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The Diheme Cytochrome c_4 from *Vibrio cholerae* Is a Natural Electron Donor to the Respiratory cbb_3 Oxygen Reductase[†]

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ABSTRACT: The respiratory chain of *Vibrio cholerae* contains three *bd*-type quinol oxygen reductases as well as one cbb_3 oxygen reductase. The cbb_3 oxygen reductase has been previously isolated and characterized; however, the natural mobile electron donor(s) that shuttles electrons between the bc_1 complex and the cbb_3 oxygen reductase is not known. The most likely candidates are the diheme cytochrome c_4 and monoheme cytochrome c_5, which have been previously shown to be present in the periplasm of aerobically grown cultures of *V. cholerae*. Both cytochromes c_4 and c_5 from *V. cholerae* have been cloned and expressed heterologously in *Escherichia coli*. It is shown that reduced cytochrome c_4 is a substrate for the purified cbb_3 oxygen reductase and can support steady state oxygen reductase activity of at least 300 e^{-1}/s. In contrast, reduced cytochrome c_5 is not a good substrate for the cbb_3 oxygen reductase. Surprisingly, the dependence of the oxygen reductase activity on the concentration of cytochrome c_4 does not exhibit saturation. Global spectroscopic analysis of the time course of the oxidation of cytochrome c_4 indicates that the apparent lack of saturation is due to the strong dependence of K_M and V_{max} on the concentration of oxidized cytochrome c_4. Whether this is an artifact of the in vitro assay or has physiological significance remains unknown. Cyclic voltammetry was used to determine that the midpoint potentials of the two hemes in cytochrome c_4 are 240 and 340 mV (vs standard hydrogen electrode), similar to the electrochemical properties of other c_4-type cytochromes. Genomic analysis shows a strong correlation between the presence of a c_4-type cytochrome and a cbb_3 oxygen reductase within the β- and γ-proteobacterial clades, suggesting that cytochrome c_4 is the likely natural electron donor to the cbb_3 oxygen reductases within these organisms. These would include the β-proteobacteria *Neisseria meningitidis* and *Neisseria gonnorhoeae*, in which the cbb_3 oxygen reductases are the only terminal oxidases in their respiratory chains, and the γ-proteobacterium *Pseudomonas stutzeri*.

The genome of *Vibrio cholerae* encodes four respiratory oxygen reductases (*1*). There are three *bd*-type oxygen reductases that use ubiquinol as the natural electron donor (*2*) and one cbb_3-type heme-copper oxygen reductase (*3*), which is presumed to use a cytochrome *c* as its natural electron donor. The motivation for this work was to identify the natural electron donor for the cbb_3-type heme-copper oxygen reductase.

The cbb_3-type oxygen reductases are members of the heme-copper superfamily that includes enzymes that perform oxygen reductase chemistry or nitric oxide reduction chemistry (*4*−*7*). The vast majority of currently identified oxygen reductases within the heme-copper superfamily are classified as being in one of three families, the A, B, and C families (*6*, *7*). The cbb_3-type oxygen reductases (*7*−*11*) are members of the C family and represent more than 20% of the sequences of heme-copper oxygen reductases from the currently known genome sequences. In the past decade, cbb_3-type oxygen reductases have been purified and characterized from several bacterial species, including *Rhodobacter sphaeroides* (*5*, *12*−*17*), *Paracoccus denitrificans* (*18*), *Rhodobacter capsulatus* (*19*−*23*), *V. cholerae* (*3*, *24*), *Bradyrhizobium japonicum* (*25*−*31*), *Rhodothermus marinus* (*32*), *Pseudomonas stutzeri* (*33*−*37*), *Helicobacter pylori* (*38*, *39*), and *Sulfurihydrogenibium azorense* (*10*). It has been demonstrated by mass spectrometry that the cbb_3-type oxygen reductases have the signature histidine-tyrosine cross-linked cofactor within their active site, but that the tyrosine comes from a different transmembrane span than for the A family and B family heme-copper oxygen reductases (*15*, *24*).

The physiological role of the cbb_3-type oxygen reductases is generally defined by a high affinity for O_2, allowing these enzymes to function at lower oxygen concentrations. Therefore, these enzymes are often expressed under microaerophilic growth conditions (*9*). These enzymes are of particular interest because they are present in a number of pathogenic species and are proposed to be important for virulence by facilitating growth under conditions of low oxygen (*40*). The cbb_3-type enzymes are the only oxygen reductases in *H. pylori* (*41*), *Neisseria gonorrheae* (*42*), and *Neisseria meningitidis* (*43*) and are, therefore, potential drug targets for these significant human pathogens.

To understand the physiological roles of the cbb_3-type oxygen reductases, it is important to know the natural electron donor(s). In most cases, the natural electron donor is not known.

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One exception is the cbb_3-type oxygen reductase from *H. pylori*, where the natural electron donor has been shown to be cytochrome c_{553} (*39*). When the electron donors are not known, the purified enzymes are usually assayed using the artificial electron donor TMPD[1] (reduced by ascorbate). In a few instances, reduced horse heart cytochrome *c* can function as an electron donor (*25*), though often these assays are also performed in the presence of TMPD.

The proteomes of *V. cholerae* strains have previously been analyzed for the presence of *c*-type cytochrome binding domains by searching for the CXXCH canonical sequence (*44*). A total of 48 CXXCH motifs were identified in 31 proteins, of which 14 have N-terminal signal sequences, indicating these are cytochrome *c*'s and are components of the bacterial envelope. Under aerobic growth conditions, six major *c*-type cytochromes are visualized by SDS−PAGE analysis using a stain for covalently bound heme (*44*). Two of these six cytochrome *c*'s have been identified as the CcoO and CcoP subunits of the cbb_3-type oxygen reductase (*44*). A third heme-containing band was tentatively identified as the YecK subunit of biotin sulfoxide reductase (*44*). It is likely that one of the heme-staining bands corresponds to the PetC gene, encoding the cytochrome c_1 component of the bc_1 complex, but this remains to be seen. The best candidates for the remaining two heme-staining bands in aerobically grown *V. cholerae* are the *cycA* and *cycB* gene products, corresponding to cytochrome c_4 and cytochrome c_5, respectively. Each of these cytochrome *c*'s is predicted to have a cleaved N-terminal signal sequence and to be located within the periplasm. The predicted molecular masses of the mature cytochromes c_4 and c_5 (19.8 and 11.5 kDa, respectively) also make these reasonable candidates for two of the heme-staining bands from aerobically grown *V. cholerae* (*44*). The *V. cholerae* genome also encodes two additional *c*-type cytochromes of unknown function that are predicted to be periplasmic (*44*). Their predicted molecular masses (9.1 and 8.5 kDa) do not match those of the major heme-staining proteins reported for aerobically grown *V. cholerae* (*44*). It is likely, therefore, that cytochrome c_4 and/or cytochrome c_5 might be an electron donor to the cbb_3-type oxygen reductase and responsible for shuttling electrons between the bc_1 complex and the heme-copper oxygen reductase. For this reason, the genes encoding the diheme cytochrome c_4 and monoheme cytochrome c_5 were each cloned and expressed heterologously in *Escherichia coli*, and the proteins were purified.

It is shown that cytochrome c_5 does not function as an electron donor to the purified cbb_3-type oxygen reductase from *V. cholerae*, whereas cytochrome c_4 can support oxygen reductase activity at a rate of at least 300 e^{-1}/s at 25 °C.

MATERIALS AND METHODS

Construction of Expression Plasmids. A heterologous *E. coli* expression system (*45*) was used to obtain recombinant cytochromes c_4 and c_5 from *V. cholerae*. Following the procedure successfully employed previously for the expression of cytochrome c_{552} from *Thermus thermophilus* (*46*), the DNA fragments encoding the predicted sequence of the mature cytochromes c_4 and c_5 were fused to a DNA fragment encoding the signal peptide (MKISIYATLAALSLALPAGA) of cytochrome c_{550} from *Thiobacillus versutus* (*47*). The genes of the mature cytochrome *c*'s were obtained by polymerase chain reaction (PCR) from genomic DNA. The following oligonucleotides were used: 5′-CAAGGCGCCCAGGCCCAAGGTAGTATCGAAG-3′ (forward primer) and 5′-AAGGATCCCTAGTGTAGGCCACCTAC-3′ (reverse primer) for *cycA* and 5′-CAAGGCGCCCAGGCTCTAACTGAAGCCGATA-3′ (forward primer) and 5′-AAGGATCCTTACAGGCCTGCGATCATA-3′ (reverse primer) for *cycB*. The primers were designed for the introduction of a 5′ *Nar*I site and a 3′ *Bam*HI site to create a full-length chimeric *T. versutus*/*V. cholerae* *cycA* or *cycB* gene and cloned into pET17b.

In addition, the 24th residue of cytochrome c_5 was changed from Leu to Gln to enhance the recognition by the *E. coli* signal peptidase in cleaving the new cutting site. Both cytochromes c_4 and c_5 are predicted to have cleaved signal peptides and to be soluble, periplasmic proteins. The predicted cleavage sites are located after Ala_{36} and Ala_{23} for cytochromes c_4 and c_5, respectively. This hybrid construct was then cloned into commercial expression vector pET17b.

Cell Growth and Enzyme Expression and Purification. Conditions of cell growth and enzyme expression were similar to those previously reported (*46*). The recombinant expression plasmids were utilized in a strain that also contained plasmid pEC86, which expresses maturation genes for cytochrome *c* in *E. coli* (*ccmABCDEFGH*) (*27*). The two plasmids were cotransformed into *E. coli* BL21(DE3) competent cells which were then streaked on a Luria-Bertani (LB) agar plate containing 50 μg/mL ampicillin and 30 μg/mL chloramphenicol (*27*, *46*). The cells were grown in 1 L of culture medium in a 2.8 L Fernbach flask to an A_{600} of 0.8−1, and then expression of the cytochrome *c* was induced with 1 mM IPTG. After 7 h, the cells were harvested by centrifugation at 7000*g* for 15 min. Generally, ∼10 g of cell paste was collected from each liter of growth medium. The cell pellet was a reddish-brown color, indicating the overexpression of cytochrome *c*.

Protein Purification. Each of the recombinant cytochrome *c*'s was purified by column chromatography, using procedures similar to those used for the purification of recombinant cytochrome c_{552} from *Th. thermophilus* (*46*). Briefly, the collected cell pellet was homogenized in 25 mM Tris-HCl (pH 7.5), 8 mM $MgSO_4$, DNase I, 0.1% Triton X-100, and protease inhibitor cocktail. The suspended cell mixture was passed through a microfluidizer three times at a pressure of 20000 psi. The cell debris was spun down at 8000 rpm for 30 min at 4 °C. The supernatant was loaded onto a 5 cm × 20 cm CM-52 cellulose column (Whatman) and eluted with a gradient from 0 to 1 M NaCl in 25 mM Tris-HCl (pH 7.5) buffer. Fractions that were reddish-brown were collected and concentrated using concentrators (Amicon) with YM-10 membranes. The concentrated protein was loaded onto a 2.6 cm × 100 cm gel filtration column (Sephacryl S-100 High Resolution, GE Healthcare) equilibrated with 100 mM potassium phosphate buffer (pH 7.0) at a flow rate of 0.4 mL/min. The protein was collected and dialyzed overnight against 25 mM Tris-HCl and then loaded onto the buffer-equilibrated 1.6 cm × 20 cm DEAE-5PW column (Toso-HaaS). The column was eluted using a gradient from 0 to 1 M NaCl in 25 mM Tris-HCl (pH 7.5) buffer. The eluted protein was dialyzed and concentrated again as described above, flash-frozen in liquid nitrogen, and stored at −80 °C. The final yields of the purified cytochromes c_4 and c_5 were ∼15 and ∼5 mg/L of cell culture, respectively.

SDS−PAGE Analysis. The purified *c*-type cytochromes were analyzed using SDS−PAGE. Protein was visualized using

[1]Abbreviations: SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; PEG, pyrolytic graphite edge; TMPD, *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine; TMBZ, 3,3′,5,5′-tetramethylbenzidine.

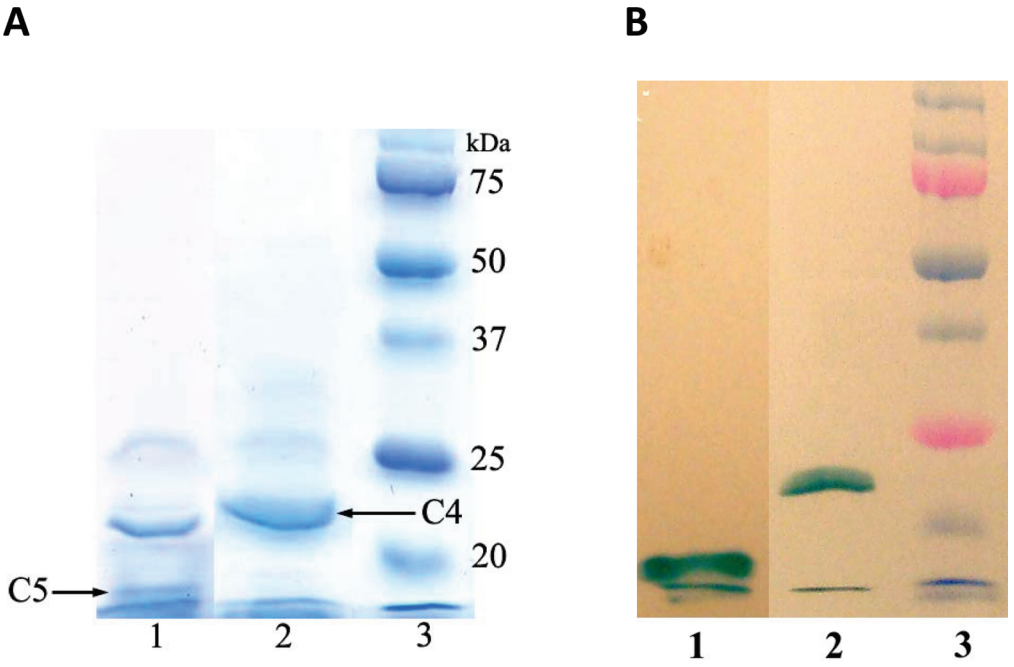

FIGURE 1: SDS−PAGE of the isolated recombinant cytochromes c_4 and c_5. (A) A 15% SDS gel stained with Coomassie blue. (B) Identical gel stained for heme. The lanes from left to right contain cytochrome c_5 (lane 1), cytochrome c_4 (lane 2), and molecular weight standards (lane 3).

Coomassie Blue, and heme staining (*48*) was used to identify proteins containing covalently attached heme *c*. Precast 15% SDS−PAGE gels from ISC BioExpress were used. After electrophoreses, the gels were then incubated in 15 mL of 6.3 mM 3,3′,5,5′-tetramethylbenzidine (TMBZ from Sigma) and 35 mL of 0.25 M sodium acetate (pH 5.0) for 1 h. The gels were then stained for heme via addition of H_2O_2 to a final concentration of 30 mM.

Spectrophotometric Measurements. Spectra of the isolated cytochromes were acquired with a Shimadzu UV−vis 2101PC spectrophotometer. The enzyme sample (3−17 μM) was examined in 25 mM Tris-HCl buffer at pH 7.5. The enzymes were oxidized with 2 μL of 1 mM $Fe(CN)_6$ and reduced with a small amount of solid dithionite, both obtained from Sigma. Spectra were scanned from 375 to 800 nm. The concentrations of the cytochrome *c*'s were estimated from the spectra of the reduced cytochromes using extinction coefficients of 40 mM^{-1} cm^{-1} at 553 nm for the diheme cytochrome c_4 (*49*, *50*) and 20 mM^{-1} cm^{-1} at 555 nm for the monoheme cytochrome c_5 (*51*).

Cyclic Voltammetry. Reduction potentials were measured using protein film voltammetry (*52*, *53*). The cytochrome c_4 was applied directly to a freshly polished pyrolytic graphite edge (PGE) electrode surface and then placed into solution in a thermostated all-glass cell encased in a Faraday cage. Because oxygen does not interfere with measurements in the range of 100−500 mV [vs standard hydrogen electrode (SHE)], measurements were performed aerobically. Analogue-scan cyclic voltammetry was performed using a Bioanalytical Systems (West Lafayette, IN) CV-27 voltammograph with an in-house amplifier, and results were recorded via an in-house program. Data were analyzed using Fourier transformation and Origin 7.5 (OriginLab Corp., Northampton, MA). The solution pH value was controlled by using 10 mM HEPES buffer containing 2 M NaCl (pH 7.0).

Purification of the cbb_3-Type Oxidase from V. cholerae. The cbb_3-type oxidase from *V. cholerae* was purified as previously described (*3*).

Steady State Kinetics Using an Oxygen Electrode. Cytochrome *c* oxidase activity was measured polarographically at 25 °C using a YSI model 53 oxygen meter. The standard reaction mixture contained 1.8 mL of 50 mM sodium phosphate (pH 6.5), 50 mM NaCl, 0.05% dodecyl maltoside, 10 mM sodium ascorbate, and the cytochrome *c* to be tested. The cytochrome c_4 and cytochrome c_5 concentrations were varied in the range of 1−100 μM. The O_2 consumption reaction was initiated by the addition of the oxidase to a final concentration of 50 nM. The dependence of activity on ionic strength was measured by varying the concentration of NaCl from 10 to 150 mM using 20 μM cytochrome c_4 reduced by 10 mM sodium ascorbate in 10 mM sodium phosphate (pH 6.5) buffer and 0.05% dodecyl maltoside.

Steady State Kinetics Assessed by Stopped-Flow Spectrophotometry. To prepare the prereduced cytochrome c_4, the sample was reduced with sodium dithionite and the excess dithionite was removed by gel filtration. The steady state kinetics between the cbb_3 oxidase from *V. cholerae* and the prereduced cytochrome c_4 was monitored at 25 °C with Applied Photophysics SX-17MV stopped-flow spectrometer as follows. One syringe of the stopped-flow apparatus was filled with 400 nM cbb_3 oxygen reductase in 50 mM sodium phosphate (pH 6.5), 150 mM NaCl, and 0.05% dodecyl maltoside, while the prereduced cytochrome c_4 in the same buffer was loaded into the other syringe. The concentration of cytochrome c_4 was varied from ~1 to 60 μM for different measurements. After the sample had been mixed, the reaction was followed spectrometrically from 400 to 700 nm for 50−1000 s using a photodiode array. Using a least-squares fitting method (*54*), the spectrum at each time point was deconvoluted into the component spectra of reduced and oxidized cytochrome c_4, which had been collected separately when cytochrome c_4 was mixed with either sodium dithionite or potassium ferricyanide. The concentrations of the reduced and oxidized cytochrome c_4 at each time point were then used to calculate the reaction rate at each point. By using different starting concentrations of cytochrome c_4 in different measurements, the reaction rates at various combinations of reduced and oxidized cytochrome c_4 concentrations were obtained.

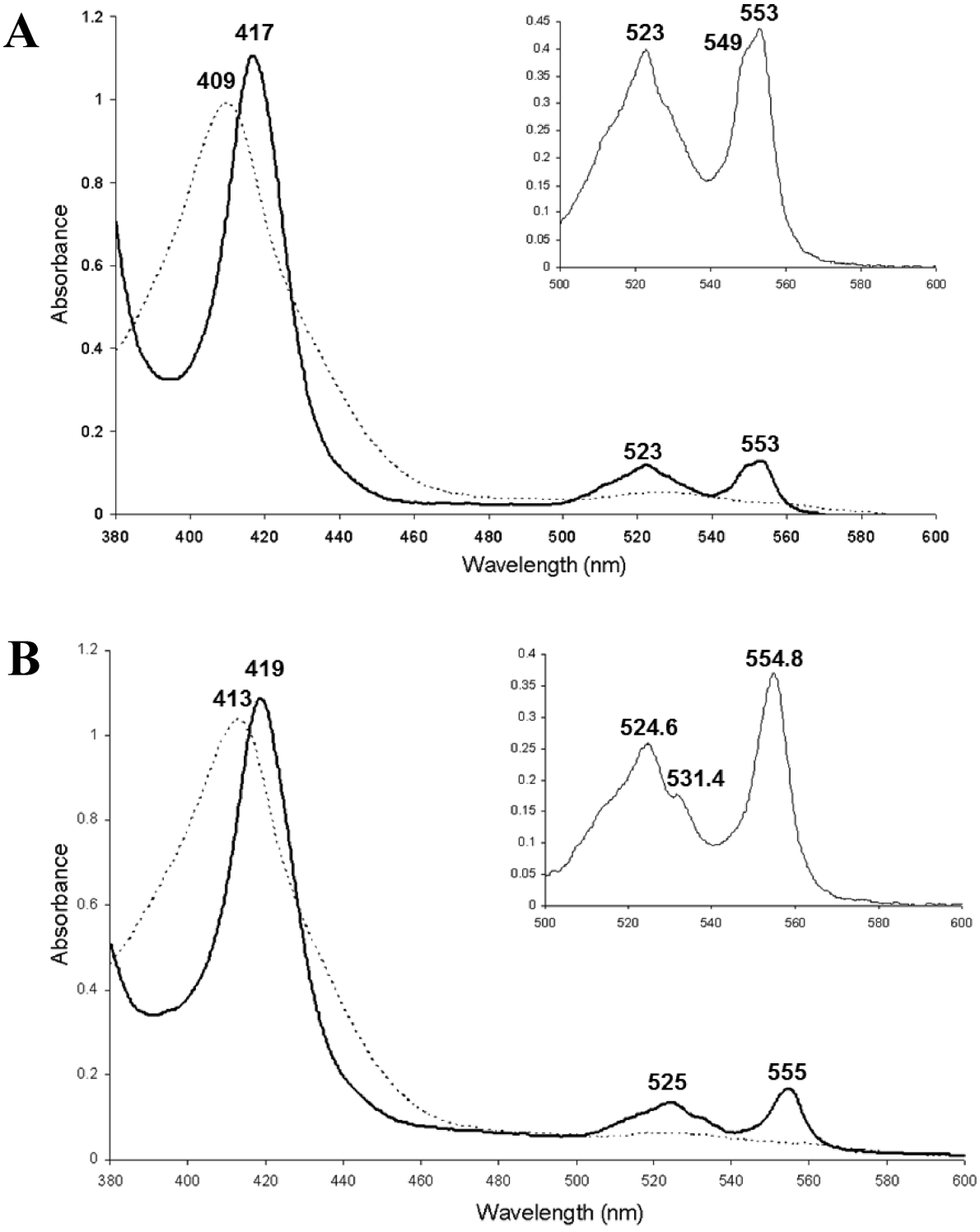

FIGURE 2: Absorption spectra of fully reduced (—) and fully oxidized (···) *V. cholerae* (A) cytochrome c_4 (3 μM) and (B) cytochrome c_5 (7 μM). The insets show the α and β bands of the fully reduced cytochrome c_4 (10 μM) and cytochrome c_5 (17 μM). All the absorption spectra were recorded in 25 mM Tris-HCl (pH 7.5) at 25 °C.

The reaction rates were plotted versus reduced cytochrome c_4 concentrations at a fixed oxidized cytochrome c_4 concentration, and the K_M and V_{max} for each plot were obtained by nonlinear least-squares fitting to the standard Michaelis−Menten kinetics model. All data processing and analyses were performed with Mathematica.

RESULTS

Expression and Purification of Recombinant Cytochrome c_4 and Cytochrome c_5. Transformants were found to express the cytochrome *c*'s upon induction (*46*), and the recombinant proteins were isolated as described in Materials and Methods. The SDS−PAGE analysis of the recombinant cytochromes is shown in Figure 1. After being stained with Coomassie Blue (Figure 1A), the purified cytochrome c_4 has a single band near 21−22 kDa that contains two covalently bound hemes (Figure 1B). The expected molecular weight of the mature cytochrome c_4 is 19791 (187 amino acid residues) with 1233 (two-heme *c* moiety). Cytochrome c_5 has a single heme-staining

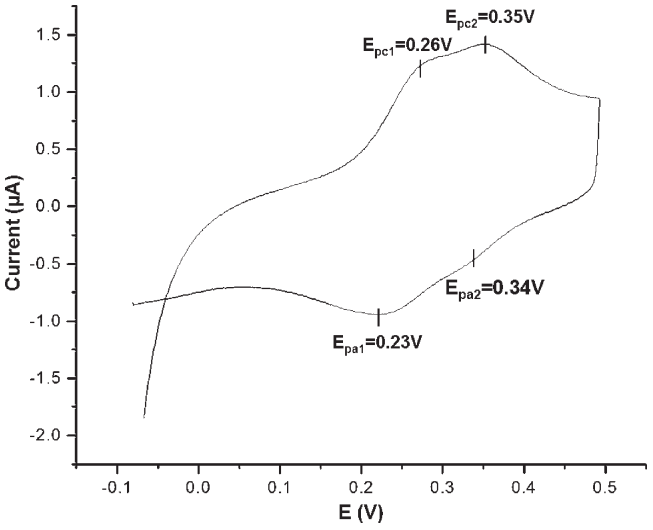

FIGURE 3: Cyclic voltammetry of the recombinant cytochrome c_4 that was applied to a freshly polished pyrolytic PGE electrode. The solution contained 10 mM HEPES buffer and 2 M NaCl (pH 7.0). The following values were obtained (vs SHE): $E_{pc1} = 0.26$ V, $E_{pc2} = 0.35$ V, $E_{pa1} = 0.23$ V, $E_{pa2} = 0.34$ V, and $E_{1/2} = (E_{pc} + E_{pa})/2 = 0.24$ and 0.34 V.

component running near the expected molecular weight of 11480, but there is a major protein contaminant that does not contain heme. Because cytochrome c_5 does not function as a reductant for the cbb_3-type oxidase (see below), no further effort was made to improve the purification.

UV−Vis Spectroscopy. Figure 2 shows the spectra of the reduced and oxidized forms of cytochromes c_4 and c_5. The spectra of each are consistent with previously published reports of homologues from other organisms (*49*−*51*). In the reduced form, the absorption spectrum of cytochrome c_4 has three maxima at 553, 523, and 417 nm, corresponding to the α, β, and γ (Soret) bands, respectively (Figure 2A). The maxima of the oxidized form of cytochrome c_4 are at 528 and 409 nm. The spectra show two diagnostic features shared by all cytochrome c_4's: (1) a split α-band with maxima at 553 and 549 nm and (2) a low ratio of the amplitude of the α band to the β band ($A_{553}/A_{523} = 1.05$).

Reduced cytochrome c_5 has maxima at 554.8, 524.6, and 419 nm, corresponding to the α, β, and γ (Soret) bands, respectively (Figure 2B). The α/β ratio $A_{555}/A_{525} = 1.5$. The spectrum of the oxidized cytochrome c_5 has two peaks at 526 and 413 nm. These features are consistent with previous reports of cytochrome c_5's (*51*).

Electrochemistry. Cytochrome c_4 was found to be amenable to direct electrochemical characterization when adhered to a carbon electrode. The midpoint potentials of the two heme components of the recombinant cytochrome c_4 were measured by cyclic voltammetry (Figure 3). Four well-defined redox peaks are observed, and the E°_m values of the two heme groups were determined to be approximately 240 and 340 mV (vs SHE).

Rates of Oxygen Reduction by the V. cholerae cbb$_3$-Type Oxidase Using Cytochromes c_4 and c_5. Figure 4A compares the rates of oxygen reduction, measured with an oxygen electrode, by the *V. cholerae* cbb_3-type oxidase using ascorbate and either (i) horse heart cytochrome *c*, (ii) recombinant cytochrome c_4, or (iii) recombinant cytochrome c_5. These assays were performed in the absence of the mediator TMPD, which can itself function as a substrate for the cbb_3-type oxidase. The data (Figure 4A) show that neither the horse heart cytochrome *c* nor cytochrome c_5 is effective as a substrate, whereas cytochrome c_4 is capable of supporting turnover at rates of > 300 e^{-1}/s at 25 °C. This turnover is comparable to what is observed with 0.5 mM TMPD in the presence of ascorbate to maintain the TMPD reduced (not shown). The turnover using recombinant cytochrome c_5 is more than a factor of 10 slower (7%) than that observed with cytochrome c_4, and horse heart cytochrome *c* is also a poor substrate (8%). Although the purity of recombinamt cytochrome c_5 was not high, it is unlikely that the contaminants are inhibitory. The oxidase activity with cytochrome c_4 is rapidly stopped upon addition of 25 μM cyanide (Figure 4B), showing that the oxidase activity is catalyzed by the cbb_3-type oxygen reductase. The data in Figure 4A do not indicate saturation even at concentrations of cytochrome c_4 as high as 100 μM. Activity was compared using 50 nM oxidase and 20 μM cytochrome c_4, varying the concentration of NaCl from 1 to 150 mM (Figure 4C). These data indicate that the apparent binding of cytochrome c_4 to the oxidase is not sensitive to ionic strength and that the lack of saturation of activity as a function of the concentration of cytochrome c_4 is not due to weak binding due to the selected ionic strength. It was determined, however, that under the conditions used for the assays with the oxygen electrode, the ascorbate does not keep the cytochrome c_4 fully reduced.

Stopped-Flow Spectrophotometry. To improve our understanding of the lack of saturation in the steady state kinetics measurements using the oxygen electrode, steady state activity was also measured by stopped-flow spectrophotometry, mixing prereduced cytochrome c_4 with a solution containing the cbb_3-type oxidase. The oxidation of cytochrome c_4 was monitored by recording the change in the absorption spectrum from 400 to 700 nm using a photodiode array. The initial concentration of cytochrome c_4 was varied from 1 to 60 μM, and spectra were recorded as a function of time for each initial condition. The spectra were deconvoluted to quantify the concentration of oxidized and reduced cytochrome c_4 in solution as a function of time. From this large matrix of data, the rate of oxidation of cytochrome c_4 could be determined for a wide range of concentrations of both the reduced and oxidized forms of cytochrome c_4. These data were plotted to show how the rate of oxidation varies as a function of reduced cytochrome c_4 concentration in the presence of a specific concentration of oxidized cytochrome c_4 (Figure 5A−E). If the oxidized cytochrome c_4 is a competitive inhibitor for the reduced cytochrome c_4, one would expect that the data would show a constant V_{max} and increasing K_M as the concentration of oxidized cytochrome c_4 increases. However, this is not the case, and both V_{max} and K_M increase as the concentration of oxidized cytochrome c_4 increases (Figure 5F). One complication is that the spectral deconvolution with the least-squares method is not satisfactory in the case of cytochrome c_4 because cytochrome c_4 contains two hemes that have slightly different spectra. The assumption that either both are oxidized or both are reduced is not realistic. Attempts were not made to analyze the data with more complex models. Despite this, our analysis indicates the following significant points.

(1) Oxidized cytochrome c_4 has a strong effect on the activity between the cbb_3-type oxidase and reduced cytochrome c_4. The apparent K_M increases in the presence of relatively small amounts of oxidized cytochrome c_4 (Figure 5F). If the concentration of oxidized cytochrome c_4 is greater than 2.5 μM, the K_M of reduced cytochrome c_4 is too large to obtain a reasonably accurate fitting to the Michaelis−Menten model.

(2) During the manipulation of the prereduced cytochrome c_4 to load the stopped-flow syringe, a significant amount of

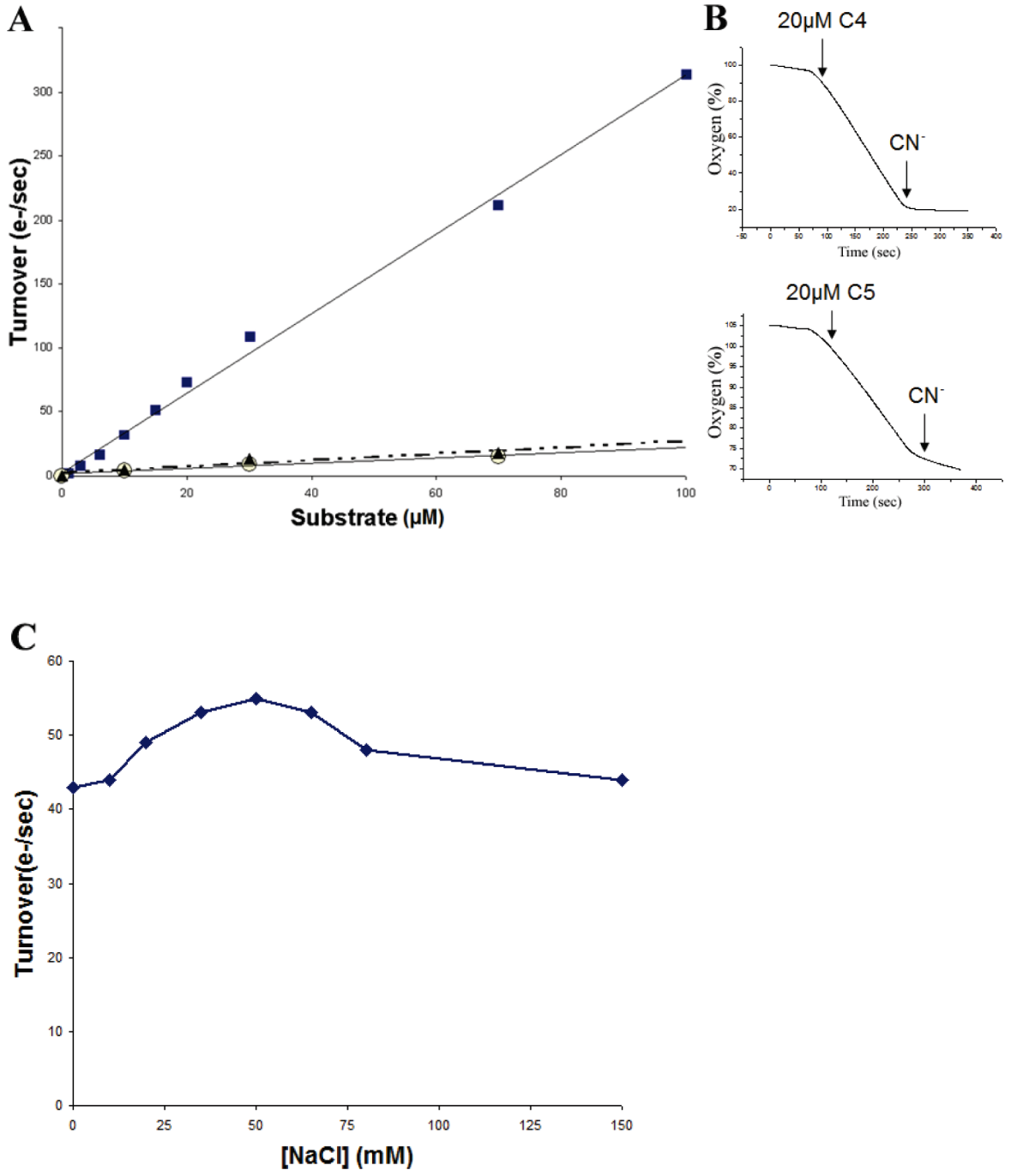

FIGURE 4: Steady state oxidase activity of the cbb_3 oxygen reductase from *V. cholerae*. (A) Dependence on the concentration of cytochrome c_4 (■), cytochrome c_5 (○), and horse heart cytochrome *c* (▲). The reaction mixture contained 50 mM sodium phosphate (pH 6.5), 50 mM NaCl, 0.05% dodecyl maltoside, 10 mM sodium ascorbate, and the indicated amount of the cytochrome *c*, in a total volume of 1.8 mL. The O_2 consumption reaction was initiated by the addition of 50 nM oxidase. (B) Sensitivity of the oxidase activity to the addition of 25 μM cyanide. (C) Dependence of the oxidase activity on the concentration NaCl, measured with 50 nM oxidase and 20 μM cytochrome c_4, in the presence of 10 mM sodium ascorbate, 10 mM sodium phosphate (pH 6.5) buffer, and 0.05% dodecyl maltoside.

autoxidation occurs. Hence, none of the data represent a starting condition under which cytochrome c_4 is actually fully reduced.

(3) Because of the spectroscopic complications of having two different heme components in cytochrome c_4, data fits are not sufficient to extrapolate to obtain true values for either V_{max} or K_M. Phenomenologically, as the concentration of oxidized cytochrome c_4 increases, both the apparent V_{max} and K_M increase. The inhibitory influence of oxidized cytochrome c_4 and the increasing concentration of oxidized cytochrome c_4 that accumulates as the reaction proceeds explain the lack of saturation under conditions used for the oxygen electrode assays (Figure 4A).

DISCUSSION

Although initially thought to be confined to proteobacteria (*7*, *11*), the cbb_3 oxygen reductases are widely distributed among bacterial phyla (*5*, *10*). Although there are exceptions, most archaea lack *c*-type cytochromes (*55*), and no example of a cbb_3-type oxidase in an archaea has been reported. Nearly all studies have focused on the role of the C family (cbb_3) oxygen reductases in the aerobic respiratory chains of proteobacteria (*8*), exceptions being the cbb_3-type oxidases from *Rh. marinus* (yet to be confirmed in the genome sequence) (*32*) and from *S. azorense* (*10*).

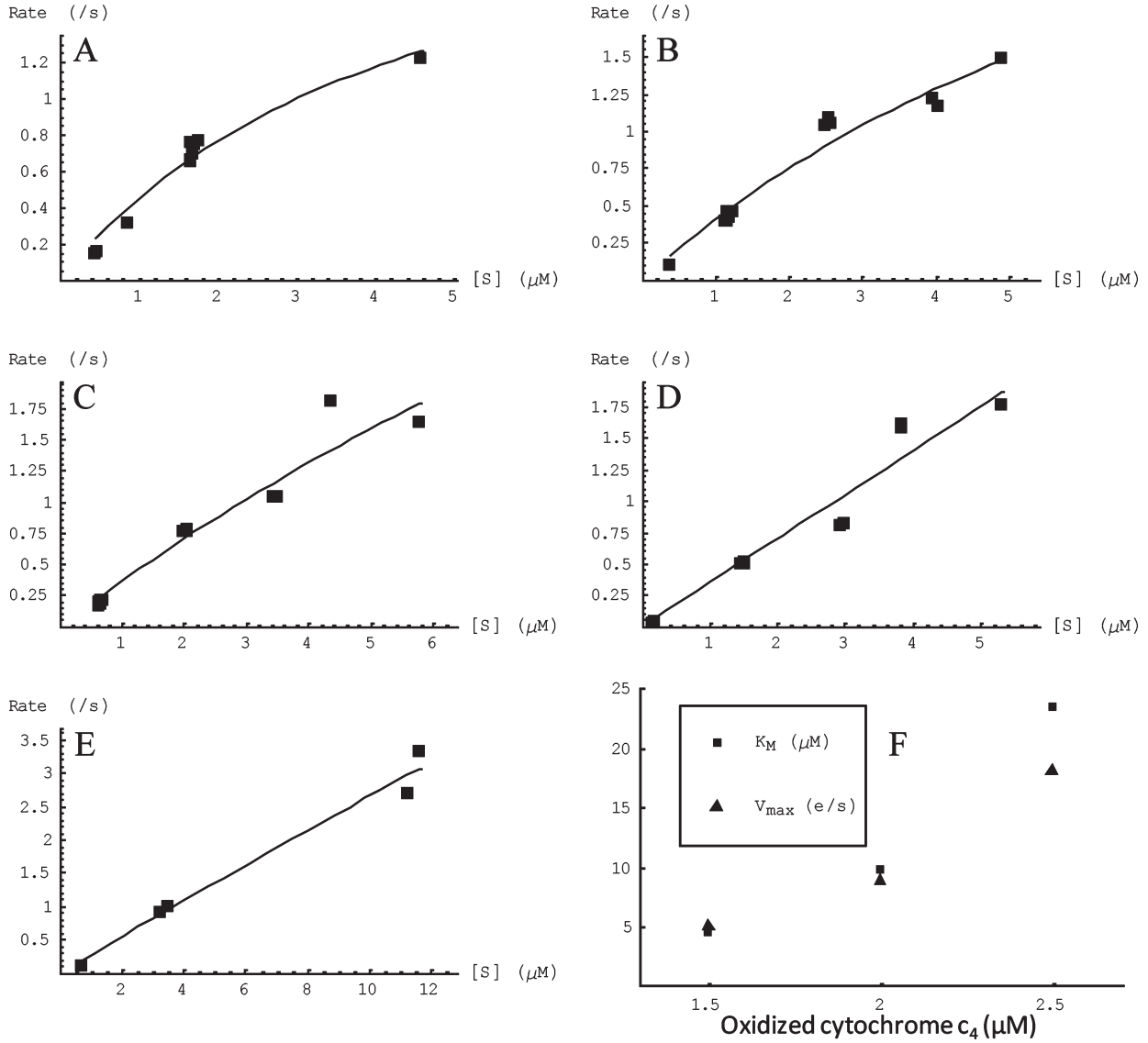

FIGURE 5: Rates of oxidation of reduced cytochrome c_4 by the cbb_3 oxygen reductase from *V. cholerae* at different oxidized cytochrome c_4 concentrations determined by stopped-flow spectroscopy and least-squares fitting. The substrate S refers to reduced (not total) cytochrome c_4. The oxidized cytochrome c_4 concentrations were (A) 1.5, (B) 2.0, (C) 2.5, (D) 3.0, and (E) 7.5 μM. The curves show the nonlinear least-squares fitting of the points to Michaelis−Menten kinetics. The K_M and V_{max} values for 1.5, 2.0, and 2.5 μM oxidized cytochrome c_4 are plotted in panel F.

In most cases, the natural electron donors for these C family oxygen reductases are not known. This work has identified the diheme cytochrome c_4 as a natural electron donor to the cbb_3-type oxygen reductase from the γ-proteobacterium, *V. cholerae*. Cytochrome c_4 is one of five cytochrome *c*'s encoded in the genome of *V. cholerae* that are predicted to be soluble, periplasmic proteins (*44*). Cytochromes c_4 and c_5 appear to be among the major cytochrome *c*'s expressed under aerobic growth conditions (*44*). Heterologous expression in *E. coli* was successful, generating soluble cytochromes c_4 and c_5. Cytochrome c_4 from *V. cholerae* has spectroscopic and electrochemical properties similar to those of the cytochrome c_4's from other organisms. These include the high midpoint potentials (240 and 340 mV, vs SHE) of the two hemes, the split α band, and the low ratio of the peak heights of the α and β bands (*50*, *56*, *57*).

Cytochrome c_4 can clearly donate electrons to the cbb_3-type oxidase, supporting steady state reduction of O_2 at a rate of at least 300 e^{-1}/s. Rates attained using either cytochrome c_5 or horse heart cytochrome *c* were 10−20-fold lower under comparable conditions. The one caveat is that the steady state rate of cytochrome c_4 oxidase activity does not saturate and quite high concentrations (100 μM) are required to reach the turnover rate of 300 s^{-1}. The lack of saturation (Figure 4A) is unusual and not expected. By comparison, the rate of steady state turnover of *H. pylori* cytochrome c_{553} by the *H. pylori* cbb_3-type oxidase is 250 s^{-1}, with a K_m of 0.9 μM. The nearly linear dependence of oxygen reductase activity as a function of the concentration of cytochrome c_4 (Figure 4A) can be qualitatively explained as being due to the influence of oxidized cytochrome c_4 (*39*). There is a significant concentration of oxidized cytochrome c_4 present at all times during the oxygen reductase assay, and the presence of ascorbate at a concentration as high as 100 mM does not alleviate this problem. The data do show that the true V_{max} for the oxidation of cytochrome c_4 must be at least 300 s^{-1}, but the true K_m cannot be measured using this assay. In the steady state assay, the velocity is measured in the presence of 100 μM reduced cytochrome c_4 with ~25 μM oxidized c_4 also present. The stopped-flow experiments cannot be performed under these conditions, but the data show that the values of both V_{max} and K_M depend on the concentration of oxidized cytochrome c_4, and

the stopped-flow data are consistent with the steady state kinetics. It is quite conceivable that the true K_m is in the range of 1−10 μM, but further work will be needed to determine this.

Because the oxidized and reduced forms of cytochrome *c* often have similar affinities for their redox partners, it is to be expected that oxidized cytochrome c_4 would competitively inhibit the activity between reduced cytochrome c_4 and the cbb_3-type oxidase. However, product inhibition can explain only the increase in the K_M but not the increase in V_{max} that is observed as the concentration of the product increases (Figure 5). Whether this kinetic behavior reflects an in vitro artifact or an unusual form of allosteric regulation is not clear. This unusual behavior might be related to the fact that cytochrome c_4 is a two-electron carrier. Cyclic voltammetry shows that the diheme cytochrome c_4 exhibits two substantially different midpoint potentials, but the extent of cooperativity between the two hemes is not known. In principle, there could be two different binding sites on cytochrome c_4 for the cbb_3 oxygen reductase, each with a distinct K_M and V_{max}. In any event, further characterization of the oxidation kinetics of the diheme cytochrome c_4 is needed to address this question.

At this point, it is safe to conclude that cytochrome c_4 is definitely a substrate for the cbb_3-type oxidase whereas cytochrome c_5 is a very poor substrate, at best, under in vitro conditions.

Roles of Cytochrome c_4 in Other Bacteria. Analysis of sequenced genomes demonstrates that the distribution of cytochrome c_4 is limited, with the majority of homologues being found within the β- and γ-proteobacterial clades. Cytochrome c_4 is also sporadically distributed within the α-proteobacterial clade as well as a few other bacterial phyla. Bacteria within the β- and γ-proteobacterial clades that contain a cbb_3 oxygen reductase are very likely also to contain a cytochrome c_4. Within these groups of bacteria, it is reasonable to assume that cytochrome c_4 donates electrons to the cbb_3 oxygen reductase. It is noted that the cbb_3 oxygen reductases within the β- and γ-proteobacterial clades all contain the auxiliary subunit CcoP, a diheme cytochrome *c* that is the likely electron acceptor from cytochrome c_4. A large number of the cbb_3 oxygen reductases outside the β- and γ-proteobacterial clades are missing the CcoP subunit, and others have an alternative subunit, CcoR (*10*). Indeed, many organisms that are outside the β- and γ-proteobacterial clades that encode a cbb_3 oxygen reductase do not contain cytochrome c_4 and, therefore, must be using a different donor of electrons to this enzyme.

Other γ-proteobacteria that contain cbb_3 oxygen reductases include *Azotobacter vinelandii* (*58*), and *P. stutzeri* (*33*−*37*). Knockout mutants of both cytochromes c_4 and c_5 in *A. vinlandii* support a role for both of these cytochromes in aerobic respiration, though there are no data concerning a specific role with the cbb_3 oxygen reductase. The cytochrome c_4 from *P. stutzeri* has been extensively characterized (*49*, *59*−*62*), but its physiological role has not yet been defined.

Acidothiobacillus ferrooxidans is a γ-proteobacterium whose genome includes several cytochrome c_4's but not a cbb_3-type oxidase (*63*−*65*). The cytochrome c_4's are strongly implicated in the aerobic respiratory chain (*66*−*68*). *Ac. ferrooxidans* is an obligate chemolithotropic bacterium that grows at acidic pH (pH 1.5−4) and uses either ferrous iron or reduced sulfur compounds as an electron source. The *cyc1* gene encodes a diheme c_4-type cytochrome c_{552} within an operon that includes an A family oxygen reductase as well as the blue copper protein rusticyanin (*69*). These respiratory components comprise the electron transport chain between the ferrous iron oxidase and O_2. The cytochrome c_4 forms a complex with rusticyanin that is likely the immediate donor of electrons to the A family oxygen reductase (*68*, *70*). The structure of the *Cyc1* cytochrome c_4 has been determined, and the complex with rusticyanin has been modeled computationally (*64*, *70*−*72*). Two additional genes encoding cytochrome c_4's, *cycA1* and *cycA2*, are in operons encoding two separate bc_1 complexes (*66*). It is suggested that when growing on reduced sulfur compounds, *CycA2* shuttles electrons from the bc_1 complex encoded by the *petII* operon to one or several terminal reductases (*66*). When growing on Fe^{2+}, *CycA1* is proposed to be part of an aerobic respiratory chain that runs in the reverse direction to generate NADH: *CycA* → bc_1 (*petI*) → Q → Complex I (*66*, *67*).

The β-proteobacterial clade includes *N. meningitidis* and *N. gonorrheae*, which are each human pathogens and are notable also for the fact that the cbb_3 oxygen reductase is the only respiratory oxidase encoded in their genomes (*42*, *43*). Knockout mutations have implicated cytochrome c_4 along with cytochrome c_x (a homologue of cytochrome c_{552} from *Th. thermophilus*) as being a donor of electrons to the cbb_3 oxygen reductase in *N. meningitidis* (*73*). Cytochrome c_5 is required for the transfer of electrons to the nitrite reductase in *N. meningitidis* (*73*). Knockout mutations have also indicated a role of cytochrome c_4 along with cytochrome c_5 as substrates of the cbb_3 oxygen reductase in *N. gonorrheae*, but not cytochrome c_2 (*42*). Interestingly, the CcoP subunit of the cbb_3-type oxidase in *N. gonorrheae* has an extra cytochrome *c* domain that is homologous to cytochrome c_5, and this domain is implicated in the transfer of electrons to the nitrite reductase that is tethered to the outer membrane (*74*).

Outside the β- and γ-proteobacterial clades, there is limited information about the native electron donors for the cbb_3 oxygen reductase. One exception is *H. pylori*, in which the monoheme cytochrome c_{553} (*39*) is the substrate for the cbb_3 oxygen reductase (*38*). *H. pylori* is an ε-proteobacterium. Closely related ε-proteobacteria that contain cbb_3 oxygen reductases and might also use homologues of cytochrome c_{553} as substrates are *Campylobacter jejuni* (*41*, *75*) and *Wolinella succinogenes* (*76*, *77*).

The cbb_3 oxygen reductases have been biochemically characterized from several α-proteobacteria: *R. sphaeroides* (*5*, *12*−*17*), *Pa. denitrificans* (*18*), *R. capsulatus* (*19*−*23*), *B. japonicum* (*25*−*31*), and *Rh. marinus* (*32*). Using knockout mutants, it was concluded that both the A and C family oxygen reductases from *R. sphaeroides* can utilize either cytochrome c_2 or membrane-anchored cytochrome c_y (*21*).

CONCLUSION

Cytochrome c_4 has been shown to be the native electron donor for the C family (cbb_3) oxygen reductase in *V. cholerae*. Within the γ- and β-protobacterial clades, there is a strong correlation for the coexistence of a C family oxygen reductase and a cytochrome c_4, suggesting that in these organisms, it is likely that cytochrome c_4 is a natural electron donor. This does not exclude other electron donors from also being substrates within these organisms. Outside the γ- and β-protobacterial clades, there is clear evidence that other cytochrome *c*'s are substrates for C family oxygen reductase, though in most cases, there are no data to identify these electron donors.

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