Journal of Bioenergetics and Biomembranes, Vol. 18, No. 4, 1986

Cytochrome *c* Oxidase from *Paracoccus denitrificans* in Triton X-100: Aggregation State and Kinetics

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Received December 23, 1985

Abstract

Cytochrome *c* oxidase from *Paracoccus denitrificans* was homogenously dispersed in Triton X-100. Using gel exclusion chromatography and sucrose gradient centrifugation analysis a molecular weight of the detergent–protein complex of 155,000 was determined. After subtraction of the bound detergent (111 mol/mol heme aa_3) a molecular weight of 85,000 resulted, which agreed well with the model of a monomer containing two subunits. This monomer showed high cytochrome *c* oxidase activity when measured spectrophotometrically in the presence of Triton X-100 ($V_{max} = 85 s^{-1}$). The molecular activity, plotted according to Eadie–Hofstee, was monophasic as a function of the cytochrome *c* concentration. A K_m of 3.6×10^{-6} M was evaluated, similar to the K_m observed in the presence of dodecyl maltoside [Nałęcz *et al.* (1985). *Biochim. Biophys. Acta* **808**, 259–272].

Key Words: Cytochrome *c* oxidase; aggregation state; steady-state kinetics; *Paracoccus denitrificans*; molecular weight.

Introduction

Bacterial heme aa_3-type cytochrome *c* oxidases (EC 1.9.3.1) show remarkable functional and spectroscopic similarities with those isolated from mitochondria [for review see Ludwig (1980) and Poole (1983)]. Interest in the prokaryotic enzymes was stimulated by their simple structure, which made them very useful as model systems for the highly complex mammalian enzymes [up to 13 polypeptides; Kadenbach *et al.* (1983)]. Cytochrome *c* oxidase from *P. denitrificans* is composed of only two subunits (Ludwig and Schatz, 1980), which show immunological cross-reactivity and strong amino acid sequence homologies (Steffens *et al.*, 1983) with subunit I and II of the mitochondrial

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0145-479X/86/0800-0277$05.00/0

oxidases. The absence of subunit III in the purified *Paracoccus* enzyme may be useful to elucidate the role of this subunit in the mammalian oxidases. Subunit III is considered to be involved in the proton translocating activity (Casey *et al.*, 1980; Saraste *et al.*, 1981; Chan and Freedman 1983; Penttilae, 1983) and may also play a role in the stabilization of the dimeric form of the enzyme (Nałęcz *et al.*, 1985). Despite the absence of subunit III, *Paracoccus* oxidase was reported to pump protons when incorporated into phospholipid vesicles, although with a lower stoichiometry (Solioz *et al.*, 1982). The aggregation state of the enzyme as determined in octyltetra/pentaoxyethylene (C_8E_{45}) (Ludwig *et al.*, 1982) and dodecyl maltoside (Nałęcz *et al.*, 1985) was proved to be monomeric.

In this study we investigated the aggregation state and molecular weight of the cytochrome *c* oxidase from *P. denitrificans* in Triton X-100. In this detergent, where the bovine enzyme proved to be dimeric (Bolli *et al.*, 1985a), the *Paracoccus* oxidase was still monomeric. We found, contrary to other reports (Ludwig *et al.*, 1982), that in Triton this enzyme had considerable electron transport activity, also in the absence of phospholipids. Eadie–Hofstee plots of the steady-state kinetics were monophasic, as was observed for the monomeric enzymes from bovine heart and *Paracoccus* measured in dodecyl maltoside as reported previously (Nałęcz *et al.*, 1983, 1975; Bolli *et al.*, 1985b).

Materials and Methods

Ultrogel AcA 34 was from LKB. Ferritin, catalase, aldolase, blue dextran, and Sephadex G-25 were products of Pharmacia. Triton X-100 was from Fluka and horse heart cytochrome *c* from Sigma. All other reagents were of the highest analytical grade commercially available.

Cytochrome *c* oxidase from *Paracoccus denitrificans* was a kind gift from Dr. B. Ludwig. The heme *a* content was 27 nmol/mg of protein and the phospholipid content below 1 mol/mol of oxidase. Beef heart cytochrome *c* oxidase was isolated according to Yu *et al.* (1975). Ferrocytochrome *c* was obtained by reduction of ferricytochrome *c* as described in Bolli *et al.* (1985b).

Spectral analysis and cytochrome *c* oxidase activity measurements were made on an Aminco DW-2a and in an Uvikon (Kontron) spectrophotometer. The following extinction coefficients ($mM^{-1}\,cm^{-1}$) were applied: $\varepsilon_{607-630} = 23.5$ (reduced–oxidized) and $\varepsilon_{424} = 163$ (oxidized) for the *Paracoccus* oxidase, $\varepsilon_{605-630} = 24$ (red.–ox.) and $\varepsilon_{422} = 168$ (ox.) for the bovine enzyme, $\varepsilon_{277} = 1.465$ for Triton X-100 (Robinson and Tanford, 1975), and $\varepsilon_{550-540} = 19.4$ for cytochrome *c* (Robinson and Capaldi, 1977).

Gel filtration was carried out at 4°C on Ultrogel AcA 34 using a 1 × 55 cm column, equilibrated with 10 mM Tris-HCl, pH 7.4, 50 mM KCl, and 0.05% Triton X-100. Before loading, 1.6–3.3 nmols of cytochrome *c* oxidase were incubated for 2 h in 120 μl of equilibration buffer containing between 0.3 and 0.75% Triton X-100. The column was calibrated with ferritin, catalase, and aldolase, and the void volume and the total volume was determined with blue dextran and ferricyanide, respectively. Fractions of 0.8 ml were collected. The binding of Triton X-100 was determined photometrically in the fractions containing protein according to the method of Robinson and Capaldi (1977) using the following formula:

$$(A_{277}/A_{424} - r) \times \varepsilon^{ox}_{424}/\varepsilon^{TX}_{277} = \text{mol Triton X-100/mol oxidase}$$

where A and ε are the absorptions and the extinction coefficients at the indicated wavelength, respectively, and r is the ratio A_{277}/A_{424} for the enzyme in a detergent, which does not absorb at 277 nm. For the *Paracoccus* oxidase, r was determined to be 1.82 in dodecyl maltoside or Tween 80 and for the bovine enzyme r was 2.51 [see also Robinson and Capaldi (1977)].

Sucrose gradient centrifugation was run in a Beckman SW 60 rotor on a Beckman L8 centrifuge. Linear sucrose gradients (5–20%) were made in 10 mM Tris-HCl, pH 7.4, 50 mM KCl, and 0.1% Triton X-100. Oxidase samples prepared the same way as for gel filtration described above were centrifuged at 55,000 rpm for 5.5 h at 5°C. Sucrose gradient analysis and the calculations of sedimentation coefficients ($s_{20,w}$), partial specific volumes (v^*), and molecular weights are described in Nałęcz *et al.* (1985).

Cytochrome *c* oxidase activity was measured spectrophotometrically at 20°C. The assay medium contained 10 mM Tris-HCl, pH 7.4, 50 mM KCl, 0.5% Triton X-100, 0.35 nm oxidase, and initially 0.3 to 30 μM ferrocytochrome *c*.

Protein was estimated according to the method of Lowry *et al.* (1951).

Results

The result of a typical gel filtration experiment of *Paracoccus* cytochrome *c* oxidase in Triton X-100 is shown in Fig. 1. The enzyme, whose elution was followed from its heme absorbance, appeared to be a homogeneous species sufficiently separated from the Triton micelles to allow estimation of the amount of the bound detergent. This could be determined by the measured increase in the absorbance ratio A_{277}/A_{424} (2.85) due to the presence of Triton X-100 in the detergent–protein complex. With other nonabsorbing detergents (i.e., in dodecyl maltoside) this ratio was 1.82. The amount of bound Triton molecules per heme aa_3 was then directly calculated according

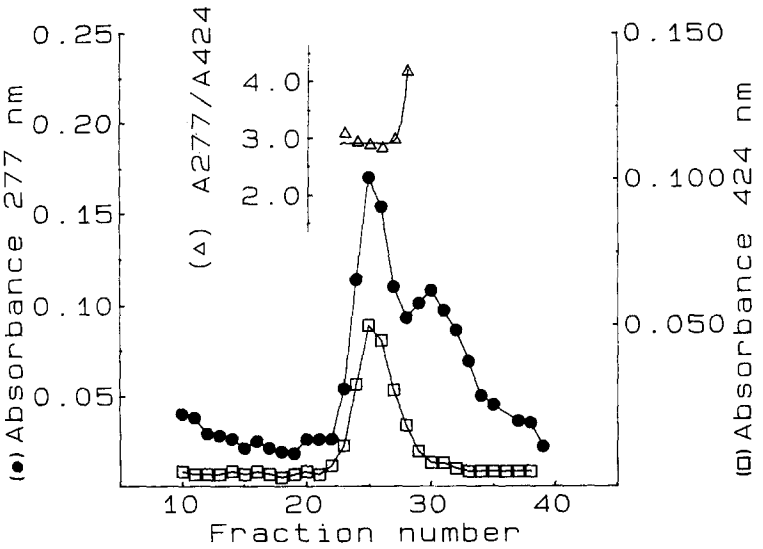

Fig. 1. Gel filtration of cytochrome *c* oxidase from *P. denitrificans* on Ultrogel AcA 34. 1.6 nmols of heme aa_3 in 120 µl was incubated with 0.3% Triton X-100, loaded and run as described in Materials and Methods. In the fractions collected the absorbance at 277 nm (●) and 424 nm (□) was measured. The A_{277}/A_{424} ratio (△) was calculated in the peak fractions considering a basic absorbance at A_{277} of 0.02.

to the formula given in Materials and Methods and is presented in Table I (111.3 mol/mol). This value was constant in four to five fractions and was not changed when the Triton concentration during incubation was increased by a factor of 2 (not shown). This result gave evidence that the amount of Triton binding to cytochrome oxidase was constant and also that, under our

Table I. Molecular Parameters of the Triton X-100 Cytochrome *c* Oxidase Complex from *P. denitrificans* and Bovine Heart Mitochondria[a]

Parameter	Value	
	Paracoccus	bovine
Stokes radius (Å)	54	63
Weight fraction of the protein (mg/mg)	0.535	0.615
Bound Triton X-100 (mol/mol)	111.3 ± 8	190 ± 12
Partial specific volume v^* (ml/g)	0.717	0.817
Sedimentation coefficient $s_{20,w}$ (S)	7.2 ± 0.6	15.5 ± 1.4
Molecular weight M^*	155,000	600,000
Molecular weight of the protein moiety	85,000	360,000
Aggregation state	Monomer	Dimer

[a] Measurements and calculations of the values are described in Materials and Methods and in the text. The values shown are the averages derived from three to seven experiments.

conditions, the protein was saturated with detergent. With the same method the binding of Triton to the bovine oxidase gave 190 mol/mol (Table I), a value comparable to the one obtained by Robinson and Capaldi (1977) (180 mol/mol). Protein and heme estimation in the peak fractions showed a constant heme-to-protein ratio of 26 nmol/mg for the *Paracoccus* oxidase, indicating that hardly any heme was lost during chromatography. From the amount of bound Triton and protein in the fractions the weight fraction of the protein moiety in the detergent–protein complex could be estimated (Table I). This value was used to calculate the partial specific volume of the complex, v^*, which is the sum of the partial specific volumes of the pure protein ($v_1 = 0.551$; Nałęcz *et al.*, 1985) and the detergent ($v_2 = 0.908$; Tanford and Reynolds, 1976) according to their weight fractions (x): $v^* = x_1 v_1 + x_2 v_2$. It might be interesting to note that the partial specific volumes and therefore the particle densities of the Triton- and dodecyl maltoside oxidase complexes from *P. denitrificans* were, within experimental error, the same, although the protein bound more than twice as much dodecyl maltoside than Triton. The difference in the partial specific volumes of the two detergents compensate for the difference in their weight fraction of the complexes.

By molecular size calibration of the Ultrogel column with proteins of known Stokes radii (R_S) according to Ackers (1967) (Fig. 2), an apparent R_S of 54 Å for the Triton protein complex of *Paracoccus* oxidase and 63 Å for the bovine enzyme was obtained (Table I). Although the Stokes radius of solubilized membrane proteins determined by gel filtration might be slightly under- or overestimated depending on their asymmetry and interaction with the resin (Tanford *et al.*, 1974), it is a reasonable measure of the size of a complex relative to another one and may be used to estimate its molecular weight provided that sedimentation analysis data are available.

The sedimentation coefficients of the Triton oxidase complexes presented in Table I were calculated from their sedimentation velocity in a 5–20% sucrose gradient at high angular velocity. The salt and buffer conditions were kept the same as for the filtration experiments, except that the Triton X-100 concentration in the gradient was increased to 0.1%. At lower detergent concentrations, the bovine enzyme, which needs more detergent for stabilization, tended to aggregate and sedimented at the bottom of the tube. The *Paracoccus* oxidase gave the same sedimentation coefficients in 0.05% and 0.1% Triton. The sedimentation coefficients were calculated using the tables of McEwen (1967). Molecular weights for the complexes and, after correcting for the bound detergent, for the protein moieties (Table I) were calculated as described in Nałęcz *et al.* (1985). The value for the *Paracoccus* oxidase of 85,000 is consistent with a monomeric enzyme composed of two subunits with M_r of 45,000 and 28,000 determined by polyacrylamide gel

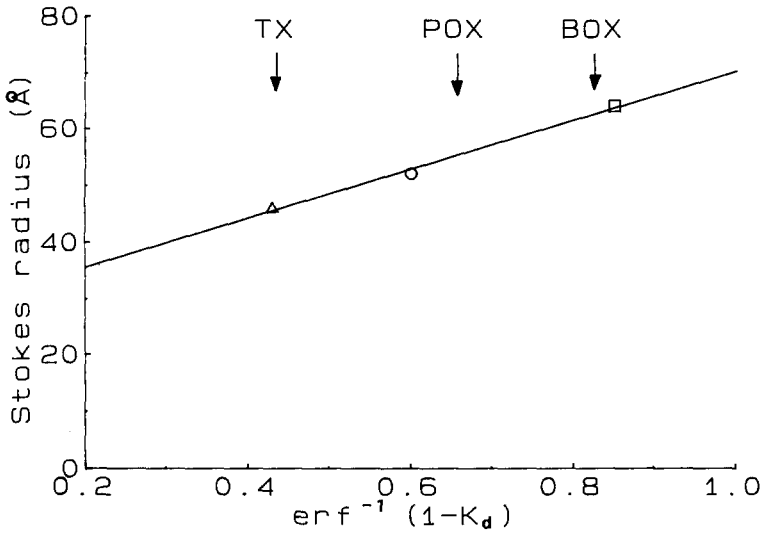

Fig. 2. Estimation of the Stokes radius (R_S) of *Paracoccus* and bovine cytochrome *c* oxidase by gel filtration on Ultrogel AcA 34. The column was calibrated with ferritin (□), catalase (○), and aldolase (△) according to Ackers (1967) taking the Stokes radii from Wang *et al.* (1985). The arrows indicate the positions of bovine (BOX) and *Paracoccus* (POX) cytochrome *c* oxidase and of the Triton X-100 micelles (TX).

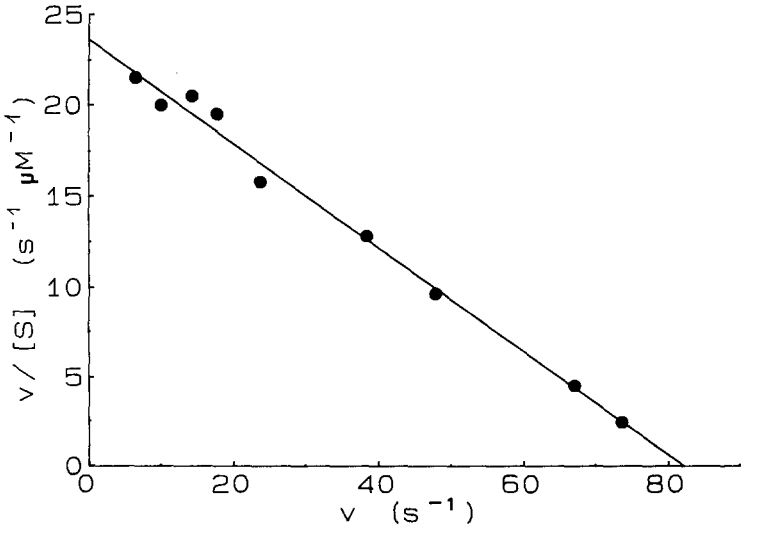

Fig. 3. Eadie–Hofstee plot of *Paracoccus* cytochrome *c* oxidase activity measured spectrophotometrically. The assay medium containing 0.05% Triton X-100 is described in Materials and Methods. The molecular activity (mol cytochrome *c*/mol heme aa_3/s) of cytochrome *c* oxidase (0.35 nM) eluted from the Ultrogel column was measured at initial ferrocytochrome *c* concentrations between 0.3 and 30 μM.

electrophoresis (Kadenbach *et al.*, 1983). Contrary to this, the bovine oxidase was dimeric, in agreement with the result obtained from sedimentation velocity and equilibrium centrifugation studies (Saraste *et al.*, 1981, Georgevich *et al.*, 1983). In the presence of 0.05% Triton X-100 *Paracoccus* oxidase was highly active before (not shown) and after gel chromatography (Fig. 3). Cytochrome *c* oxidase activity measured spectrophotometrically gave a linear Eadie–Hofstee plot over the wide range of 0.3–30 μM ferrocytochrome *c*. The extrapolated V_{max} of 81 s^{-1} was about three times lower than in the presence of dodecyl maltoside (260 s^{-1}) observed previously (Nałęcz *et al.*, 1985). However, the affinity of the detergent oxidase complex for cytochrome *c* seemed not to be influenced by the type of detergent, (K_m 4 $\times$ 10^{-6} M). This phenomenon might be interpreted in two ways: either the electrostatic interaction between cytochrome *c* and the oxidase, supposed to be the main force for a successful electron transfer reaction, is fortuitously similar in both detergents or this interaction is in fact not the rate-limiting step in the assay employed here. A similar observation has already been made previously in case of the subunit III-depleted bovine oxidase (Nałęcz *et al.*, 1985)

A much lower activity (V_{max} = 8 s^{-1}) and a biphasic Eadie–Hofstee plot for the bovine cytochrome *c* oxidase in Triton X-100 was reported elsewhere (Bolli *et al.*, 1985a).

Discussion

The sedimentation coefficient, molecular weight, aggregation state, and kinetics of a membrane protein solubilized in detergents might be modified, depending on the experimental conditions (i.e., protein concentration, ionic strength, kind of detergent) (Bolli *et al.*, 1985a). Molecular size and sedimentation analysis as well as the kinetics of cytochrome *c* oxidase from *Paracoccus denitrificans* were analyzed in Triton X-100 in the same medium. Because of technical reasons the enzyme concentration for the activity measurements had to be much lower than that used for the molecular weight determination. It was previously noted (Bolli *et al.*, 1985a) that dilution of the enzyme might dissociate rather than aggregate the protein detergent complex. Therefore we conclude that the monophasic kinetics observed for the *Paracoccus* oxidase activity in Triton X-100 are obtained with a monomeric enzyme.

Three different detergents [C_8E_{45} (Ludwig *et al.*, 1982), dodecyl maltoside (Nałęcz *et al.*, 1985), and Triton X-100 (this study)] were used up to now to study the aggregation state of *Paracoccus* cytochrome *c* oxidase. Three different sedimentation coefficients ranging from 4.1 to 12.2 were obtained, but in all cases the enzyme proved to be monomeric. It seems thus far that,

for this enzyme, the monomer is the stable and most active form, while in the mammalian oxidase the dimer is considered to be most active. Whether the differences in the electron transfer and proton pumping activities are a consequence of the different aggregation states and/or the presence of the smaller subunits (specially of subunit III) cannot be resolved at present and is the subject of future investigation.

Acknowledgment

We would like to thank Dr. B. Ludwig from the Medizinischen Hochschule Luebeck for providing us with cytochrome *c* oxidase from *Paracoccus denitrificans* and for his helpful discussion. We are grateful to Dr. A. Boschetti from the Institute of Biochemistry of the University of Bern for making available to us the equipment for sucrose gradient centrifugation and analysis. This work was supported by the Schweizerische Nationalfonds (N.3.739-0.80), the Sandoz Stiftung, and the Emil-Barrel Stiftung.

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