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# CYTOCHROME OXIDASE FROM *PSEUDOMONAS AERUGINOSA*

## I. PURIFICATION AND SOME PROPERTIES*

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SUMMARY

A new purification procedure for the isolation of *Pseudomonas* cytochrome oxidase (ferrocytochrome $c_2$:$O_2$ oxidoreductase, EC 1.9.3.2) is described. The product is purified 16-fold over the crude cell extract and contains equimolar amounts of heme *c* and heme *d*.

The molecular weight of the electrophoretically homogeneous enzyme is about 121200 as determined by sedimentation equilibrium. Acrylamide gel electrophoresis in the presence of sodium dodecyl sulfate results in a single protein-staining band with a molecular weight of 58000. The minimal molecular weight on the basis of heme-iron content is 55600.

With $O_2$ as the final electron acceptor and at 30 °C in 0.1 M phosphate at the optimum pH of 6.0 the $K_m$ for reduced *Pseudomonas* cytochrome *c*-551 as substrate is 10.5 $\mu$M whereas with *Pseudomonas* copper protein as the electron source the $K_m$ is 17.2 $\mu$M under identical conditions.

The absorption spectrum of purified *Pseudomonas* cytochrome oxidase shows maxima at 640, 525 and 412 nm in the oxidized state and at 625–655, 544, 549, 521, 460 and 418 nm in the reduced state.

The EPR spectrum taken at 10 °K has signals with *g* values of 4.30, 2.93, 2.45, 2.31, 2.08, 1.71 and 1.4. Evidence indicates that those signals at $g=2.93$, 2.31 and 1.4 arise from the heme *c* while those at $g=2.45$ and 1.71 arise from heme *d*. No signals can be seen after reduction of the enzyme.

## INTRODUCTION

*Pseudomonas* cytochrome oxidase (ferrocytochrome $c_2$:$O_2$ oxidoreductase, EC 1.9.3.2) is an enzyme that functions as a terminal electron carrier in the bacterium, *Pseudomonas aeruginosa*. This enzyme has been purified to a state of homogeneity and crystallized by Okunuki and co-workers[1–8] who have also studied many of its physical and enzymic properties. Their work indicates that *Pseudomonas* cytochrome oxidase is a protein with a molecular weight of about 90000 that contains

* A preliminary account of this work has been reported (see Gudat, J. C. and Wharton, D. C. (1971) *Fed. Proc.* 30, 1190).

two heme prosthetic groups, one a heme *c* and the other a heme *d*. No metal besides Fe has been detected. The prosthetic groups on the oxidase can be reduced by either of two electron donors that are present in the organism, cytochrome *c*-551 and a copper protein; the reduced oxidase can be oxidized either by $O_2$ or by nitrite.

Because *Pseudomonas* cytochrome oxidase contains two distinct heme groups on a homogeneous protein and since it can be obtained in crystalline form, we believe that this enzyme is an excellent candidate with which to study electron transfer mechanisms. In order to undertake these studies and to lay the necessary groundwork, copious amounts of *Pseudomonas* cytochrome oxidase are required. Although we proceeded initially on the assumption that the purification procedure of Horio *et al.*[4] would supply us with sufficient quantities of highly purified oxidase, we found that, in our hands, their procedure resulted in quite variable and unpredictable results. For that reason a new purification procedure was developed. This new procedure appears to be highly reproducible and yields a product in large quantity that has high enzymic activity and appears to be homogeneous. In addition, the electron donors of the oxidase, *Pseudomonas* cytochrome *c*-551 and *Pseudomonas* copper protein, can be purified easily from the cell extract. Furthermore, the new procedure avoids the use of highly flammable acetone that is utilized in the procedure of Horio *et al.*[4].

In this communication we present details of the new purification procedure, including those for the isolation of the other electron transfer components, and describe properties of the purified *Pseudomonas* cytochrome oxidase. These properties are compared to those reported by the Japanese workers for their purified enzyme. In a later paper we shall discuss the properties of the other electron carriers purified from *P. aeruginosa*.

## EXPERIMENTAL

### *Materials*

All inorganic reagents were of analytical reagent grade. $(NH_4)_2SO_4$ was the special enzyme grade and Tris, the ultrapure grade; both were purchased from Schwarz-Mann. Deoxyribonuclease (beef pancreas, amorphous) was a product of Sigma Chemical Co. Sephadex G-100 and carboxymethyl Sephadex C-50 were obtained from Pharmacia. DEAE-cellulose and CM-cellulose were purchased from Reeve Angel and Co. in the pre-swollen form. Ascorbic acid was a product of Merck and Co. Bathocuproine (2,9-dimethyl-4,7-diphenyl-1,10-phenanthroline) and bathophenanthroline (4,7-diphenyl-1,10-phenanthroline) were obtained from G. Frederick Smith Chemical Co. $Na_2S_2O_4$ was a product of Hardman and Holden, Miles Platting, Manchester, England. Reagents for acrylamide gel electrophoresis were purchased from Canalco, Inc. Benzidine·2HCl and sodium dodecyl sulfate were obtained from Fisher Scientific; the sodium dodecyl sulfate was then recrystallized from 95% ethanol. CO (reagent grade) was a product of Matheson. Trypsin (hog pancreas, 1:300) was supplied by Nutritional Biochemicals Corp. Anthrone was purchased from Eastman.

### *Methods*

#### *Estimation of protein*

Protein concentration was determined using either the biuret method of

Gornall *et al.*[9] or by the method of Lowry *et al.*[10]. Crystalline bovine serum albumin was employed as the standard with both methods.

*Estimation of iron and copper*

Total iron was determined according to the extraction method of Doeg and Ziegler[11]. This method was found to give results that were in good agreement with those obtained using the wet digestion procedure of Van de Bogart and Beinert[12]. Non-heme iron was estimated by the method of Doeg and Ziegler[11]. Cu was assayed by the method of Wharton and Rader[13].

*Lipid determination*

Lipid was determined colorimetrically by the method of Johnson[14] after extraction with chloroform–methanol (4:1, v/v).

*Estimation of flavin*

Flavin content was determined spectrophotometrically by acid extraction before and after tryptic digestion.

*Estimation of carbohydrate*

Total carbohydrate was assayed by the anthrone method of Hassid and Abraham[15].

*Polyacrylamide gel electrophoresis*

Electrophoretic homogeneity of *Pseudomonas* cytochrome oxidase was determined by acrylamide gel electrophoresis according to the method of Davis[16]. A Buchler disc gel electrophoresis apparatus with a Gelman Model 38206 power supply was used. The upper buffer contained 0.0025% (w/v) Bromophenol blue as a tracking dye. After electrophoresis the gels were removed and fixed overnight in a few ml of a solution containing 10% (w/v) trichloroacetic acid and 10% (w/v) sulfosalicyclic acid. For the detection of protein bands the gel was immersed for 3 h in a solution of 0.05% (w/v) Coomassie brilliant blue in 12.5% trichloroacetic acid. The gels were destained by immersion for 24 h in 7.5% acetic acid. Several changes of acetic acid were made during the destaining period.

Heme groups were detected in the gels by means of the reaction with benzidine·2HCl. For this reaction the gels, after the destaining with 7.5% acetic acid, were transferred to test tubes (13 mm × 100 mm) containing 1 ml of 1% (w/v) aqueous benzidine·2HCl, 1 ml of 7.5% acetic acid, and sufficient distilled water to cover the gels. After incubation for 1 h at room temperature a few ml of the solution were removed and replaced with a similar volume of 5% $H_2O_2$. After the brown color had reached a maximum intensity the gels were removed, rinsed in 7.5% acetic acid, and stored in 7.5% acetic acid.

Molecular weight of protein components was determined by acrylamide gel electrophoresis in the presence of 1% (w/v) sodium dodecyl sulfate according to the method of Weber and Osborn[17] with the exception that only one-half of the usual concentration of cross-linkage was used. Staining was done as described by Weber and Osborn. A standard curve to determine molecular weight was constructed from data collected from the identical electrophoresis of proteins of known molecular weight.

*Sedimentation equilibrium*

For determinations of particle weight, samples of *Pseudomonas* cytochrome oxidase were subjected to sedimentation equilibrium in a Beckman Model E analytical ultracentrifuge according to the method of Yphantis[18]. The sample of oxidase was

used at protein concentrations of 0.1 and 0.3 mg per ml of 0.05 M Tris–HCl, pH 8.0. Samples were centrifuged at 4 °C using an Yphantis-type cell in an AN-D rotor at a speed of 18000 rev./min. The boundary region was measured by means of a photoelectric scanner at a wavelength of 280 nm.

*Absorption spectra*

Absorption spectra of the respiratory components of *P. aeruginosa* were obtained using either a Beckman Model DK-2A ratio recording spectrophotometer or a Cary Model 14 recording spectrophotometer. Low temperature spectra were obtained in a Cary Model 14 spectrophotometer equipped with a scattered transmission accessory.

*Determination of Pseudomonas electron carriers*

The concentration of *Pseudomonas* cytochrome oxidase was determined from the absorbance of the dithionite-reduced enzyme at 554 nm using an extinction coefficient of 30.2 $mM^{-1} \cdot cm^{-1}$ (ref. 4). The concentration of cytochrome *c*-551 was calculated from the absorbance of its α-band at 551 nm after reduction with dithionite using an extinction coefficient of 28.3 $mM^{-1} \cdot cm^{-1}$ (ref. 19). The concentration of copper protein was obtained from the absorbance of the oxidized protein at 630 nm using an extinction coefficient of 6.95 $mM^{-1} \cdot cm^{-1}$ (ref. 20).

*Assay of activity*

*Pseudomonas* cytochrome oxidase activity was measured spectrophotometrically by observing the rate of oxidation of reduced *Pseudomonas* cytochrome *c*-551 or *Pseudomonas* copper protein. Where *Pseudomonas* cytochrome *c*-551 was used the reaction mixture, in a final volume of 1 ml, routinely contained 60 μM reduced cytochrome *c*-551 in 0.1 M phosphate buffer, pH 6.0. The stock solution of cytochrome *c*-551 was reduced by adding a few grains of $Na_2S_2O_4$; excess dithionite was removed by gently shaking the solution for a few minutes. After the mixture had equilibrated at 30 °C for 4 min, the reaction was initiated by adding 10 μl of enzyme solution of suitable concentration and the absorbance change at 551 nm followed by means of a Heath Model EU-20B recorder attached to a Beckman Model DU-2 spectrophotometer modified with a Gilford Model 222 photometer. Initial rates were used to determine specific activity which was expressed as μmoles of cytochrome *c*-551 oxidized per min per mg of enzyme protein.

When *Pseudomonas* copper protein was used in the assay system the reaction mixture, in a final volume of 1 ml, routinely contained 45 μM of reduced Cu protein in 0.1 M phosphate buffer, pH 6.0. The stock solution of Cu protein was reduced by adding a few grains of $Na_2S_2O_4$; excess dithionite was removed by dialyzing the copper protein overnight at 4 °C *vs* 500 volumes of the phosphate buffer. The assay was carried out exactly as in the case of the cytochrome *c*-551 except that a wavelength of 620 nm was used to monitor the reaction. Specific activity was expressed as μmoles of *Pseudomonas* copper protein oxidized per min per mg of enzyme protein.

*Electron paramagnetic resonance spectroscopy*

EPR spectroscopy was performed either in a Varian Model E-3 spectrometer equipped with a Model V-4557 variable temperature accessory or in a Varian Model V-4500 spectrometer with modifications described by Beinert and co-workers[21–24]. The Model E-3 spectrometer was used for experiments at temperatures of liquid

$N_2$ while the Model E-4500 spectrometer was used chiefly for experiments near the temperature of liquid He.

The spectra shown in this paper represent the first derivatives of the absorption curves. The *g* values given on these spectra do not necessarily represent the true *g* values but are given as a means of quickly locating prominent points in the spectra.

*Organism*

A culture of *P. aeruginosa* was obtained from the Section of Microbiology, Cornell University, through the courtesy of Professor E. Delwiche. The organism was maintained routinely on an agar slant (2%) containing bouillon–peptone. Slants were stored in a refrigerator and transfers made to new slants every two months.

*Culture conditions*

For the large-scale production of cells of *P. aeruginosa* the organism was grown in three stages. In the first stage 30 ml of the bouillon-peptone medium (5 g beef extract, 10 g peptone, and 2.5 g NaCl per 1 of solution adjusted to pH 7.0 with NaOH) were inoculated from an agar slant of the culture. The flask was incubated for 24 h at 37 °C with shaking. This culture was then added to 12 l of the bouillon–peptone medium and the cells grown under the same conditions as for the 30-ml volume.

The final stage of cell production was accomplished by adding the 12-l culture to 190 l of nitrate medium (10 g beef extract, 10 g peptone, 20 g $KNO_3$, 6.4 g $KH_2PO_4$, 3.6 g $Na_2HPO_4$ per 1 of solution) in a large fermentor. After stirring in the inoculum the cells were grown at 37 °C without aeration or agitation. When the pH of the culture reached 8, the cells were harvested using a Sharples centrifuge. Growth curves indicated that at pH 8 the culture of *P. aeruginosa* was in the late log state of growth. After harvesting the cell paste was frozen and stored at −20 °C.

The same culture of *P. aeruginosa* can now be obtained by special order as a frozen cell paste from General Biochemicals, Chagrin Falls, Ohio.

*Purification of Pseudomonas cytochrome oxidase*

All operations were performed at 4 °C unless stated otherwise.

*Step 1. Preparation of crude extract.* The frozen cell paste of *P. aeruginosa* was thawed and suspended uniformly in 0.1 M phosphate buffer, pH 6.0, at a concentration of 1 g of paste per 3 ml of buffer. After filtering the suspension through two layers of cheesecloth the cells were broken by passing the filtered suspension through a Manton–Gaulin laboratory homogenizer (Manton–Gaulin Manufacturing Company, Inc., Everett, Mass.) at a pressure of 9000 lb/inch$^2$. The effluent from the homogenizer, which was noticeably darker in color and much more viscous than the original suspension, was collected in a 3-l beaker immersed in a salted ice bath. Deoxyribonuclease was added to the effluent at a concentration of 1 mg per 100 ml and within 5 min had eliminated the increased viscosity .When the temperature of the suspension of broken cells reached 10 °C the homogenization was repeated. Following the second homogenization solid $(NH_4)_2SO_4$ was added to give a concentration of 10% of saturation (7.2 g/100 ml). The addition of $(NH_4)_2SO_4$ increased the yield of *Pseudomonas* cytochrome oxidase as well as other oxidation–reduction compounds. After 30 min the suspension was centrifuged for 40 min at $18000 \times g$ in the GSA rotor of a Sorvall Model RC-2B centrifuge. The brown supernatant was decanted and stored at 4 °C. The residue was resuspended

in one-half the original volume of 0.1 M phosphate buffer, pH 6.0, and this suspension was rehomogenized twice in the Manton–Gaulin homogenizer as described before. This homogenate was then centrifuged as outlined above except no further $(NH_4)_2SO_4$ was added. The supernatant was decanted and combined with the first supernatant. The residue was discarded. Of the total *Pseudomonas* cytochrome oxidase in the combined supernatant solution, approximately 90% came from the first homogenization.

*Step 2. $(NH_4)_2SO_4$ fractionation.* The combined extract was brought to 40% of saturation by adding solid $(NH_4)_2SO_4$ (21.5 g/100 ml). This solution was then centrifuged for 30 min at $18000 \times g$ in the GSA rotor of a Sorvall Model RC-2B centrifuge. The pinkish-brown residue was discarded and the golden-brown, almost clear supernatant was brought to 95% of saturation by adding solid $(NH_4)_2SO_4$ (39.8 g/100 ml). This solution was then centrifuged for 20 min at $18000 \times g$ as before. The supernatant was discarded and the light tan pellet was suspended in cold glass-distilled water at a concentration of 35 ml of water per 100 g of original cell paste. The suspension was dialyzed with agitation for 12 h in half-filled No. 30 dialysis tubing (Visking) against 40 volumes of glass-distilled water. The dialysis medium was changed twice during the 12-h period.

*Step 3. Chromatography on Sephadex G-100.* The dialyzed $(NH_4)_2SO_4$ fraction was applied to the base of a column (5.0 cm × 100.0 cm) of Sephadex G-100 equilibrated with 0.01 M phosphate buffer, pH 6.0. The column was operated by upward flow. Protein was eluted from the column with 0.01 M phosphate buffer, pH 6.0, and was collected in 6-ml fractions using a LKB Model 7000A fraction collector. The elution pattern of this column, which is illustrated in Fig. 1 shows a separation of the $(NH_4)_2SO_4$ fraction into two broad fractions differing in molecular weight.

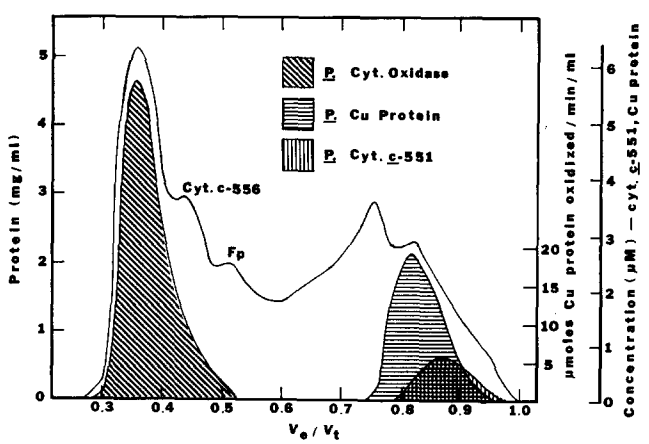



Fig. 1. Elution diagram of respiratory components of *P. aeruginosa* from a column of Sephadex G-100. Elution was achieved with 0.01 M phosphate buffer, pH 6.0, and samples were collected in 6-ml fractions. *Pseudomonas* cytochrome oxidase was assayed by means of its oxidation of reduced *Pseudomonas* copper protein as described in Methods. The concentrations of *Pseudomonas* cytochrome *c*-551 and *Pseudomonas* copper protein were determined spectrophotometrically as described in Methods. $F_p$, flavoprotein.

The first of these contains *Pseudomonas* cytochrome oxidase as well as a *c*-type cytochrome (*c*-556) and a flavoprotein; the second broad fraction contains cytochrome *c*-551 and a blue Cu protein.

*Step 4. Chromatography on CM-Sephadex C-50.* The fractions obtained from the Sephadex G-100 column that contained *Pseudomonas* cytochrome oxidase activity were combined and applied to a column (2.5 cm × 40 cm) of CM-Sephadex C-50 that had been equilibrated with 0.01 M phosphate buffer, pH 6.0. As the sample was applied to the column a tight reddish-orange band formed at the top while the bulk of the greenish brown oxidase passed through. Most of the *Pseudomonas* cytochrome oxidase was eluted from the column with the equilibrating buffer while the reddish-orange band adhered to the top of the column. About one-third of the *Pseudomonas* cytochrome oxidase applied to the CM-Sephadex column was not eluted by the equilibrating buffer. This oxidase was eluted subsequently with 0.1 M sodium phosphate, pH 7.0, following elution of the reddish-orange band with 0.01 M sodium phosphate, pH 7.0. The *Pseudomonas* cytochrome oxidase eluted with the 0 1 M phosphate was dialyzed overnight against 0.02 M Tris–HCl, pH 8.0, and then combined with the remainder of the oxidase that had been eluted earlier from the DEAE-cellulose column (see Step 5).

*Step 5. Chromatography on DEAE-cellulose.* The greenish brown effluent obtained from the column of CM-Sephadex C-50 using the equilibrating buffer was adjusted to a pH of 8.1 by adding a suitable volume of 0.05 M Tris. This solution was then applied to the top of a column (2.5 cm × 25.0 cm) of DEAE-cellulose that had been equilibrated with 0.05 M Tris–HCl, pH 8.0. It was found that a bed-height of 6 cm was required for each 100 g of cell paste that had been extracted. The oxidase was eluted from the column as a broad green band using 0.05 M Tris–HCl, pH 8.0, and was collected as 3-ml fractions by means of the LKB fraction collector. Each fraction was assayed for protein concentration and for *Pseudomonas* cytochrome oxidase activity; the absorbance at 280 and 410 nm was measured for each fraction. Those fractions with oxidase activity and an $A_{280\,nm}/A_{410\,nm}$ of less than 1.0 were pooled.

*Step 6. Gradient elution chromatography on DEAE-cellulose.* The pooled fractions of Step 5 along with the dialyzed fractions obtained by elution of the CM-Sephadex C-50 with 0.1 M phosphate (see Step 4) were applied to a column (2.0 cm × 20 cm) of fresh DEAE cellulose equilibrated with 0.02 M Tris–HCl, pH 8.0. The oxidase was eluted from the column by means of a linear gradient of 0.02 M to 0.05 M Tris–HCl, pH 8.0. The total volume of the elution buffer was 1000 ml. Fractions containing the oxidase were pooled and concentrated by vacuum dialysis followed by dialysis overnight against 0.05 M Tris–HCl, pH 8.0.

*Purification of other electron transfer components. Purification of Pseudomonas cytochrome c-551 and Pseudomonas copper protein.* The second broad fraction obtained earlier from the column of Sephadex G-100 (See Step 3 above) was adjusted to a pH of 3.9 by adding a suitable volume of 0.05 M acetic acid. The whitish precipitate that formed on acidification was removed by centrifuging the turbid solution for 20 min at 20000 × *g* in the SS-34 rotor of a Sorvall Model RC-2B centrifuge. The clear supernatant was applied to the top of a column (1.5 cm × 25 cm) of CM-cellulose that had been equilibrated with 0.05 M acetate–$NH_4OH$ buffer, pH 3.9. The *Pseudomonas* cytochrome *c*-551 was eluted from the column as a red band

using the 0.05 M acetate–$NH_4OH$ buffer, pH 4.45. After the *Pseudomonas* cytochrome *c*-551 had eluted, the column was washed with an additional 100 ml of the same buffer. The *Pseudomonas* copper protein, which had by now moved half-way through the column, was eluted as a blue band using 0.05 M acetate–$NH_4OH$ buffer, pH 4.65.

The red effluent containing the cytochrome *c*-551 was concentrated by precipitation with solid $(NH_4)_2SO_4$. The effluent solution was first taken to 60% saturation (43 g per 100 ml) and the precipitate discarded after centrifugation for 30 min at $20000 \times g$. The supernatant was made 75% saturated (10.7 g per 100 ml) and the resulting precipitate collected by centrifugation as above. The clear, very light red supernatant was discarded; the pellet was dissolved in a few ml of 0.1 M phosphate, pH 6.0, and the solution was dialyzed for 12 h in No. 8 dialysis tubing (Visking) against a 30-fold excess of the suspending buffer.

The blue fractions eluted from the column of CM-cellulose were combined and concentrated by vacuum dialysis using a Schleicher and Schuell model No. 100/21 apparatus. The concentrated solution of copper protein was then dialyzed for 12 h in No. 8 dialysis tubing against 30 volumes of 0.1 M phosphate, pH 6.0.

## RESULTS

### *Purification of Pseudomonas cytochrome oxidase*

The results of a typical fractionation procedure for the purification of *Pseudomonas* cytochrome oxidase are summarized in Table I.

A 14- to 16-fold increase in the specific activity over the initial crude cell extract was attained with a recovery of about 5–10%.

### *Composition of Pseudomonas cytochrome oxidase*

The iron and copper content of purified preparations of *Pseudomonas* cytochrome oxidase is summarized in Table II. These preparations usually contained

TABLE I

PURIFICATION OF *PSEUDOMONAS* CYTOCHROME OXIDASE

| *Fraction* | *Vol. (ml)* | *Protein* | | *Activity* | | *Specific activity (μmoles Cu protein oxidized/min per mg protein)* | *% Yield* | *Purification ratio* |
|---|---|---|---|---|---|---|---|---|
| | | *(mg per ml)* | *(total mg)* | *(units* per ml)* | *(total units)* | | | |
| Crude extract | 1760 | 16.9 | 29 744 | 1.30 | 2288 | 0.077 | 100 | 1.0 |
| $(NH_4)_2SO_4$, 40–95% | 380 | 27.6 | 10 488 | 2.18 | 828 | 0.079 | 36 | 1.03 |
| Sephadex G-100 | 560 | 8.7 | 4 872 | 1.27 | 711 | 0.145 | 31 | 1.88 |
| CM-Sephadex | 615 | 6.4 | 3 936 | 0.97 | 597 | 0.152 | 26 | 1.97 |
| DEAE-cellulose | 177 | 3.5 | 620 | 1.40 | 248 | 0.400 | 11 | 5.20 |
| Gradient elution from DEAE-cellulose | 10 | 9.3 | 93 | 11.5 | 115 | 1.24 | 5 | 16.1 |

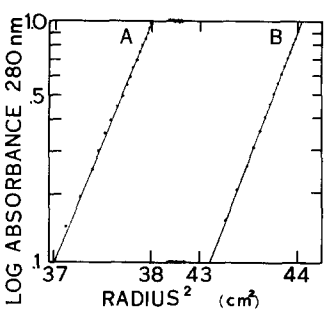
* Units are defined as μmoles of copper protein oxidized per min.

TABLE II

CONCENTRATION OF HEME, IRON, AND COPPER IN PURIFIED *PSEUDOMONAS* CYTOCHROME OXIDASE

| *Component* | *Concentration (μmoles per g protein)* |
|---|---|
| Heme | 18.0 |
| Total iron | 19.4 |
| Non-heme iron | 1.2 |
| Copper | 1.5 |

a small amount of copper and non-heme iron. The great bulk of the iron, however, was present in the heme groups of the protein. Dialysis of the enzyme against 0.01 M KCN, pH 8.0, resulted in the complete removal of the copper and partial loss of the non-heme iron. The content of heme iron was not affected by the cyanide treatment nor was there a significant loss of activity.



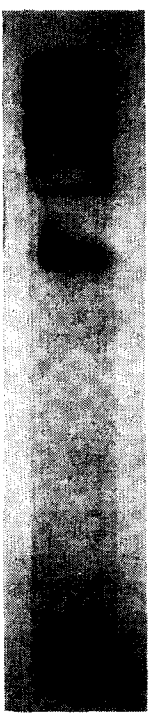



Fig. 2. Sedimentation equilibrium of purified *Pseudomonas* cytochrome oxidase showing the relationship of the logarithm of the concentration of the enzyme in 0.05 M Tris buffer, pH 8.0, *versus* the square of the radius of the absorption maximum at 280 nm. A, protein concentration of 0.1 mg per ml; a slope of 2.43 was calculated from these data. B, protein concentration of 0.3 mg per ml; a slope of 2.52 was calculated from these data.

Fig. 3. Polyacrylamide gel electrophoresis of *Pseudomonas* cytochrome oxidase. Gels contained 7% acrylamide.

No acid extractable flavin was detected before or after tryptic digestion. No carbohydrate or lipid could be found in purified preparations of the oxidase.

*Physical properties of Pseudomonas cytochrome oxidase*

*Sedimentation equilibrium.* Only one species was evident during the ultracentrifugation of purified *Pseudomonas* cytochrome oxidase. This species, at a protein concentration of 0.1 and 0.3 mg/ml, was calculated to have a molecular weight of 121 215 and 121 272 for the two concentrations, respectively, and with $\bar{v}$ equal to 0.73 $cm^3/g$. The slopes obtained from these runs and shown in Fig. 2 reflect the effect of changes in concentration on the sedimentation equilibrium. The fact that the slopes for two different initial concentrations are straight lines indicates that the enzyme is homogeneous.

*Acrylamide gel electrophoresis.* When purified *Pseudomonas* cytochrome oxidase underwent polyacrylamide gel electrophoresis only one protein-staining band was observed as shown in Fig. 3.

Acrylamide gel electrophoresis of the same enzyme preparation in the presence of 1% sodium dodecyl sulfate resulted in only one protein-staining band as shown in Fig. 4. The band, which was also stained with the heme-specific benzidine·2HCl, was calculated to have a molecular weight of 58000. Increasing the concentration of sodium dodecyl sulfate up to three times the normal concentration failed to produce any further dissociation of the protein.

A minimal molecular weight of 55600 was calculated on the basis of hemeron content. No significant amounts of non-heme iron were detected.

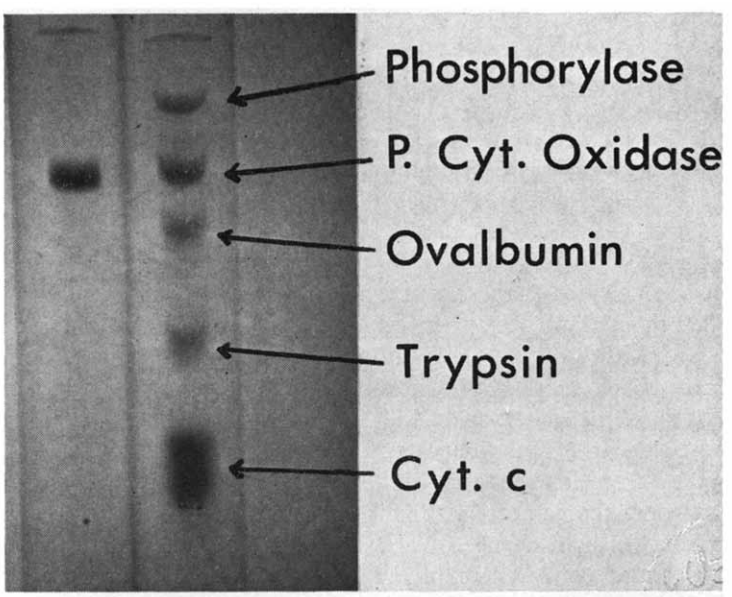



Fig. 4. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of *Pseudomonas* cytochrome oxidase and protein standards. Gels contained 10% acrylamide and 1% sodium dodecyl sulfate. The following standards were employed: glycogen phosphorylase (mol. wt 94000), ovalbumin (mol. wt 43000), trypsin (mol. wt 23700), and cytochrome *c* (mol. wt 12200).

*Absorption spectrum*

The absorption spectrum of purified *Pseudomonas* cytochrome oxidase at pH 8.0 is illustrated in Fig. 5. The oxidized form has absorption maxima at 640, 525 and 412 nm and a shoulder at 360 nm. After reduction with sodium dithionite maxima are present at 625–655, 554, 549, 521, 460 and 418 nm. These spectra are similar to those reported by Okunuki and co-workers[1,3–5] for *Pseudomonas* cytochrome oxidase prepared by a different procedure.

The position and absorbance of the bands associated with the heme *c* component at 554, 549, 521 and 418 nm were uniform from one preparation to another. However, some variability was observed in the shape of the absorption maximum of the heme *d* component which appeared to be associated with the relative absorbances of two bands, one at 655 nm and the other at 625 nm. Most often a plateau was observed where both bands contributed equally to the absorption.

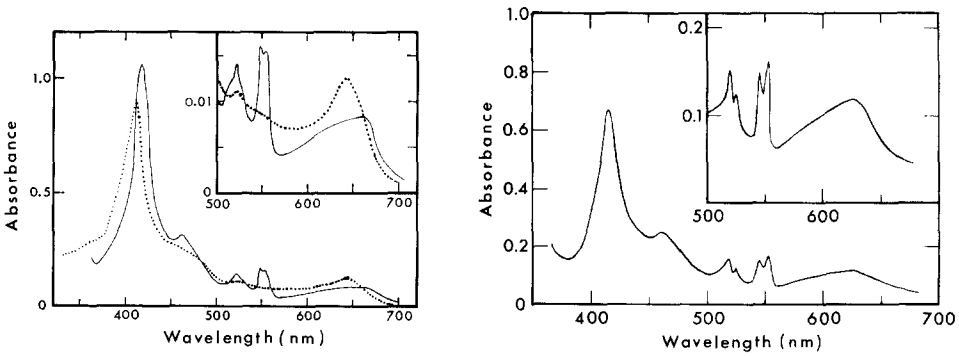



Fig. 5. Absorption spectra of *Pseudomonas* cytochrome oxidase. The enzyme was dissolved in 0.05 M Tris–HCl buffer, pH 8.0. ———, the oxidized preparation; ·····, the preparation reduced with $Na_2S_2O_4$.

Fig. 6. Absorption spectrum of *Pseudomonas* cytochrome oxidase at 87 °K. The enzyme was dissolved in 0.05 M Tris–HCl buffer, pH 8.0, and was reduced with $Na_2S_2O_4$ before freezing in liquid $N_2$.

The absorption spectrum of the *Pseudomonas cytochrome* oxidase at 77 °K is shown in Fig. 6. The principal difference between this spectrum and that obtained at room temperature is that the doublet observed in the α-band at 549 and 554 nm of the spectrum became much more pronounced at the lower temperature. In addition, the β-band of the heme *c* component split into a doublet. The bands of the heme *d* component were similar at both temperatures.

The pH altered the absorption spectrum of the heme *d* portion of *Pseudomonas* cytochrome oxidase at room temperature although that of the heme *c* component was not significantly modified by a change of pH. Thus, lowering the pH from 8.0 to 5.8 decreased the absorption band at 460 nm and increased the overall absorbance in the red region chiefly because of an increase in the absorbance of the band at 625 nm.

The absorption spectra of reduced *Pseudomonas* cytochrome oxidase in the presence of cyanide or CO were similar to that previously reported by Yamanaka

and Okunuki[7]. The spectrum of the heme *c* moiety was not altered but that of the heme *d* component was modified.

*Pyridine hemochrome*

The pyridine hemochrome of the heme *d* component of *Pseudomonas* cytochrome oxidase which was liberated from the protein by treatment with acidified acetone had an absorption spectrum with bands at 630 and 455 nm and a shoulder near 415 nm.

The pyridine hemochrome of the hematoheme derivative of heme *c* after cleavage from the protein by treatment with $Ag_2SO_4$ had absorption maxima at 549, 420 and 422 nm.

The spectra of these hemochromes are identical to those obtained by Okunuki and co-workers[4] from the *Pseudomonas* cytochrome oxidase that they purified.

*Enzyme activity*

The maximum activity of *Pseudomonas* cytochrome oxidase with its two native electron donors, *Pseudomonas* cytochrome *C*-551 and *Pseudomonas* copper protein, was observed at pH 6.0. With $O_2$ as the final electron acceptor the $K_m$ for the *Pseudomonas* cytochrome *c*-551 was 10.5 $\mu$M at pH 6.0 and 30 °C while the $K_m$ for the copper protein was 17.2 $\mu$M under the same conditions. The enzymic activity was inhibited 100% by $10^{-4}$ M KCN with either $O_2$ or nitrite as the terminal acceptor and with either electron donor. Carbon monoxide ($CO$-$O_2$, 9:1) caused a 90% inhibition of activity when only $O_2$ was the electron acceptor CO, even at a concentration of 100%, did not significantly inhibit the reaction with nitrite.

*Electron paramagnetic resonance spectra*

*Pseudomonas* cytochrome oxidase exhibited an EPR spectrum which reflected the presence of two different paramagnetic species, *i.e.* the iron of heme *d* and of heme *c*. The entire spectrum could only be observed when the temperature approached that of liquid He as illustrated in Fig. 7. This spectrum has signals with *g* values of

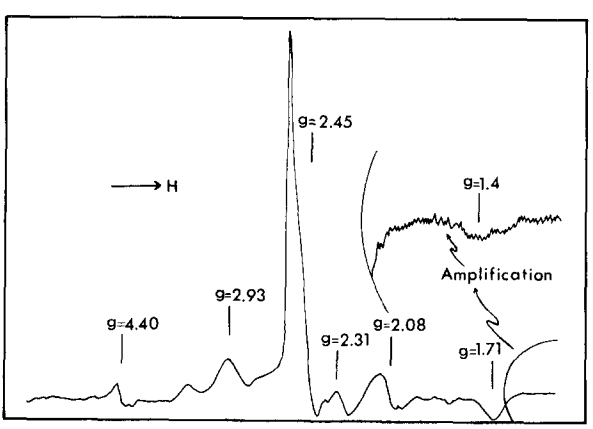



Fig. 7. EPR spectrum of oxidized *Pseudomonas* cytochrome oxidase. The spectrum was obtained using a Varian Model V-4500 spectrometer with modifications for work at ultra-low temperatures. The instrument settings were: power output, 0.3 mW; field modulation, 5 G; temperature, 13 °K; microwave frequency, approximately 9250 MHz.

4.40, 2.93, 2.45, 2.31, 2.08, 1.71 and 1.4. The signals at $g = 2.93$, 2.45, 2.31, 1.71, and 1.4 could be associated with components of the oxidase. The signal at $g = 4.40$ is similar to that frequently observed in biological preparations and in certain types of glass and results from iron thought to be present due to degradation of other iron-containing structures or to contamination[25]. The signal at $g=2.08$ is due to contamination by copper[26] which was also shown to be present by chemical assay methods. When the EPR spectrum was taken at the temperature of liquid $N_2$ (87 °K) the signals with $g$ values at 2.93, 2.31 and 1.4 could not be seen.

The addition of $Na_2S_2O_4$ to preparations of *Pseudomonas* cytochrome oxidase caused a complete loss of the signals attributed to components of the oxidase. No new signals were observed.

The addition of KCN to the oxidized enzyme caused a loss of the EPR signals with $g$ values of 2.45 and 1.71. Since cyanide has been observed to alter the absorption spectrum of the heme *d* moiety we interpret the effect of cyanide on the EPR spectrum to indicate that the family of signals with $g$ values of 2.45 and 1.71 result from the heme *d* in the enzyme. This hypothesis was reinforced by the observation that the EPR spectrum of the oxidase from which heme *d* had been removed lacked signals at $g=2.45$ and 1.71 while they were restored when the protein was reconstituted with heme *d*.

## DISCUSSION

The new purification procedure reported in this communication results in a 14- to 15-fold purification of *Pseudomonas* cytochrome oxidase from the aqueous cell extract of *P. aeruginosa*. The purified product is homogeneous as determined by acrylamide gel electrophoresis and by ultracentrifugation. Furthermore, no other cytochromes or flavoproteins are present on the basis of absorption spectra. No carbohydrate or lipid could be detected in the purified preparation. The absence of lipid is interesting since it is suspected that, *in vivo*, *Pseudomonas* cytochrome oxidase is bound to a membrane. Thus, in that respect, this oxidase appears to bear a closer similarity to cytochrome *c* of mitochondrial systems than it does to mitochondrial cytochrome oxidase. The presence of small amounts of copper and non-heme iron in purified preparations probably results from contamination which occurs during the cell breakage of purification procedures. The copper and most of the non-heme iron can be removed without significant loss of activity.

The present purified preparation of *Pseudomonas* cytochrome oxidase appears to be similar in many respects to the enzyme purified by Okunuki and co-workers[1–4]. Both preparations contain heme *c* and heme *d* as the only detectable prosthetic groups and both have similar purification ratios over the cell extracts. Both preparations are oxidized by $O_2$ or nitrite and both are reduced by *Pseudomonas* cytochrome *c*-551 and *Pseudomonas* copper protein as well as by the same chemical reducing agents. Inhibition of the activity of both preparations occurs at similar concentrations of cyanide and CO. A small difference in pH optimum has been observed using either *Pseudomonas* cytochrome *c*-551 or *Pseudomonas* copper protein as electron donors: pH 5 with the preparation of Horio *et al.*[4] and pH 6 with our preparation.

Our data collected by sedimentation equilibrium, sodium dodecyl sulfate acrylamide gel electrophoresis, and heme-iron content indicate that *Pseudomonas*

cytochrome oxidase, purified to electrophoretic homogeneity, has a molecular weight of about 120000 and is composed of two subunits, each of which has a molecular weight of approximately 60000. Whether the heme *c* and heme *d* are located on the same or on different subunits is presently under investigation. The molecular weight of 120000 is slightly higher than the value of 90000 obtained by Horio *et al.*[4] using a sedimentation velocity method and that of 85000 reported by Newton[27] who employed a gel filtration method. The value is considerably higher than that in the more recent report of Nakada *et al.*[28] who calculated a molecular weight of 67500 for their crystalline preparation on the basis of the amino acid composition and heme content. The reasons for the discrepancy between these values is still unexplained.

The EPR spectrum of *Pseudomonas* cytochrome oxidase is of particular interest since it has not been described previously. This spectrum is complex because it contains the components of two heme groups. Furthermore, without treatment to remove contaminating iron and copper, these metals contribute their signals as well. We tentatively assign the signals with *g* values of 2.45 and 1.71 to the heme *d* moiety and those with *g* values of 2.93, 2.31, and 1.4 to the heme *c* component. These assignments are based on the following evidence: (1) removal of heme *d* from the protein results in a loss of the signals at $g=2.45$ and $g=1.71$ and reconstitution produces their reappearance; (2) cyanide, which is known to react with heme *d*, causes a disappearance of the signals at $g=2.45$ and $g=1.71$; (3) at various redox equilibria the signals at $g=2.93$, 2.31, and 1.4 are diminished more than the other set of signals in agreement with the relative mid-point potentials of heme *c* and heme *d*, respectively[4,29].

The positions of these *g* values indicate that each paramagnetic species is a ferric iron in the low-spin state[30]. Similar *g* values have been found associated with other cytochromes[26,31–33] or have been predicted on theoretical grounds[34,35]. With respect to theoretical considerations it is of interest to compare the present *g* values to those calculated by Loew[34] as a function of tetragonal and rhombic distortions. For the *g* values of the heme *d* moiety, Loew would predict the following *g* values: $g_z=2.46$; $g_y=2.46$; $g_x=1.78$. These values are in close agreement with the observed values of 2.45 and 1.71 and would also explain why only two clear signals are seen for this species since both the $g_z$ and $g_y$ values would be identical. In the case of the second species, *i.e.* heme *c*, the calculations of Loew yield $g_z=2.93$, $g_y=2.31$, and $g_x=1.4$, all of which are identical to the experimental values. Further quantitative work on the assignment of these *g* values is in progress and will be reported in a later communication.

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REFERENCES

1 Horio, T. (1958) *J. Biochem. Tokyo* 45, 195–205
2 Horio, T. (1958) *J. Biochem. Tokyo* 45, 267–279
3 Horio, T., Higashi, T., Matsubara, H., Kusai, K., Nakai, M. and Okunuki, K. (1958) *Biochim. Biophys. Acta* 29, 297–302
4 Horio, T., Higashi, T., Yamanaka, T., Matsubara, H. and Okunuki, K. (1961) *J. Biol. Chem.* 236, 944–951
5 Yamanaka, T., Kijimoto, S., Okunuki, K. and Kusai, K. (1962) *Nature* 194, 759–760
6 Yamanaka, T. and Okunuki, K. (1963) *Biochim. Biophys. Acta* 67, 379–393
7 Yamanaka, T. and Okunuki, K. (1963) *Biochim. Biopyhys. Acta*, 67, 394–406
8 Yamanaka, T. and Okunuki, K. (1963) *Biochim. Biophys. Acta* 67, 407–416
9 Gornall, A. G., Bardawill, C. J. and David, M. M. (1949) *J. Biol. Chem.* 177, 751–766
10 Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* 193, 265–275
11 Doeg, K. A. and Ziegler, D. M. (1962) *Arch. Biochem. Biophys.* 97, 37–40
12 Van de Bogart, M. and Beinert, H. (1967) *Anal. Biochem.* 20, 325–334
13 Wharton, D. C. and Rader, M. (1970) *Anal. Biochem.* 33, 226–229
14 Johnson, M. J. (1949) *J. Biol. Chem.* 181, 707–711
15 Hassid, W. Z. and Abraham, S. (1957) in *Methods in Enzymology*, Vol. 3, pp. 34–50, Academic Press, New York
16 Davis, B. J. (1964) *Ann. N.Y. Acad. Sci.* 121, 404–427
17 Weber, K. and Osborn, M. J. (1969) *J. Biol. Chem.* 244, 4406–4412
18 Yphantis, D. A. (1964) *Biochemistry*, 3, 297–317
19 Horio, T., Higashi, T., Sasagawa, M., Kusai, K., Naki, M. and Okunuki, K. (1960) *Biochem. J.* 77, 197–201
20 Horio, T., Sekuzu, I., Higashi, T. and Okunuki, K. (1961) in *Haematin Enzymes* (Falk, J. E., Lemberg, R. and Morton, R. K., eds) pp. 302–311, Pergamon Press, London
21 Hansen, R. E. and Beinert, H. (1966) *Anal. Chem.* 38, 484–487
22 Beinert, H. and Kok, B. (1964) *Biochim. Biophys. Acta* 88, 278–288
23 Beinert, H. and Palmer, G. (1964) *J. Biol. Chem.* 239, 1221–1227
24 Palmer, G. (1965) in *Methods in Enzymology*, Vol. 10, pp. 595–609, Academic Press, New York
25 Van Gelder, B. F. and Beinert, H. (1969) *Biochim. Biophys. Acta* 189, 1–24
26 Beinert, H., Griffiths, D. E., Wharton, D. C. and Sands, R. H. (1962) *J. Biol. Chem.* 237, 2337–2346
27 Newton, N. (1969) *Biochim. Biophys. Acta* 185, 316–331
28 Nagata, Y., Yamanaka, T. and Okunuki, K. (1970) *Biochim. Biophys. Acta* 221, 668–671
29 Kamen, M. D. and Horio, T. (1970) *Annu. Rev. Biochem.* 39, 673–700
30 Ehrenberg, A. (1962) *Ark. Kemi* 19, 119–128
31 Salmeen, I. and Palmer, G. (1968) *J. Chem. Phys.* 48, 2049–2052
32 Watari, H., Groudinsky, O. and Labeyrie, F. (1967) *Biochim. Biophys. Acta* 131, 592–594
33 Boris-Poltoratsky, R. and Ehrenberg, A. (1967) *Eur. J. Biochem.* 2, 361–365
34 Loew, G. H. (1970) *Biophys. J.* 10, 196–212
35 Blumberg, W. E. and Peisach, J. (1971) in *Structure and Function of Macromolecules and Membranes* (Chance, B., Yonetani, T. and Mildvan, A., eds) pp. 215–229, Academic Press, New York