

## Solubilized Cytochrome *c* Oxidase from *Paracoccus denitrificans* Is a Monomer\*

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Cytochrome *c* oxidase purified from the bacterium *Paracoccus denitrificans* was analyzed by analytical ultracentrifugation. In the detergent octyltetra/pentaoxyethylene (C<sub>8</sub>E<sub>45</sub>), the isolated enzyme exhibits a molecular weight of 79,000 to 84,000. The detergent-solubilized enzyme is thus a monomer which contains one copy of each of the two subunits.

Bacterial cytochrome *c* oxidases have recently attracted increasing interest because their polypeptide structure is much simpler than that of the mitochondrial oxidases (1). The oxidase from the bacterium *Paracoccus denitrificans*, for example, has kinetic and spectral properties (2, 3) that are very similar, if not identical, to those of bovine heart oxidase, and also catalyzes proton translocation coupled to electron transport (4). Yet, this bacterial oxidase is composed of only two subunits of apparent molecular weights 45,000 and 28,000 (3) whereas mitochondrial oxidases have at least seven subunits (5). Cytochrome *c* oxidase from bovine heart, both in a detergent-solubilized state (6, 7) or when incorporated into densely packed vesicles (8, 9), has been described to exist as a monomer (heme *a*<sub>3</sub>) or a dimer, depending on the choice of detergent. In a native membrane environment, the size of the functional unit of the eukaryotic enzyme is not known.

We have shown previously (3) that the minimal structural unit of the oxidase from *P. denitrificans* is composed of two nonidentical subunits, two heme groups (*a* and *a*<sub>3</sub>), and two copper ions. In this study, we investigated by analytical ultracentrifugation whether solubilized oxidase is a monomer or an oligomer. Sedimentation equilibrium analysis, and the hydrodynamic study were carried out in the detergent octyltetra/pentaoxyethylene, whose partial specific volume allows determination of molecular properties without knowledge of the amount of detergent bound. Solubilized cytochrome *c* oxidase from *Paracoccus* proved to be a monomer with a mass of 79,000 to 84,000 daltons.

### MATERIALS AND METHODS

Growth of *P. denitrificans* (ATCC 13 543) and purification of its cytochrome *c* oxidase were performed as published (3). Enzyme activity was measured spectroscopically (3) in the presence of 25 μM reduced horse heart cytochrome *c* after addition of phospholipid (Asolectin) and an at least 500-fold dilution with 50 mM KP<sub>i</sub>, pH 7.0. Phospholipid bound to the isolated oxidase was determined according to Refs. 10 and 11; the presence of carbohydrate was assessed by

periodic acid-Schiff stain (12) or by testing<sup>1</sup> for binding of <sup>125</sup>I-concanavalin A. Heme was determined spectroscopically as in (3), based on Δε (millimolar, 587–620 nm) = 21.7 for the pyridine hemochromogen (13).

**Determination of Molecular Weight and Hydrodynamic Parameters**—All experiments were performed in standard buffer (20 mM NaP<sub>i</sub>, 0.1 M NaCl, pH 7.0) containing 1% C<sub>8</sub>E<sub>45</sub>,<sup>2</sup> a detergent obtained by distillation from octylpolyoxyethylene (14). It consists to ≥90% of octyltetra- and pentaoxyethylene; the remaining ≤10% are octylhexa- and traces of other homologues (not shown). The density of the buffer, including C<sub>8</sub>E<sub>45</sub>, is 1.0055 g·cm<sup>-3</sup>; its relative viscosity 1.07 cP. The critical temperature of this fraction is 58 °C. The partial specific volume of the detergent micelles ( $\bar{v}_{DM}$ ) is 0.992 cm<sup>3</sup>·g<sup>-1</sup>; the product  $\bar{v}_{DM} \cdot \rho_{solvent} = 0.997$  cm<sup>3</sup>/g. The buoyant density mass,  $M(1 - \phi\rho)$ , therefore reduces to  $M_p(1 - v_{pp})$  (14), since neither phospholipids (detection limit below 1 mol of P<sub>i</sub>/mol of *aa*<sub>3</sub>) or carbohydrate moieties (as determined by periodic acid Schiff stain or concanavalin A binding) were detected in purified preparations. Cytochrome *c* oxidase was freed from Triton X-100 by preparative ultracentrifugation (4 h at 4 or 20 °C; 100,000 × *g*) in 5–20% sucrose gradients containing 1% C<sub>8</sub>E<sub>45</sub>. If <sup>3</sup>H-labeled Triton X-100 (0.246 mCi/g; Roehm and Haas, Philadelphia) was added in a 70-fold molar excess over oxidase, no radioactivity above background was detected in protein-containing fractions after centrifugation (detection limit < 0.06 mol of Triton/mol of *aa*<sub>3</sub>). These fractions were then pooled and used for all subsequent experiments. Analytical ultracentrifugation was performed at 4 °C in a Beckman Model E analytical ultracentrifuge equipped with a photoelectric scanning system. Speeds were 12,000 or 14,000 rpm for sedimentation equilibrium, and 56,000 rpm for sedimentation velocity experiments in AnG or AnD rotors, respectively. Zonal ultracentrifugations in sucrose density gradients were performed according to Martin and Ames (15). Details and the validity of the procedure are described elsewhere (16). Estimation of diffusion coefficients was performed (17) by thin layer gel filtration in standard buffer (*cf.* above). Assays for enzymic activity and SDS-polyacrylamide gel electrophoresis (3) were performed before and after each experiment.

### RESULTS

Fig. 1 shows the result of sedimentation equilibrium centrifugation carried out and presented according to standard procedures. Evaluation of the results of all equilibrium sedimentation experiments, as well as of the hydrodynamic properties of cytochrome *c* oxidase are shown in Table I. Examination of both sedimentation and diffusion coefficients reveals values lower than those of soluble proteins with comparable polypeptide mass, since the hydrodynamic properties of the protein-detergent complex (18), rather than those of the protein moiety, are observed. Nevertheless, their combination yields the protein mass, rather than that of the complex (19). The results of enzymic activities after molecular size determination are shown in Table II. All assays were performed upon reconstitution with phospholipids (Asolectin), since the

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<sup>1</sup> Schneider, G. and Daum, G., unpublished observations.

<sup>2</sup> The abbreviations used are: C<sub>8</sub>E<sub>45</sub>, a mixture of octyltetra- and octylpentaoxyethylene; SDS, sodium dodecyl sulfate.

enzyme exhibits very low, if any, activity in either Triton X-100 or  $C_8E_{45}$ .

## DISCUSSION

The estimated mass of cytochrome *c* oxidase from *P. denitrificans* is  $84,000 \pm 9,000$  daltons in the nonionic detergent  $C_8E_{45}$  (Table I). The values observed are likely to be correct to within about 10%, as shown in Table I, and discussed in detail elsewhere (19). Together with the stoichiometry of its subunits (20), these results strongly suggest that the monomer of cytochrome *c* oxidase consists of one large and one small subunit. It is interesting to compare the values presented here with the molecular weight estimated from SDS-polyacrylamide gel electrophoresis and with the values derived from determinations of the tightly bound ligands, heme and copper. The sum of the subunit molecular weights, as determined by gel electrophoresis in SDS, is lower (Table III). This is not surprising, as apparent molecular weights of membrane pro-

teins estimated from migration rates in gels containing SDS are notoriously unreliable. A relevant example is subunit I of mammalian oxidase for which the molecular weight, calculated from its DNA sequence, is considerably higher than the apparent molecular weight, estimated from SDS-polyacrylamide gel electrophoresis (23). Since subunits I of the mitochondrial and the *Paracoccus* oxidase appear to be homologous (3), the value of 45,000 for the molecular weight of

TABLE II

## Oxidase activity before and after molecular weight determination

Activities were measured as described under "Materials and Methods." Care was taken to minimize exposure of the enzyme to the detergent since prolonged exposure, especially at room temperature, led to a considerable and apparently irreversible loss of activity.

Enzyme fraction	Specific activity $\mu\text{mol of cytochrome } c \text{ oxidized}/(\text{min} \times \text{mg of protein})$
Stock enzyme	30.5
After sucrose gradient centrifugation	16.7
After sedimentation velocity centrifugation	19.4
After sedimentation equilibrium centrifugation	12.7
After thin layer gel filtration (peak fractions)	11.1-16.8

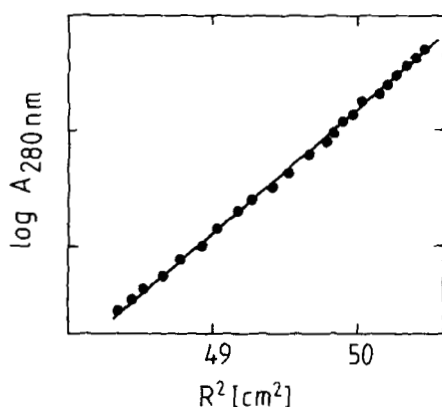


FIG. 1. Determination of cytochrome *c* oxidase mass by analytical ultracentrifugation to equilibrium. After conclusion of the experiment, the enzyme still exhibited 42% of the specific activity of the stock enzyme (Table II). The molecular weight, derived from the slope shown, is 81,000. Simultaneous experiments with enzyme whose activity was reduced after sedimentation exhibited tendencies to dissociate and to aggregate. Centrifugations were performed in standard buffer (see "Materials and Methods"). Absorbance was scanned at 280 nm. The detergent  $C_8E_{45}$  was present at a concentration of 1%.

TABLE III

## Molecular weight estimation by several independent methods

Method	Molecular weight
Ultracentrifugal analyses (this study)	79,000-84,000
Apparent molecular weight from SDS-polyacrylamide gel electrophoresis (3)	71,000-74,000
Heme to protein ratio	69,500 <sup>a</sup> 65,500 <sup>b</sup> 67,000 <sup>c</sup>
Copper to protein ratio	67,000 <sup>d</sup>

<sup>a</sup> Heme determined spectroscopically (see "Materials and Methods") and protein according to Lowry *et al.* (21) in the presence of 0.5% SDS.

<sup>b</sup> As *a*, but iron determination by atomic absorption (3).

<sup>c</sup> As *a*, but protein measured by quantitative amino acid determination (22) in presence of a norleucin standard added before hydrolysis.

<sup>d</sup> As *a*, but copper determined by atomic absorption (3).

TABLE I

Molecular weight and hydrodynamic properties of cytochrome *c* oxidase from *P. denitrificans* in the presence of  $C_8E_{45}$ 

The partial specific volume of the protein ( $\bar{v}_p$ ) was calculated from the amino acid composition. Values of a redetermination were within 10% of those published previously (3), except for Asx (new determination, 7.3 mol %), Ile (3.7 mol %), Leu (9.0 mol %), Tyr (3.7 mol %), and Met (3.6 mol %). In addition, the contents of Cys and Trp were determined to be 1.0 mol % and 2.0 mol %, respectively. These data yield a value of  $\bar{v}_p = 0.742$ .

Method	Hydrodynamic properties <sup>a</sup>	$M_r \pm$ standard deviations	Number of Experiments
Sedimentation equilibrium (analytical ultracentrifugation)		$84,000 \pm 9,000^b$	5 <sup>c</sup>
$s_{20,w}^d$ (analytical ultracentrifugation)	$4.1 \pm 0.3$ S		4
$D_{20,w}$ (calculated from $M_r$ and $s_{20,w}$ ; cf. above)	$4.7 \cdot 10^{-7}$ cm <sup>2</sup> s <sup>-1</sup>		NA <sup>e</sup>
$s_{\text{obs}}$ (zonal centrifugation in sucrose gradients)	$4.2 \pm 0.25$		4
$D_{\text{obs}}$ (thin layer gel filtration)	$5.1 \pm 0.3 \cdot 10^{-7}$ cm <sup>2</sup> s <sup>-1</sup>		4
$M_r$ , calculated by the Svedberg equation from the observed $s$ , $D$		$79,000 \pm 15\%$	NA

<sup>a</sup> Hydrodynamic properties are those of the protein-detergent complex (cf. text).

<sup>b</sup> The standard deviation of the values obtained from sedimentation equilibrium analyses is about 5,000. In view of the dissociation and aggregation phenomena mentioned in the legend to Fig. 1, a value of 10% appears more appropriate.

<sup>c</sup> 5 experiments of two independent preparations.

<sup>d</sup> Determinations at concentrations from 0.2 to 0.25 mg/ml were normalized to water and 20 °C by standard procedures. They were not extrapolated to zero concentrations.

<sup>e</sup> Not applicable.

*Paracoccus* oxidase subunit I may well represent an underestimation. Heme *a* can be quantitated spectroscopically based on the pyridine hemochromogen, but different literature values for the extinction coefficient of the pyridine hemochromogen (see Ref. 24) do not allow conclusive evaluation of the results obtained. Values for copper and (heme-) iron, as determined by atomic absorption analysis, are biased by the assumption that the metal content only reflects specifically bound metal ions, and that no heme or copper had been lost during purification.

We conclude that, in the nonionic detergent used, cytochrome *c* oxidase from *P. denitrificans* exists as a monomer containing one copy of each of two subunits, two copper atoms and two heme *a* groups. Of course, this study does not answer the question whether monomers or oligomers represent the functional entity, since *Paracoccus* oxidase exhibits very low enzymic activity in the detergent used in this study as well as in all other detergents tested so far. Nevertheless, if oligomerization is required for activity, the structure of the enzyme in a monomeric state is not irreversibly perturbed in the detergents used. Lack of activity in the solubilized state could either reflect inhibition of cytochrome *c* binding, or reversible structural alterations of the enzyme.

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