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Functional Importance of a Pair of Conserved Glutamic Acid Residues and of Ca^{2+} Binding in the cbb_3-Type Oxygen Reductases from *Rhodobacter sphaeroides* and *Vibrio cholerae*

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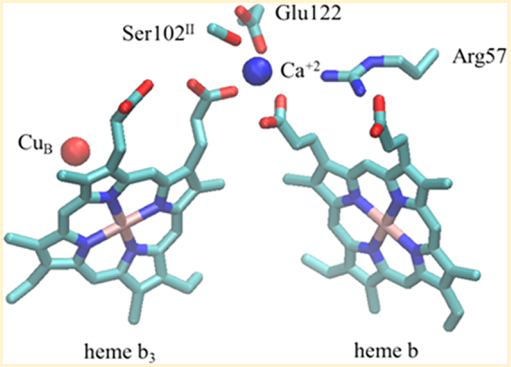
ABSTRACT: The cbb_3-type cytochrome *c* oxidases are members of the family of heme-copper proton pumping respiratory oxygen reductases. The structure of the cbb_3-type oxidase from *Pseudomonas stutzeri* reveals that, in addition to the six redox-active metal centers (two *b*-type hemes, three *c*-type hemes, and Cu_B), the enzyme also contains at least one Ca^{2+}. The calcium bridges two propionate carboxyls at the interface between the low-spin heme *b* and the active-site heme b_3 and, in addition, is ligated to a serine in subunit CcoO and by a glutamate in subunit CcoN. The glutamate that is ligated to Ca^{2+} is one of a pair of glutamic acid residues that has previously been suggested to be part of a proton exit pathway for pumped protons. In this work, mutations of these glutamates are investigated in the cbb_3-type oxidases from *Vibrio cholerae* and *Rhodobacter sphaeroides*. Metal analysis shows that each of these wild-type enzymes contains Ca^{2+}. Mutations of the glutamate expected to ligate the Ca^{2+} in each of these enzymes (E126 in *V. cholerae* and E180 in *R. sphaeroides*) result in a loss of activity as well as a loss of Ca^{2+}. Mutations of the nearby glutamate (E129 in *V. cholerae* and E183 in *R. sphaeroides*) also resulted in a loss of oxidase activity and a loss of Ca^{2+}. It is concluded that the Ca^{2+} is essential for assembly of the fully functional enzyme and that neither of the glutamates is likely to be part of a pathway for pumped protons within the cbb_3-type oxygen reductases. A more likely role for these glutamates is the maintenance of the structural integrity of the active conformation of the enzyme.



The heme-copper superfamily includes respiratory oxygen reductases, which are proton pumps, as well as NO reductases, which are not pumps.[1−4] The superfamily is defined by sequence homology within the integral membrane subunit that contains the catalytic bimetallic center where either O_2 is reduced to water or NO is reduced to N_2O. In the case of the oxygen reductases, the active site is comprised of a high-spin heme and a nearby copper, called Cu_B. For the NO reductases, the bimetallic center consists of a high-spin heme and a nearby Fe, called Fe_B. All of the members of the superfamily also contain a second, low-spin heme within the same subunit that is required for the transfer of an electron into the active site.

The respiratory oxygen reductases can be further subdivided into three major families, denoted families A−C.[1−3,5−7] The two families of NO reductases that have been biochemically and structurally characterized, called the cNOR and qNOR families,[1,4,8−11] are phyllogenetically most closely related to the C-family of oxygen reductases.[12−16] The qNOR and cNOR enzymes are very closely related to each other;[4,8,9,17] however, the cNOR enzymes use a cytochrome *c* as an electron donor,[4] whereas qNORs use a quinol substrate.[10]

It has been noted that in sequence alignments, the cbb_3-type oxygen reductases, along with the cNOR and qNOR families, have in common a pair of conserved glutamic acid residues that were predicted, prior to the recently available X-ray structures,[10,11] to be near the periplasmic surface of the proteins.[18,19] Site-directed mutagenesis of the cNOR from *Paracoccus denitrificans* showed that these glutamates are each necessary for function but not for assembly of the enzyme.[18,20,21] Similar experiments with the cbb_3-type oxidases from both *Vibrio cholerae* and *Rhodobacter sphaeroides* also indicate that these glutamates are important for function.[7] It was suggested that these glutamates might function as part of a proton pathway in the NO reductases to deliver protons to the

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active site from the periplasm and, in the cbb_3-type oxidases, to deliver pumped protons in the opposite direction, from the active site to the periplasm.

X-ray structures are now available for the cbb_3-type oxygen reductase from *Pseudomonas stutzeri*,[22] the cNOR from *Pseudomonas aeruginosa*,[11] and the qNOR from *Geobacillus stearothermophilus*.[10] The qNOR is in an inactive form with Zn^{2+} bound in place of the non-heme Fe_B. In each case, there is a single Ca^{2+} that bridges between propionates of the low-spin and high-spin heme components and, in addition, is ligated to one of the glutamates (e.g., E122 in the *P. stutzeri* cbb_3, E126 in the *V. cholerae* cbb_3, E180 in the *R. sphaeroides* cbb_3, E135 in the *P. aeruginosa* cNOR, and E429 in the *G. stearothermophilus* qNOR). In each of the structures, the second glutamate of each pair, located three residues on the C-terminal side, is not ligated to the Ca^{2+} but is engaged in a network of hydrogen bonds, more clearly shown in the higher-resolution structures of the NO reductases. In the structure of the cbb_3-type oxygen reductase, there is extra electron density near (but not adjacent to) the second glutamate that has been suggested as a possible second Ca^{2+} bound to the enzyme, but there is no indication of a second Ca^{2+} in the qNOR or cNOR structures.

Figure 1 shows the Ca^{2+} binding site between the two hemes in the cbb_3 oxygen reductase from *P. stutzeri*. In addition to the Ca^{2+} bridging the D-propionates of heme *b* and heme b_3, an arginine (Arg57) is hydrogen bonded to the A- and D-propionates of the low-spin heme *b*.

In the A- and B-family oxygen reductases, there is also an arginine that hydrogen bonds to both of the propionates of the low-spin heme, but in place of the Ca^{2+}, there is a second arginine that bridges the low-spin and active-site hemes by hydrogen bonding to two propionates.[23,24] Figure 2 shows the structure near the low-spin heme *a* and active-site heme a_3 of the A-family cytochrome *c* oxidase from *R. sphaeroides* and compares this to the corresponding region in the cbb_3 oxygen reductase from *P. stutzeri*. Arg481 takes the place of the Ca^{2+} found in the cbb_3 oxygen reductases, and the adjacent Arg482 bridges the two propionates of the low-spin heme. The low-spin heme propionate that is ligated to the Ca^{2+} in the C-family (cbb_3) oxygen reductases (circled in Figure 2), as well as in the cNOR and qNOR families, has a rotomeric configuration different from that of the equivalent propionate that is hydrogen bonded to the adjacent arginine residues in the A- and B-family oxygen reductases. It is of interest that some of the A-family oxygen reductases, including the *R. sphaeroides*[25] and bovine[26] cytochrome *c* oxidases, also bind to a single Ca^{2+}, but this cation is not ligated to the heme propionates. Mutagenesis studies have shown that this Ca^{2+} is not essential for either assembly or function.[25,27]

In the A- and B-family oxygen reductases, the two adjacent arginines that interact with the heme propionates (R481 and R482 in Figure 2) are nearly totally conserved, but there are no conserved acidic residues corresponding to the glutamate pair found in the C-family oxygen reductases and in the cNOR and qNOR enzymes.

In this work, mutations of each of the glutamates of the conserved pair in the cbb_3 oxygen reducatases from *V. cholerae* and *R. sphaeroides* are further investigated, and it is shown that the loss of activity of the enzymes correlates with the loss of Ca^{2+}. We conclude that the mutagenesis data cannot be used to support the hypothesis that these glutamates are important for the delivery of a proton to or from the active site in the cbb_3 oxygen reductases. It is more likely that these residues are important for maintaining the structures of these enzymes in an active conformation.

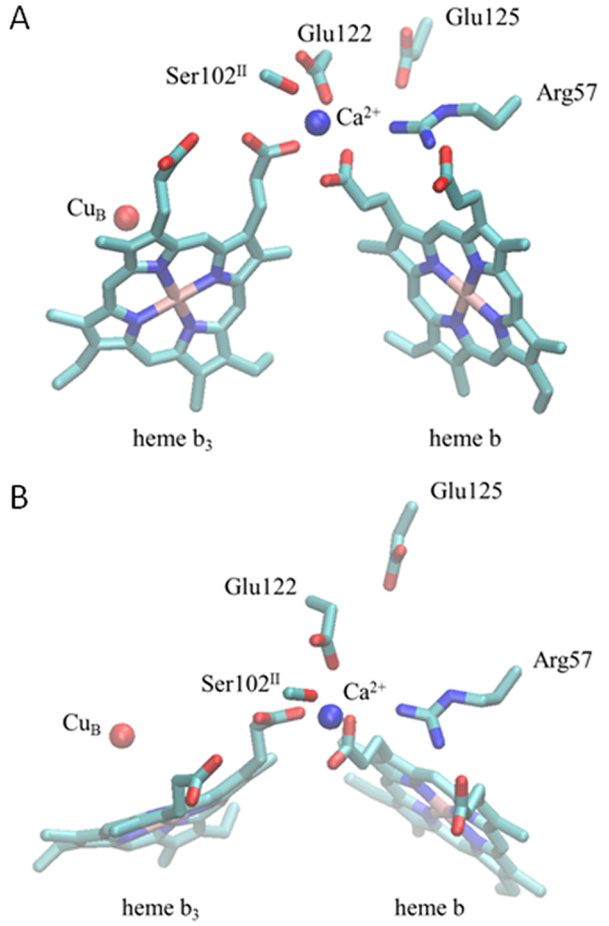

Figure 1. Structure of the Ca^{2+} binding site in the C-family oxygen reductase from *P. stutzeri* (Protein Data Bank entry 3MK7). (A) Ca^{2+} binding site, located at the interface between subunits I (CcoN) and II (CcoO), as seen from the side. The Ca^{2+} ion (blue) is ligated to the D-propionates of both the low-spin heme *b* and the active-site high-spin heme b_3, along with two conserved amino acid side chains, Glu122 in subunit I (CcoN) and Ser102 in subunit II (CcoO). The A- and D-propionates of the low-spin heme are both hydrogen bonded to a conserved arginine (Arg57) in subunit I. (B) View of the same region of the protein as seen from the top (periplasmic side). The residue numbering corresponds to that of the *P. stutzeri* cbb_3 oxygen reductase.

MATERIALS AND METHODS

Mutagenesis of the cbb_3-Type Oxidases from *R. sphaeroides* and *V. cholerae*. Site-directed mutagenesis and cloning were performed as previously reported [28]

Cell Growth and Purification of the His-Tagged cbb_3-Type Oxidases. *R. sphaeroides* was grown semiaerobically at 30 °C in Sistrom medium with 2 μg/mL tetracycline (Sigma). *V. cholerae* was grown aerobically at 37 °C in modified M9 medium (0.1% glucose and 1 mM $MgSO_4$ supplemented with 10 mg/L thiamine, 10 μM $CuSO_4$, and 50 μM $FeSO_4$) with 100 μg/mL ampicillin (Fisher Biotech) and 100 μg/mL streptomycin (Sigma). It was found that the expression of the *V. cholerae* cbb_3 was equally good if the cells were grown in minimal medium as in LB. However, protein purified from cells grown in minimal medium had fewer metal contaminants and more consistent metal analyses.

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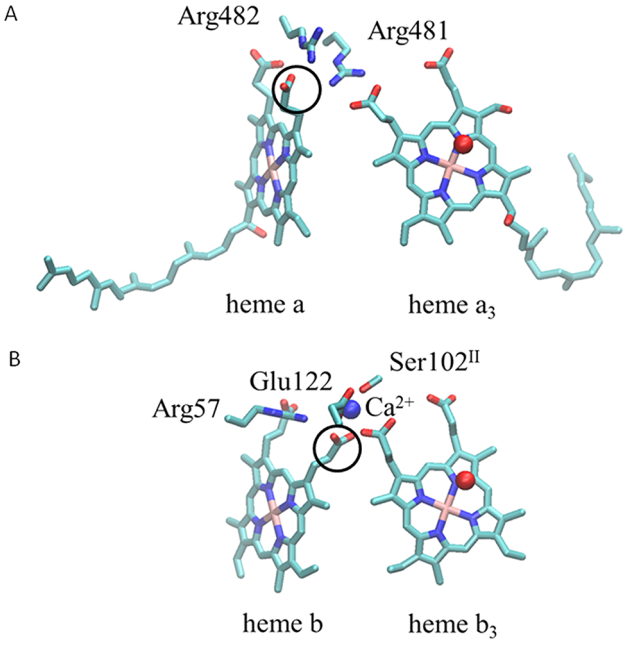

Figure 2. Comparison of heme propionate interactions in different heme-copper oxidoreductase families. (A) In the A- and B-families, represented by the structure of the A-family cytochrome *c* oxidase from *R. sphaeroides* (Protein Data Bank entry 1M56), the heme propionates interact with a pair of conserved, adjacent arginine residues. The first arginine forms hydrogen bonds with propionates of the low-spin heme (heme *a*) and the active-site, high-spin heme (heme a_3). The second arginine forms hydrogen bonds with the two propionates of the low-spin heme. (B) In the C-family oxidase from *P. stutzeri* (Protein Data Bank entry 3MK7), the Ca^{2+} binding site plays a role analogous to that of one of the arginines (R481 in the top panel). Note the difference in the rotational configurations of the low-spin heme propionates (circled) in the two panels. In the A- and B-families, the propionate is rotated out to form a hydrogen bond with the arginine, whereas in the C-family, it is rotated in to ligate the Ca^{2+} and to allow room for the arginine to form hydrogen bonds with the low-spin heme propionates. This structural feature is also found in the cNOR and qNOR families. The residue numbering in panel B is from the cbb_3 oxygen reductase from *P. stutzeri*.

The wild-type and mutant cbb_3 proteins (with polyhistidine tags) from *R. sphaeroides* and *V. cholerae* were overexpressed and purified as previously described[28] using the detergent dodecyl maltoside (DDM). GeneMate Express sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) gels from ISC BioExpress were used to evaluate the isolated protein complexes, using Coomassie Brilliant Blue staining. The CcoO and CcoP subunits were visualized by heme staining[29] because each of these subunits contains covalently bound heme *c*.

UV/Vis Spectroscopic Analysis. A Shimadzu UV/vis-2101PC spectrophotometer was used to obtain the spectra of wild-type and mutant enzymes. The concentrated protein samples were diluted with 10−20 mM Tris buffer and 0.05% DDM at pH 7.5−8.0. The enzymes were oxidized by air and reduced with sodium dithionite (Sigma). Spectra were measured from 300 to 800 nm and analyzed using Origin.

Pyridine Hemochrome Assay. The concentrations of heme *b* and heme *c* were determined as previously described;[30] 0.5 mL of a stock solution containing 200 mM NaOH and 40% (by volume) pyridine and 3 μL of 0.1 M $K_3Fe(CN)_6$ were placed in a 1 mL cuvette. A 0.5 mL aliquot of the protein sample (~5 μM) was added with thorough mixing, and the spectrum of the oxidized hemes was recorded within 1 min. Solid sodium dithionite (2−5 mg) was then added, and several successive spectra of the reduced pyridine hemochromes were recorded. The absorbance differences at selected wavelengths were used to obtain the concentrations of heme *b* and heme *c*. In some cases, after reduction by dithionite, turbidity resulted in a distorted baseline. This was corrected by fitting the baseline to either an exponential or a third-order polynomial and subtracting this from the experimental spectrum.

Steady-State Activity. A YSI model 53 oxygen monitor was used to polarographically measure steady-state oxidase activity at 25 °C. To the sample chamber were added 20 μL of 1 M ascorbate (pH 7.4), 10 μL of 0.1 M TMPD, and 1.8 mL of 50 mM sodium phosphate buffer (pH 6.5) containing 100 mM NaCl and 0.05% DDM. Horse heart cytochrome *c* (Sigma) was added as the substrate to a final concentration of 40 μM in the case of *R. sphaeroides* cbb_3. The reaction was initiated by adding 10 μL of a solution containing 1 μM enzyme, and the rate of oxygen consumption was monitored.

Metal Analysis. The metal content of the protein was determined by inductively coupled plasma-optical emission spectroscopy (ICP-OES) using a Spectro Genesis spectrometer as previously described.[31] The buffer containing the purified *V. cholerae* cbb_3 protein was exchanged using a 100 kDa cutoff Amicon concentrator (Millipore). The buffer was exchanged three times with 10 mM Tris buffer (pH 8) containing 0.05% DDM, 5% glycerol, 0.25 mM EDTA, and 0.1 mM EGTA, followed by another three times with the same buffer without EDTA and EGTA. Purified *R. sphaeroides* cbb_3 protein samples were incubated with 1 mM EDTA and 0.1 mM EGTA and then dialyzed against 20 mM Tris buffer (pH 7.5) containing 30 mM KCl, 1 mM EDTA, and 0.05% DDM. After dialysis, the protein was concentrated using the 100 kDa cutoff Amicon concentrator. Metal concentrations were calculated from regression lines of element standards run as samples rather than the regression lines produced by the Spectro software to avoid a 10% underestimation of element concentrations at low levels of the metals.

RESULTS

Conservation of the Glutamate Residues at or near the Ca^{2+} Binding Site. The one Ca^{2+} clearly observed in the structure of the cbb_3-type oxidase from *P. stutzeri* is ligated to the carboxyl of E122 (CcoN subunit) and to a serine, S102, located in the CcoO subunit, in addition to the two D-propionates from the low-spin heme *b* and the active-site heme b_3, which are located in the CcoN subunit. Sequence alignments show that E122 (CcoN) is >99% conserved, replaced with a glutamine in the few exceptions, and S102 (CcoO) is completely conserved. The nearby glutamate, E125 (CcoN), is not ligated to the Ca^{2+} but is also very highly conserved (>99%). These data are included in Table 1. Also shown is the pattern of conservation of the equivalent residues in the qNOR and cNOR families. In the cNOR enzymes, as in the C-family oxygen reductases, the Ca^{2+} has ligands that bridge two separate subunits (NorC and NorB), and it is the ligand from the subunit that contains cytochrome *c* (CcoO or NorC), which is most highly conserved. The two glutamates are less conserved in the cNOR family than in the cbb_3 oxygen reductases and even less conserved in the qNOR family.

Metal and Heme Content of the Wild-Type cbb_3-Type Oxygen Reductases. The metal contents of the cbb_3 enzymes from *V. cholerae* and *R. sphaeroides* are listed in Table 2. These

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Table 1. Conservation of Residues Important for Calcium Binding in the C-Family of Oxygen Reductases and the cNOR and qNOR Families of NO Reductases[a]

family	E122[b] (in CcoN), Ca^{2+} ligand	E125[b] (in CcoN)	S102[b] (in CcoO), Ca^{2+} ligand
C-family	E (Q), >99%	E (D, Q, K), 99%	S, T, 100% (in CcoO)
cNOR	E (K, P, H, V, G), 85%	E (D), 95%	Y, >99% (in NorC)
qNOR	E (A, S, D, P), 80%	D (E, S, Q, N, T), 45%	Y, 90%

[a]The percent conservation of the residues is given, along with the variants for each family. [b]The numbering of the residues refers to the cbb_3 oxygen reductase from *P. stutzeri*.

enzymes contain three *c*-type hemes, two *b*-type hemes, and one Cu_B, so the Fe/Cu ratio should be 5/1. The observed ratios are higher than this (Fe/Cu = 7.5 and 10.1 for the *V. cholerae* and *R. sphaeroides* enzymes, respectively). These high values could be due to loss of Cu_B, contaminating Fe, or both. The ratio of heme *c* to heme *b* should be 3/2 (=1.5), and the measured values are 1.9 and 1.5 for the *V. cholerae* and *R. sphaeroides* enzymes, respectively (Table 2).

The Ca contents of the enzymes are listed in Table 2 as either a ratio to Cu or Fe. If the Ca stoichiometry is taken as a ratio to the total iron content, the data indicate a Ca content of 1.1 and 1.4 equiv/mol of enzyme (five Fe atoms per enzyme molecule) for the *V. cholerae* and *R. sphaeroides* cbb_3 oxygen reductases, respectively. The Ca stoichiometry that is determined from the Ca/Cu ratio is higher in both cases, ~1.8 for the *V. cholerae* enzyme and 2.4 for the *R. sphaeroides* enzyme. It is noted that the possible presence of a second Ca^{2+} in the cbb_3 enzyme from *P. stutzeri* is also unclear, both from the crystal structure itself and from the metal analysis.[22]

At this point, with the current enzyme preparations, it is not possible to conclude with confidence whether these enzymes contain 1 or 2 equiv of Ca^{2+}. For the sake of convenience, the Ca^{2+} stoichiometries of various mutants are reported as the values determined based on the amount of Fe present (assuming 5 Fe atoms/mol of enzyme).

Site-Directed Mutagenesis of the *V. cholerae* cbb_3 Oxygen Reductase. *E126 (Ca^{2+} ligand).* The E126D, E126Q, and E126G mutations were characterized. As shown in Table 2, each of these mutations is virtually inactive (2, <1, and <1%, respectively). The ratio of heme *c* to heme *b* of the E126D mutant is similar to that of the wild type, but both E126Q and E126G have a lower ratio. SDS−PAGE analysis shows that both of the heme *c*-containing subunits,[22] CcoO and CcoP, are present in each mutant, though this is qualitative and not quantitative. The Ca stoichiometry was measured for the E126Q mutant and is approximately half that of the wild type. Hence, mutation of the Ca ligand results in both the loss of Ca and the loss of activity.

E129. The E129D, E129Q, E129G, and E129A mutants were characterized. The E129D and E129Q mutants have 11 and 18% of the wild-type activity, respectively, whereas the E129G and E129A mutants have 2% of the wild-type activity (Table 2). The heme *c*/heme *b* ratio ranges from 1.3 to 1.8 (expected, 1.5). The Ca stoichiometry was determined for the E129Q mutant and was shown to be approximately half the wild-type value (Table 2), 0.63 versus 1.11 equiv of Ca/mol of enzyme. Even though E129 is not directly ligated to the bound Ca, an E129 mutation can still cause loss of this metal, apparently by an indirect interaction.

Site-Directed Mutagenesis of the *R. sphaeroides* cbb_3 Oxygen Reductase. *E180 (Ca^{2+} ligand).* The E180D, E180Q, and E180G mutants were examined. All were virtually inactive (Table 2), and the yield of E180G was very low, implying instability or an assembly problem. The E180G mutant was not examined further. Metal analysis of both E180D and E180Q showed not only that there was no Ca but also that there was no Cu. In addition, the dithionite reduced-minus-oxidized UV/visible spectra (Figure 3) show that the loss of Cu from E180Q and E180D is coincident with the lack of reduction of heme *b*. The content of heme *b* in the mutant enzymes is similar to that of the wild-type enzyme, as shown by the pyridine−

Table 2. Characteristics of Wild-Type and Mutant cbb_3-Type Oxygen Reductases

subunit	strain	mutant	activity (% WT)	[C]/[B] ratio	Ca/Cu	5(Ca/Fe)
CcoN	*R. sphaeroides*	WT	100[a]	1.5	2.4 ± 0.3 (n = 3)	1.44 ± 0.5 (n = 3)
	R. sphaeroides	E180D	<1	1.7[b]		0 (n = 1)
	R. sphaeroides	E180Q	1	1.6[b]		0 (n = 1)
	R. sphaeroides	E180G	<1	2		
	R. sphaeroides	E183D	1	1.6[b]		0 (n = 1)
	R. sphaeroides	E183Q	7	1.5	1.92 ± 0.34 (n = 3)	1.13 ± 0.23 (n = 3)
	R. sphaeroides	E183G	1	4		
	V. cholerae	WT	100[a]	1.9	1.82 ± 0.05 (n = 7)	1.11 ± 0.2 (n = 7)
	V. cholerae	E126D	2	1.8		
	V. cholerae	E126Q	<1	1.2		0.55 ± 0.33 (n = 7)
	V. cholerae	E126G	<1	1.2		
	V. cholerae	E129D	11	1.3		
	V. cholerae	E129Q	18	1.7		0.63 ± 0.4 (n = 8)
	V. cholerae	E129G	2	1.8		
	V. cholerae	E129A	2	1.5		
CcoO	*R. sphaeroides*	S105T	<1			
	R. sphaeroides	S105A	<1			
	R. sphaeroides	S105G	<1			

[a]The activities of the *R. sphaeroides* and *V. cholerae* cbb_3-type oxygen reductases were 800 and 500 e^-/s, respectively. See Materials and Methods for details. [b]For these mutants, the low-spin heme *b* component of the oxidase, though present, was not reduced by dithionite in the assembled enzyme.

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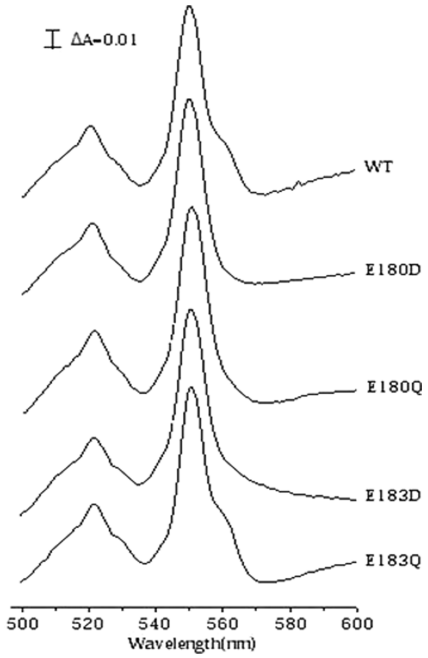

Figure 3. Dithionite-minus-air-oxidized difference spectra of the *R. sphaeroides* cbb_3-type oxygen reductases. The 551 nm peak is due to *c*-type hemes, and the 561 nm peak is mostly due to the low-spin heme *b*. The data show the absence of the spectroscopic signature for reduced heme *b* in the E180D, E180Q, and E183D mutants.

hemochrome analysis (Table 2), but the low-spin heme is not reduced by dithionite in the assembled enzyme. This indicates substantially more damage due to the alteration of the Ca ligand in the *R. sphaeroides* enzyme than in the *V. cholerae* enzyme. The nature of this perturbation to the low-spin heme *b* was not explored further. It is noted, however, that this unusual mutant phenotype of a heme that cannot be reduced by dithionite has been previously observed in a mutant of the cytochrome *bd* quinol oxidase from *Escherichia coli*.[32]

E183. The E183D, E183Q, and E183G mutants were characterized. All the mutants have very low (or no) oxidase activity, with the highest activity (7%) observed with E183Q (Table 2). E183G was obtained in very low yield and was not examined further. The E183D mutant had properties similar to those of the E180 mutants insofar as there was loss of both Ca and Cu_B, and the low-spin heme *b* component was not reduced by dithionite. The E183Q mutant retains a small amount of oxidase activity (7%) and undergoes a partial loss of Ca^{2+} but no loss of Cu (Table 2). This is the only *R. sphaeroides* mutant of those examined in which loss of activity is not correlated with a massive loss of Ca^{2+}.

S105 (CcoO). The S105T, S105A, and S105G mutations were made, but no expressed protein was obtained for any of the mutants. This Ca^{2+} ligand appears to be absolutely necessary for protein assembly and stability.

DISCUSSION

Prior to the availability of X-ray structures, the conserved pair of glutamates in a putative periplasmic "loop" appeared to be reasonable candidates as participants in proton translocation to and from the active sites of the cbb_3-type (C-family) oxygen reductases as well as the related cNOR and qNOR families of NO reductases. Models suggested that these glutamates might be located near the perisplasmic surface of the proteins,[18,19] and site-directed mutagenesis indicated that the glutamates are functionally important in both the cbb_3 oxygen reductases and cNOR enzymes.[18,20,21] In the case of the cNOR enzymes, protons required for the chemistry at the active site come from the periplasm[18,33,34] and the limited data were consistent with these glutamates playing a role in providing a pathway for the transfer of a proton to the active site.[34] For the cbb_3 enzymes, protons for the chemistry at the active site must come from the opposite side of the membrane,[2,7] so the glutamates were proposed to provide a pathway for the exit of pumped protons to the periplasm.[7] The X-ray structures[10,11,22] as well as recent functional information, including the data presented in this work, make these proposals much less likely.

The X-ray structures of the cbb_3 oxygen reductase, the cNOR, and the qNOR each reveal a single Ca^{2+} that bridges propionates on heme *b* and heme b_3 and is also ligated to a glutamate carboxyl,[10,11,22] E122 in the case of the *P. stutzeri* cbb_3 enzyme. There is extra electron density in the *P. stutzeri* cbb_3 structure[22] that suggests the possibility of a second Ca^{2+} near E125. Metal analysis also indicates that there might be a second Ca^{2+} present in the *P. stutzeri* cbb_3 oxidase.[22] The current data on the *V. cholerae* and *R. sphaeroides* cbb_3 enzymes are similarly ambiguous about the possibility of a second Ca^{2+} (Table 2). Metal impurities in the protein preparations, present even after three column chromatography steps, as well as possible loss of metals, contribute to the uncertainty.

The X-ray data[10,11,22] show that in each of these enzyme families (cbb_3, cNOR, and qNOR), the two glutamates are not near the surface in contact with the bulk aqueous phase but are more interior. The upstream member of the pair (lower residue number) is, in each case, ligated to a Ca^{2+}. The downstream glutamate is not ligated to the Ca^{2+} or part of a salt bridge but participates in a network of hydrogen bond interactions in each protein. The possible second Ca^{2+} in the cbb_3 structure is located near this downstream glutamate.[22] There is only one Ca^{2+} in the structures of cNOR and qNOR.[10,11]

The very high (>99%) degree of conservation of each of the two glutamates in the sequences of the cbb_3 oxygen reductases makes it evident that they are both important in these enzymes (Table 1). This is confirmed by the site-directed mutagenesis presented here (Table 2). Regardless of the uncertainty of whether there are one or two Ca^{2+} ions in the wild-type enzymes, the pattern for both the *V. cholerae* and *R. sphaeroides* cbb_3 mutants is one in which substitution of either of the glutamates results in a substantial loss of activity as well as a partial or complete loss of Ca^{2+} (Table 2). Changing the Ca^{2+} ligand, E126 (*V. cholerae*) or E180 (*R. sphaeroides*), results in the virtually complete loss of function as well as the partial or complete loss of Ca^{2+} (Table 2). In the *R. sphaeroides* mutants, the extent of evident damage is greater, including loss of Cu_B. Note that if the wild-type enzyme is interpreted to contain two Ca^{2+} ions, then a single mutation (E180D or E180Q) results in the loss of both Ca^{2+} atoms. The loss of function due to these mutations can be most easily interpreted as a consequence of gross conformational changes. The mutagenesis data do not rule out a role for this glutamate in proton translocation but, importantly, cannot be used in support of such a role. If E180 were to cycle through a state during catalysis in which it were protonated and not ligated to the Ca^{2+}, one might not expect the E180Q mutation to cause so much damage because glutamine to some extent resembles a protonated glutamate.

Changing the downstream glutamate, E129 (*V. cholerae*) or E183 (*R. sphaeroides*), also invariably results in a loss of

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function, though the results are more varied and not as absolute as those observed with the mutations of the Ca^{2+}-ligating glutamate. The *V. cholerae* protein is more tolerant of mutations at this position than the *R. sphaeroides* enzyme. E129D and E129Q mutants of the *V. cholerae* enzyme retain 11 and 18% activity, respectively, whereas E183D and E183Q mutants of the *R. sphaeroides* oxidases retain only 1 and 7% activity, respectively. In all cases, either partial or complete loss of Ca^{2+} is observed.

The following are reasonable conclusions that can be drawn from the data.

(1) The cbb_3 oxygen reductases contain either one or two Ca^{2+} ions. Our data do not resolve this uncertainly, raised initially by the work on the enzyme from *P. stutzeri*.[22]

(2) The Ca^{2+} at the binding site that is apparent in the X-ray structure is essential for the function of the cbb_3 oxygen reductases. Mutations that result in the loss of Ca^{2+} or disruption of the Ca^{2+} binding site result in the loss of enzyme activity. It is likely that the Ca^{2+} is important for maintaining the protein in a conformation in which the hemes and Cu_B are functional. The role of the Ca^{2+} to form a bridge between the CcoN and CcoO subunits is also critical, as evidenced by the lack of protein assembly when the Ca^{2+}-liganding amino acid in the CcoO subunit (S105) is substituted with another amino acid. The essential nature of the Ca^{2+} in the cbb_3 oxygen reductases is in contrast to the nature of the Ca^{2+} that binds near the low-spin heme in some of the A-family oxygen reductases but is not required for assembly or enzyme activity.[25,27]

(3) Depending on the mutation and which enzyme is studied, via mutation of the nonliganding glutamate (downstream member of the pair), the cbb_3 enzyme can be inactivated along with partial or complete loss of Ca^{2+}. There is no reason to attribute the loss of function to blocking proton transfer of the pumped proton away from the cbb_3 active site. It is not evident, in any event, that blocking the pumped proton would necessarily inhibit oxidase activity..

The conclusion that the role of the Ca^{2+} is primarily to stabilize the heme and protein for optimal activity has also been reached for the related NOR enzymes.[4,8−10] Note that the two glutamates examined in this work are not nearly as well conserved in the cNOR and qNOR sequences as in the sequences of the cbb_3 oxygen reductases (Table 1). This is particularly true for the qNORs. When present, the Ca^{2+} ligands are important, as shown by the fact that mutants of each of the Ca^{2+} ligands (E429 and Y93) in the qNOR from *G. stearothermophilus* lose most of their activity as well as Ca^{2+}.[10] It is possible that in the qNOR and cNOR variants lacking the equivalent glutamate, the Ca^{2+} has a different ligand or, alternatively, the role of Ca^{2+} is filled by alternative structural features. There are credible hydrophilic pathways that bypass the two glutamates for delivering protons from the periplasm to the active site in the cNOR enzymes, so there is no need to propose any role of the glutamate pair in proton translocation in either the cNOR or qNOR enzymes.[4,9] Experimental tests will be required, however, to determine the input pathway(s) for protons to reach the active sites of the qNOR and cNOR enzymes.

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