Purification and Properties of Cytochrome Oxidase from _Pseudomonas aeruginosa_

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Yaoi and Tamiya (1) found that _Escherichia coli_ and _Shigella dysenteriae_, grown aerobically, exhibited an absorption band which differed from those of previously described cytochromes in that it lay within the red region of the spectrum. Keilin (2) attributed this band to a cytochrome component, designated in that it lay within the red region of the spectrum. Keilin (2) found spectroscopic evidence that cytochrome a$_2$ was autoxidizable and could combine with carbon monoxide and cyanide. Fujita and Kodama (7) independently found spectroscopic evidence that cytochrome a$_2$ was autoxidizable and could combine with carbon monoxide and cyanide. Fujita and Kodama also showed that this cytochrome was widely distributed in other bacteria, _e.g._ _Acetobacter chroococcum_, _Proteus vulgaris_, _Acetobacter pasteurianum_, _Eberthella typhosa_, and _Salmonella paratyphi_. The oxidized form in whole cells showed the a$_2$-type band at 645 mn in the visible region of the spectrum.

With the basis mainly on the spectroscopic examination of cytochrome a$_2$ in whole cells, it is generally assumed that in these organisms, cytochrome a$_2$ functions as a cytochrome oxidase (8). Most recently, Barrett (9) has extracted and purified a green heme from _Acetobacter aerogenes_ and several other bacteria, all of which contain cytochrome a$_2$, which he has designated as hemin a$_2$.

In 1958, Horio (10, 11) extracted and partially purified four different kinds of soluble respiratory components from _Pseudomonas aeruginosa_ without the use of any detergent or organic solvent. Among the purified components, there was a greenish brown fraction which possessed a complex absorption spectrum that appeared to contain both a so-called cytochrome a$_2$ and a c-type cytochrome(s). This component exhibited a typical cytochrome oxidase activity, which was entirely inhibited by carbon monoxide and cyanide. During the early purification, this oxidase was called cytochrome a$_2$ (10, 11) because of its greenish brown color; later (11, 12) it was renamed _Pseudomonas_ cytochrome oxidase.

Horio et al. later succeeded in crystallizing two soluble factors, namely, _Pseudomonas_ cytochrome c-551 (13, 14) and _P._ blue protein (11), and also in obtaining in very pure form _P._ cytochrome-554 (10) and _P._ cytochrome oxidase (12). Even after extensive purification, the _P._ cytochrome oxidase preparation continued to show both the absorption bands of c-type cytochrome(s) and so-called cytochrome a$_2$. This indicated either that two different kinds of heme moieties were contained in one molecule of _P._ cytochrome oxidase, or that the oxidase was a tightly bound complex of two different heme proteins. _P._ cytochrome oxidase could rapidly oxidize _P._ cytochrome c-551 and _P._ blue protein in their reduced form and very slowly oxidize _P._ cytochrome-554.

In this paper, we present a detailed procedure for purifying _P._ cytochrome oxidase from _P. aeruginosa_. The best preparation, approximately 70% pure, has been used to study the physicochemical properties of _P._ cytochrome oxidase.

**EXPERIMENTAL PROCEDURE**

**Organism**—The strain of _Pseudomonas aeruginosa_ was the same as in previous reports (10-14). It produced large amounts of respiratory components when grown in submerged culture with an oxygen tension of less than 20% (15, 16).

**Medium and Growth of Organism**—The medium has been described in an earlier report (14). The organism was cultured for 1 day in approximately 100 liters of medium in a deep steel tank at 37° without shaking in air. At the end of incubation, the medium contained an average of approximately 8 mg wet weight of the organism per ml.

**Preparation of Resin**—The methods were the same as for the purification of _P._ cytochrome c-551 (14): Duolite CS-101, H-form, powdered from 150 to 200 mesh was suspended in water and its pH adjusted to approximately 6 with aqueous ammonia. Then the resin was washed with a large amount of ammonium phosphate buffer, 0.1 m in ammonium ion, pH 6.0, and washed with water. The resulting resin was called the pH 6 resin. Alternatively, Amberlite IRC-50 (CG-50, type 2) could be used but with somewhat lower efficiency.

**Zone Electrophoresis on Vertical Starch Column**—Zone electrophoresis was carried out on a vertical column packed with raw potato starch powder (30 × 3 cm in diameter) under the following experimental conditions: 0.1 m sodium phosphate buffer, pH 7.0; approximately 20 milliamperes at 350 to 400 volts with the anode at the bottom of the column; temperature, 4-5°. The starch powder (Wako Pure Chemical Industries, Company, Ltd., Osaka, Japan) was washed three times, first with a large amount of 1 N HCl, then with water to remove acid, and finally with a large amount of buffer. Only the buffered powder which sedimented on standing within half an hour was used.

**Electrophoretic Analysis**—A tiselius apparatus was used. The cells contained equilibrated buffer (0.02 m sodium phosphate buffer, ionic strength 0.10, adjusted with NaCl) as the overlying solution. A sample of _P._ cytochrome oxidase was dialyzed at 4-5° against the buffer for three days with continuous stirring. Determinations of purity of _P._ cytochrome oxidase were carried...
out in a melting ice bath (1°), and electrophoretic mobilities in a water bath regulated at 20°. Calculations were based upon movement of descending boundaries.

**Ultra-centrifugal Analysis**—Sedimentation measurements were made with a Spinco model E ultracentrifuge (rotor type AN-A). The runs were carried out at 350,700 × g at room temperature (approximately 20°). The average of initial and final temperatures was used for calculation. The ηsp,v was determined by the method of Svedberg and Pedersen (17).

**Diffusion Analysis**—Diffusion was studied by the cylindrical lens spheroid method with the use of cells of the Neurath type; the temperature of the thermostat was regulated at 20 ± 0.01°. Dm,v was determined by a standard procedure (18).

**Partial Specific Volume**—The densities of the solution and the solvent were measured at 20 ± 0.05° with an Ostwald type of pycnometer (approximately 3 ml capacity), and V calculated in the usual way (19).

**Absorption Spectra**—A Cary model 11 recording spectrophotometer with silica cuvettes was used (1-cm optical path). Protein concentration was simply expressed in terms of absorbancy at 280 μμ.

**Manometric Assay**—A conventional Warburg manometric apparatus was used, and reactions were carried out at 30°.

**Analysis of Iron, Copper, and Nitrogen**—A sample of P. cytochrome oxidase was dialyzed with stirring against glass-distilled water for 72 hours at 4-5° with several changes of outside water. During the dialysis, some of the P. cytochrome oxidase was precipitated. The dialyzed solution containing the precipitate was desiccated at 60° over P2O5 until the dried residue attained constant weight. Iron was estimated by the o-phenanthroline method (20-22), and copper by the dithizone method (22).

Nitrogen was assayed by a micro-Kjeldahl procedure.

**Preparation of Crystalline P. Cytochrome c-551 and P. Blue Protein**—P. cytochrome c-551 and P. blue protein were crystallized according to the methods of Horio et al. (9-11).

### RESULTS

#### Purification of P. Cytochrome Oxidase

**Preparation of Acetone Dried Cells**—The culture medium (96 liters), which had been estimated to contain approximately 660 g of cells in wet weight, was filtered through a large Büchner funnel with the aid of 1 kg of Hyflo Super-Cel, and the grown cells were collected together with the Celite. After chilling to 4-5° in a cold room, the cells collected together with Celite were thrown into 5 liters of acetone which had been chilled to -3° in dry ice bath. During the acetone treatment, the mixture was continuously stirred and its temperature was maintained at 0-3°. After 10 minutes, the mixture was swiftly filtered on a chilled Büchner funnel. The collected cells with Celite were washed with 2 liters of cold acetone, and dried at room temperature (10-20°) for 1 day under a continuous stream of air. Alternatively, the cells collected by centrifugation were lyophilized. P. cytochrome oxidase could be purified from either lyophilized or acetone-dried cells by the following procedure.

**Extraction of P. Cytochrome Oxidase from Acetone-dried Cells**—The acetone-dried cells with Celite were mixed well with 10 liters of 0.1 M sodium citrate, pH 7.6, with a Waring Blender. The mixture was heated at 40° for 10 minutes. After heating, and without cooling, the mixture was filtered at room temperature on a large Büchner funnel. The filtration was usually completed within 1 day. The filtrate, 8.3 liters, pH 6.4, contained 107,000 units of P. cytochrome oxidase, and its specific enzyme activity was 0.20 (Table 1).

**Treatment of Enzyme Extract with Rivanol**—The brown-colored extract, 8.3 liters, was mixed with 1 liter of 4% aqueous rivanol,2 which resulted in the formation of a large amount of viscous, fibrous precipitate. The precipitate was easily removed by filtration through a glass fiber sheet.

**Precipitation of Enzyme by Ammonium Sulfate**—To the rivanol-treated extract, 9.3 liters, 6.3 kg of ammonium sulfate were added (approximately 95% saturation), and the mixture was allowed to stand overnight in a cold room (4-5°). The resulting precipitate was collected by centrifugation (10,000 × g for 30 minutes). The brown precipitate was washed with 90% saturated aqueous ammonium sulfate, pH 7, dissolved in 500 ml of water, and then centrifuged at 10,000 × g for 1 hour. The resulting supernatant was dialyzed against water for 2 days in a cold room. During the dialysis, the outside water was changed frequently, and the dialyzing tubes were replaced three times because a brown viscous substance precipitated and adhered to the tube membrane, thereby interfering with continued dialysis. The P. cytochrome oxidase, which was partially precipitated during the dialysis, was stored together with all of the dialyzing tubes used. The dialyzed solution was centrifuged. The resulting precipitate and the stored tubes were well suspended in 100 ml of 0.1 M sodium phosphate buffer, pH 7.0, and centrifuged at high speed. The resulting supernatant was dialyzed against 10-4 M sodium phosphate buffer, pH 6.0, and centrifuged. The supernatants from both centrifugations were combined; the

2 Also known as “acrinol” (6,9-diamino-2-ethoxy-2-ethylenylamine lactate).

**Preparation of P. Cytochrome oxidase from 660 g wet weight of P. aeruginosa cells**

Manometric vessels contained the following: In the main compartment, 0.4 ml of 0.2 M sodium phosphate buffer, pH 6.4, an appropriate amount of enzyme solution, and water to make a total volume of 2.0 ml; in the side arm, 0.2 ml of 0.5 M hydroquinone. Reactions were carried out at 30° in the presence and absence of 10-4 M potassium cyanide, because the preparations before the resin procedure were contaminated with cyanide-insensitive hydroquinone oxidase (12, 14). The activity of P. cytochrome oxidase was regarded as the difference in oxygen uptake occurring in the absence and presence of cyanide. One unit of P. cytochrome oxidase was defined as the amount of enzyme catalyzing the consumption of 1 μl of oxygen during the first 5 minutes of reaction. Specific enzyme activity was expressed by the ratio of units of the enzyme to E 260 μμ.

### Table 1

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Volume</th>
<th>Total Enzyme</th>
<th>Total units</th>
<th>Specific Activity</th>
<th>Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>5,500</td>
<td>338,500</td>
<td>107,900</td>
<td>0.20 (100)</td>
<td></td>
</tr>
<tr>
<td>Rivanol-treated</td>
<td>9,300</td>
<td></td>
<td>96,300</td>
<td></td>
<td>90</td>
</tr>
<tr>
<td>Saturated (NH4)2SO4, ppt. and dialysis</td>
<td>1,300</td>
<td>78,800</td>
<td>72</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Resin eluate</td>
<td>200</td>
<td>36,900</td>
<td>77,500</td>
<td>2.10 72</td>
<td></td>
</tr>
<tr>
<td>Zone electrophoresis First run</td>
<td>54</td>
<td>17,200</td>
<td>55,000</td>
<td>3.20 51</td>
<td></td>
</tr>
<tr>
<td>Second run</td>
<td>12</td>
<td>540</td>
<td>17,420</td>
<td>32.3 16</td>
<td></td>
</tr>
</tbody>
</table>

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mixture, 1.3 liters, contained 78,000 units of P. cytochrome oxidase, a yield of 72%.

If the first dialysis was done against $10^{-4}$ M sodium phosphate buffer, pH 6.0, almost all of the P. cytochrome oxidase remained in a water-soluble state, but excessive losses occurred in subsequent steps.

Chromatography on pH 6 Resin Column—The mixture (1.3 liters) was passed through a pH 6 resin column (60 x 4 cm in diameter) and the eluate was stored for further purifications of P. cytochrome c-551 (14) and P. blue protein (11). P. cytochrome oxidase was adsorbed on the column, forming a greenish brown zone at the top part of the column just below a yellow zone of residual rivanol. The adsorbed oxidase was eluted with 2 M ammonium phosphate buffer, pH 7.0. The resulting eluate (200 ml) was greenish brown, and contained 77,500 units of P. cytochrome oxidase, a yield of 72%. This eluate was still contaminated with a small amount of rivanol.

Fractionation by Ammonium Sulfate—An equal volume of saturated aqueous ammonium sulfate, pH 7, was added to the eluate and was immediately followed by centrifugation. The resulting supernatant, saturated aqueous ammonium sulfate was then added to achieve 70% saturation. After standing overnight in a cold room, the mixture was centrifuged. The greenish brown precipitate was dissolved in 50 ml of water and clarified by centrifugation. The supernatant contained 55,000 units, a yield of 51%.

Zone Electrophoresis—The enzyme solution was dialyzed against water for 3 days in a cold room. During the dialysis, a large amount of greenish brown precipitate was formed. The dialyzed solution containing the precipitate was lyophilized, dissolved in 2.5 ml of 0.1 M sodium phosphate buffer, pH 7.0, and then centrifuged. The resulting dark brown precipitate was washed with 1.5 ml of the buffer. The supernatant fluid and the washings were mixed. The mixture was placed in the middle part of a column packed with raw potato starch powder (30 x 3 cm in diameter), where it formed a greenish brown zone, 3 to 4 cm long. Electrophoresis was carried out for 72 hours at 3-4°. During the electrophoresis, a large quantity of brown impurities migrated to the bottom of the column (anode) and flowed out from the starch column to another vessel which contained a large amount of buffer used to maintain the pH constant during the long electrophoresis. The P. cytochrome oxidase slowly migrated downward, a distance of approximately 5 cm, forming an intense green zone. Just below the green zone, a yellow-colored zone was formed, which was followed by a brown zone extending down to the bottom. No colored substance appeared to migrate above the green zone. At the end of electrophoresis, the starch column was eluted with the buffer, and the eluate was collected in 3 ml fractions (Fig. 1). Fractions of P. cytochrome oxidase with specific activity greater than 20 were combined; the collected mixture showed a specific activity of 25.3, and contained 33,500 units, a yield of 31%.

If the electrophoresis was repeated with the use of the lyophilized powder of the enzyme, the specific activity rose to 32.3 with a yield of 16%.

Storage of P. Cytochrome Oxidase—The purified P. cytochrome oxidase could be stored for several months without a notable

![Absorption spectrum of P. cytochrome oxidase. Approximately 1.3 mg of the sample purified up to the final step of the purification procedure was dissolved in 1 ml of 0.1 M sodium phosphate buffer, pH 6.0. The absorption spectrum was measured at room temperature (22°). ——, oxidized form; ···, dithionite-reduced form. To obtain molecular extinction, multiply ordinate by 23.5 x 10^6 (250 to 500 m\(\mu\)) or 7.2 x 10^6 (500 to 700 m\(\mu\)).](attachment:image.png)

![Figure 1. Fractionation of P. cytochrome oxidase by zone electrophoresis on a vertical starch column. ——, total absorbancy. E_{280}^-; ···, specific enzyme activity of each fraction.](attachment:image.png)
Molecular extinction of absorption peaks of P. cytochrome oxidase

<table>
<thead>
<tr>
<th>Form</th>
<th>Absorption maximum (nm)</th>
<th>Molecular extinction (× 10^4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxidized</td>
<td>630</td>
<td>30.2</td>
</tr>
<tr>
<td></td>
<td>408</td>
<td>149</td>
</tr>
<tr>
<td>Reduced</td>
<td>625</td>
<td>36.0</td>
</tr>
<tr>
<td></td>
<td>554</td>
<td>30.2</td>
</tr>
<tr>
<td></td>
<td>549</td>
<td>30.2</td>
</tr>
<tr>
<td></td>
<td>521</td>
<td>29.9</td>
</tr>
<tr>
<td></td>
<td>418</td>
<td>182</td>
</tr>
</tbody>
</table>

Physicochemical Properties of P. Cytochrome Oxidase

Absorption Spectrum—Absorption spectra of the P. cytochrome oxidase in its purest form were measured in 0.1 M sodium phosphate buffer, pH 6.0. Absorption maxima were found at 630, 554, 408, 360, and 278 nm in the oxidized form, and at 625, 554, 549, 521, and 418 nm in the dithionite-reduced form (Fig. 2). The molecular extinctions of P. cytochrome oxidase were calculated after correction for purity (see following) (Table II).

Electrophoretic Behavior—The behavior of the purified P. cytochrome oxidase during electrophoresis in sodium phosphate buffer of various pH values between pH 5.95 and 7.31 was investigated. The peak was overlapped by a shadow which appeared to result from the green color of the oxidase. However, the peak always exhibited irregularities on the anodic side after 12 hours of electrophoresis. The results of zone electrophoresis purification suggested that these irregularities might be due mainly to brown impurities mentioned previously. By the conventional area method, the purity was estimated to be approximately 70%.

Sedimentation Behavior—Sedimentation studies were carried out with the use of protein concentrations between 0.5 and 1.0% (weight per volume). One of the patterns obtained is shown in Fig. 4. These runs showed that the sample consisted of protein almost homogeneous as regards sedimentation. The s, was calculated to be 5.8 × 10^{-13} second and was essentially independent of the protein concentrations tested. A critical run performed with the highest concentration of the sample showed that the sample was contaminated with a molecule slightly smaller than P. cytochrome oxidase (s, = 5 × 10^{-13} second), but not with a molecule as small as P. cytochrome c-551 (s, = 1.34 × 10^{-13} second).

Diffusion Behavior—Diffusion measurements were performed with the same sample solution used in sedimentation analysis. D was calculated to be 5.8 × 10^{-7} cm² per second, and was almost independent of the protein concentration tested.

Partial Specific Volume (V) —With the same sample as in the sedimentation and diffusion studies, the apparent partial specific volume was determined to be 0.73 ml per g.

Calculation of Molecular Weight—From the sedimentation and diffusion coefficients and partial specific volume, the molecular weight of P. cytochrome oxidase was calculated to be ~90,000.

Content of Iron, Copper, and Nitrogen—Iron, 1 g atom, was detected in each 67,000 g of the P. cytochrome oxidase sample, but copper was not detected. The nitrogen content was 16.3%. If the sample contained 30% impurity, as was indicated by the electrophoretic results, then there would be 1 g atom of iron per 46,900 g of protein. Inasmuch as the molecular weight calculated from s, D, and V was ~90,000, it was concluded from the iron analysis that 2 iron atoms were contained in 1 molecule of P. cytochrome oxidase of molecular weight ~94,000.

Properties of Heme Moiety of P. Cytochrome Oxidase

Acid-Acetone Treatment—To 1 volume of P. cytochrome oxidase solution which had been completely dialyzed against water, were added 4 volumes of cold acetone containing 0.5 M HCl. The mixture was allowed to stand for a few minutes in a melting
ice bath and was then centrifuged. The supernatant was vividly green, the precipitate brownish red. The precipitate was washed with a small volume of a mixture consisting of 1 volume of water and 4 volumes of HCl-acetone. The washing was combined with the first supernatant, the mixture adjusted to ~pH 8 with the use of 5 n NaOH, and then assayed spectrophotometrically (Fig. 5). Absorption spectra of the extracted hemin a2 varied greatly in repeated experiments. These variations may have resulted from slight differences in pH of the sample solution and from the extreme lability of hemin a2 itself. It was apparent that the linkage(s) binding the heme a2 moiety to its protein were markedly acid-labile, so that without use of organic solvents, the hemin a2 could be cleaved from protein at a pH lower than 5. Lowering the pH was found to liberate increasing amounts of hemin a2.

The brownish red precipitate from the HCl-acetone treatment was easily dissolved in water, although its solubility was not high. As 1 n NaOH was added to the solution, turbidity developed near pH 6; further addition of alkali completely clarified the solution and greatly increased the solubility of the precipitate. The solution was adjusted to pH 8, and the red solution that resulted was sufficiently stable for spectrophotometric assay (Fig. 5). Its absorption spectrum was very similar to that seen with a typical c-type cytochrome, except that the α-absorption maximum of its dithionite-reduced form had a flat peak from 550 μm to 553 μm, thus differing from the case of P. cytochrome oxidase which exhibited a distinct trough (Fig. 2). The extinction ratio of α-peak to β-peak was 1.1, notably different from that of typical cytochrome c. Furthermore, if this protein moiety carrying a c-type heme was treated with acetone containing a higher concentration of acid, the color of the protein changed from reddish brown to white, the c-type absorption spectrum was lost, and almost all the iron was found in the supernatant and not in the protein moiety.

The HCl-acetone extract of P. cytochrome oxidase, green in color, was adjusted to about pH 10 with 1 n NaOH, thus forming a green layer at the bottom part of the solution. A few drops of pyridine were then added with vigorous stirring for 5 minutes. The pyridine hemochrome separated as a green layer at the bottom of the solution, and it was removed from the supernatant layer.

The pyridine hemochrome was easily dissolved in water, in this respect being different from that of other kinds of pyridine hemochromes. After dilution with water to an appropriate concentration, it was assayed spectrophotometrically (Fig. 6). The cyanide hemochrome of hemin a2 was prepared in a manner similar to that used in making the pyridine hemochrome (Fig. 6). In contrast with the heme a2 extracted into HCl-acetone, the pyridine hemochrome was considerably stable when reduced anaerobically; however, when reduced in air, its color faded rapidly from green to pale yellow. The reduced pyridine hemochrome showed absorption maxima at 620, 550, and 414 μm. Except for the optical density at 620 μm, the absorption peaks did not shift with a change of pH. When carbon monoxide was bubbled through the reduced pyridine hemochrome, the resulting CO-pyridine hemochrome showed absorption maxima at 630 μm and 412 μm, the shoulder around 460 μm became more definite than that of the pyridine hemochrome, and the small peak at 548 μm which was observed with the pyridine hemochrome disappeared. The cyanide hemochrome showed absorption maxima at 635, 554, 476, and 419 μm, and was more stable than the pyridine hemochrome.

Iron determinations revealed that the supernatant and the
precipitate resulting from treatment at low temperature with
0.5 \text{n HCl-acetone contained 57 and 41\%}, respectively, of the
total iron present in the purified \textit{P. cytochrome oxidase}. Based
upon the difference in absorption spectra of the supernatant and
of the precipitate (Fig. 5), and the strong possibility that \textit{P.}
cytochrome oxidase possessed two iron atoms in each molecule, it
was concluded that \textit{P. cytochrome oxidase} had two heme irons
and no nonheme iron in each molecule. As for the reasons why
the supernatant of the HCl-acetone extraction contained more
iron than the precipitate, at least two explanations could be ad-
vanced: (a) The \textit{P. cytochrome oxidase} sample purified up to the
final step was contaminated with hemin \text{a2} which had been lib-
erated from the protein moiety during the course of purification
because of its weak binding to the protein moiety; (b) The HCl-
acetone treatment partially broke the c-type heme and liberated
some heme iron into the supernatant. However, when the
brownish red precipitate which had been separated from hemin
\text{a2} was treated again with 0.5 \text{n HCl-acetone at low temperature,
no more iron was liberated. The spectrum of this precipitate,
both in shape and extinction, also was not altered. Thus, the
latter possibility appeared to be eliminated, leaving the alterna-
tive that the purified oxidase had been contaminated with hemin
\text{a2}. However, another possibility, that of contaminating sub-
stances carrying nonheme iron which were extractable into the
acidic acetone, could be imagined.

\textbf{Physiological Properties of \textit{P. Cytochrome Oxidase}}

\textit{Oxidations by \textit{P. Cytochrome Oxidase} of Reduced \textit{P. Cytochrome
c-551, Reduced \textit{P. Blue Protein, and Hydroquinone}.—The pH
optima for oxidations of reduced \textit{P. cytochrome c-551}, reduced
\textit{P. blue protein}, and hydroquinone were re-examined with the use
of the highly purified \textit{P. cytochrome oxidase} (Figs. 7, 8, and 9).
The pH optimum was 5.1 for oxidations of reduced \textit{P. cytochrome
c-551} and reduced \textit{P. blue protein}, and 6.4 for oxidation of hydro-
quinone. These pH optima were very similar to those previously

\textbf{Fig. 7. pH activity curve for oxidation of reduced \textit{P. cyto-
chrome c-551} by \textit{P. cytochrome oxidase}. The enzyme activity was
assayed by following the decrease in optical density at 551 m\text{\textmu}m
(\(\alpha\)-absorption maximum of reduced \textit{P. cytochrome c-551}). The
cuvette contained 0.11 M sodium phosphate buffer (H\text{\textsubscript{2}}PO\text{\textsubscript{4}}, Na-
HPO\text{\textsubscript{4}} or NaH\text{\textsubscript{2}}PO\text{\textsubscript{4}}-Na\text{\textsubscript{2}}HPO\text{\textsubscript{4}}), 1.7 \times 10^{-4} M reduced \textit{P. cytochrome
c-551}, and the purified enzyme, in 3.5 ml of total volume. Reac-
tions were started by adding the enzyme and measuring at room
temperature (18\textdegree). Initial linear reaction rates were compared.

\textbf{Fig. 8. pH activity curve for the oxidation of reduced \textit{P. blue
protein} by \textit{P. cytochrome oxidase}. The experimental conditions
were the same as for Fig. 6, except that reduced \textit{P. blue protein}
(4.3 \times 10^{-4} M) was used instead of reduced \textit{P. cytochrome c-551}.

\textbf{Fig. 9. pH activity curve for oxidation of hydroquinone by \textit{P.}
cytochrome oxidase}. The experimental conditions were the same
as for Table I, except that buffers of different pH were used, the
enzyme had been purified up to the final step, and cyanide was
omitted.
Pseudomonas Cytochrome Oxidase

**Fig. 10.** $1/V - 1/S$ curves for oxidations of reduced P. cytochrome c-551, and P. blue protein by P. cytochrome oxidase. The experimental conditions were the same as for Figs. 6 and 7, except that phosphate buffer, pH 5.1, was used.

**Fig. 11.** $1/V - 1/S$ curve for oxidation of hydroquinone by P. cytochrome oxidase. The experimental conditions were the same as for Fig. 8, except that phosphate buffer, pH 6.4, was used.

obtained with a cruder enzyme preparation, but they were generally a little more acidic.

$K_m$ was determined to be $1.9 \times 10^{-4}$ M for oxidation of reduced P. cytochrome c-551, $3.9 \times 10^{-4}$ M for oxidation of reduced P. blue protein (Fig. 10), and $4.2 \times 10^{-2}$ M for hydroquinone oxidation (Fig. 11). The turnover number was calculated to be 96 (in moles per mole of P. cytochrome oxidase per minute) at pH 5.1 and at 18° for P. cytochrome c-551, 100 at pH 5.1 and at 18° for P. blue protein, and 54 at pH 6.4 and at 30° for hydroquinone.

**DISCUSSION**

Verhoeven and Takeda (23) have described a 70-fold purification of a cytochrome-nitrite oxidase achieved by ammonium sulfate fractionation and Celite treatment, using Pseudomonas aeruginosa which had been cultured anaerobically in a nitrate-containing medium. Their purified sample showed another enzymic activity, cytochrome-oxygen oxidase, and the ratio of a cytochrome-nitrite oxidase, which was precipitated by the acetone. The hemoprotein, free of hemin $a_2$, could be dissolved in water and showed an absorption spectrum similar but notably different from that of the c-type cytochromes.

Together with the result of iron analysis of the highly purified P. cytochrome oxidase, it can be safely concluded that P. cytochrome oxidase contains two different kinds of heme in 1 molecule: heme $a_2$ and a c-like heme. In contrast with mammalian cytochrome c oxidase (24, 25), there is no copper present.

As already mentioned, a cytochrome which is found in a number of bacteria and which has a absorption band at around 630 m$\mu$, has been called cytochrome $a_2$. However, no cytochrome, other than P. cytochrome oxidase, which has the absorption band of cytochrome $a_2$, has been purified. P. cytochrome oxidase has a c-type heme as well as heme $a_2$ on one molecule. Therefore, even P. cytochrome oxidase is not cytochrome $a_2$. Inasmuch as cytochrome $a_2$, which has only heme $a_2$, has not yet been isolated and purified, it is not established that cytochrome $a_2$, which has only heme $a_2$ as a prosthetic group, exists as such in any bacteria.

P. cytochrome oxidase may be said to be the first c-type cytochrome to be extracted and purified without the aid of any detergent. The high solubility in water of the cytochrome apparently is associated with the hydrophilic behavior of heme $a_2$ liberated from the cytochrome. When P. cytochrome oxidase is deprived of heme $a_2$, the remaining protein moiety precipitates easily if any salt is present. This may be caused by acid treatment (acid denaturation), but it is also possible that P. cytochrome oxidase behaves in this manner because of the marked hydrophilic nature of heme $a_2$.

**SUMMARY**

A water-soluble cytochrome oxidase has been extracted from Pseudomonas aeruginosa. The solubilized enzyme can be purified by a method which consists mainly of rivanol treatment, chromatography on an Amberlite IRC-50 or Duolite CS-101 column, ammonium sulfate fractionation, and zone electrophoresis on a vertical starch column. The purity of the best preparations, as estimated from examination of diffusion and sedimentation patterns, is approximately 70%.

The isoelectric point of the oxidase is at pH 5.8. Coefficients of sedimentation and diffusion, and apparent partial specific volume are: $\xi_{30,0} = 5.8 \times 10^{-13}$ second; $D_{30,0} = 5.8 \times 10^{-7}$ cm$^2$ per second; $V = 0.73$ ml per g. From these values, the molecular weight can be calculated to be approximately 90,000.

P. cytochrome oxidase appears to possess two different kinds of heme in one molecule; one is an $a_2$-type heme (hemin $a_2$), and the other an unknown heme similar to hemin c. Iron analysis suggests that these two heme iron are contained in approximately

(11, 14). These remaining as impurities in the highly purified P. cytochrome oxidase sample may be responsible for the absorption peaks similar to those of c-type cytochromes. However, this is very unlikely because of the following facts: (a) P. cytochrome c-551 and P. cytochrome-554 could be separated easily from P. cytochrome oxidase in the course of purification, because of marked differences in isoelectric points, in solubility in ammonium sulfate, and in behavior on the cationic resins. (b) Sedimentation studies showed that the sample of P. cytochrome oxidase was not contaminated with such a small molecule as P. cytochrome c-551. (c) Acidic acetone could solubilize hemin $a_2$ and separate it from another heme still bound on the protein moiety of the oxidase, which was precipitated by the acetone. The hemoprotein, free of hemin $a_2$, could be dissolved in water and showed an absorption spectrum similar but notably different from that of the c-type cytochromes.

In addition to P. cytochrome oxidase, P.-cytochrome c 551 and P. cytochrome-554 have been purified from the same organism

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94,000 g of protein, the latter value having been determined after correcting for 30% impurity. The α₂-type heme is easily cleaved from its protein moiety by acidic acetone, whereas the ε-like heme is not released.

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