuge at a temperature of 6°C. From the clear portion of the supernatant 1 ml was removed and mixed with 0.2 ml cytochrome oxidase solution, which had been dialyzed against detergent-free buffer to remove Tween 80 (initial concentration 0.5%). The mixture was sonicated at 0°C for 1 min, followed by 1 min of cooling and an additional 1 min sonication. The opalescent dispersion was then dialyzed overnight to remove any remaining detergent. Sample solutions made in this way typically contained about 30 μM cytochrome oxidase and 5–6 mg/ml of phosphatidylcholine as determined by the method [7].

Reduced cytochrome *c* was prepared by reduction with excess ascorbate, which was removed by passing the solution through a column of Sephadex G-25. The concentration of oxidase and cytochrome *c* was determined spectrophotometrically in a Beckman spectrophotometer model Acta M IV with the extinction coefficient given [8].

For electron microscopy a drop of the sample was applied to platinized grids with round holes of 70 μm (Agar G215) covered with a supporting carbon film. Excess fluid was sucked off with filter paper, and the grid was allowed to dry. A drop of solution for negative staining was then added, and again the excess liquid was removed with filter paper. The stain solution was 2% ammonium molybdate, pH 7, or 2% phosphotungstic acid–sodium tungstosilicate, pH 7. The specimens were examined in a Philips 300 electron microscope with a primary magnification of 28 000 – 70 000 with an accelerating voltage of 80 kV and a 30 μm objective aperture.

Vesicle suspensions with and without cytochrome oxidase were examined by proton-NMR spectroscopy in a Bruker 270 MHz spectrometer at 10°C.

Molecular activity was determined as described [3]. The stopped-flow system used for transient kinetics was as described [9] except that the amplified photomultiplier signal was fed into a minicomputer (Nova 3, Data General Corp.) for conversion to absorbance units and subsequent plotting. The sampling rate was 20–2000 readings.s^{-1}.

Reductive titrations with cytochrome *c* were carried out as described [2]. The samples were incubated for 20–30 min at room temperature before freezing in the EPR tube. EPR spectra were recorded at 12°K in a Varian E-9 spectrometer.

3. Results and discussion

The electron micrograph in fig.1 shows that it is

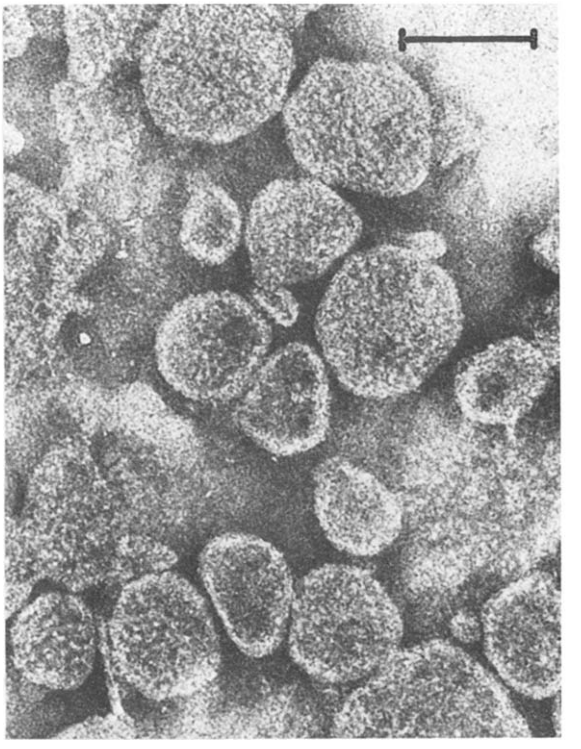

Fig.1. Electron micrograph of phosphatidylcholine–cytochrome oxidase vesicles. The sample contained 30 μM oxidase; the phospholipid concentration was 5.6 mg/ml. The total magnification is 180 000 ×; the bar corresponds to 100 nm.

possible to obtain relatively small, closed vesicles with a high concentration of cytochrome oxidase. Most vesicles have diameters in the range of 50–100 nm, but a number of larger ones are also present. Compared to the vesicles without oxidase there are evenly dispersed 'buds' covering the surface of the vesicles. From micrographs with a higher magnification these can be estimated to have diameters of around 6 nm and a center-to-center distance of approx. 10 nm. Particles are seen projecting from the surface of the vesicle profiles. These particles are smaller than the whole oxidase molecule, suggesting that the enzyme is to a large extent embedded in the membrane. NMR measurements are consistent with the oxidase spanning the lipid bilayer. The spectra show narrow resonances of the choline methyl groups and of the hydrocarbon side chains, characteristic of small vesicles [10]. There is some increase in linewidth, however, compared to vesicles without oxidase, but the relative intensities of the lines remain unchanged. This means that the oxidase vesicles are small but that the resonances of most phospholipid molecules have been broadened by immobilization or by the relaxation effect of the paramagnetic metal sites.

A few calculations show that the interpretations given are reasonable. The vesicles sample used for fig.1 contained 30 μM oxidase and 7.6 mM phosphatidylcholine, corresponding to 0.92 mg lipid/mg protein. Using the molecular dimensions reported [11], which agree with the approximate particle size found here, it can be estimated that a 70 nm vesicle contains 140 oxidase molecules with a space of 5 nm between adjacent molecules, provided the distribution is statistical. Again this figure is close to the estimate from the electron micrographs. On the basis of these short distances one would expect extensive broadening of the NMR lines by both mechanisms mentioned.

The oxidase preparation used showed a molecular activity of 25 s^{-1} in 0.5% Tween 80, at pH 7.4 and 25°C. On incorporation into the phospholipid vesicles this activity increased to 97 s^{-1}, K_m being 6.9 μM. The actual augmentation in activity is even greater, as only about half the oxidase molecules are available for reaction with cytochrome *c*, probably due to a statistical orientation in the membrane. This is demonstrated in fig.2 by the decrease in the extent of the burst phase associated with the rapid initial reaction between cytochromes c^{2+} and a^{3+} [12]. As expected because of the decreases in diffusion velocity and in the effective concentration of the oxidase, the rate of the second-order burst reaction is also decreased.

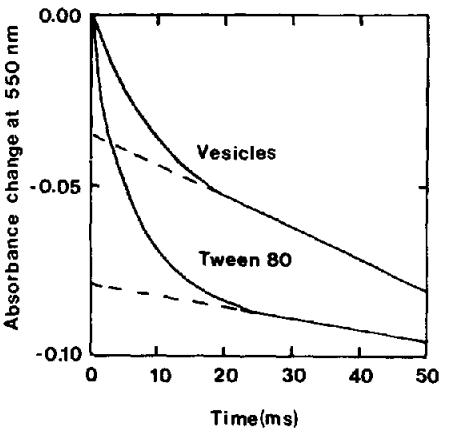

Fig.2. Time course of cytochrome c^{2+} oxidation with cytochrome oxidase in Tween 80 solution and in phosphatidylcholine vesicles. The formation of cytochrome c^{3+} was followed at 550 nm in a stopped-flow apparatus (2 cm cell) at 25°C in the same buffer as used for vesicle preparation (see Materials and methods). In both cases the oxidase concentration was 4.1 μM and that of cytochrome c^{2+} 9.0 μM, after mixing; the initial reaction mixture also contained 2.6 μM cytochrome c^{3+} and 125 μM O_2. The initial absorbance of the vesicle solution was 0.2 higher than the Tween solution, but the curves were normalized to the same starting point. Total ΔA_{550} was −0.38.

The linear second phase in the reaction curves of fig.2 reflects the intramolecular electron transfer from cytochrome a^{2+} to other sites [12], which is rate-limiting under turnover conditions. It can be seen that its rate is also considerable increased in the vesicle preparation. This finding supports the suggestion [3] that there is a blockade of electron transport between cytochrome *a* and a_3 in purified cytochrome oxidase.

The EPR spectra of oxidized and partially-reduced cytochrome oxidase in the vesicle preparation are shown in fig.3. The spectrum of the oxidized enzyme is indistinguishable from that of good preparations in detergent solution [2]. It has, however, been noticed that spectra of the partially-reduced enzyme are more sensitive indicators of heterogeneity [2]. For example, with most preparations in detergent solution, including the one used here, a minor low-spin heme component

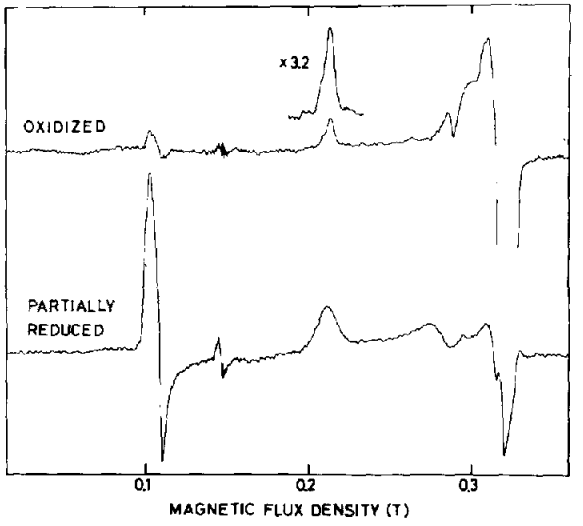

Fig.3. EPR spectra of oxidized and partially-reduced cytochrome oxidase in phosphatidylcholine vesicles. The sample was prepared as described in Materials and methods except that the buffer used was 0.05 M potassium phosphate, pH 7.0, with 0.5 M NaCl. The reduction was made with 4 equiv. cytochrome c^{2+}. The spectra were recorded at microwave frequency 9.1 GHz, microwave power 2 mW, modulation amplitude 2 mT and temperature 12°K. The spectrum was corrected for contributions from a non-linear base-line with the aid of a computer; the signal at 0.145 T represents an artifact of this procedure.

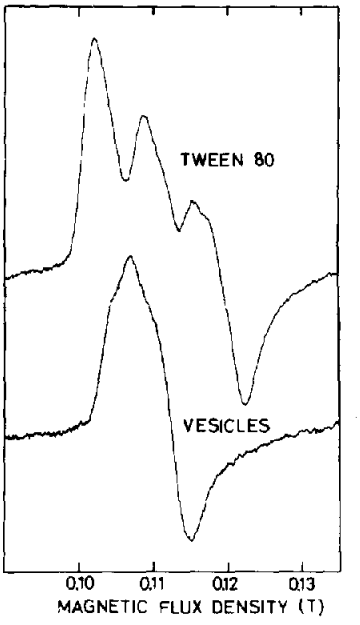

Fig.4. EPR spectra of the high-spin heme of the partially-reduced cytochrome oxidase in Tween 80 and in phosphatidylcholine vesicles. The spectrum was recorded with the same conditions as fig.3 except that the modulation amplitude was 1.25 mT.

at g 2.6 [2] is observed. This signal is, however, entirely absent in titrations of vesicle oxidase (fig.3). The high-spin signal at g 6 is composed of at least one axial and two rhombic components in detergent solution. In the vesicle, on the other hand, these rhombic components are not formed at all (fig.4). The signal, which corresponds to as much as 0.5 heme/functional unit, is still not homogeneous, however, consisting of at least two nearly axial species. The EPR results of figs 3 and 4 show that the increase in catalytic activity, observed when purified cytochrome oxidase is incorporated into an artificial membrane, is associated with changes in structure and improvement in homogeneity.

In recent years there has been considerable interest in the enzymatic properties of cytochrome oxidase vesicles with a high lipid and low protein content (lipid : protein weight ratio $\geq$ 10) [11,13]. These vesicles thus differ markedly in composition from the mitochondrial membrane, which contains more than 50% protein. In addition, their protein concentration is too low to allow a physical–chemical characterization of the oxidase. Our vesicles, however, contain an approx. equal weight of lipid and protein. They are in many respects similar to the low-lipid vesicles first prepared by [4] and later studied [11], the main difference being the smaller vesicle size. This makes them more suitable for studies of the redox equilibria and kinetics in the reaction between the oxidase and cytochrome c, which could be extensively blocked by the structure of the low-lipid oxidase membranes [11]. In view of the increased homogeneity and catalytic activity of cytochrome oxidase in our vesicle preparation, compared to the purified enzyme in detergent solution, it would seem profitable to characterize the spectroscopic and redox properties, as well as the kinetic behaviour of the enzyme in these artificial membranes. We are pursuing such investigations in detail.

Acknowledgements

We wish to thank Mrs Barbro Bejke and Miss Elisabeth Svahn for assistance in the enzyme preparation and activity measurements, Dr Karl-Erik Falk for his participation in the NMR experiments, and Drs Lars-Erik Andréasson and Bengt Reinhammar for operating the stopped-flow system. This investigation has been supported by grants from Statens naturvetenskapliga forskningsråd, Statens medicinska forskningsråd and Knut och Alice Wallenbergs stiftelse.

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