O'Leary, T. J. (1987) *Biophys. J. 52*, 137–139.
Perham, R. N. (1978) *Tech. Life Sci., [Sect.]: Biochem. B110*, 1–39.
Peters, F. A. L. J., Van Wielink, J. E., Wong Fong Sang, H. W., De Vries, S., & Kraayenhof, R. (1983) *Biochim. Biophys. Acta 722*, 460–470.
Staehelin, L. A., & Arntzen, C. J. (1983) *J. Cell Biol. 97*, 1327–1337.
Steinback, K. E., Bose, S., & Kyle, D. J. (1982) *Arch. Biochem. Biophys. 216*, 356–361.
Todhunter, J. A., & Purich, D. L. (1977) *Biochim. Biophys. Acta 485*, 87–94.
Walker, A. I., Hunt, T., Jackson, R. J., & Anderson, C. W. (1985) *EMBO J. 4*, 139–145.
Whitelegge, J. P., Millner, P. A., Gounaris, K., & Barber, J. (1987) in *Proceedings of the VIIth International Congress on Photosynthesis* (Biggins, J., Ed.) Vol. 2, pp 809–812, Martinus Nijhoff, Dordrecht, The Netherlands.
Widger, J., Farchaus, J. W., Cramer, W. A., & Dilley, R. A. (1984) *Arch. Biochem. Biophys. 233*, 72–79.

Purification and Partial Characterization of the Membrane-Bound Cytochrome *o*(561,564) from *Vitreoscilla*†

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ABSTRACT: Cytochrome *o*(561,564) terminal oxidase was solubilized from the membrane fraction of the bacterium *Vitreoscilla* sp., strain C1, and purified by differential pH dialysis, gel filtration chromatography, and ion-exchange chromatography. Subunit molecular weights, determined on sodium dodecyl sulfate–polyacrylamide gels by the Ferguson plot method, were 49 500 and 23 500. There were two protohemes IX, two coppers, and 45 mol of phosphorus per mole of protomer (73 000). The molecular weight of the cytochrome *o* complex estimated by chromatography on Sephacryl-400 in deoxycholate was 265 000, which is consistent with the enzyme complex under these conditions being a dimer (146 000) with the remaining molecular weight contribution arising from bound phospholipid, deoxycholate, and possibly other, smaller subunits. Difference spectra of the dithionite-reduced enzyme have split α absorption maxima at 561 and 564 nm at room temperature and 558 and 561 nm at 77 K. The CO difference spectrum at room temperature has absorption maxima at 570, 534, and 416 nm. Dissociation constants for CO and cyanide binding to the reduced and oxidized forms of the oxidase are 5.2 μM and 3.5 mM, respectively. The hemes in the cytochrome are one electron accepting centers, both with midpoint potentials around +165 mV at pH 7.0. The enzyme is highly autoxidizable, and its menadiol oxidizing activity is stimulated by phospholipids.

Cytochrome *o* was first identified in bacteria more than 30 years ago (Chance et al., 1953). There now appear to be two general types of *o*-type terminal oxidases (Wood, 1984): oxidases of the *c*–*o* type, like those purified from *Azotobacter vinelandii* (Jurtshuk et al., 1981) and *Methylophyilus methylotrophus* (Carver & Jones, 1983), and the terminal oxidases of the *b*–*o* type, e.g., the cytochrome *b*-562–*o* purified from *Escherichia coli* (Kita et al., 1984; Matsushita et al., 1984). The "soluble cytochrome *o*" from *Vitreoscilla* sp. was formerly believed to be a unique member of this latter group, but recent experiments have shown it to be a bacterial hemoglobin (Orii & Webster, 1986; Wakabayashi et al., 1986). Photolysis experiments on intact cells of *Vitreoscilla* had previously indicated the existence of a second CO-reactive *b*-type cytochrome (DeMaio et al., 1983). This second *o*-type cytochrome could be differentiated from the cytoplasmic hemoglobin because its CO compound underwent detectable photolysis at temperatures lower than −80 °C and its reaction with oxygen at −100 °C to form an oxygenated intermediate was similar spectrally and kinetically to that of the membrane-bound cytochrome *o* from *E. coli* (Poole et al., 1979). Additional evidence for the existence of a membrane-bound cytochrome *o* in *Vitreoscilla* came from studies on the supernatant and respiratory membrane fractions from cells disrupted by osmotic lysis after lysozyme treatment (DeMaio & Webster, 1983). CO difference spectra showed that this membrane fraction contained a CO-binding cytochrome with a Soret maximum at 416 nm, significantly different from the Soret band (420 nm) of the hemoglobin found in the supernatant fraction. Membranes free of soluble hemoglobin showed rapid respiratory rates with either NADH or ascorbate–DCPIP as substrate. Here we report on the purification and characterization of the membrane-bound cytochrome *o* from *Vitreoscilla*.

Materials and Methods

Purification of Cytochrome o(561,564). The organism used for these studies, *Vitreoscilla* sp., strain C1, was obtained originally from R. G. E. Murray, University of Western Ontario, and is his culture 389. The growth of *Vitreoscilla*, the preparation of respiratory membrane fragments, and their solubilization with sodium deoxycholate have been described

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elsewhere (Georgiou & Webster, 1987). All of the purification procedures were performed at 0–4 °C. The membrane fragments were suspended in 0.02 M Tris[1] buffer at pH 8.5 containing 0.25 M sucrose, 1.0 M KCl, and 1% (w/v) sodium deoxycholate with a protein to detergent ratio of 1:2.5 (w/w). The suspension was incubated for 30 min and centrifuged for 1 h at 48000*g*. The supernatant was dialyzed against 50 volumes of 0.02 M potassium phosphate buffer, pH 6.0, overnight. The cloudy dialyzate was centrifuged at 48000*g* for 1 h, and a pellet was obtained that consisted of a top red, jelly-like layer and a solid white bottom layer. The red layer was carefully removed, washed in 0.02 M potassium phosphate buffer, pH 7.2, and resuspended (15% wet weight/v) in 0.02 M Tris buffer, pH 8.0. Solid ammonium sulfate was added to 50% saturation while the pH was maintained at 8.0 by the addition of 0.1 M ammonium hydroxide. The suspension was incubated for 30 min and then centrifuged at 48000*g* for 30 min; the precipitate was suspended (20% wet weight/v) in 0.02 M Tris buffer, pH 8.0, and dialyzed overnight in 10 volumes of the same buffer. The dialyzate was centrifuged at 48000*g* for 1 h; the pellet was washed with the Tris buffer and suspended in 0.02 M Tris, pH 8.0, containing 0.25 M sucrose, 1.0 M KCl, and 1% (w/v) sodium deoxycholate at a protein to detergent ratio of 1.5:1 (w/w). The suspension was incubated for 30 min and centrifuged at 48000*g* for 1 h. This extraction with sodium deoxycholate was repeated 2 more times, and the three combined supernatants were dialyzed against 20 volumes of 0.02 M Tris, pH 8.0, containing 0.5% (w/v) sodium deoxycholate. The dialyzate was concentrated in an Amicon cell concentrator using a 25PM10 Diaflo ultrafiltration membrane to give a solution of approximately 25 mg of protein/mL and centrifuged for 1 h at 150000*g*.

Ten milliliters of this cytochrome solution was applied to a column of Sephadex G-200 superfine (111 × 2.2 cm) that was equilibrated with 0.02 M Tris, pH 8.0, containing 1% (w/v) sodium deoxycholate and eluted with a flow rate of 11 mL/h, and 4-mL fractions were collected. Cytochrome fractions that contained more than 4 nmol of heme *b* per milliliter (Figure 1, second elution peak) were combined, adjusted to 7.5 mg of protein/mL and 1% (w/v) Triton X-100, and dialyzed overnight against 20 volumes of 0.02 M Tris, pH 8.0, containing 1% (w/v) Triton X-100 and 0.005 M EDTA. Six milliliters of this solution was applied to a DEAE-Sepharose CL-6B column (28.5 × 1.9 cm) that was equilibrated with the latter buffer. The cytochrome was eluted with 200 mL of a linear gradient, 0–0.2 M NaCl in equilibration buffer, at a flow rate of 12 mL/h in 3-mL fractions. Cytochrome *o*(561,564) eluted from the column at 0.08 M NaCl. Fractions containing 15–16 nmol of heme *b* per milligram of protein (Figure 2) were combined, dialyzed against 0.01 M Tris, 0.005 M EDTA, and 1% Triton X-100, pH 8.0, and then rechromatographed on the same column and under same elution conditions. Fractions that contained approximately 19 nmol of heme *b* per milligram of protein were pooled and dialyzed against 0.02 M Tris, pH 7.2, containing 1% Triton X-100. The ratio of nanomoles of heme *b* per milligram of protein was used to evaluate the purity of the cytochrome.

Assays and Spectroscopy. Protein concentration in samples that contained sodium deoxycholate or Triton X-100 was estimated by a modified Lowry method (Dulley & Grieve, 1975) with bovine serum albumin (fraction V) as protein standard. Phosphorus was estimated by the Bartlett method (Dittmer & Wells, 1969). Hemes *b* and *c* were measured by the pyridine hemochromogen method (Furhop & Smith, 1975). Heme was stained on PAGE and isoelectric focusing gels according to the tetramethylbenzidine method (Thomas et al., 1976). Total iron and copper were estimated by atomic absorption. The visible–Soret spectra at room and low temperature were performed as described previously (Georgiou & Webster, 1987).

Determination of Molecular Weight. The molecular weight of the cytochrome in sodium deoxycholate was estimated by gel filtration (Whitaker, 1963) with a Sephacryl 400 column (115 × 0.9 cm) equilibrated with 0.02 M Tris, pH 8.0, containing 1% (w/v) sodium deoxycholate at 4 °C. The column was calibrated with 5 mg each of bovine serum albumin (M_r 66 000), aldolase (M_r 158 000), catalase (M_r 232 000), ferritin (M_r 440 000), and thyroglobulin (M_r 669 000). The subunit molecular weight was determined by SDS–PAGE (Laemmli, 1970) at 11.5, 13, 14.5, and 16% acrylamide concentrations in combination with the Ferguson plot method (Hedrick & Smith, 1968). Slab gels were calibrated with lysozyme (M_r 14 300), β-lactoglobulin (M_r 18 400), trypsinogen (M_r 24 000), egg albumin (M_r 45 000), and bovine serum albumin (M_r 66 000). These were run in a Protean slab gel electrophoresis chamber (Bio-Rad Labs) with a Brinkman voltage–current-regulated power supply. Gels were stained for protein with Commassie Brilliant Blue R-250 and scanned with an Isco Model 959 gel scanner and an Isco Model UA-5 absorbance monitor.

Cytochrome o(561,564) Subunit Composition and pI. The minimum subunit composition of cytochrome *o*(561,564) was determined by first dimension isoelectric focusing in the presence of the zwitterionic detergent CHAPS followed by SDS–PAGE (Laemmli, 1970) as the second dimension. The isoelectric focusing was a modification of a previously described method (Manrique & Lasky, 1981). The isoelectric focusing gels (125 × 93 × 1.5 mm) were composed of 2.5% (w/v) Sephadex G-200 superfine, 0.75% (w/v) isoelectric focusing Agarose, 10% (v/v) glycerol, 3% (w/v) ampholytes (pH range 3.5–10.0), and 2% (w/v) CHAPS or 1% (v/v) Triton X-100. The anode solution was 0.1 N sulfuric acid, and the cathode solution was 0.1 N NaOH. The gels (two per run) were prefocused for 2 h at 200 V. The sample (10–20 μg of protein) was mixed with Sephadex G-200 superfine to form a slurry and applied to the top of the gel adjacent to the cathode. The gel was focused at 100 V for 13 h at 10 °C. The cytochrome *o*(561,564) was identified in the isoelectric focusing gels by heme staining (Thomas et al., 1976). For the second dimension, slices (0.5–1.0 cm wide) from the isoelectric focusing gel were incubated for 30 min in 0.0625 M Tris, pH 6.8, containing 5% (w/v) SDS, 5% (v/v) β-mercaptoethanol, and 10% (w/v) sucrose and then placed on top of an SDS–PAGE gel 1.5 mm thick. The slices were secured with a 0.5 cm thick layer of 1% (w/v) agarose in stacking gel buffer. The p*I* of the cytochrome complex was estimated by determination of the pH gradient of an isoelectric focusing gel by measuring the pH of successive 0.5 × 1 cm gel slices each of which had been suspended in 0.01 M KCl that had been boiled to remove dissolved carbon dioxide. The isoelectric focusing gels were run in an LKB Bromma flat-bed Multiphor-2117 using an LKB power supply, Model 3371-E.

Determination of CO and Cyanide Binding. The dissociation constant (K_d) for the binding of CO to the purified cytochrome *o*(561,564) was estimated in room temperature CO

[1] Abbreviations: CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; DCPIP, 2,6-dichlorophenolindophenol; DEAE, diethylaminoethyl; EDTA, ethylenediaminetetraacetate; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; Tris, tris(hydroxymethyl)aminomethane.

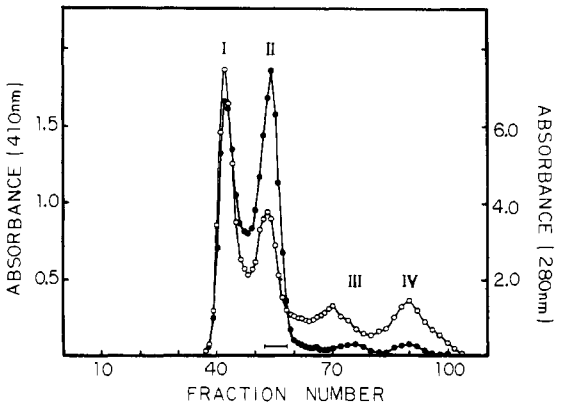

FIGURE 1: Chromatography of cytochrome *o*(561,564) on Sephadex G-200 superfine. Experimental details are described under Materials and Methods. The fractions that contained cytochrome were pooled as indicated by the bar. (●) Absorbance at 410 nm; (○) absorbance at 280 nm.

difference spectra by the absorbance difference $A(416) - A(430)$. The cytochrome solution (1 mL) containing 2.12 nmol of heme *b* in 0.1 M sodium phosphate, pH 7.4, 45% (v/v) glycerol, and 1.2% sodium cholate was reduced with solid sodium dithionite and titrated by the addition of CO-saturated phosphate buffer. The K_d for cyanide binding was estimated by recording the absorbance difference $A(420) - A(400)$ in cyanide difference spectra at room temperature. The cytochrome solution (1 mL) contained 4.24 nmol of heme *b* in the same buffer that was used for the CO titration. It was titrated by microliter additions of 0.1 and 1.0 M buffered KCN for the initial and final stages of the titration, respectively.

Determination of Oxidation–Reduction Potential. The oxidation–reduction potential of cytochrome *o*(561,564) was determined by the dye equilibrium method (Gunsalus & Sligar, 1976; Tyree & Webster, 1978) utilizing EDTA, riboflavin, and a mixture of redox mediator dyes, specifically DCPIP, toluylene blue, and thionin with midpoint potentials of 217, 115, and 63 mV (Wilson & Dutton, 1970; Loach, 1968), respectively, at concentrations ranging from 1 to 10 μM. The dyes were recrystallized from water prior to use. The anaerobic solution of dyes and cytochrome *o* in an argon atmosphere at 23 °C contained 4.5 nmol of heme *b* in 2 mL of 0.1 M potassium phosphate and 0.2% (v/v) Triton X-100, pH 7.0. The cytochrome sample in a 23 °C water bath was illuminated for 5–60 s with a 100-W tungsten bulb, and the oxidation–reduction state of the cytochrome complex was estimated from the absorbance changes at 429 nm, that occurred in the difference spectra on the stepwise photochemical reductions.

Manadiol Oxidase Activity. Cytochrome *o*(561,564) (22 μmol) was preincubated with varying concentrations of *Vitreoscilla* crude phospholipids (0–1 mM final concentration based on phosphorus) in 100 μL of 0.1 M potassium phosphate, pH 7.0, for 5 min at 4 °C. The reaction volume was brought to 1 mL with the same phosphate buffer, and the menadiol oxidase activity was measured at 25 °C by recording the absorbance change of menadiol at 262 nm. The concentration range of menadiol used was 0–1 mM. The rates were corrected for autoxidation of menadiol in the absence of any added enzyme. The phospholipids were prepared by suspending *Vitreoscilla* cells in acidified acetone (1% 6 N HCl, v/v), 10 mL/g of cells being used, and by stirring for 1 h at room temperature, evaporating the acetone under a light vacuum, extracting the aqueous residue twice with an equal volume of light petroleum ether, and then extracting the phospholipids from the petroleum ether into 1 volume of 95% methanol.

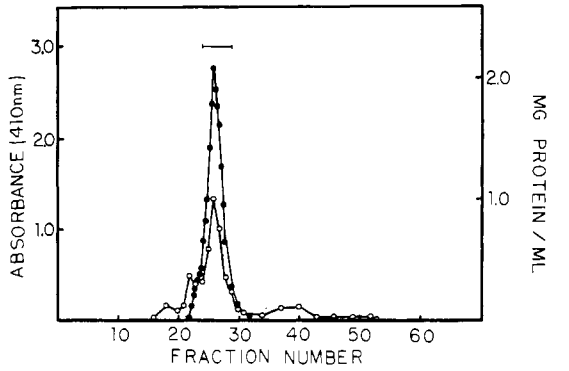

FIGURE 2: First chromatography of cytochrome *o*(561,564) on DEAE-Sepharose CL-6B. Experimental details are described under Materials and Methods. The pooled cytochrome fractions are indicated by the bar. (●) Absorbance at 410 nm; (○) milligrams of protein per milliliter.

Table I: Purification of Cytochrome *o*(561,564)

step	total heme (nmol)	total protein (mg)	heme/protein (nmol/mg)	yield (%)
membranes[a]	14360	20400	0.7	100
red pellet[b]	3270	2280	1.4	23
Sephadex G-200 pool	657	116	5.6	4.6
DEAE-Sepharose pool (first run)	373	24	15.5	2.6
DEAE-Sepharose pool (second run)	241	11	22.0	1.7

[a] In solubilizing buffer [0.02 M Tris, pH 8.0, containing 0.25 M sucrose, 1.0 M KCl, and 1% (w/v) sodium deoxycholate], 166 g wet weight. [b] In solubilizing buffer.

RESULTS

Purification of Cytochrome o(561,564). The elution pattern of the Sephadex G-200 chromatography step contained four peaks, which had absorption at 410 nm (Figure 1). The first peak consisted of cytochrome *o*(561,564) contaminated with minor amounts of cytochrome *c*-556 as judged by second-order derivative spectra of difference and pyridine hemochromogen spectra. The second peak contained cytochrome *o*(561,564). When fractions from the first elution peak were rechromatographed on Sephadex G-200 in the presence of higher (2%) deoxycholate in the elution buffer, it was partially converted to the second peak, indicating that this first peak contained some cytochrome *o*(561,564) in an aggregated state. The third elution peak contained cytochrome *c*-556, which is described elsewhere (Georgiou & Webster, 1987). The fourth elution peak was greenish and eluted with the V_t of the column. The pooled fractions of the second peak were chromatographed twice on DEAE-Sepharose CL-6B (Figure 2). Cytochrome *o*(561,564) eluted at 0.08 M NaCl and contained 22 nmol of heme *b* per milligram of protein after the second DEAE-Sepharose chromatography. The purification of cytochrome *o*(561,564) is summarized in Table I.

Spectral Characterization of Cytochrome o(561,564). The room temperature reduced minus oxidized spectrum of cytochrome *o*(561,564) showed absorption peaks at 590 (minor broad band), 561, 530, and 430 nm (Figure 3A). The second-order derivative spectrum (Figure 3B) of the latter spectrum resolved the α peak into a high-intensity peak at 561 nm and a peak with lower intensity at 564 nm. These spectra did not unambiguously resolve the Soret peak, but shoulders were visible at 429 and 431 nm (not shown). The peaks were all blue shifted at low temperature (77 K): 586, 561, 558, 528, and 426 nm (Figure 4). The CO difference spectrum of the

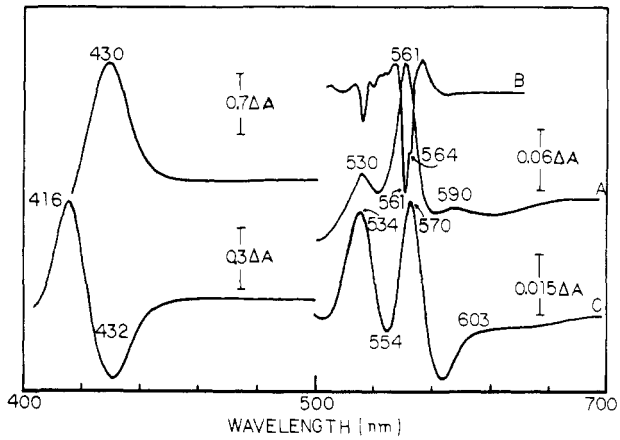

FIGURE 3: Room temperature difference spectra of cytochrome *o*-(561,564). The cytochrome was dissolved in 0.02 M Tris buffer, pH 7.5, containing 0.5% sodium deoxycholate. The protein concentration was 1.8 mg/mL, and the heme *b* concentration was 10.5 μM. (A) Reduced minus oxidized spectrum; (B) finite second-order derivative spectrum of spectrum A; (C) reduced plus CO minus reduced spectrum.

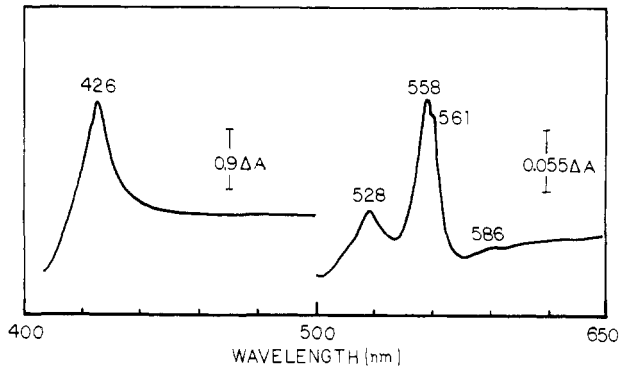

FIGURE 4: Low-temperature reduced minus oxidized spectrum of cytochrome *o*(561,564). The cytochrome was dissolved in 0.02 M potassium phosphate, pH 7.2, containing 1% (v/v) Triton X-100 and 1.0 M sucrose. The protein concentration was 1.0 mg/mL, and the heme concentration was 5.5 μM. Plexiglass cuvettes with a 1.5-mm light path were inserted into the holder of an Oxford instruments Model D-10200 cryostat cell and were immersed in liquid nitrogen (77 K) for about 2 min before being transferred to the cryostat unit. Difference spectra were obtained from absolute spectra recorded with a single cryostat cell in a Varian Cary-210 split-beam spectrophotometer using an interfaced Apple II+ computer and a Varian Spectral Calculations Program.

cytochrome showed a weak, broad band at 603 nm, peaks at 570, 534, and 416 nm, and troughs at 554 and 432 nm (Figure 3C). The reduced-CO absolute spectrum and its second-order derivative spectrum for cytochrome *o*(561,564) had absorption maxima at 564, 561, 530, 429, and 417 nm (Figure 5).

Chemical Composition of Cytochrome o(561,564). First-dimension isoelectric focusing and second-dimension SDS-PAGE established the presence of at least two subunits in the purified cytochrome. The molecular weights of the subunits were determined on SDS-PAGE gels of different acrylamide concentrations by Ferguson plot analysis (Hedrick & Smith, 1968): the relative mobility of each protein was plotted vs the total percentage of acrylamide, and the slopes of these lines were plotted versus the log of their molecular weights. From this standard curve it was estimated that the two subunits of the cytochrome had molecular weights of 49 500 ± 3500 and 23 500 ± 3500. The protein staining pattern of the cytochrome after SDS-PAGE was the same whether or not it was treated with β-mercaptoethanol. When the sample was boiled prior to electrophoresis, there was protein aggregation. The protein densitogram of the SDS-PAGE (Figure 6) showed the pres-

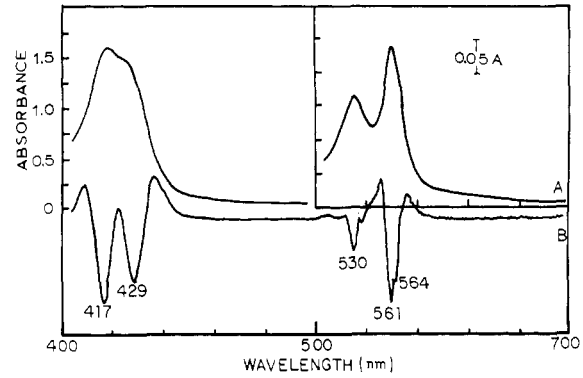

FIGURE 5: Room temperature reduced plus CO spectrum of cytochrome *o*(561,564). The sample composition and experimental conditions were the same as those described in the legend for Figure 3. (A) Absolute reduced plus CO spectrum of cytochrome *o*(561,564); (B) finite second-order derivative spectrum of spectrum A.

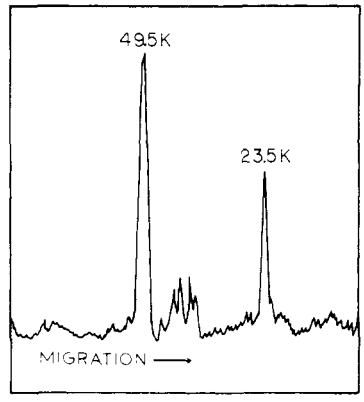

FIGURE 6: Densitometric scan of cytochrome *o*(561,564) on a SDS-11.5% polyacrylamide gel. The molecular weights, 49 500 and 23 500, of the subunits were estimated by Ferguson plot analysis as described in the text.

ence of four or five minor bands in the cytochrome *o*(561,564) preparation, which may be impurities or additional subunits. The ratio of the peak area of the M_r 49 500 subunit to that of the M_r 23 500 subunit was 2.2:1. If the subunit ratio is 1:1 and the amount of dye bound per residue is the same for each subunit, then a peak area ratio (equivalent to the molecular mass ratio) of 2.1:1 would be expected. The sum of these two subunit peak areas was approximately 75% of the total protein peak area. The apparent molecular weight of cytochrome *o*(561,564) estimated by chromatography on Sephacryl-400 in the presence of deoxycholate as described under Materials and Methods was 265 000 ± 36 000 (not shown). The phosphorus content was 45 mol/mol of protomer, which would contribute around 34 000 (assuming an average phospholipid M_r of 750) to the protomer molecular weight (73 000) for a total of 107 000. The protein most likely exists as a dimer of the protomer under these conditions with the remaining 51 000 molecular weight contribution coming from bound deoxycholate and possibly small subunits.

The prosthetic group of cytochrome *o*(561,564) was identified as protoheme IX by the pyridine hemochromogen method (Furhop & Smith, 1975), and there was 20 nmol of heme/mg of protein. Assuming a protomer M_r of 73 000 for the cytochrome, the molar ratio of heme *b* to protein in the preparation was 1.5. If there are two hemes per protomer of the cytochrome *o*, then the protomers comprise 75% of the protein in the cytochrome preparation, which is in agreement with the estimate from the electrophoresis densitogram above. The ratio of iron to copper was 1.16:1 and of heme to copper was 1.1:1 in the preparation. The isoelectric pH of the cy-

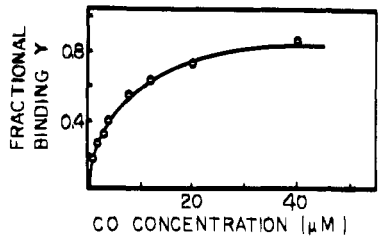

FIGURE 7: Carbon monoxide binding curve of cytochrome *o*(561,564). The titration of the reduced cytochrome with CO is described under Materials and Methods. *Y* is the fractional binding of CO to the cytochrome.

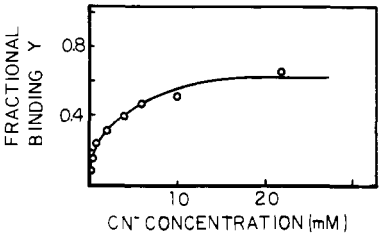

FIGURE 8: Cyanide binding curve of cytochrome *o*(561,564). The titration of the oxidized cytochrome with cyanide is described under Materials and Methods. *Y* is the fractional binding of cyanide to the cytochrome.

tochrome complex was 6.7 in Triton X-100 and 6.5 in CHAPS.

Cytochrome *o*(561,564) exhibited menadiol oxidase activity that was stimulated by *Vitreoscilla* total phospholipids. The apparent K_m for menadiol oxidation was 404 μM in the absence and 168 μM in the presence of the optimum phospholipid concentration, approximately 500 mol of phospholipids/mol of heme. The corresponding turnover numbers were 17 and 64 mol of menadiol oxidized per mole of cytochrome *o* per second, respectively.

Interactions of Cytochrome o(561,564) with CO and Cyanide. The binding curve of the dithionite-reduced cytochrome to carbon monoxide was hyperbolic (Figure 7) with a dissociation constant K_d = 5.2 μM and a Hill coefficient of 0.91, which was estimated from a Hill plot (not shown). The binding curve of the air-oxidized cytochrome to cyanide was pseudohyperbolic (Figure 8) with a K_d = 3.5 mM and a Hill coefficient of 0.6.

Oxidation–Reduction Potential of Cytochrome o(561,564). Purified cytochrome *o*(561,564) was reduced stepwise by photochemical titration in the presence of the redox mediators DCPIP, toluylene blue, and thionin (Figure 9). In the initial stages of the titration, the α absorption peak was at 564 nm with a shoulder at 561 nm, which fused to form a composite symmetrical peak at 562 nm as the titration progressed through the midpoint. At the end of the titration the peak was at 561 and the shoulder at 564 nm, which is characteristic of the α absorption band of the cytochrome when it is completely reduced by sodium dithionite (Figure 3). The straight line was drawn through the experimental points on the basis of the assumption that there were two equipotential (+165 mV) electron-accepting centers present in equimolar concentrations.

Discussion

The only *b*–*o*-type oxidase that has been studied in any detail in vivo and in vitro until now is the *E. coli* enzyme, *b*-562–*o*. The purified protein, according to one group of investigators (Kita et al., 1984) possesses two subunits of M_r 55 000 and 33 000. Two *b*-type hemes and two copper atoms were present per complex of M_r 88 000. However, in two earlier studies (Kranz & Gennis, 1983; Matsushita et al., 1983) the presence of two additional smaller subunits with M_r 18 000–22 000 and

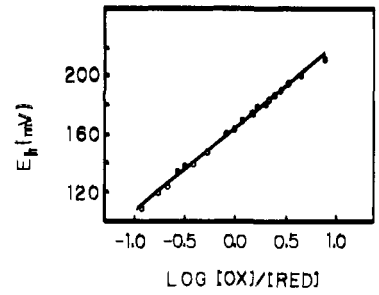

FIGURE 9: Nernst plot for the photochemical equilibrium titration of cytochrome *o*(561,564). The midpoint potential of the cytochrome was estimated by photochemical equilibrium titration in a mixture of the redox mediators DCPIP (3.0 μM), toluylene blue (5.0 μM), and thionin (5.0 μM), as described under Materials and Methods. The experimental points show the dependence of the state of reduction of the cytochrome complex on the oxidation–reduction potential (Eh). The theoretical one electron equiv line was drawn through the points on the basis of the assumption that the cytochrome complex consists of two redox centers present in equal proportions.

12 700–17 000 was reported. *Vitreoscilla* cytochrome *o*-(561,564) contains two subunits with M_r of 49 500 and 23 500 and has two *b*-type hemes and two copper atoms per complex of M_r 73 000. Our results do not exclude the possible existence of smaller subunits.

Experiments on CO-liganded cells of *E. coli* photolyzed at −100 °C under aerobic conditions identified an oxygen intermediate of the cytochrome *o* (Poole et al., 1979). Similarly, an oxygen intermediate of the membrane cytochrome *o* was found in *Vitreoscilla* cells that was spectrally and kinetically similar to the one in *E. coli*, but there were differences in reactivities of the two cytochrome intermediates (DeMaio et al., 1983). The absorption bands of both purified cytochromes are split in the α region, supportive evidence for the presence of two independent hemes, but the absorption maxima of these bands and other bands in the various spectra of the *Vitreoscilla* cytochrome differ from those observed for the *E. coli* cytochrome (Kranz & Gennis, 1983; Kita et al., 1984). The *Vitreoscilla* enzyme also shows a similar strong affinity for CO and exhibits a menadiol oxidase activity similar to that of the *E. coli* enzyme (Kita et al., 1984). On the other hand, there is a marked difference in the cyanide concentration required for 50% inhibition of oxidase activity, the *Vitreoscilla* enzyme being approximately 1000 times less sensitive than that of the *E. coli* enzyme (Kita et al., 1984; Lis & Webster, 1986). Titration of intact membranes from *Vitreoscilla* with cyanide revealed two cyanide binding pigments with different affinities (Georgiou & Webster, 1987). The major pigment had binding properties (K_d = 2.14 mM, Hill coefficient = 0.49) similar to those of the purified cytochrome *o* (3.5 mM and 0.6, respectively).

The experimental values for K_d and Hill coefficient for CO binding to the purified *Vitreoscilla* enzyme compared to those estimated for the CO-binding compound of the membranes are 5.2 μM and 0.91 and 3.9 μM and 0.80, respectively, evidence for identity of the two pigments. Both the cyanide- and CO-binding studies indicate that there is only one binding site per protomer or that there are two essentially identical binding sites per protomer. The evidence supports the former. The reduced cytochrome has α absorption maxima at 561 and 564 nm and Soret maxima at 429 and 431 nm (Figure 3). The absolute reduced plus CO spectrum has a peak at 417 nm, but a peak persists at 429 nm, which suggests the presence of a heme *b* component that does not bind CO (Figure 5). In addition, the two heme electron-accepting centers had midpoint potentials that were too close to be resolved, but the *b*-564 component appeared more reduced during the initial stages

of the photochemical equilibrium titration of the cytochrome complex and may have a slightly higher midpoint potential than the *b*-561 component. These data suggest that it is the *b*-564 component that binds CO and is the final electron donor to oxygen and that the *b*-561 component does not bind CO and may act as electron donor to the *b*-564 component, which would be analogous to the *a* and a_3 components of cytochrome *c* oxidase. The *Vitreoscilla* cytochrome *o* could consequently be called cytochrome *b*-561–*o*, but we feel that further evidence is necessary before we can make this final designation.

Vitreoscilla cytochrome *o* exhibited quinol oxidase activity that, like that of the *E. coli* enzyme (Kita et al., 1984), was stimulated by phospholipids. The similar physical and enzymatic properties of these two cytochromes suggest similar roles for the two proteins. A *d*-type cytochrome terminal oxidase is also present in the membranes of *Vitreoscilla* and is possibly similar to the cytochrome *d* complex purified from *E. coli* (Miller & Gennis, 1983; Kita et al., 1984b). The latter appears when the oxygen media concentration is low (i.e., in the early stationary phase) and is present at even higher concentrations in cells grown anaerobically (Miller & Gennis, 1983) whereas cytochrome *o* is the only terminal oxidase present in cells harvested from highly oxygenated media (i.e., in the early log phase) (Kita et al., 1984). *Vitreoscilla*, on the other hand, did not show such an adaptation of its two terminal oxidases when grown in oxygen-rich and oxygen-limited media (Georgiou and Webster, unpublished results). It appears to adapt to oxygen-poor environments by synthesizing relatively large amounts of *Vitreoscilla* hemoglobin (Wakabayashi et al., 1986). A cyanide-resistant mutant of *Vitreoscilla* did show a fivefold increase in the amount of membrane cytochrome *d* relative to the wild type (Lis & Webster, 1986). Cells of *E. coli* in which cytochrome *d* was the primary terminal oxidase were also more resistant to cyanide (Pudek & Bragg, 1974). The cyanide-binding affinity for both of these *d* cytochromes is less than that of the corresponding cytochrome *o*.

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REFERENCES

Carver, A. M., & Jones, C. W. (1983) *FEBS Lett. 155*, 187–191.

Chance, B., Smith, L., & Castor, L. N. (1953) *Biochim. Biophys. Acta 12*, 289–298.

DeMaio, R. A., & Webster, D. A. (1983) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 42*, 2072.

DeMaio, R. A., Webster, D. A., & Chance, B. (1983) *J. Biol. Chem. 258*, 13768–13771.

Dittmer, J. C., & Wells, M. A. (1969) *Methods Enzymol. 14*, 482–528.

Dulley, J. R., & Grieve, P. A. (1975) *Anal. Biochem. 64*, 136–141.

Furhop, J. H., & Smith, K. M. (1975) *Porphyrins and Metalloporphyrins* (Smith, K. M., Ed.) pp 804–807, Elsevier/North-Holland Biomedical Press, Amsterdam.

Georgiou, C. D., & Webster, D. A. (1987) *Arch. Microbiol.* (in press).

Gunsalus, I. C., & Sligar, S. G. (1976) *Biochimie 58*, 143–147.

Hedrick, J. L., & Smith, A. J. (1968) *Arch. Biochem. Biophys. 126*, 155–164.

Jurtshuk, P., Mueller, T. J., & Wong, T. Y. (1981) *Biochim. Biophys. Acta 637*, 374–382.

Kita, K., Konishi, K., & Anraku, Y. (1984) *J. Biol. Chem. 259*, 3368–3374.

Kranz, R. G., & Gennis, R. B. (1983) *J. Biol. Chem. 258*, 10614–10621.

Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.

Lamba, P., & Webster, D. A. (1980) *J. Bacteriol. 142*, 169–173.

Loach, A. P. (1968) *Handbook of Biochemistry* (Sober, H. A., Ed.) Part J, pp 27–34, The Chemical Rubber Co., Cleveland, OH.

Manrique, A., & Lasky, M. (1981) *Electrophoresis (Weinheim, Fed. Repub. Ger.) 2*, 315–320.

Matsushita, K., Patel, L., Gennis, R. B., & Kaback, H. R. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 4889–4893.

Matsushita, K., Patel, L., & Kaback, H. R. (1984) *Biochemistry 23*, 4703–4713.

Miller, J. M., & Gennis, R. B. (1983) *J. Biol. Chem. 258*, 9159–9165.

Orii, Y., & Webster, D. A. (1986) *J. Biol. Chem. 261*, 3544–3547.

Poole, R. K. (1983) *Biochim. Biophys. Acta 726*, 205–243.

Poole, R. K., Waring, A. J., & Chance, B. (1979) *Biochem. J. 184*, 379–389.

Pudek, M. R., & Bragg, P. D. (1974) *Arch. Biochem. Biophys. 164*, 682–693.

Tamura-Lis, W., & Webster, D. A. (1986) *Arch. Biochem. Biophys. 244*, 285–291.

Thomas, P. E., Ryan, D., & Levin, W. (1976) *Anal. Biochem. 75*, 168–176.

Tyree, B., & Webster, D. A. (1978) *J. Biol. Chem. 253*, 7635–7637.

Wakabayashi, S., Matsubara, H., & Webster, D. A. (1986) *Nature (London) 322*, 481–483.

Whitaker, J. R. (1963) *Anal. Chem. 35*, 1950–1953.

Wilson, D. F., & Dutton, P. L. (1970) *Biochem. Biophys. Res. Commun. 39*, 59–64.

Wood, P. M. (1984) *Biochim. Biophys. Acta 768*, 293–317.

Yamanaka, T., & Okunuki, K. (1974) *Macrobial Iron Metabolism* (Neilands, J. B., Ed.) pp 349–400, Academic, New York.