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Spectral and catalytic properties of cytochrome oxidase in organic solvents

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Isolated bovine heart cytochrome oxidase has been extracted into *n*-hexane, probably in reverse micelles, by the use of asolectin and calcium. The diluted extracts are composed of particles with the hydrodynamic radius of 42 nm. Spectral characteristics of the extracted oxidase are similar to those in aqueous solutions. At the high molar ratio of water to phospholipid ($W_0 = 8$) in an organic solvent both cytochrome *a* and a_3 are reducible and oxygen uptake is observed. However, at low W_0 ($W_0 = 1.8$) the rate of cytochrome *a* reduction is decreased and reduction of cytochrome a_3 is inhibited.

Introduction

Cytochrome oxidase is an integral membrane protein which mediates electron transfer from ferrocytochrome *c* to molecular oxygen in mitochondria, reducing oxygen to water. The electron transfer occurs through four redox centres: two groups containing haem *a*, denoted cytochrome *a* and a_3, and two copper ions, Cu_A and Cu_B [1]. The electrons are first accepted by cytochrome *a* and Cu_A, and subsequently transferred to the binuclear cytochrome a_3-Cu_B site, to which the oxygen is bound. An additional function of the multicentre enzyme is to translocate protons across the inner mitochondrial membrane [1,2].

Recently, it has been suggested that cytochrome *a* is accessible to water molecules [3] and the electron transfer from cytochrome *a* and Cu_A to cytochrome a_3-Cu_B site is supposed to be associated with solvent (water) movement [4]. To elucidate the role of the aqueous medium on the oxidase spectral and catalytic properties it would be interesting to study the enzyme in organic media with low water content.

Many enzymes can be transferred from water into organic solvents in reverse micelles of surfactants. The properties of entrapped enzymes and proteins in reverse micelles are strongly dependent on the molar ratio of H_2O to surfactant (W_0) [5,6,7,8]. In the case of cytochrome oxidase in the organic medium, only the thermal stability and catalytic activity have been investigated [9–12].

In the present paper we have studied in greater detail the spectral and catalytic properties of cytochrome oxidase in organic solvents. The spectral responses of the oxidase in hexane on the cyanide binding and on the reduction have been found to be essentially the same as in the water medium. The reduction of cytochrome oxidase and the oxygen uptake at two W_o have been compared.

Materials and Methods

Soybean asolectin (95–98% phosphatide content) and 2,5,3,6-tetramethyl-*p*-phenylenediamine (DAD) were purchased from Fluka, *n*-hexane p.a. from PS Park (UK), *n*-decane p.a. and dioctyl sulphosuccinate sodium salt (AOT) from Sigma, Fisher reagents (sulphur dioxide in pyridine and iodine in methanol) from Lachema (Czechoslovakia). Bovine heart cytochrome oxidase was isolated according to Yonetani [13] and stored in 50 mM Hepes/0.5% Tween 80 (pH 7.4) under liquid nitrogen.

The procedure used for the extraction of oxidase in the organic solvent was modified from that given in Refs. 9,14. Usually the oxidised oxidase was dissolved in 2 ml of 50 mM Tris-HCl buffer/0.5% Tween 80 (pH 7.4) to a concentration of about 75 μM. 400 mg of asolectin was then added and the sample was shaken for 5 min on a Vortex mixer at room temperature. The

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resulting suspension was sonicated in a Dynatech sonic disintegrator for 10 min under a stream of N_2 at 0°C; 0.2 ml of 1 M $CaCl_2$ was added to the sonicated sample (final $CaCl_2$ concentration, 75 mM), and mixed rapidly. Then 2 ml of *n*-hexane was layered and the whole sample was mixed thoroughly again on the Vortex mixer for 3 min at room temperature. Phase separation was aided by centrifugation in a clinical centrifuge (5000 rpm, 5 min). The hexane phase with the extracted oxidase was cooled to −30°C several times to decrease the water content of the sample; during this procedure water and some phospholipids are precipitated. The extracted oxidase was stored in hexane at −20 to −30°C. The water content of the extracted oxidase in hexane was measured by the Fisher method [15]. The mixture of pyridine, sulphur dioxide, iodine and methanol was titrated by the extracts. The end point of the titration was determined by optical spectroscopy.

The total quantity of phosphate in the oxidase preparation was determined by the method of Ref. 16. The amount of the organic and inorganic phosphate was measured in the following way. The oxidase preparation was diluted 50-fold in a bidistilled water and boiled for 2 min. The sample was then centrifuged in a clinical centrifuge at 5000 rpm for 20 min. The pellet of precipitated oxidase and the supernatant were separated for determination of the total amount of phosphate. We assumed that the precipitated oxidase contained mainly organic phosphate, and the supernatant the inorganic one.

The asolectin concentration in the extracts has been determined indirectly. We have measured the total quantity of the phosphate in the extracts and calculated asolectin concentration using the experimentally found amount of the total phosphate in 1 mg of asolectin.

The content of the enzyme, asolectin and water in the extracts is dependent on the initial ratio of oxidase, asolectin and $CaCl_2$ [14]. Under the conditions of our experiments the extracts usually contained 130–150 mg of asolectin, 27–29 mg of water per ml and 40–43 μM of cytochrome oxidase. The extraction yield, defined as the ratio of the oxidase in hexane to the initial quantity of the enzyme in the aqueous phase of the extraction mixture, was about 35%. If we assume the average molecular mass of asolectin to be 750 Da, then the molar ratio of water to the surfactant in the extracts is about 8–10.

W_0 of extracts was changed by the dissolution of asolectin in organic solvent or by the brief sonication of the extracts with the corresponding amount of the buffer solution (50 mM Tris-HCl buffer (pH 7.4)/0.5% Tween 80). Extraction of HCN into hexane was performed by adding 10 ml of hexane to a freshly prepared 5 ml of 2 M KCN in water, adjusted with concentrated HCl to pH 7.4 and mixing the sample for 5 min.

The extinction coefficients of cytochrome oxidase for the water solutions were used without corrections in this work. Oxidase concentration (aa_3 unit) in both solvents has been calculated from the absorption spectrum of oxidised oxidase taking an extinction difference at 600 minus 630 nm, 13 $mM^{-1} \cdot cm^{-1}$ [17]. To determine the oxidase concentration in hexane extracts the asolectin absorption was subtracted from the optical spectrum of oxidase extracts. The asolectin spectral contribution was measured after bleaching of the oxidase absorption by mixing 1.2 ml of extracts with 0.05 ml of 30% hydrogen peroxide.

The oxygen uptake was measured with the aid of the Clark-type electrode in a closed electromagnetically stirred vessel of 2.5 ml volume filled with the saturated solution of DAD in decane. The electrode was covered with the thin polyethylene membrane. The oxygen uptake was registered in the water film (water solution of 0.1 M KCl and 0.1 M $NaHCO_3$) which filled the space between the surface membrane and the electrode. The reaction was started by addition of the extracted oxidase to the air saturated DAD solution at 22°C.

Optical measurements were carried out in a Shimadzu UV/VIS-3000 spectrophotometer.

Quasielastic and elastic light scattering techniques were used to investigate the structure of extracted oxidase solutions. Extracts were diluted to the concentration of 1 μM oxidase in hexane. The content of asolectin and water was 5.7 mg/ml and 2.9 mg/ml, respectively. In order to remove dust particles, the samples were filtered through a Millipore filter with a 0.22 μm pore size into glass cuvettes, which had been cleaned thoroughly with the acetone vapour. Cuvettes were thermally fused in after filling. A homodyne spectrometer was used for quasielastic light scattering measurements [18]. 50 mW of the 514.5 nm line of an Argon ion laser (ILA 120–1, Carl Zeiss Jena) was used as an incident light. The sample was placed in a temperature-controlled chamber, optically matched with xylene. A laboratory-built digital correlator enabled to measurement of homodyne correlation functions over a wide time-scale from 0.1 μs to several seconds. The experimental data were analysed by the CONTIN program [19]. Elastic light scattering measurements were carried out on a Sofica 42000 instrument using a He-Ne laser with 632.8 nm line as a light source. Measurements were performed in the angular range 30–150°.

Results

The results of quasielastic light scattering measurement showed that the homodyne correlation function consists of two exponentials with corresponding bimodal distribution of characteristic times obtained by the CONTIN program. The faster mode with the characteristic time of several microseconds contributes only 5% to the total scattered light intensity and thus it is

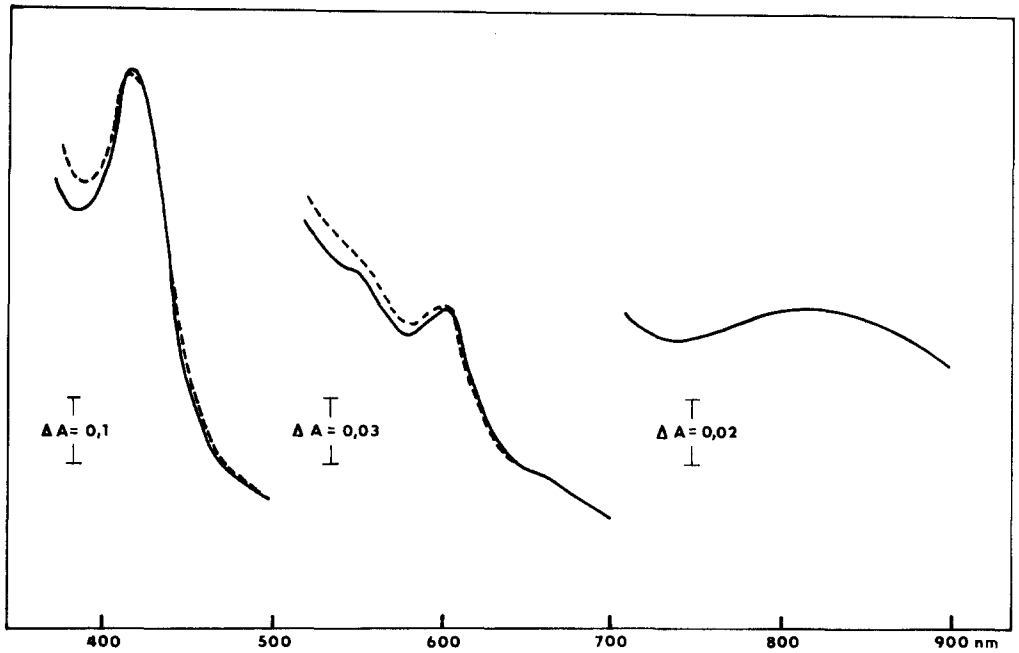

Fig. 1. Absolute spectra of oxidised oxidase in hexane (———) and in aqueous (– – – – –) solution. In hexane, the spectrum of oxidised oxidase extract after dilution to the concentration of 2.8 μM and 20 μM in the visible and near-infrared region, respectively. For the spectrum of oxidase in aqueous solution (visible range), the aliquot of hexane extract was evaporated, dissolved and sonicated in 50 mM Hepes buffer (pH 7.4)/0.5% cholate for 5 min under the stream of N_2 at 0° C. Because of the higher light scattering of the aqueous sample the spectrum was corrected in two regions. In the region 350–500 nm and 500–700 nm the absorption values at 500 and 700 nm, respectively, were taken to be the same as for the hexane extract.

difficult to analyze the origin of this small contribution. It possibly originates from a small amount of asolectin micelles. The slower mode, with the characteristic time of order 100 μs, has a diffuse character (based on angular dependence of the characteristic time) and corresponds to the diffusion of particles formed by enzyme, lipid and water residuals in the hexane phase. The corresponding diffusion coefficients has a relatively narrow distribution. The hydrodynamic radius of particles $R_H = 42$ nm was calculated from the extrapolated value of the diffusion coefficient to the zero angle using the Stokes-Einstein formula. The radius of gyration, R_G, calculated from the angular dependence of the diffusion coefficient is in the range $48 < R_G < 58$, according to the assumed architecture of scatterers (for details of calculation see Ref. 20). Elastic light scattering measurement yields the value of $R_G = 54$ nm, calculated from the linear angular dependence of the reciprocal scattered light intensity. Both elastic and quasielastic light scattering experiments were repeated with the same samples stored at 4° C 2 weeks later. Identical results were obtained, indicating that samples are stable during this period. A more detailed study is in progress and will be published later.

The optical spectra of oxidised oxidase in the aqueous solution and hexane are given in Fig. 1. The spectra are essentially the same, but the spectrum of the extracted oxidised oxidase was stable for hours at room temperature, in contrast to some evolution of the absorption in aqueous solutions [21,22]. This observation is in accordance with the increased stability of the native structure of the enzyme in the organic solvent when the water content is decreased [11].

A more sensitive approach to test the spectral characteristics of the individual redox centres of oxidase can be based on the determination of the spectral responses of oxidase on ligand binding and on the selective reduction. We have used the binding of cyanide to oxidised cytochrome a_3 and the reduction of cytochrome a and Cu_A in the oxidase cyanide complex.

Addition of cyanide to the oxidised oxidase in hexane (see Materials and Methods) brings about spectral changes giving rise to the peaks at approx. 434, 540 and 588 nm, and the minimum at 414 and about at 650 nm in the difference spectrum (Fig. 2). The half-time of the reaction under these conditions is about 90 min. The absolute spectrum of the complex of the oxidised oxidase with cyanide in hexane is similar to the spectrum of oxidised oxidase (Fig. 1), but the maxima are at 424 and 600 nm with a decreased shoulder at 660 nm.

The similarity of spectral changes induced by cyanide in hexane extracts (Fig. 2) to those in the water solutions [23] shows that cyanide binding to oxidised cytochrome a_3 is associated with the transition of the haem iron from the high to the low spin state. Using the difference extinction coefficient, $\Delta\epsilon_{432-411} = 58\ mM^{-1} \cdot cm^{-1}$ [23], for the oxidised oxidase cyanide complex minus oxidised oxidase, it can be calculated that prob-

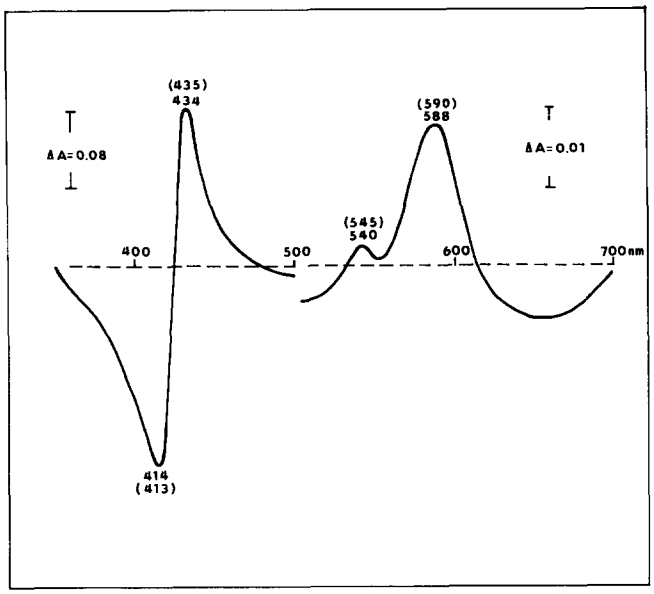

Fig. 2. The difference spectrum of oxidised oxidase plus cyanide minus oxidised oxidase in hexane. The extracted oxidase was diluted to 7.7 μM in 2.4 ml of hexane. After addition of 0.1 ml of HCN extract to the sample the spectrum was stored in the computer memory of the spectrophotometer. The difference spectrum was registered 3 h after the storage. For comparison the minimum and the maxima of the corresponding difference spectrum in aqueous medium are given in parentheses. For this spectrum the aliquot of hexane extract was evaporated, dissolved and sonicated in 50 mM Tris-HCl buffer (pH 7.4), 0.5% cholate and 0.1 mM ferricyanide for 2 min under the stream of N_2 at 0 °C. The spectrum was measured in the way described above with a final 5 mM concentration of HCN.

ably all molecules of cytochrome a_3 reacted with cyanide.

The cyanide complex in hexane or decane is reducible by DAD. The reduction of cytochrome *a* of the extracted oxidase cyanide complex by DAD in hexane gives rise to the characteristic spectral changes with the minimum at 428 nm and the maxima at 448 and 607 nm (Fig. 3). The reduction of Cu_A is indicated by the absorption decrease at 830 nm (Fig. 3). All cytochrome *a* and Cu_A centres appear to be also reducible by DAD in hexane, which can be calculated from the magnitude of the absorption changes at 428, 448, 607 and 830 nm using the difference extinction coefficients for the enzyme in water media [17,24].

Spectral responses of the uninhibited oxidase induced by DAD in hexane at two W_0 values are given in Fig. 4. When DAD is added to the oxidised enzyme at low W_0 ($W_0 = 1.8$) (Fig. 4A), a difference spectrum is observed with the minimum at 428 nm and maximum at 448 nm. The spectral change is a result of cytochrome *a* reduction [17]. The simple enlargement of the absorption difference in time (Fig. 4C) with the one stable minimum at 428 nm indicates the slow reduction of cytochrome *a*, but not cytochrome a_3. In the measurement mentioned, W_0 was decreased to 1.8 by the addition of asolectin to the sample with $W_0 = 8$. As the same spectral response is observed in the sample with W_0 decreased to 1.8 by the addition of the AOT surfactant the properties of the enzyme at low W_0 are not dependent on the type of surfactant used.

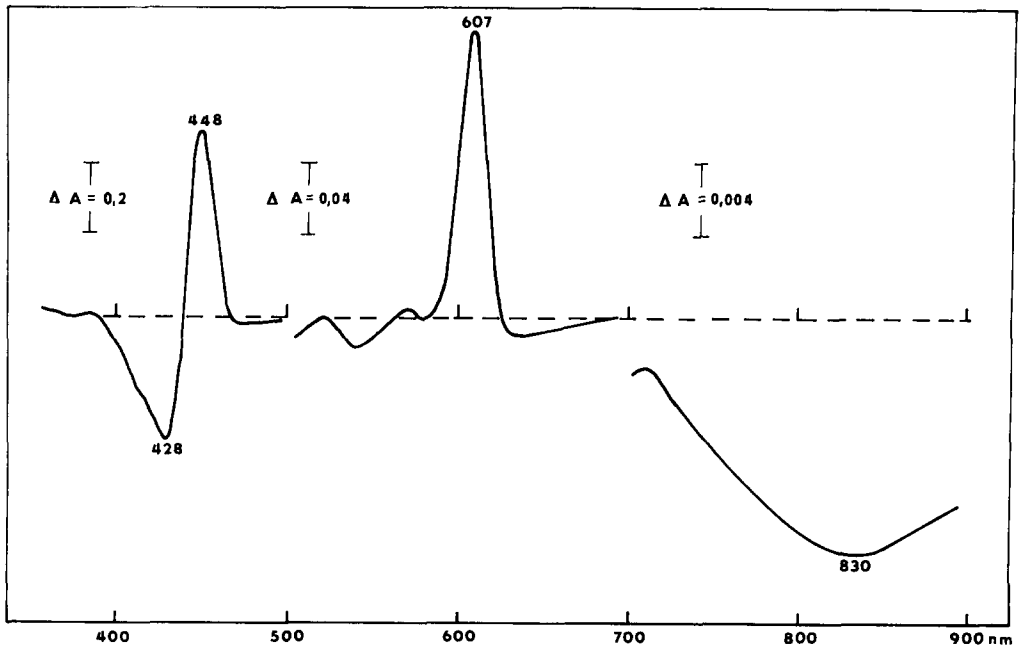

Fig. 3. The difference spectrum of reduced-minus-oxidised oxidase cyanide complex in hexane. The extracted oxidase was diluted to 8.6 μM by the hexane extract of HCN and incubated for 3 h at room temperature to prepare the complex. The spectrum of oxidised oxidase cyanide complex was stored in the computer memory of the spectrophotometer and the sample (1.2 ml) was reduced by the saturated hexane solution of DAD (0.04 ml). The difference spectrum was measured 5 min after the initiation of the reduction.

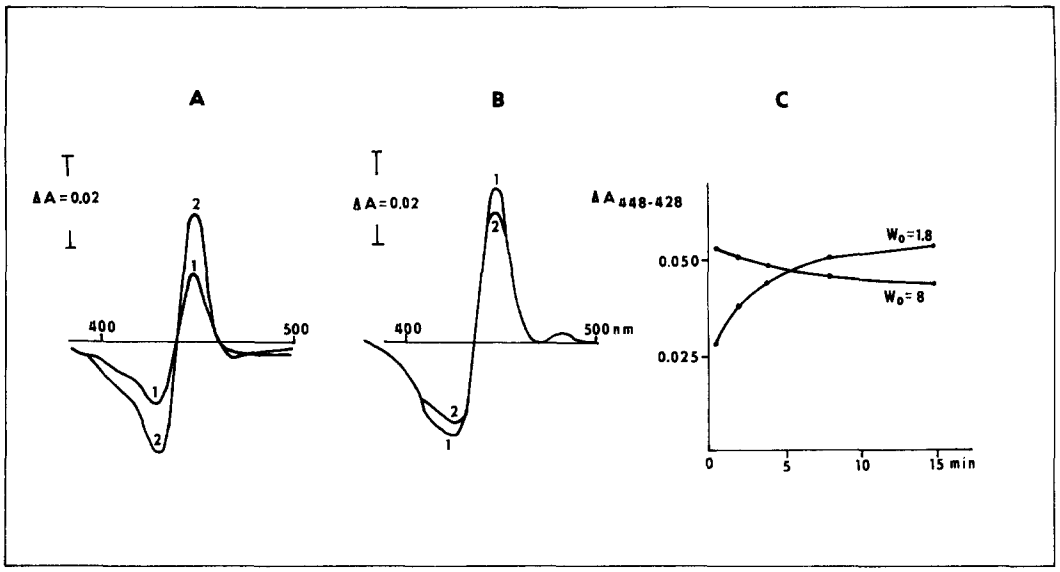

Fig. 4. The spectral responses of oxidase in hexane induced by DAD at two W_0. (A) $W_0 = 1.8$ (concentration of water 1 mg/ml, and asolectin 24.4 mg/ml). (B) $W_0 = 8$ (concentration of water 1 mg/ml, and asolectin 5.5 mg/ml). The oxidase concentration in both cases is 0.8 μM. The spectrum of oxidised oxidase was stored in the computer memory of the spectrophotometer and the difference spectra were registered 30 s (1) and 8 min (2) after addition of the saturated solution of DAD (0.02 ml) to the sample (1.2 ml). (C) Kinetics of the spectral responses determined from the evolution of difference spectral change, ΔA(448–428), at $W_0 = 1.8$ and $W_0 = 8$.

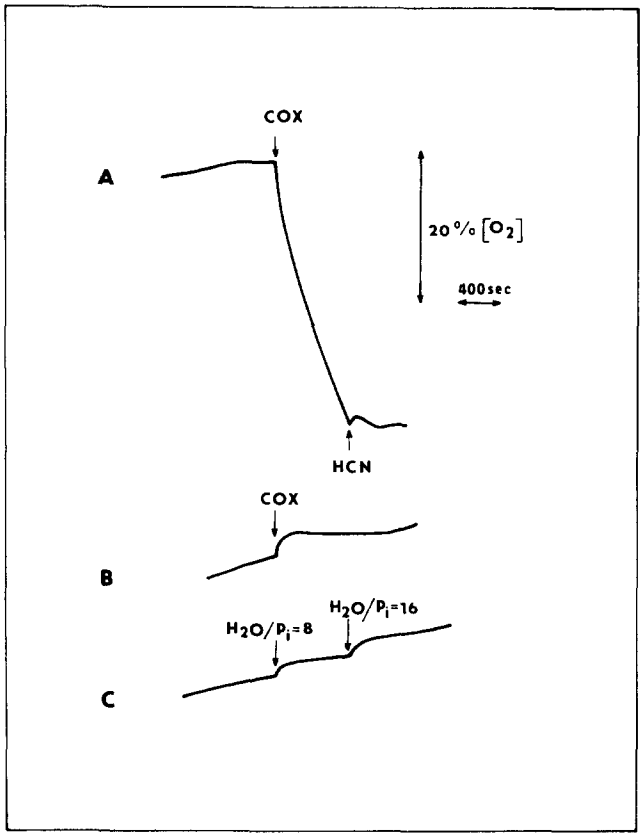

Fig. 5. Polarographic measurement of oxygen consumption by cytochrome oxidase in decane. (For experimental conditions see 'Materials and Methods'.) Indicated additions: COX, 0.1 ml of 80 μM oxidase in hexane, (A) $W_0 = 8$ (concentration of water 2.4 mg/ml and asolectin 13.1 mg/ml), (B) $W_0 = 1.8$ (concentration of water 0.6 mg/ml and asolectin 13.1 mg/ml); HCN, 0.1 ml of the extracted HCN in hexane; $H_2O/P_i = 8$ or 16, 0.1 ml of asolectin reverse micelles (without oxidase) in hexane with $W_0 = 8$ or 16 (concentration of water 2.4 mg/ml or 4.8 mg/ml, and asolectin 13.1 mg/ml in both cases.)

At high W_0 ($W_0 = 8$) (Fig. 4B) a minimum at 428 nm with the shoulder at 414 nm and a maximum at 448 nm are observed. The spectral change quickly develops and slowly decreases in time after the DAD addition (Fig. 4C). The presence of the minimum with the shoulder shows that both cytochrome *a* and a_3 are reduced at the higher W_0. The slow decrease of the initial absorption difference is supposed to correspond either to the reoxidation of the reduced cytochrome a_3 or both cytochrome *a* and a_3 by molecular oxygen.

These conclusions are supported by the measurement of the oxygen uptake determined by the Clark oxygen electrode in decane (Fig. 5). The uptake of oxygen was measurable at micromolar oxidase concentration (3.2 μM) only in the sample with $W_0 = 8$ (Fig. 5A) but not in the one with $W_0 = 1.8$ (Fig. 5B). It was inhibited by cyanide extracts (Fig. 5A) and was not observed after the injections of the asolectin reverse micelles (without oxidase) with $W_0 = 8$ and even with $W_0 = 16$ (Fig. 5C). The reference measurement (Fig. 5C) showed that the oxygen consumption was not a simple autooxidation of DAD in the water phase of the reverse micelles.

The presence of the oxygen consumption at high W_0, its sensitivity to HCN and its absence at low W_0 offer the same conclusion as the one derived from the spectral measurement: at high W_0 cytochrome a_3 is probably reoxidised by O_2, which is not the case at low W_0.

Discussion

The procedure used for the extraction of oxidase in *n*-hexane is similar to phase transfer methods [8]. The

enzyme is transferred from water to the organic solvent by the use of phospholipids and calcium probably in reverse micelles. In the reverse micelle, polar groups of phospholipids are directed towards the interior of the aggregate and form a polar core which can solubilize water, whereas the hydrophobic chains are exposed to the solvent.

Despite the similar spectral responses of the oxidised oxidase in the aqueous [17,23] and in the organic media to the cyanide binding and the reduction, the position of the minima and the maxima in the corresponding difference spectra are slightly (1–5 nm) shifted. Because of the large hydrophobicity of the enzyme the spectral shifts could be a consequence of the protein exposition to the organic solvent and/or interaction with phospholipids.

The next factors that can influence the enzyme spectral properties could be a high concentration of Ca^{2+} [25,26] used for the protein extraction and the amount of water in the reverse micelle. The optical and catalytic properties of enzymes in reverse micelles are strongly dependent on the molar ratios of H_2O to surfactant. This ratio, rather than the absolute amount of water or surfactant present in the organic solvent, determines most of the properties of the entrapped proteins [5,6,8].

The observed reduction of cytochrome a, a_3 and the oxygen uptake at high W_0 (Figs. 4, 5), which is in agreement with the recent observation of the catalytic activity of oxidase in toluene [12], can be interpreted in two ways. Firstly, the enzyme is extracted into organic solvent in the state when the true catalytic activity is retained i.e., the reduction of cytochrome a and Cu_A is followed by the intramolecular electron transfer to cytochrome a_3-Cu_B site and then to molecular oxygen. Secondly, the structure of the extracted oxidase particles in hexane [12] can be similar to the proposed one for the other extracted integral membrane protein – bacteriorhodopsin [27]. Two hydrophilic (cytosol and matrix) parts are covered by the asolectin reverse micelles and the hydrophobic part is delipidated and exposed to the organic solvent. In this case the electron transport from cytochrome a to a_3 can be inhibited, as has been shown for the delipidated oxidase in the water medium [28]. Accordingly, the observed reduction of cytochrome a and a_3 are independent processes and the oxygen consumption is a consequence mainly of the reduction and the reoxidation of the cytochrome a_3-Cu_B site.

At low W_0 the reduction of cytochrome a_3 and the oxygen uptake are inhibited and the rate of cytochrome a reduction is decreased (Figs. 4, 5). There are at least two reasons for the observed properties at low W_0. (i) Increase of the surfactant concentration at a constant water content decreases both W_0 and the micellar size [5,8]. Possibly the decrease of the micellar dimension changes the exposure of the enzyme to the organic solvent, which is connected with the change of its properties. (ii) The water in the water pool of the reverse micelle is partially bound to the polar heads, and is relatively free only above some critical concentration. At low W_0 the amount of water in the extracts is probably lower than critical concentration and the water is in the 'bound' state. The 'bound' water in the micelle could restrict or inhibit the conformational changes associated with the redox reactions of cytochrome oxidase [4,29,30], which influences its catalytic properties.

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